The Acute Regulation of Intestinal Chylomicron Secretion by Glucagon-Like Peptides

by

Joanne Hsieh

A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy
Graduate Department of Biochemistry

University of Toronto

© Copyright by Joanne Hsieh (2012)
ABSTRACT

Postprandial overproduction of apolipoprotein B48 (apoB48)-containing lipoproteins has been observed in states of insulin resistance and is important to the sequelae of cardiovascular disease, but little is understood about factors that regulate their secretion. The glucagon-like peptides (GLPs) are released from ileal enteroendocrine L-cells following lipid ingestion. I hypothesized that the GLPs could acutely affect the production of apoB48-containing triglyceride (TG)-rich lipoproteins (TRL) in the small intestine. Using the Syrian golden hamster, I first characterized the gross effects of the GLPs on TRL secretion in response to an oral fat load and then continued to dissect the mechanisms of these changes using primary intestinal cell cultures and a variety of knockout mouse models. An exogenous GLP-1 receptor (GLP-1R) agonist was found to acutely inhibit chylomicron secretion in both hamsters and mouse models, and extending the bioactivity of endogenously-secreted GLP-1 with a dipeptidyl peptidase-4 inhibitor had suppressive effects in insulin-resistant fructose-fed hamsters. The insulinotropic and delayed gastric emptying functions do not completely account for the hypolipidemic effect of GLP-1R agonism, and the effect of the GLP-1R agonist exendin-4 could
be seen directly in the apoB48 secretion of primary enterocytes. In contrast, the sister peptide GLP-2 was a potent acute stimulator of chylomicron secretion in hamsters and mice. The hyperlipidemic effect of GLP-2 could be attributed to an increased rate of luminal FA uptake mediated by the posttranslational modification of the FA transporter CD36, and CD36-deficient mice were found to be refractory to the stimulatory effects of GLP-2. The activity of nitric oxide synthase was also found to be essential to the hyperlipidemic action of GLP-2. I identified a set of intercellular communications that could contribute in mediating the action of GLP-2, in which GLP-2 secreted from the enteroendocrine L-cell stimulates intestinal subepithelial myofibroblasts to release vascular endothelial growth factor, which directly activated the enterocyte to secrete apoB48. In summary, this thesis demonstrates that two co-secreted postprandial hormones have considerable but completely opposite influences on chylomicron production. Changing the balance of the GLPs’ actions in vivo could provide a therapeutic strategy to combat postprandial dyslipidemia.
ACKNOWLEDGMENTS

This Ph.D. thesis was a long endeavour that could not have been accomplished without the support and input of many people. It began with my parents Tse Kao and Yu Sze in supporting my decision to move from beautiful British Columbia to Toronto to pursue my studies at the University of Toronto. I have to thank my husband Edward who, despite not being trained in science, was indispensable to the completion of the thesis. He was my motivation, respite from the frustrations of problematic experiments, and even in a couple of instances, my financial bailout when annual tuition fees were due. I would also like to express gratitude to my sisters Vivian and Yvonne, and my parents-in-law Thomas and Rebecca, for all making this journey a little easier.

My supervisor Dr. Khosrow Adeli always managed to provide project funding, review manuscripts, input on oral presentations, and mentorship on my academic career all while having the busiest professional and travel schedule of anyone I personally know. Much of the confidence I have come to acquire over the years have come from his encouragement. I came with an interest in lipid biochemistry, and I can honestly say I am leaving the lab with a love for lipids. My committee members Dr. Amira Klip and Dr. Patricia Brubaker have provided invaluable suggestions for experiments during each and every one of my supervisory committee meetings, and I’d hate to imagine where I would be without the immense knowledge of GLP physiology and intestinal biology of Dr. Brubaker. The technical support of Angelo Izzo with the conditioned media studies presented in Chapter 4 is also very much appreciated. Dr. Daniel Drucker has also been an integral part of this thesis with his involved collaboration in these projects, and the successful publication of the papers in this thesis would not have been possible with some of the experiments conducted by Dr. Christine Longuet.

Many people over the years have commented that the lab has an exceptional environment, and I couldn’t agree more. My fellow lab members were not only brainstormers, troubleshooters, and extra hands during technically-demanding experiments; they were my closest friends in Toronto. A list of everyone would necessitate its own thesis chapter, but standouts have included past (Julie Tsai, Angela Rutledge, Elaine Xu, Rita Kohen, Bolin Qin, Diana Wong, Joanna Nelken, Jennifer Webb and Amanda Hayashi) and present (Chris Baker,
Mark Naples, Rianna Zhang, Mark Dekker, Wei Qiu, Qiaozhu Su, and Man Khun Chan) members. In addition, my classmates in the Molecular Structure and Function department of The Hospital for Sick Children could always be counted on for fun, relaxation, and reagents in a pinch.

Finally, I would like to express my gratitude to the Department of Biochemistry of the University of Toronto and The Hospital for Sick Children. My thesis project has been financially supported by The Hospital for Sick Children’s Restracomp program, the National Sciences and Engineering Research Council of Canada Canada Graduate Scholarship M, and the Frederick Banting and Charles Best Canada Graduate Scholarship D and an operating grant from the Canadian Institutes of Health Research.
TABLE OF CONTENTS

Abstract ........................................................................................................................................... ii
Acknowledgments ......................................................................................................................... iv
Table of Contents .......................................................................................................................... vi
List of Tables .................................................................................................................................. xi
List of Figures ................................................................................................................................. xii
List of Abbreviations ..................................................................................................................... xiv

Chapter 1: Introduction ................................................................................................................. 1
  1.1 Clinical significance ................................................................................................................ 2
  1.2 Intestinal Fat Absorption and Chylomicron Assembly ......................................................... 3
    1.2.1 Dietary lipid uptake ........................................................................................................ 3
    1.2.2 Chylomicron assembly and secretion ........................................................................... 5
    1.2.3 Naturally occurring mutation in fatty acid transporters ............................................. 11
    1.2.4 Insulin action and insulin resistance .......................................................................... 14
    1.2.5 Hormonal regulation of chylomicron secretion ........................................................... 16
    1.2.6 Other fates of dietary lipid in the intestine ................................................................. 20
    1.2.7 Chylomicron catabolism ............................................................................................. 21
  1.3 Glucagon-Like Peptides ....................................................................................................... 23
    1.3.1 Glucagon-like peptide structure .................................................................................. 23
    1.3.2 Intestinal GLP secretion .............................................................................................. 25
  1.4 Biology of GLP-1 ................................................................................................................ 27
    1.4.1 Physiological functions of GLP-1 .............................................................................. 27
    1.4.2 GLP-1 receptor ............................................................................................................ 27
1.4.3 GLP-1 and lipid metabolism.................................................................28
1.5 Biology of GLP-2 .................................................................................30
  1.5.1 Biological functions of GLP-2.........................................................30
  1.5.2 GLP-2 and intestinal nutrient absorption...........................................31
  1.5.3 GLP-2 receptor.................................................................................35
1.6 Hypothesis.............................................................................................37

Chapter 2: The glucagon-like peptide-1 receptor regulates postprandial lipoprotein synthesis and secretion.................................................................38
  2.1 Summary............................................................................................39
  2.2 Introduction........................................................................................40
  2.3 Materials and Methods.......................................................................42
    2.3.1 Animals........................................................................................42
    2.3.2 Assessment of intestinal lipoprotein production by in vivo Triton-WR1339 infusion .................................................................42
    2.3.3 Isolation of triglyceride-rich lipoproteins (TRL)............................43
    2.3.4 Chemiluminescent immunoblotting.............................................43
    2.3.5 Ex vivo metabolic labeling of intact primary enterocytes............44
    2.3.6 Immunoprecipitation, SDS-PAGE, and fluorography...............44
    2.3.7 Plasma measurements................................................................45
    2.3.8 Fast protein liquid chromatography of plasma lipoproteins.......45
    2.3.9 Statistical analysis.........................................................................45
  2.4 Results................................................................................................46
    2.4.1 A DPP-4 inhibitor attenuates dyslipidemia in fructose-fed hamsters.........................................................................................46
    2.4.2 A DPP-4 inhibitor decreases intestinal production of TRL-TG and TRL-cholesterol.................................................................49
    2.4.3 Pharmacological activation of the GLP1R mimics the effects of sitagliptin on intestinal lipid absorption.................................53
2.4.4 Endogenous GLP-1R signalling is required for control of postprandial lipemia..57
2.4.5 Intact GLP-1R signalling is required for the hypolipidemic action of sitagliptin.................................................................57
2.4.6 Exendin-4 directly reduces enterocyte ApoB48 secretion.................................60
2.5 Discussion...........................................................................................................62

Chapter 3: GLP-2 increases intestinal lipid absorption and chylomicron production via CD36 .................................................................65
3.1 Summary.............................................................................................................66
3.2 Introduction..........................................................................................................67
3.3 Materials and Methods........................................................................................69
  3.3.1 Antibodies and chemicals ..............................................................................69
  3.3.2 Animals............................................................................................................69
  3.3.3 Determination of triglyceride-rich lipoprotein apoB48 secretion in vivo in hamsters ........................................................................69
  3.3.4 Determination of TG-rich lipoprotein apoB48 secretion in vivo in mice ....70
  3.3.5 Isolation of TRL...............................................................................................70
  3.3.6 FPLC of plasma lipoproteins ..........................................................................70
  3.3.7 Density ultracentrifugation of plasma lipoproteins.........................................71
  3.3.8 Metabolic labelling of primary jejunal fragments ex vivo .............................71
  3.3.9 Monitoring labelled triolein secretion in vivo .................................................71
  3.3.10 In situ apical membrane protein biotinylation .............................................72
  3.3.11 Statistical analysis..........................................................................................72
3.4 Results.................................................................................................................73
  3.4.1 GLP-2 acutely increases circulating levels of apoB48-containing TRL in hamsters .................................................................73
  3.4.2 GLP-2 accelerates secretion of luminal fatty acids.......................................78
  3.4.3 Acute GLP-2 treatment increases apoB48-containing TRL production in mice...81
| 3.4.4 | GLP-2-stimulated chylomicron secretion requires CD36 | 83 |
| 3.5 | Discussion | 86 |
| Chapter 4: Intercellular communications mediating GLP-2-stimulated chylomicron secretion | 90 |
| 4.1 | Summary | 91 |
| 4.2 | Introduction | 92 |
| 4.3 | Materials and Methods | 94 |
| 4.3.1 | Animals | 94 |
| 4.3.2 | Determination of dietary fat absorption in vivo | 94 |
| 4.3.3 | Determination of postprandial TRL production in vivo | 95 |
| 4.3.4 | Determination of intestinal apoB48 secretion ex vivo | 95 |
| 4.3.5 | ISEMF-conditioned media | 96 |
| 4.3.6 | Steady state labelling of apoB48 production ex vivo | 96 |
| 4.3.7 | Other biochemical measurements | 96 |
| 4.3.8 | Kinex phosphoprotein screen | 97 |
| 4.3.9 | Statistical analysis | 97 |
| 4.4 | Results | 98 |
| 4.4.1 | In vivo role of NO in GLP-2-stimulated postprandial TRL secretion | 98 |
| 4.4.2 | Ex vivo role of NO in apoB48 secretion | 100 |
| 4.4.3 | Role of eNOS in GLP-2-stimulated chylomicron secretion | 102 |
| 4.4.4 | Role of ISEMFs in GLP-2-stimulated apoB48 secretion | 104 |
| 4.4.5 | VEGF action in enterocyte apoB48 secretion | 106 |
| 4.4.6 | VEGF as a mediator linking ISEMFs and enterocytes | 108 |
| 4.5 | Discussion | 110 |
| Chapter 5: Discussion and conclusions | 115 |
5.1 Summary of Results ..................................................................................................................116
5.2 GLP-1 as a Direct Regulator of Intestinal Lipoprotein Metabolism ...........................................117
5.3 CD36 as a Hormone-Sensitive FA Transporter in the Gut .........................................................118
5.4 VEGF and NO as Modulators of Intestinal Function .................................................................120
5.5 Concerted GLP-1 and GLP-2 Action ..........................................................................................122
5.6 Future Directions .......................................................................................................................128
5.7 Conclusions ...............................................................................................................................129

Permission to Publish Copyrighted Material .................................................................................130

References .........................................................................................................................................148
LIST OF TABLES

Table 1-1. Biological functions of GLP-1 and GLP-2................................................................. 34

Table 2-1. Body mass and blood glucose following sitagliptin treatment................................. 48

Table 2-2. TRL fraction measurements in chow-fed mouse studies ............................................. 51
LIST OF FIGURES

Figure 1-1. Dietary lipid absorption and chylomicron assembly in the enterocyte.............................. 9

Figure 1-2. Insulin signalling pathway depicted with perturbations known to occur in the enterocyte during insulin resistance........................................................................................................ 18

Figure 1-3. Differential proglucagon polypeptide processing in pancreatic alpha-cells and intestinal enteroendocrine L-cells........................................................................................................ 24

Figure 2-1. Changes in plasma lipids following administration of sitagliptin............................................ 47

Figure 2-2. Changes in TRL lipid mass following chronic sitagliptin administration ............................... 50

Figure 2-3. Changes in postprandial lipid secretion following a single acute administration of sitagliptin in chow-fed mice........................................................................................................ 52

Figure 2-4. Pharmacological activation of the GLP1R mimics the effect of sitagliptin on post-prandial lipid excursion.................................................................................................................... 55

Figure 2-5. Role of glucose-modulating hormones in mice ................................................................. 56

Figure 2-6. Role of GLP-1 in postprandial lipemia and sitagliptin action........................................ 58

Figure 2-7. Direct effects of exendin-4 on intestinal apoB48 metabolism ex vivo .............................. 61

Figure 3-1. In vivo effects of GLP-2 on apoB48-containing TRL secretion........................................ 75

Figure 3-2. Plasma lipoprotein profiling by salt-density sedimentation in hamsters ....................... 76

Figure 3-3. Ex vivo effects of GLP-2 on apoB48-containing lipoprotein secretion.......................... 77

Figure 3-4. GLP-2 accelerates secretion of luminal fatty acids in hamsters .................................. 79

Figure 3-5. GLP-2 enhances intestinal CD36 action in hamsters...................................................... 80
Figure 3-6. Stimulatory effects of GLP-2 on intestinal lipid absorption and apoB48-chylomicron production in wild type mice .................................................................................................................. 82

Figure 3-7. Lack of GLP-2 mediated stimulation of intestinal lipoprotein secretion in Cd36<sup>−/−</sup> mice ............................................................................................................................................. 84

Figure 4-1. The role of nitric oxide in apoB48 production in hamsters in vivo .............................................. 99

Figure 4-2. The effect of an NO donor on apoB48 production in hamster enterocytes ex vivo 101

Figure 4-3. Effect of GLP-2 on dietary fat absorption and postprandial lipoprotein secretion in C57Bl/6J and eNOS KO mice ........................................................................................................................................... 103

Figure 4-4. Effect of ISEMF conditioned media on apoB48 production in primary hamster enterocytes .................................................................................................................................................. 105

Figure 4-5. The role of VEGF in intestinal apoB48 secretion in mice ............................................................ 107

Figure 4-6. The role of VEGF in GLP-2-stimulated ISEMF-mediated intestinal apoB48 secretion in enterocytes ........................................................................................................................................ 109

Figure 4-7. Intercellular interactions resulting in GLP-2-stimulated chylomicron secretion .... 111

Figure 5-1. Physiological contributions of GLP-1 and GLP-2 to chylomicron secretion ........ 126
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG5</td>
<td>ATP binding cassette, sub-family G, member 5</td>
</tr>
<tr>
<td>ACAT2</td>
<td>acyl-CoA:cholesterol acyltransferase 2</td>
</tr>
<tr>
<td>ACCα</td>
<td>acetyl-CoA carboxylase α</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>apoAI</td>
<td>apolipoprotein AI</td>
</tr>
<tr>
<td>apoAIV</td>
<td>apolipoprotein AIV</td>
</tr>
<tr>
<td>apoB48</td>
<td>apolipoprotein B48</td>
</tr>
<tr>
<td>apoCII</td>
<td>apolipoprotein CII</td>
</tr>
<tr>
<td>apoCIII</td>
<td>apolipoprotein CIII</td>
</tr>
<tr>
<td>BBM</td>
<td>brush border membrane</td>
</tr>
<tr>
<td>CD36</td>
<td>cluster of differentiation CD36</td>
</tr>
<tr>
<td>CPT-1</td>
<td>carnitine palmitoyltransferase-I</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DGAT</td>
<td>acyl-CoA:diacylglycerol acyltransferase</td>
</tr>
<tr>
<td>DPP-4</td>
<td>dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular related kinase 1/2</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FATP4</td>
<td>fatty acid transporter 4</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>GIP</td>
<td>glucose-dependent insulinoitropic peptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>glucagon-like peptide-1</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>glucagon-like peptide-1 receptor</td>
</tr>
<tr>
<td>GLP-2</td>
<td>glucagon-like peptide-2</td>
</tr>
<tr>
<td>GLP-2R</td>
<td>glucagon-like peptide-2 receptor</td>
</tr>
<tr>
<td>GLUT2</td>
<td>glucose transporter 2</td>
</tr>
<tr>
<td>GPR119</td>
<td>G protein-coupled receptor 119</td>
</tr>
<tr>
<td>GPR120</td>
<td>G protein-coupled receptor 120</td>
</tr>
<tr>
<td>GPR40</td>
<td>G protein-coupled receptor 40</td>
</tr>
<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase-3</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitroso-L-glutathione</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HFD</td>
<td>high fat diet</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>ISEMF</td>
<td>intestinal subepithelial myofibroblast</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KGF</td>
<td>keratinocyte growth factor</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>L-FABP</td>
<td>liver fatty acid binding protein</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N(^G)-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>L-N(^G)-monomethyl-L-arginine</td>
</tr>
<tr>
<td>LpL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>MAG</td>
<td>monoacrylglycerol</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MGAT</td>
<td>acyl-CoA:monoacrylglycerol acyltransferase</td>
</tr>
<tr>
<td>MTP</td>
<td>microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>Niemann-Pick C1-like 1</td>
</tr>
<tr>
<td>NTS</td>
<td>nucleus of the solitary tract</td>
</tr>
<tr>
<td>PC1/3</td>
<td>prohormone convertase 1/3</td>
</tr>
<tr>
<td>PCTV</td>
<td>prechylomicron transport vesicle</td>
</tr>
<tr>
<td>PI3-K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC(\zeta)</td>
<td>protein kinase C(\zeta)</td>
</tr>
<tr>
<td>PPAR(\alpha)</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>SCD-1</td>
<td>stearoyl-CoA desaturase-1</td>
</tr>
<tr>
<td>SGLT-1</td>
<td>sodium-glucose cotransporter-1</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble N-ethylmaleimide sensitive factor attachment protein</td>
</tr>
<tr>
<td>SR-BI</td>
<td>scavenger receptor class B type 1</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>sterol responsive element binding protein-1c</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TGF-(\beta)</td>
<td>transforming growth factor-(\beta)</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>tumour necrosis factor-(\alpha)</td>
</tr>
<tr>
<td>TRL</td>
<td>triglyceride-rich lipoprotein</td>
</tr>
<tr>
<td>VAMP</td>
<td>vesicle-associated membrane protei</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
</tbody>
</table>
Chapter 1: INTRODUCTION

Figure 1-2 is reproduced from:


**Author contribution:** J. Hsieh produced all text and figures in this chapter.
1.1 Clinical significance

Cardiovascular disease (CVD) continues to be the largest cause of mortality in the world (1). While CVD is traditionally thought of as a problem limited to developed nations, the prevalence of risk factors for CVD, such as obesity, is rising worldwide (2). Low density lipoprotein (LDL) has long been targeted as a causative factor of atherosclerosis, but growing evidence suggest that intestinally-derived lipoproteins also play a role in the pathogenesis of atherosclerotic heart disease (3-6). There is pathological and experimental evidence to indicate that intestinally-derived apolipoprotein B48 (apoB48)-containing lipoproteins accumulate in the arterial wall to deposit cholesterol (7; 8) and induce endothelial dysfunction (9). Postprandial hyperlipidemia is also an inherent feature of diabetic dyslipidemia (10), thereby including it as part of a major complication of a disease that has been identified as a growing epidemic. Moreover, given typical Western eating patterns are based on three meals a day, humans spend the majority of their day with some degree of postprandial lipemia (11). Understanding the mechanisms regulating postprandial lipid metabolism is thus clearly important in elucidating the pathways underlying the pathogenesis of atherosclerosis and its cardiovascular complications. However, our knowledge of lipid handling in the intestine pales in comparison to what is known about hepatic lipoprotein metabolism, and even more lacking is our understanding of how intestinal lipoprotein secretion is regulated.
1.2 Intestinal Fat Absorption and Chylomicron Assembly

1.2.1 Dietary lipid uptake

The digestion of ingested fat in the intestine begins with its lipolysis by pancreatic juices and emulsification by bile in the duodenum and upper jejunum. The action of pancreatic lipase and colipase on triglyceride (TG) results in free fatty acid (FFA) and sn2-monoacylglycerol (MAG), and the micellization of these products with bile acids and phospholipids prepares lipid for uptake across the apical membrane of the enterocyte (reviewed in (12)). Because of the high efficiency of fat absorption in the mammalian gut, and the high luminal concentrations in the postprandial state, fatty acids (FA) and MAG were thought to enter the enterocyte by simple diffusion across the phospholipid bilayer. However, there is a large body of evidence in different cell lines and organs that indicate FA transport is also a saturable protein-facilitated process.

For the intestine, a number of proteins have been suggested to transport FA at the apical membrane. Cluster of differentiation 36 (CD36)/fatty acid translocase is a widely expressed scavenger type B receptor, with a hairpin topology that has short cytoplasmic tails but the large extracellular loop contains a stretch of hydrophobic residues that could serve to bind fatty acids. CD36 has three extracellular disulphide bridges and 10 putative N-linked glycosylation sites that confer this 53 kDa protein an apparent mass of 88 kDa (reviewed in (13)). CD36 expression has been documented to be on the cell surface in caveolae, where its fatty acid transport activity occurs (14; 15), and is important for FA transport into myocytes (16), adipocytes (17), and hepatocytes (18). Because CD36 has been demonstrated to bind multiple ligands in other tissues, including oxidized LDL, thrombospondin, and malaria-infected erythrocytes, it is not surprising it exhibits some ligand promiscuity in the intestine and has been implicated in the transport of both long chain FA and cholesterol. The role of CD36-mediated FA uptake is most prominent in the proximal intestine (19), although its expression along the gastrointestinal tract has been reported to be highest in the ileum (20). Also in the proximal intestine, CD36 participates in the uptake of the less efficiently absorbed cholesterol (21). CD36 transport activity appears to be selective to very long chain FA, especially those over 24 carbons in length (22), while Cd36−/− mice remain capable of absorbing most dietary FA. With regards to the absorption of the more common 18 carbon-long oleic acid, CD36 plays a key role in its output from the intestinal
epithelium, where CD36 deficiency resulted in lipid trapping in the intestinal epithelium (21; 23). Also in the gastrointestinal tract, CD36’s affinity for long chain FA is thought to be involved in conferring a gustatory disposition for lipids. CD36 is expressed in taste buds and CD36-deficient mice do not exhibit any preference for long chain FA-enriched solutions (24).

Another scavenger type B receptor, scavenger receptor type B-I (SR-BI) has been implicated in intestinal lipid absorption. SR-BI is known for its role in high density lipoprotein (HDL)-binding and reverse cholesterol transport, but has been found to be concentrated in the microvilli of enterocytes (25) and has a structure very similar to CD36. Intestine-specific overexpression of SR-BI has been shown to increase absorption of both oleic acid and cholesterol (26). However, SR-BI is typically regarded as a cholesterol transporter (27), given its documented high affinity for cholesterol in the brush border membrane (BBM) (28). During the absorptive state, SR-BI shows signs of internalization and trafficking to intracellular lipid droplets in the enterocyte (29). Although SR-BI increases cholesterol across the BBM, it does not form the rate limiting step in cholesterol absorption (30). Nieman-Pick C1 Like 1 (NPC1L1) is also integral to cholesterol transport, as it was identified as the pharmacological target of ezetimibe, a small molecule that inhibits intestinal cholesterol absorption (31). A deficiency in NPC1L1 was clearly associated with a drastic reduction in dietary sterol absorption (32). Like SR-BI, intracellular movements are essential to NPC1L1-mediated cholesterol transport, as the mechanism of NPC1L1 action involves vesicular endocytosis, and ezetimibe affects its cycling between endosomal compartments and the plasma membrane (33). In what is beginning to appear as a common feature of membrane lipid transporters, NPC1L1 has been localized to specific lipid raft domains (34).

Even if appreciable amounts of dietary FA likely pass through the apical membrane by simple diffusion, the efficiency of absorption would suggest that is vectorial in nature, that is, lipid traverses only in the apical to basolateral direction in enterocytes, which implicates a protein-mediated mechanism. There are six members in the fatty acid transport protein (FATP) family, but only FATP1 and FATP4 have been reported to be detected in the small intestine (35; 36). FATP4 is abundantly expressed in the small intestine in the microvilli (36) and endoplasmic reticulum (ER) (37) of epithelial cells. Deleting one allele of Fatp4 almost halves the FA uptake in enterocytes (38), but FATP4 also exhibits acyl-CoA synthetase activity, with greater
specificity for very long chain FA (39). It is argued that the acyl-CoA synthetase activity drives FA uptake (37), presumably because the conjugation of the coenzyme A helps to maintain the FA inside enterocytes. Mice with two mutant alleles of Fatp4 suffer from neonatally lethal skin defects, but rescued Fatp4-/- mice that only express FATP4 under a keratinocyte-specific promoter do not have compromised dietary FA absorption and uptake, although FATP4 may be necessary to target FA for secretion as TG on a high fat diet (40). FATP1 also possesses acyl-CoA synthetase activity, and its expression in cells drives oleic acid import and assimilation into TG (41). As for FATP1, though expressed in the small intestine in moderate amounts (35), no function has been ascribed to it in the organ. Liver fatty acid binding protein (L-FABP) is found in jejunal BBM and can bind FA starting from 16 carbons in length (42). Hepatocytes also express high amounts of L-FABP, where its presence facilitates FA uptake (43). L-FABP is also found in the cytoplasm, where it accelerates the intracellular movement of its bound FA, possibly directing the FA for esterification (44). Each L-FABP molecule can bind two FA molecules (45), and L-FABP efficiently sequesters the bulk of luminal-derived FA in the enterocyte cytoplasm (46). These studies depict L-FABP as an intestinal sink for FA, which may contribute to the directionality of lipid transport. Indeed, while there is no fat malabsorption, Lfabp-/- mice exhibit delayed TG appearance in the plasma following an oral fat challenge, and this is accompanied by increased lipid accumulation in the proximal intestine (47). Intestinal fatty acid binding protein (I-FABP) is expressed specifically in the intestine, but has been deemed non-essential for dietary lipid absorption (48). However, I-FABP does appear to play a role in targeting dietary-derived FA to TG synthesis as opposed to β-oxidation, while the directing of dietary-derived MAG to TG synthesis is done by L-FABP (49). Despite their striking structural similarities, the functions of L-FABP and I-FABP do not appear to have overlapping functional role, as the other protein is not grossly upregulated when one is knocked out (50; 51).

1.2.2 Chylomicron assembly and secretion

Assembling a very large particle (up to 500 nm in diameter) comprised chiefly of hydrophobic molecules in the aqueous environment of the cell requires a complex and coordinated array of activities. To summarize, the chylomicron assembly process begins with FA uptake at the apical membrane, which needs to be reesterified and targeted to the secretory pathway. The lipids are then assembled with the nascent aggregate-prone apoB48 polypeptide,
followed by further expansion of the lipid core. To continue along the secretory pathway, the large cargo necessitates specialized export machinery to exit the ER, followed by further maturation before leaving the enterocyte at the basolateral membrane. Details of these processes are outlined below.

Once FA is made available inside the enterocyte by diffusion and/or one or more of the transporters described above, it is assimilated into TG at the ER membrane. In the intestine, most TG synthesis follows the acyl-CoA:monoacylglycerol acyltransferase (MGAT)/acyl-CoA:diacylglycerol acyltransferase (DGAT) pathway, where fatty acyl-CoA is sequentially added to MAG to form diacylglycerol (DAG), then to DAG for the final TG product. The acyl CoA likely arises from the activity of FATP4 at the ER, as discussed above. MGAT2 predominates in the proximal small intestine (52), while MGAT3 is more abundant in the distal small intestine (53). These two isoforms appear to be capable of compensating for each other’s activity, for while MGAT2-deficient mice do not exhibit gross fat malabsorption, the kinetics of absorption are delayed distally (54). Both DGAT1 and DGAT2 are expressed in the small intestine, but currently data is available only for DGAT1. While DGAT1 accounts for 89% of TG synthesis initiated from MAG in the intestinal mucosa (55), it is dispensable for esterifying dietary FA with DAG to form TG (56). However, DGAT1 does play an important role in dietary nutrient handling, for intestine-specific DGAT1 expression in Dgat1−/− mice was sufficient to confer susceptibility to high fat diet-induced obesity (57). The TG synthesized by DGAT1 may be more preferentially targeted for chylomicron assembly, as Dgat1−/− mice exhibit large neutral lipid droplets that persist in the enterocyte following feeding (56). Cholesterol is also esterified at the ER, and the action of acyl CoA:cholesterol acyltransferase 2 (ACAT2) is essential to intestinal cholesterol absorption (58).

Newly-synthesized lipids are required to cotranslationally lipidate the nascent apoB48 polypeptide, the large, amphipathic, nonexchangeable structural protein of the chylomicron. The apoB48 arises from posttranscriptional editing of apoB mRNA by apoB mRNA editing enzyme catalytic polypeptide 1, a cytidine deaminase that introduces a premature stop codon to yield a truncated apoB (59). ApoB gene transcription is generally considered to be constitutive (reviewed in (60)), but transforming growth factor β (TGF-β) can activate ApoB gene transcription through SMADs in the human epithelial colorectal adenocarcinoma cell line, Caco-
Also, recently there is evidence of a p53 response element in the ApoB promoter (62) and interleukin-6 (IL-6) can increase apoB mRNA levels (63). The translation of apoB mRNA is also subject to regulation in the liver, given the highly structured 5’ and 3’ untranslated regions (64-67), but such studies have not yet been performed in the intestine.

Currently, a three-step model is proposed for chylomicron assembly (68). The first step is the formation of a “primordial lipoprotein” which begins with the recruitment of phospholipids to the N-terminal domain of apoB48 both intrinsically and by microsomal triglyceride transfer protein (MTP) (69). Newly-synthesized apoB48 initially resides in the smooth ER membrane, but moves to the lumen in an MTP-dependent step (70). At this point, the phospholipid-rich apoB48 particle is still TG-poor and high density lipoprotein (HDL)-sized. In the liver, insufficient lipids for continued lipidation would result in the apoB polypeptide being targeted for degradation and prevent VLDL secretion. However, there is no strong evidence for apoB48 degradation in the intestine. There is evidence in models of intestinal lipoprotein oversecretion, such as the fructose-fed hamster or Psammomys obesus, of attenuated degradation of newly-synthesized apoB48 (71; 72). While inhibiting proteasomal degradation with N-carbobenzoxy-L-leucyl-L-leucyl-L-norleucinal (MG132) raised intracellular levels of newly-synthesized apoB48, it did little to drive apoB48-containing particle secretion in chow-fed hamster enterocytes (71). In the Caco-2 cell model, apoB is normally not degraded unless an MTP inhibitor is added and only minimal levels of intracellular apoB are found conjugated to ubiquitin (73). Imaging of this cell model has suggested that immature apoB48-containing lipoproteins pass through the trans-Golgi and stored in an apical compartment, from which they proceed basolaterally upon exposure to luminal lipid micelles (74). When lipid micelles are included ex vivo, apoB48 can be recovered quantitatively from primary murine enterocytes, suggesting that apoB48 degradation is not an active pathway in the postprandial condition (75). Moreover, apoB48-containing particles exhibit far greater heterogeneity in size than apoB100 particles, suggesting that less lipid is permissive to the secretion of the truncated form of apoB (75). After all, under fasting conditions, the enterocyte can take up circulating albumin-bound nonesterified FA, which will be referred to henceforth as free fatty acids (FFA), to form and secrete small HDL-sized particles (76). The second step is the formation of lipid droplets, comprised of neutral lipids synthesized by the enzymes outlined above. The third step is core expansion, with
MTP facilitating the incorporation of lipids into the nascent lipoprotein. Apolipoprotein AIV (apoAIV) is thought to be involved in this step (77). Core expansion occurs in the smooth ER lumen and, probably by virtue of the preformed lipid droplets, is a rapid step that allows the apoB48 particle to acquire the bulk of its TG (78). This step also allows each apoB48 polypeptide to transport ten-fold more TG in the postprandial state compared to fasting (79).

By the time it is ready to exit the ER, the apoB48-containing lipoprotein has almost attained the size of a mature chylomicron. To shuttle this immensely-sized cargo between the ER and Golgi, a specialized COPII vesicle called the prechylomicron transport vesicle (PCTV) has been identified. Notably, the PCTVs also contain the GTPase Sar1, which is necessary for fusion with the Golgi, thus providing the mechanistic explanation for chylomicron retention disease, a disorder of fat absorption characterized by malnutrition and hypocholesterolemia (80). Other COPII proteins Sec23 and more importantly Sec24 are necessary for docking onto the cis-Golgi (81), followed by fusion that is mediated by a v-N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex comprised of vesicle associated membrane protein 7 (VAMP7), syntaxin 5, Bet1 and vti1a (82). Interestingly, PCTV budding involves factors that are involved in fatty acid uptake at the apical membrane, L-FABP and CD36 (83; 84), thus highlighting the mobility in the subcellular localization of these proteins. Notably, I-FABP was not observed to have the same PCTV-budding activity as L-FABP (84). There is evidence that PCTV budding can be regulated. Protein kinase C ζ (PKCζ), which can be activated by a variety of lipid species including ceramide, phosphorylates a 9 kDa ER-associated protein to drive PCTV budding (85). In the Golgi, chylomicron maturation is completed with the inclusion of the exchangeable apolipoproteins, apolipoprotein AI (apoAI), apolipoprotein CII (apoCII), and apolipoprotein CIII (apoCIII), followed by secretion as a particle encased in a phospholipid monolayer and with a density less than 1.006 g/mL (86). Dietary lipid uptake and chylomicron assembly and secretion are summarized in Figure 1.1.
Figure 1-1. Dietary lipid absorption and chylomicron assembly in the enterocyte.

Following intraluminal hydrolysis, FA or MAG is made available at the BBM by the action of CD36, followed possibly by desorption from the membrane bilayer facilitated by L-FABP/I-FABP. SR-BI appears to play a role in cholesterol uptake at the BBM but the rate-limiting step involves the endocytosis of cholesterol with NPC1L1. SREBP-1c action induces expression of ACC and FAS, which produce FA that can also be incorporated into chylomicron particle. FA is activated to fatty acyl-CoA at the ER by FATP4, and then subsequently used to esterify MAG by MGAT2. TG synthesis culminates with the transfer of a fatty acyl-CoA to DAG. Cholesterol transported into the cell by the action of SR-BI and NPC1L1 and destined for secretion is also esterified through the action of ACAT2 at the ER. Meanwhile, the nascent apoB48 polypeptide
acquires lipid cotranslationally with the help of MTP in the ER lumen. The primordial, phospholipid-rich apoB48 particle continues to acquire additional TG and cholesteryl ester from lipid droplets, with MTP facilitating lipid transfer between these unilamellar entities. The apoB48 particle also acquires apoAIV in the ER, which aids in the lipoprotein’s core expansion. By the time the apoB48 particle is ready to exit the ER, it is nearly the size of a mature chylomicron, so a specialized transport vesicle called the PCTV is necessary to accommodate this immensely-sized cargo. PCTV budding from the ER is a process that is L-FABP- and CD36-dependent, while COPII proteins and Sar1 GTPase are necessary for docking and fusion at the cis-Golgi. The apoB48 particle undergoes the final stages of maturation once the exchangeable apolipoproteins are added as it progresses through the Golgi.
1.2.3 Naturally occurring mutation in fatty acid transporters

Mutations resulting in either defective CD36 expression or function are quite common in the human population. Most reported deletions and insertions result in frameshift mutations that reveal a premature stop codon or exon-skipping, often producing a truncated or unstable product, thereby creating a phenotype of CD36 deficiency (87). The C268T substitution occurs with over 50% frequency among mutated Cd36 alleles in the Japanese population (88). Replacement of Pro90 with Ser leads to an 81 kDa protein product indicative of incomplete posttranslational glycosylation, and this mutated CD36 is not efficiently expressed on monocyte cell surface but rather degraded in the cytoplasm (89). The Pro90Ser mutation was associated with higher FFA levels (90), but not the insulin resistance, elevated fasting TG, and greater postprandial apoB48 levels observed in older individuals with rare Cd36 mutations (91; 92). Similar abnormalities were observed in CD36-null mice (93). Another haplotype, 30294G>C, was also linked to FFA elevation in non-diabetic Italians (94), but the effect of this polymorphism in the 3’ UTR has not been fully characterized. Given the ubiquitous expression and ligand-promiscuity of CD36, the lipid and lipoprotein abnormalities in these individuals could be a result of CD36 deficiency in multiple tissues. For example, subjects with two mutated alleles have compromised myocardial long chain FA uptake (95), and this defective uptake in the heart and peripheral tissues may contribute to the greater circulating FFA levels. Consequently, this FFA elevation inhibits lipoprotein lipase action (discussed in ‘Chylomicron catabolism’) , thereby contributing to the persistence of chylomicron remnants (96). Moreover, CD36-mediated LCFA uptake in myocytes helps maintain insulin sensitivity (97), which may provide an explanation for the insulin resistance observed in CD36-deficient individuals. Lymphatic cannulations revealed intestinal overproduction of apoB48-containing lipoproteins in CD36-deficient mice which may explain the lipoprotein abnormalities (98), which is in disagreement with other published studies in these mice. However, intestinal insulin resistance may be contributing to the chylomicron overproduction (discussed later in ‘Hormonal regulation of chylomicron secretion’), as genes involved in de novo lipogenesis were upregulated (98). A nonsense mutation in Cd36 that presented with autosomal dominant diabetes was identified in the French population (99). The mechanism for the diabetes is unresolved, as the truncated product, while capable of trafficking to the cell surface, was incompetent in binding modified LDL (99) but still included the putative
FA binding domain (100). However, the loss of the second transmembrane domain would have prevented CD36 from assuming the hairpin conformation and possibly affected the folding of the FA binding domain.

Metabolic abnormalities have also been noted for naturally occurring polymorphisms in L-FABP. A mutation in the highly conserved Thr94 position was found in the French Canadian population, in which the Ala94 allele was found to occur with 32.3% frequency (101). Even after adjustments for age, body mass index, smoking habit, menopausal status, and ApoE genotype, the Thr94Ala mutation was significantly associated with higher fasting TG and LDL-cholesterol in women (102). Thr94 is in the FA-binding β-barrel of L-FABP, but it comprises a β-strand involved in hydrogen bonds atypical of a β-barrel (45). Chang liver cells transfected with the Thr94Ala variant have been noted to transport less FA (103), and McA-RH7777 rat liver cells overexpressing wild type L-FABP secrete more apoB100 (104), so the etiology of the fasting hyperlipidemia observed in Thr94Ala individuals remains unclear. However, Ala94 carriers who have a greater percentage of caloric intake from fat have lower plasma apoB levels than Thr94 homozygotes (101), so the gene-diet interaction in this particular context may be due to compromised FA transport in the intestine. Carriers of the Ala94 allele were also more likely to have plasma TG above the therapeutic target following treatment with the PPARα agonist fenofibrate (105). While the 5’ flanking region of the Lfabp gene contains a PPAR response element, intestinal L-FABP expression appears to be regulated specifically by PPARδ (106), so the fenofibrate response of Ala94 carriers was likely attributable to aberrant lipid metabolism occurring at the hepatic level.

Though the absence of IFABP in mice does not affect their ability to amass lipids in their tissues, a naturally occurring single nucleotide polymorphism in IFABP is accompanied with observable metabolic phenotypes in humans. A polymorphism in codon 54 was first discovered in Pima Indians in an attempt to identify the genetic factor determining insulin action in the exceptionally type 2 diabetes-prone population (107), and then later detected in many other ethnicities. The IFABP Ala54Thr missense mutation confers two-fold greater affinity for long chain FA (107), possibly due to stabilization of noncovalent interactions in the lipid entry portal which may prohibit FA dissociation from the protein (108). Non obese and non-diabetic male Japanese-American carriers of the Thr54 alleles have higher fasting TG (109). Type 2 diabetic
Caucasian male Thr54 homozygotes have fasting and postprandial hypertriglyceridemia, with the postprandial TG attributed to elevated chylomicron levels (110). In a French-Canadian paediatric population, Thr54 homozygotes had a steeper increase in fasting apoB with rising TG levels, an interaction that was not even associated with MTP genotype (111). Consistent with these observations in humans, Caco-2 cells transfected with this mutant IFABP have enhanced long chain FA uptake and TG secretion (112). The Thr54 allele may thus render an individual to be more sensitive to the FA content of their diet. Young healthy normolipidemic subjects more readily exhibited signs of insulin resistance following 28 days of a saturated fat-enriched diet than Ala54 homozygotes (113). Pima Indian Thr54 carriers mount a greater insulin response to a high fat meal yet have higher postprandial FFA concentrations suggesting insulin resistance (114). However, a study in Korean men showed that neither heterozygotes nor homozygotes of the Thr54 allele had different peak serum $^3$H-activity following ingestion of $^3$H-labelled oleic acid (115). Likewise, the Thr54Ala polymorphism did not affect the postprandial mean TG following a fat tolerance test in males from the European Atherosclerosis Study (116). Therefore, rather than being essential to intestinal lipid absorption, IFABP may be important in adapting to dietary stresses.

Naturally occurring FATP4 polymorphisms are rare in the human population, considering that even missense mutations in the ER localization signal, AMP binding domain, or the noncatalytic C-terminal domain all result in ichthyosis prematurity syndrome, which is characterized by premature birth, neonatal asphyxia, and lifelong skin abnormalities (117). The defective barrier function observed in infants with these FATP4 polymorphisms is reminiscent of the phenotype of Fatp4<sup>-/-</sup> mice (118). A missense mutation in exon 3 of Fatp4 was found to occur in Swedish men with an allele frequency of 0.05. The Gly209Ser polymorphism resulted in lower body mass index, plasma TG, VLDL-TG, insulin, HOMA index, and systolic blood pressure. And oral fat tolerance test revealed that Ser209 carriers and homozygotes also tended to have lower plasma TG, chylomicron apoB48, VLDL<sub>1</sub>-apoB100, VLDL<sub>2</sub>-apoB100 and FFA concentrations in the postprandial state, which suggests FATP4 is involved in intestinal lipid handling (119). A structural model of FATP4 suggested the variable residue 209 is flanked by proline residues and located within an exposed hydrophobic loop, implicating this region in protein-protein interactions, although the interacting partner was not identified (119). While
FATP1 has not been functionally characterized in the small intestine, there is evidence that a common intronic single nucleotide polymorphism may affect postprandial lipid metabolism. 50-year-old Swedish male homozygotes of the A/A genotype in intron 8 have significantly higher plasma TG concentrations in response to an oral fat tolerance test, with the TG appearing to reside in the chylomicron and larger VLDL1 fractions, concomitant with a failure to suppress FFA levels in the postprandial state (120). However, with such a postprandial lipid profile and the high FATP1 expression in adipose tissue (35), it was likely the postprandial dyslipidemia was due to defective uptake of FA released by LpL-mediated hydrolysis of circulating TRL. This FA would have inhibited further LpL action and/or induced hepatic VLDL production.

1.2.4 Insulin action and insulin resistance

Since the discovery of insulin 90 years ago, it was clear the pancreas-derived hormone works to decrease blood glucose while having other anabolic effects (121). The actions of insulin proceed via its binding and the autophosphorylation of the insulin receptor on tyrosine residues, which then recruits insulin receptor substrates (IRS) that also become phosphorylated on tyrosine residues. The phosphotyrosine residues on IRS-1/2 recruit phosphatidylinositol-3-kinase (PI3-K) via the p85 regulatory subunit. The p110 catalytic subunit of PI3-K phosphorylates phosphatidylinositol (4,5)-disphosphate, which consequently recruits Akt/protein kinase B to the plasma membrane for phosphorylation by phosphoinositide-dependent kinase-1 (PDK1). The insulin receptor can also activate the mitogen-activated protein kinase/ERK kinase (MEK)/extracellular related kinase (ERK) pathway by recruiting the adaptor protein Src homology 2 domain-containing protein (Shc) and protein growth factor receptor-bound protein 2 (Grb2), which brings the Ras GTPase activator Son of Sevenless (SOS) to the membrane (122). Through a combination of glucose transporter 4 (GLUT4)-mediated glucose uptake in muscle and fat (123), suppressed gluconeogenesis in the liver (124), and stimulated glycogen storage (124), insulin counteracts postprandial hyperglycemia. Insulin also inhibits the secretion of its counterpart in glucose homeostasis, glucagon, from pancreatic α-cells (125). While the effects on glycemia are among its most immediately appreciable effects, insulin is integral to signalling nutrient abundance in the postprandial state and is thus an important regulator of lipid metabolism. In fat tissue, insulin stimulates the assimilation of carbohydrate-derived substrates into TG stores and inhibits lipolysis (126). In adipocytes, lipid droplet-associated proteins such
as perilipin A (PLINA) and hydrolases such as hormone sensitive lipase (HSL) are among the substrates for protein kinase A (PKA). The phosphorylation of PLINA recruits HSL to lipid droplets, and the phosphorylated HSL associates with FABP4 for activation. Together with the activities of adipose triglyceride lipase (ATGL) and monoacylglycerol lipase (MGL), the activated HSL releases nonesterified FA (FFA) and glycerol from adipose storage depots into the circulation. Insulin signalling suppresses FFA release in the postprandial state by activating phosphodiesterase 3B to degrade cyclic adenosine monophosphate (cAMP) and remove the stimulatory signal for PKA (127). Insulin also promotes FA synthesis through the induction of lipogenic genes by activating sterol regulatory element binding protein-1c (SREBP-1c) in an Akt2-dependent manner (128). The sterol regulatory element is found in the promoters of fatty acid synthase (FAS) and acetyl-CoA carboxylase α (ACCα) (129). Moreover, insulin’s lipogenic effects can be achieved more acutely by the phosphorylation of ACCα (130).

Insulin is also a strong lipogenic signal in the liver. The liver-specific transcript insulin-induced gene 2a (INSIG-2a) is an ER-localized protein present in limiting amounts that sequesters SREBP cleavage-activating protein (SCAP). In an Akt2-dependent manner, insulin suppresses Insig2a expression, thereby releasing SCAP from the ER and allowing it to escort SREBP-1c to the Golgi for proteolytic processing and activation (131). The products of de novo lipogenesis are then used to lipidate VLDL (132). Despite the stimulation of lipogenesis, insulin actually reduces hepatic VLDL secretion (133-136). While the lowering effect is partly attributable to diminished FFA supply due to suppressed lipolysis in adipose tissue, insulin appears to have effects on apoB metabolism itself (137). Insulin may decrease VLDL secretion by excluding forkhead box O1 (FoxO1) from the nucleus to inhibit Mttp transcription (138), enhancing apoB degradation (139), and inhibiting ApoB mRNA translation (64), all in a PI3-K-dependent manner.

The etiology of insulin resistance is under debate, but there is evidence that inflammatory signalling that arise from tumour necrosis factor-α (TNF-α) action through its receptor TNFR may impair insulin signalling through the action of c-Jun N-terminal kinase (JNK) which negatively affects IRS-1 activity. Also, protein tyrosine phosphatase-1B (PTP-1B) attenuates insulin action by dephosphorylating the insulin receptor tyrosine residues (122). In insulin resistance, there is impaired glucose tolerance, hyperinsulinemia and hypertriglyceridemia. The
paradox in insulin resistance is that while glucose uptake and suppression of gluconeogenesis is blunted, the lipogenic effect of insulin proceeds unabated (140). Coupling this selective insulin resistance in lipoprotein-producing organs with defective insulin action in peripheral issues sets the state for dyslipidemia. Both increased FA flux due to dysregulated lipolysis in adipose depots and increased de novo lipogenesis provide more substrate for VLDL lipidation (141; 142). Meanwhile in the liver, other steps regulating TRL secretion become refractory to insulin action such as MTP expression (143; 144) and apoB degradation (145; 146). VLDL overproduction strains an overburdened lipolytic system, in which the increased FFA inhibits lipoprotein lipase (LpL) and prevent TRL clearance (147). In the postprandial state, the problem is amplified with the influx of chylomicrons competing for common lipolytic pathways (further discussed in ‘Chylomicron catabolism’). However, the nature of dysregulated lipoprotein overproduction in the intestine in insulin resistant states is less well understood.

1.2.5 Hormonal regulation of chylomicron secretion

The regulation of intestinal lipoprotein production by endocrine signals is a relatively new notion, as apoB48 secretion was traditionally regarded as a constitutive process. Initially, there were multiple human studies to suggest aberrant postprandial intestinal lipoprotein metabolism in insulin resistance (148-151). Indeed, a kinetic study indicated that hyperinsulinemic insulin resistant patients have increased apoB48 production rates, as opposed to defective TG-rich lipoprotein (TRL) clearance (152). Prior to this observation, evidence from multiple rodent models emerged to indicate that insulin resistance is associated with TRL overproduction from the intestine and have provided clues regarding the mechanism. The fructose-fed hamster, a model of diet-induced insulin resistance and hepatic VLDL overproduction (145), secretes not only more apoB48-containing lipoproteins, but larger particles as well (71; 145). The apoB48 overproduction can be partially normalized with the insulin-sensitizing agent rosiglitazone, which restored intestinal MTP expression to the levels of chow-fed hamsters (153). The enterocyte is itself sensitive to insulin, and fructose-feeding blunts insulin signaling as evidenced by decreased IRS-1 tyrosine phosphorylation, an increase in PTB-1B expression, and attenuated Akt response to insulin while maintaining mitogen-activated protein kinase (MAPK) signalling (154). The consequence is increased SREBP-1c activation, which may explain the increased lipogenesis observed by Haidari et al (71). A summary of the
signaling derangements that could lead to apoB48 oversecretion in insulin-resistant enterocytes is depicted in Figure 1-2. Fructose-feeding is also associated with upregulated DGAT2 expression to accompany the increased intestinal lipid content (155) and the enterocyte endoplasmic reticulum appears to be modified, as PCTV’s isolated from fructose-fed hamster microsomes had increased chaperone content to suggest possible ER stress (156).
Figure 1-2. Insulin signalling pathway depicted with perturbations known to occur in the enterocyte during insulin resistance.

Certain components of the system are upregulated (↑) or downregulated (↓) with respect to either mass or phosphorylation, as determined in the fructose fed hamster model. TNF-α signalling plays a role in inducing intestinal insulin resistance, and appears to be mediated by both TNF-α receptor 1 (TNFR1) and TNF-α receptor 2 (TNFR2). Points at which TNF-α interrupts insulin action are shown, with a putative interaction between inflammatory signalling (via JNK) and insulin signalling (on IRS-1 phosphorylation) represented by a dotted line.
Another rodent model of insulin resistance and type 2 diabetes is the sand rat *Psamommys obesus*, which also oversecretes intestinally-derived apoB48-containing lipoproteins. Aside from amplified apoB48 biogenesis and *de novo* lipogenesis, chylomicron assembly is also modulated with heightened MGAT and DGAT activities, along with increased L-FABP expression (72). ApoB48 oversecretion has also been observed in the leptin receptor-deficient Zucker obese fa/fa rats, a phenomenon that was attributed to upregulated intestinal MTP expression (157). Another insulin-resistant rodent, the JCR:LA-cp rat, has been characterized with apoB48-containing TRL oversecretion (158). While the Zucker obese fa/fa and JCR:LA-cp rats are of interest primarily for their insulin resistance, the defective leptin action also complicates these models, as leptin itself affects intestinal lipid metabolism. For example, leptin can acutely reduce intestinal apoAIV mRNA (159), thereby implicating a role for the hormone in regulating chylomicron size, as apoAIV has been shown to facilitate chylomicron core expansion (77). Moreover, db/db mice with a defective leptin receptor have inefficient intestinal lipid secretion due to decreased MTP expression, in a manner that involves a gut-intrinsic melanocortin pathway (160). The effect of diet-induced insulin resistance on intestinal lipoprotein secretion in mice has not been characterized in great detail. The B6D2F1 mouse strain, which becomes diabetic within three weeks on a high fat diet (HFD), absorb and secrete dietary fat as chylomicrons at a faster rate, which is then more efficiently cleared by increased intestinal production of apoCII (161). Enterocytes isolated from HFD-fed C57BL/6 mice secrete newly-synthesized apoB48 at double the rate of enterocytes from chow-fed mice. However, it cannot be discerned if this difference is attributed to the insulin resistance induced by the diet, or the fat content of the diet itself, as bile salt lipid micelle supplementation can ameliorate the differences in apoB48-containing particle secretion (75).

While the above mentioned studies look at a chronic deficiency in insulin action, there is also evidence that insulin can acutely modulate intestinal lipoprotein secretion. Acute hyperinsulinism delayed the postprandial rise in apoB48 by two hours in healthy men (162). The ability of insulin to lower apoB48 output is partly attributable to its suppression of circulating FFA (163), as an acute elevation in FFA is enough to significantly increase TRL-apoB48 secretion (164). Another hormone that can acutely modulate intestinal apoB48 metabolism through the insulin pathway is the inflammatory cytokine TNF-α. In hamsters, a 4 h infusion
suppressed intestinal insulin signalling while stimulating p38, extracellular related kinase 1/2 (ERK1/2), and c-jun N-terminal kinase JNK signalling, and increasing TRL-apoB48 secretion, likely by raising MTP expression (165). There is evidence that other mitogenic signals can regulate intestinal lipoprotein metabolism. Epidermal growth factor (EGF) has been shown to stimulate apoB48 biogenesis in human fetal intestine (166). TGF-β1 has also been shown to augment apoB secretion in the Caco-2 cell model (167), but in addition to being an intestinal cell line that secretes both apoB100 and apoB48 isoforms, observations in this model have been inconsistent with the aforementioned studies in primary cultures. For example, TNF-α was noted to decrease apoB-containing lipoprotein secretion from Caco-2 cells (168), while the aforementioned study on primary hamster enterocytes suggests that TNF-α is a stimulatory signal for secretion (165). Therefore, our current understanding of how endocrine factors can regulate intestinal lipid metabolism is still in its infancy.

1.2.6 Other fates of dietary lipid in the intestine

Although most of the ingested lipid is packaged into chylomicrons for secretion, some of the lipids are catabolized or even stored. Generally speaking, β-oxidation occurs at a low rate in enterocytes for both fasting and fed states. Glutamine constitutes the primary fuel for enterocytes, where it is catabolized to CO₂ and alanine (169; 170). During food deprivation, approximately 31% of FA taken up at the serosal side is oxidized in enterocytes, but this figure drops to 12% for luminally-derived FA. The fed intestine oxidizes FA at half the rate, with only 12% of circulating FA and 5% of dietary FA being utilized for fuel (49). However, FA oxidation can be induced in fat-enriched diets, perhaps as an early adaptation to increased caloric intake. Polyunsaturated FA-enriched diet is associated with increases in intestinal β-oxidation (171), and the guts of both the obesity-resistant A/J and the obesity-prone C57BL/6J mouse strains have upregulated β-oxidation and carnitine palmitoyl transferase-1 (CPT-1) activity after only two weeks on a HFD (172). While FA play only a minor role in providing energy for the enterocyte, there is evidence that the metabolites of FA oxidation plays an important role in signalling satiety. Inhibiting intestinal long-chain acyl-CoA dehydrogenase with an intrajejunal infusion of mercaptoacetate stimulated food intake in sham-operated rats, but not in rats with subdiaphragmatic vagal deafferentation, suggesting that vagal afferents are important in the sensing of signals elicited by enterocytic FA oxidation (173).
Considering the enterocyte’s short lifespan, it seemed unlikely that this cell type would store lipids for later use. However, it has been observed in humans that 13% of chylomicron TG is derived from FA ingested the previous evening, indicating that dietary lipid continues to be used for intestinal TG synthesis 18 h after ingestion (174). Glucose ingestion five hours after an oral fat load is followed by a peak in chylomicron-TG and apoB48 45 min later, along with less lipid staining in the jejunal mucosa, suggesting the lipoproteins were assembled from stored lipids in the enterocytes (175). Indeed, imaging studies have shown that following lipid ingestion, there is a cytoplasmic pool of lipid coincident with the lipid droplet protein tail-interacting protein of 47 kDa (TIP47) that forms in enterocytes. The diameter of these cytoplasmic lipid droplets increase up to 3 h following an oral gavage of oil, and then diminish in size until the pool is depleted by 12 h (176). However, the acyltransferase responsible for assimilating the TG in this temporary storage pool, or the hydrolase that mobilizes these stored lipids for secretion, have yet to be identified.

1.2.7 Chylomicron catabolism

Chylomicrons are involved in ‘forward’ lipid transport – the delivery of lipids from the intestine to peripheral tissues. After traversing the lymphatic system, chylomicrons enter the circulation where some remodelling occurs to allow these particles to deposit FA in peripheral tissues. Upon reaching the circulation, apoAI and apoAIV along with some phospholipids are rapidly transferred from nascent chylomicrons to circulating HDL particles (177). In exchange, chylomicrons acquire apoCII and apoE from HDL, which are important for its catabolism and removal (178; 179). The apoCII molecules on the chylomicron surface are necessary to activate LpL bound to the vascular endothelial cell surface by heparin sulphate proteoglycans. The enzymatic action of LpL is to hydrolyze 1(3)-ester linkages which liberates FA and 2-monoacylglycerol from the TG transported in the chylomicron core (180). The heart is the main site of hydrolysis and uptake of the dietary lipid transported by chylomicrons. In fact, whole body chylomicron-TG clearance proceeds at only 40% of the wild type rate in heart-specific LpL knockout mice (181). Chylomicrons therefore are critical to providing the predominant energy substrate to the heart, where LpL deficiency results in cardiac dysfunction (182). Insulin raises and depresses LpL activity in adipose tissue and skeletal muscle, respectively, thereby directing chylomicron-TG away from muscle tissues and towards storage depots in the postprandial state.
However, the FA liberated by LpL is not efficiently taken up by local tissues, and about 36% escape into the systemic circulation. This “spillover” is consequently an important contributor to postprandial FFA levels. The efficient lipolysis of chylomicron-TG is also important to vascular health, as evidenced by glycosylphosphatidylinositol-anchored high-density lipoprotein binding-protein 1 (GPIHBP1)-deficient mice. GPIHBP1 is expressed on vascular endothelial cells and binds chylomicrons and LpL to mediate LpL’s hydrolytic activity. GPIHBP1 deficiency resulted in severe chylomicronemia even on a low-fat diet, and GPIHBP1 knockout mice spontaneously developed lipid-rich atherosclerotic lesions on a chow diet. A chylomicron particle remains in circulation until 80% of its TG content has been catabolized peripherally. The remaining chylomicron remnant still retains its apoB48 polypeptide, almost all of its original cholesteryl ester content, and surface apoE and is cleared by the liver. Chylomicron remnants are endocytosed either by the low density lipoprotein receptor (LDLR) or the low density lipoprotein receptor-related protein 1 (LRP1) via recognition of apoE. Notably, these receptors are also involved in the clearance of apoB100-containing hepatic-derived lipoproteins, so chylomicron remnants compete for these receptors in the postprandial state, which ultimately has implications for dyslipidemia. There is also evidence of impaired hepatic chylomicron remnant clearance in LRP5-deficient mice fed a high fat diet.
1.3 Glucagon-Like Peptides

1.3.1 Glucagon-like peptide structure

Glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) arise from posttranslational proteolytic processing of the preproglucagon polypeptide by prohormone convertase 1/3 (PC1/3) in intestinal enteroendocrine L-cells and in the brainstem. GLP-1 and GLP-2 occur sequentially in preproglucagon, preceded by glicentin-related polypeptide and glucagon with short intervening peptide sequences (190). The differential processing that occurs in the gut and pancreas to produce different peptides from the same proglucagon polypeptide is illustrated in Figure 1-3. Although PC1/3 cleaves at pairs of basic residues, GLP-1 is N-terminally truncated, with the biologically active peptides being GLP-1(7-37) and more abundant GLP-1(7-36) amide (191). GLP-2 is similarly-sized at 33 amino acids.

Human GLP-1 peptide sequence: HAEGTFTSDVSSYLEGGAAKEFIAWLVKGRG

Human GLP-2 peptide sequence: HADGSFSDEMNTILDNLAARDFINWLIQTKITD

The sequences of both GLPs are highly conserved across mammalian species, with the sequence of GLP-1 being identical between humans, mice, rats, and other multiple other species. Both GLPs have an alanine at the N-terminal penultimate position, rendering them susceptible to degradation by dipeptidyl peptidase-4 (DPP-4), also known as CD26 (192). Exendin-4 which was originally isolated from the venom of Heloderma suspectum has 53% amino acid sequence identity with GLP-1. Exendin-4 also agonizes the GLP-1 receptor (GLP-1R) but is notable for the fact that it is DPP-4-resistant (193). Therefore, exendin-4 serves as a peptide that exacts similar functions as GLP-1, but in a more sustained manner. While exendin-4 is a GLP-1R agonist, exendin9-39 is the antagonist (194). As for GLP-2, replacing the penultimate alanine with glycine to make Gly2-GLP-2 is enough to confer resistance to DPP-4 (195). The N-terminally truncated GLP-2, GLP-23-33 has been identified as a GLP-2 receptor (GLP-2R) antagonist (196), but the peptide also exhibits weak agonist qualities at higher concentrations (197). Although both have high amino acid identity with glucagon, hence their names, these sister peptides exhibit divergent physiological functions, as will be further discussed below.
Figure 1-3. Differential proglucagon polypeptide processing in pancreatic alpha-cells and intestinal enteroendocrine L-cells

The proglucagon polypeptide is a Type IV precursor protein that is processed at basic doublets by proprotein convertases, while the C-terminal basic residues of the cleaved fragments are removed by carboxypeptidase E (CpE). The pancreatic α-cell-specific prohormone convertase 2 (PC2) cleaves to generate glucagon and the major proglucagon fragment. In ileal enteroendocrine L-cells, prohormone covertase 1 (PC1, also known as PC3) simultaneously cleaves the proglucagon polypeptide to generate GLP-1 and GLP-2, along with oxyntomodulin. GLP-1 can exist in a glycine-extended form or amidated by peptidylglycine alpha-amidating monooxygenase.
1.3.2 **Intestinal GLP secretion**

Because both GLPs are simultaneously processed from the same proglucagon polypeptide, GLP-1 secretion is always mirrored by that of GLP-2. Therefore, for purposes of discussion, it is assumed that documented observations regarding GLP-1 secretion would also apply similarly to GLP-2. As postprandial hormones, the GLPs have low circulating levels during fasting, with the low picomolar range for GLP-1 (198; 199), and values from 39 pM (200) to over 200 pg/mL (201) for GLP-2 in healthy humans. Upon meal ingestion, GLPs are secreted in a biphasic manner, with the first peak occurring quickly at about 15 min after meal ingestion, followed by a larger and more prolonged elevation for the next 2 hours (198; 200; 202). The first phase may arise from neural signals mediated by the vagus (203; 204) or cholecystokinin and GIP originating from the proximal intestine (205). The second phase of secretion likely results from nutrients directly interacting with enteroendocrine L-cells of the distal intestine. The magnitude of the increase is dependent on both caloric content and nutrient composition (206; 207). While the GLPs are found and cleared extensively in the circulation, there is some evidence that the L-cells also secrete the peptides into the lymphatic system, where there is protection from DPP-4-mediated degradation and the opportunity to interact with enteric neurons (208).

It has been noted that the ingestion of fat is a stimulus for more prolonged and sustained GLP secretion than carbohydrate or protein (199; 202). While sugars are very efficiently absorbed in the proximal intestine, FA can persist more distally in the lumen of the gastrointestinal tract, allowing them to be sensed by the enteroendocrine L cells in the ileum, especially 60 min after ingestion during the second, more extended phase of GLP secretion (209). Multiple studies assessed the role of FA saturation on GLP secretion, with monounsaturated fat being more potent stimulus than saturated FA (210-212). A set of orphan G protein-coupled receptors have been implicated in mediating FA-induced GLP-1 secretion. Two \( G_q \)-coupled receptors, G protein-coupled receptor 40 (GPR40) which binds long chain FA, and G protein-coupled receptor 120 (GPR120), which binds very long chain FA, have been shown to increase GLP-1 secretion through the increase in cytosolic calcium. GPR40 is expressed on enteroendocrine L cells and Grp40\(^{−/−} \) mice have impaired GLP-1 secretion 60 min following an oral fat challenge (213). GPR120 signalling induces GLP-1 release from STC-1 and NCI-H716.
intestinal cell lines (214), but a diet enriched in a natural agonist, \( \omega-3 \) FA, failed to increase postprandial GLP-1 in vivo (215). Oleylethanolamide, a lipid-derived satiety factor generated from CD36-mediated oleic acid transport (216), has also been shown to stimulate GLP-1 secretion through a G protein-coupled receptor 119 (GPR119)- and PKA-dependent pathway in enteroendocrine L cells (217). PKC\( \zeta \) also has a role in oleic acid-induced GLP-1 secretion (209; 218). Moreover, a G protein-coupled receptor (GPCR) for bile acids, TGR5, is expressed on the surface of enteroendocrine L cells and its activity mobilizes intracellular calcium for GLP-1 secretion (219). Therefore it is evident that multiple aspects of intestinal lipid metabolism are intricately linked to postprandial GLP-1 and GLP-2 secretion.

Instances of pathology, particularly in insulin resistance and diabetes, have been associated with modified GLP secretion. In obese women, there was an attenuated GLP-1 response to an oral carbohydrate load (220). The postprandial GLP-1 response, especially within the first hour, correlated with insulin sensitivity in non-diabetic men (221), and a blunted GLP-1 response was noted even when insulin resistance was not associated with obesity (222). A defect in GLP-1 secretion is also observed in type 2 diabetes (223), which appears to worsen with disease severity (224). The importance of insulin sensitivity to GLP-1 levels may be attributable to the stimulatory effect of insulin on enteroendocrine L cell GLP-1 secretion (225). Leptin is also a stimulus for GLP-1 secretion and likewise leptin resistance has also been connected to decreased GLP-1 levels in multiple rodent models, including high fat diet-fed mice (226). Observations of attenuated GLP-1 secretion have also been noted in the JCR:LA-cp rats (227) and high fat-fed Wistar rats (228). On the other hand, patients with type 1 diabetes have a preserved GLP-1 response (207), and ostensibly normal levels of GLP-2, although studies in streptozocin (STZ)-treated rodents suggest otherwise. The hyperphagia that accompanies STZ-induced diabetes results in hypersecretion of both GLP-1 and GLP-2 to mediate the intestinal growth (further discussed later) (229; 230).
1.4 Biology of GLP-1

1.4.1 Physiological functions of GLP-1

GLP-1 was discovered in the quest to identify the second incretin hormone to gastric inhibitory peptide (GIP) (reviewed in (231)). As such, GLP-1 stimulates insulin secretion in a glucose-dependent manner in response to enteral glucose (232; 233), but can also regulate glycemic excursion in response to nonenteral glucose (234). In pancreatic islets, GLP-1 upregulates pro-insulin gene transcription (235; 236), sensitizes to glucose (237), suppresses inflammation (238), restores protein synthesis in the recovery from ER stress (239), and of great therapeutic interest, exerts protective and proliferative effects (240-244). GLP-1 also has a glucagonostatic effect on pancreatic \( \alpha \)-cells, which elaborates on its glucose-lowering ability (245-247). GLP-1 also has myriad extrapancreatic effects. To complement its ability to augment insulin secretion, chronic GLP-1R agonism also has insulin-sensitizing effects on other tissues (248; 249). Notably, exogenous GLP-1 can slow gastric emptying (250), to which part of its postprandial glucose-lowering ability is attributable (251). In the central nervous system, GLP-1 has the ability to limit food intake (252), which is of great interest in combating obesity. Thus in studies looking at the chronic effects of GLP-1, it is essential to dissociate the other physiological functions of GLP-1 from the weight loss induced by its anorectic ability. There is also a growing appreciation for the cardiovascular effects of GLP-1, which includes increases in arterial pressure and heart rate when it is administered peripherally (253) and centrally (254).

1.4.2 GLP-1 receptor

The GLP-1 receptor (GLP-1R) is a 7-transmembrane GPCR and classified as a part of the family B secretin-receptor family. As to be expected of its insulinotropic function, the GLP-1R is expressed on the pancreatic \( \beta \)-cell, from which the cDNA was originally cloned (255). Much of the current understanding of GLP-1R signalling comes from work in various \( \beta \)-cell lines. The GLP-1R is a \( \mathrm{G}_{\alpha \rm{s}} \)-coupled protein, and so its agonism is associated with an increase in cAMP levels (256). Thus, it is no surprise that GLP-1 signalling can proceed through PKA (257; 258). However, the cAMP formed from GLP-1R agonism can also bind cAMP-regulated guanine nucleotide exchange factor-II (Epac2), which too can lead to a rise in intracellular calcium and insulin secretion (259; 260). Consistent with its growth factor-like role on the \( \beta \)-cell, GLP-1 also
activates PI3-K (261) and Erk (258). Many of the same pathways also appear to be applicable to GLP-1 action in neurons (262; 263), along with PKA-mediated inhibition of 5’-adenosine monophosphate-activated protein kinase (AMPK) (262).

The GLP-1R is also widely expressed in other tissues, including the lung, kidney, brain, heart and throughout the gastrointestinal tract (264-266). Its expression in the central nervous system has been localized to neurons of the nucleus of the solitary tract (NTS), paraventricular nucleus, central nucleus of the amygdala, anterodorsal thalamic nucleus, and the arcuate nucleus among other nuclei in the hypothalamus (252; 267), as well as other regions in the hypothalamus (268). In the gastrointestinal tract, GLP-1R has been identified in vagal afferent nerves terminating in the hepatic portal vein (269), and in myenteric neurons in the duodenum and colon (270). The GLP-1R is also found in a specific population of enterocytes which number highest in the jejunum, and the number of GLP-1R-positive enterocytes increases after ileal transposition (271). It is not unlikely that GLP-1 exerts appreciable effects in the organ of its origin, given that DPP-4 is highly expressed in the gastrointestinal tract and degrades the majority of GLP-1 before it enters the systemic circulation (reviewed in (272)).

1.4.3 GLP-1 and lipid metabolism

In adipose tissue, the conclusions are mixed on whether GLP-1 has an anabolic or catabolic effect on lipid depots. It appears that at very high levels, GLP-1 has a lipolytic effect on adipocytes associated with cAMP generation, while it is appears to have a direct lipogenic effect at more physiological concentrations and exhibits synergism with insulin (273; 274). Moreover, GLP-1R-deficient mice are relatively resistant to HFD-induced weight gain, thus suggesting a role for GLP-1 signalling in lipid deposition, although the resistance to weight gain may be attributable to increased energy expenditure arising from greater locomotor activity (275). On the other hand, central GLP-1 indirectly decreases adiposity in a mechanism involving the sympathetic nervous system. Through the action of β-adrenergic receptors, intracerebroventricular GLP-1 suppresses mRNA levels of the lipogenic genes FAS, ACCα, and stearoyl-CoA desaturase-1 (SCD-1) (276).

Noguieras et al also noted that central GLP-1R activation also decreased lipogenic gene expression in the liver, although this was attributable entirely to the weight loss associated with
the anorexigenic effect (276). There is mounting evidence that peripheral GLP-1 can exert an effect on hepatic lipid metabolism. GLP-1R mRNA has been detected in primary hepatocytes and the receptor exhibits agonist-induced internalization (277), where it activates both Akt and MAPK pathways (278). Exendin-4 directly reduced TG stores in these cells (277) because of reduced lipogenesis, as evidenced by decreased SREBP-1c, ACC and SCD-1 mRNA (279). Upregulated β-oxidation may also play a part, as exendin-4 increased peroxisome proliferator activator receptor α (PPARα) and acetyl-CoA oxidase mRNA levels, which was also observed in vivo in the livers of ob/ob mice (279), while 100 nM GLP-1 has been demonstrated to increase carnitine palmitoyl transferase-1 (CPT-1) expression in primary hepatocytes. There is evidence that GLP-1R action can lead to attenuated VLDL secretion, although whether this is consequential to its insulin effect requires further investigation (280; 281).
1.5 Biology of GLP-2

1.5.1 Biological functions of GLP-2

As a consequence of its receptor’s limited expression throughout the body, the biological actions of GLP-2 are restricted to the gastrointestinal tract, the central nervous system and pancreatic α-cells. The GLP-2 receptor (GLP-2R) has been identified in neurons in the NTS, a structure in the brainstem that regulates food intake, and like GLP-1, central GLP-2 inhibits food intake (282). Opposite to GLP-1 though, there are reports that GLP-2 has glucagonotropic effects by acting directly on pancreatic α-cells (283; 284). GLP-2 action in the gut has received the most attention, and induction of small intestinal epithelial proliferation was the first function identified for GLP-2 (285), followed by efforts to detail its ability to increase intestinal mucosal mass. Chronic exposure to exogenous GLP-2 has proliferative and protective effects in crypt cells, with the result of maintaining mucosal cellularity when faced with such challenges as parenteral nutrition and radiation (reviewed in (286)). GLP-2 also has anti-apoptotic effects in epithelial cells further up the villus, thereby increasing villus height upon repeated administration (287; 288). Such trophic effects of exogenous GLP-2 are also observed, though to a lesser extent, in the colon (289; 290). Studies done in vitro demonstrate that GLP-2 also promotes intestinal epithelial restitution (291), and in vivo GLP-2 has been shown to improve intestinal epithelial barrier function (292). In addition to preventing protein catabolism (287), GLP-2 stimulates protein synthesis in the intestinal epithelium (293; 294). Physiologically, GLP-2 has been demonstrated to be important to intestinal adaptive growth in response to feeding (196) and after bowel resection (295).

A variety of other gut functions are modulated by GLP-2. In the stomach, GLP-2 inhibits gastric acid secretion (284; 296), ghrelin secretion (297), and modestly slows antral emptying at higher doses (298). The effect of GLP-2 on gastrointestinal motility is also observed in the small intestine, where it slows transit time (299). Pharmacological amounts of GLP-2 have anti-inflammatory effects in rodent models of intestinal injury, including limiting the infiltration of immune cells and reducing pro-inflammatory cytokine levels (289; 300). At physiological levels, GLP-2 also modulates the inflammatory response to reduce intestinal permeability (301; 302).
Moreover, pharmacological levels of GLP-2 acutely stimulates intestinal blood flow (303; 304), specifically in the superior mesentery artery, as demonstrated in pigs, rats, and humans (305-307).

1.5.2 GLP-2 and intestinal nutrient absorption

Of interest is the ability of GLP-2 to promote nutrient absorption, because these effects can typically be observed in an acute setting prior to any appreciable changes in mucosal architecture. Though an increase in nutrient absorption could be attributable to greater digestive enzyme activity in animals chronically-administered with GLP-2, such as sucrose-isomaltase in rats (308; 309), such change in the chronic setting is likely secondary to the hyperplastic effect of chronic GLP-2 treatment (310). The effect of GLP-2 on carbohydrate absorption has perhaps received the most attention. GLP-2 can acutely increase enteral hexose transport, due to upregulated sodium-dependent glucose transporter-1 (SGLT-1) and glucose transporter-2 (GLUT2) insertion into the BBM, the former being a PI3-K-dependent process (311; 312). An effect of GLP-2 is also observed on the uptake of circulating glucose, possibly attributable to increased GLUT2 expression on the basolateral membrane (303; 313; 314). The improvement in dietary glucose uptake can still be observed in cases of chronic GLP-2 administration (315-317), although it has also been observed that repeated exposure to exogenous GLP-2 does not increase enteral hexose intake due to decreases in SGLT-1 and GLUT2 mRNA levels (303; 310). In piglets receiving total parenteral nutrition, 6-day treatment with GLP-2 conditioned the intestine to increase capacity for glucose and galactose transport, with increased SGLT-1 providing greater apical transport activity and decreasing glucose metabolism to lactate (318). To complement its protein anabolic effect, chronic GLP-2 treatment also stimulates amino acid uptake in mice and pigs (310; 316). Intravenous delivery of GLP-2 for 6 days to fetal pigs increased lysine uptake as determined by assays of intact intestinal tissue (319). A 14-day infusion of GLP-2 by a subcutaneously-placed osmotic pump also dose-dependently upregulated glycine absorption per small intestinal surface area (320). GLP-2’s influence on dietary lipid absorption has mostly been investigated in a chronic setting, where adult mice exhibited greater uptake of orally administered triolein (310). However, in suckling rats, GLP-2 increased FA uptake in the ileum, but only in conjunction with dexamethasone (321). One acute study in humans did note that GLP-2 infusion augmented postprandial TG and FFA, although the authors surmised this was the result of decreased peripheral lipoprotein clearance as opposed to
modulated intestinal function (284). Therefore, there remains much to be characterized with regards to GLP-2 action on intestinal lipid transport. Some functions of GLP-1 and GLP-2 are summarized and compared and contrasted in Table 1-1.
<table>
<thead>
<tr>
<th>Function</th>
<th>GLP-1</th>
<th>GLP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insulin secretion</strong></td>
<td>Stimulates (322; 323)</td>
<td>No effect (323; 324)</td>
</tr>
<tr>
<td><strong>Glucagon secretion</strong></td>
<td>Inhibits (194)</td>
<td>Stimulates (283; 284)</td>
</tr>
<tr>
<td></td>
<td>Inhibits in type 2 diabetes (245; 246)</td>
<td>No effect in type 2 diabetes (246)</td>
</tr>
<tr>
<td><strong>Intestinal growth</strong></td>
<td>Increases mucosal area in Goto-Kakizaki rats (325)</td>
<td>Colonic epithelial growth (326)</td>
</tr>
<tr>
<td></td>
<td>No effect (285)</td>
<td>Crypt cell proliferation (327-329)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduction in epithelial apoptosis (287; 293)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Enhances barrier function (292; 330)</td>
</tr>
<tr>
<td><strong>Pancreatic β-cell growth</strong></td>
<td>Anti-apoptotic in humans (241)</td>
<td>No effect (334)</td>
</tr>
<tr>
<td></td>
<td>Differentiation in humans (331; 332)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neogenesis in rodents (243; 333)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proliferation in rodents (240; 242-244)</td>
<td></td>
</tr>
<tr>
<td><strong>Gastric emptying</strong></td>
<td>Inhibits (198)</td>
<td>Inhibits (298; 335)</td>
</tr>
<tr>
<td><strong>Intestinal motility</strong></td>
<td>Inhibits (247; 336; 337)</td>
<td>Inhibits in the presence of GLP-</td>
</tr>
<tr>
<td>Function</td>
<td>GLP-1 Effects</td>
<td>GLP-2 Effects</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Glucose uptake</strong></td>
<td>Potentiates insulin-stimulated glucose uptake in peripheral tissues (334; 339; 340)</td>
<td>Stimulates luminal glucose uptake in the intestine (312)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stimulates circulating glucose uptake in the intestine (303)</td>
</tr>
<tr>
<td><strong>Neurotropism</strong></td>
<td>Protective (341; 342)</td>
<td>Protective (345)</td>
</tr>
<tr>
<td></td>
<td>Neurogenic (343; 344)</td>
<td>Proliferative (346)</td>
</tr>
<tr>
<td><strong>Food intake</strong></td>
<td>Centrally and peripherally inhibits (252; 347)</td>
<td>Centrally inhibits (282)</td>
</tr>
<tr>
<td><strong>Arterial blood flow</strong></td>
<td>Stimulates (253; 348)</td>
<td>Stimulates mesenteric blood flow (303; 349)</td>
</tr>
<tr>
<td><strong>Heart rate</strong></td>
<td>Increases (253; 348)</td>
<td>No effect (253; 305)</td>
</tr>
<tr>
<td><strong>Bone resorption</strong></td>
<td>Inhibits (350)</td>
<td>Inhibits (351; 352)</td>
</tr>
</tbody>
</table>

**Table 1-1. Biological functions of GLP-1 and GLP-2**

Differences and similarities in the biological actions of GLP-1 and GLP-2 as inferred from studies with agonists, antagonists, and knockout mice. References included in the table are often just a sampling of the studies that characterized these actions.
1.5.3 GLP-2 receptor

Efforts to detail the mechanisms of GLP-2-improved intestinal function have been hindered largely by the highly specific expression of the GLP-2R in the intestine. Although radiolabelled GLP-2 has been found to bind the villus epithelium in high density (353), subsequent studies indicated that GLP-2R is conspicuously absent on the absorptive enterocyte. Rather, to date the GLP-2R has been identified on intestinal subepithelial myofibroblasts (ISEMF) (354), specific enteroendocrine cells (304; 355), enteric neurons (356; 357), and the vagal afferent nerve (358). ISEMFs are positive for α-smooth muscle actin and vimentin, but do no express desmin like smooth muscle (359). ISEMFs are found throughout the lamina propria, but GLP-2R-positive ISEMFs appeared to be limited to the population sitting under the basement membrane of the intestinal epithelium at the villus tip (354). Moreover, GLP-2R expression is limited to particular populations of these cell types, as it was colocalized with vasoactive intestinal peptide (VIP)-positive and endothelial nitric oxide synthase (eNOS)-positive enteric neurons, and specific enteroendocrine L-cells that have been detected to be positive for serotonin, chromagranin A, in addition to enteroendocrine L-cells and K-cells (304; 355). These cells are of relatively low abundance in the gastrointestinal tract and for this reason many earlier studies on GLP-2R signalling has relied on transfected cell lines. The GLP-2R is a $G_{as}$-coupled receptor, and so its agonism is associated with cAMP accumulation and PKA activation (360; 361). The cAMP accumulation is important to reducing apoptotic signalling, such as caspase-3 cleavage and mitochondrial cytochrome C release (362), while PKA activation is necessary to inhibit the pro-apoptotic activities of Bad and glycogen synthase kinase 3 (GSK-3) (363). GLP-2 also activates the PI3-K pathway in the intestinal epithelium, which may explain its proliferative actions, although this likely occurs indirectly of GLP-2R signalling (293; 364). The PI3-K/Akt pathway has also been implicated in the upregulation of insulin-like growth factor-1 (IGF-1) mRNA in intestinal subepithelial myofibroblasts (359), and protein synthesis in transfected HEK-293 cells (365). The proliferative action of GLP-2 can also occur through ERK1/2 signalling, stimulated putatively by the GLP-2R coupling to $G_{i/G_o}$ proteins (366). Like the GLP-1R (367), the GLP-2R exhibits desensitization induced by its agonist and internalizes in a manner requiring lipid rafts (368).
Despite the knowledge of GLP-2R signalling, given that the absorptive enterocyte does not express the receptor, there is still much to understand about GLP-2’s mechanism of action on the intestinal epithelium. GLP-2 must bind its receptor on a certain intermediary cell, stimulating it to secrete a mediating signal that would act directly on the enterocyte to bring about chylomicron overproduction. IGF-1/2 (369), keratinocyte growth factor (KGF) (370), and EGF (328) have been suggested as mediators of the proliferative and protective effects of GLP-2 on the intestinal epithelium. Vascular endothelial growth factor (VEGF) has been implicated in mediating intestinal epithelial restitution (371). VIP mediates the relaxation of fundic smooth muscle induced by GLP-2 (372). To exact its anti-inflammatory effects, GLP-2 has also been shown to activate VIP-positive enteric neurons in the submucosal plexus (300). However, the peptide mediator for GLP-2’s stimulation of enteral nutrient uptake has not yet been identified.
1.6 Hypothesis

The intestine is evidently sensitive to hormonal signals, and its handling of dietary lipid is a process that can be actively regulated. GLP-1 and GLP-2 are two postprandial hormones that have been shown to have a number of biological effects in the gut. The fact that lipid ingestion serves as a stimulus for the secretion of these two peptides hints at their potential role in postprandial lipid metabolism. Moreover, GLP-1 can modulate lipid metabolism in other tissues while GLP-2 has been shown to promote the absorption of various nutrients in the gut. Therefore, my general hypothesis is that the GLPs are critical acute regulators of intestinal lipid absorption and apoB48-containing lipoprotein secretion. This hypothesis was examined in this thesis using multiple rodent models and primary intestinal cell cultures. There was a particular reliance on the Golden Syrian hamster *Mesocricetus auratus*, which similar to humans has minimal apoB mRNA editing in the liver and therefore secretes apoB48 exclusively from the intestine (373). The use of the hamster model therefore allows for the ready delineation between hepatic and intestinal contributions to circulating apoB levels in vivo.
Chapter 2: THE GLUCAGON-LIKE PEPTIDE-1 RECEPTOR REGULATES POSTPRANDIAL LIPOPROTEIN SYNTHESIS AND SECRETION

Text and Figures in this chapter is reproduced from:


Author contributions:

Sitagliptin-treated hamster data was generated by J. Hsieh with the assistance of C.L. Baker. In vivo mouse experiments and mouse hormones measurements were performed by C. Longuet while biochemical analyses were performed by J. Hsieh. Exendin-4-treated hamster and enterocyte data was generated by B. Qin, and J. Hsieh performed FPLC analysis. Data analysis and writing were completed by J. Hsieh and C. Longuet.
2.1 Summary

Background: GLP-1 receptor agonists and DPP-4 inhibitors attenuate postprandial lipemia through mechanisms that remain unclear. As dyslipidemia is a contributing risk factor for cardiovascular disease in type 2 diabetes, we examined the mechanisms linking pharmacological and physiological regulation of GLP-1 action to control of postprandial lipid metabolism.

Methods: Postprandial lipid synthesis and secretion was assessed in normal and fructose-fed hamsters and in wildtype mice, treated with or without sitagliptin. ApoB48 synthesis and secretion was also examined in primary enterocyte cultures. The importance of exogenous vs. endogenous GLP-1 receptor signaling for regulation of intestinal lipoprotein synthesis and secretion was assessed in mice and hamsters treated with the GLP-1R agonist exendin-4, the GLP-1R antagonist exendin9-39 and in Glp1r<sup>+/+</sup> vs. Glp1r<sup>-/-</sup> mice.

Results: Sitagliptin decreased fasting plasma TG, predominantly in the VLDL fraction, as well as postprandial TRL-TG, TRL-cholesterol and TRL-apoB48 in both hamsters and mice. GLP-1R activation with exendin-4 alone also decreased plasma and TRL apoB48 in hamsters and mice and reduced secretion of apoB48 in hamster enterocyte cultures. Conversely, blockade of endogenous GLP-1R signaling using the antagonist exendin9-39 or genetic elimination of GLP-1R signaling in Glp1r<sup>-/-</sup> mice enhanced TRL-apoB48 secretion in vivo.

Conclusions: Potentiation of endogenous incretin action via DPP-4 inhibition, or pharmacological augmentation of GLP-1 receptor signaling reduces intestinal secretion of TG, cholesterol and apoB48. Moreover, endogenous GLP-1R signaling is essential for the control of intestinal lipoprotein biosynthesis and secretion.
2.2 Introduction

The growing incidence of type 2 diabetes (T2DM) is a major problem (374) and may be associated with a variety of lipid abnormalities that pose as cardiovascular disease risk factors, including hypertriglyceridemia, increased levels of LDL, and low levels of HDL (375). At the top of this cascade are intestinally-derived, TG-rich apoB48-containing chylomicrons, which are secreted following fat ingestion and peripherally catabolized to generate remnant particles (376). Insulin resistance has been found to be positively correlated with remnant-like particle cholesterol (377) and remnant-like particle TG is greatly increased in some diabetic populations (378). Thus, it is of interest to identify therapeutic strategies that can limit postprandial TRL secretion.

The ingestion of nutrients, including fat, provokes the secretion of gut-derived hormones, including two incretins: GIP from duodenal K cells (379) and GLP-1 from ileal enteroendocrine L cells (218). These peptides stimulate the secretion of insulin in a glucose-dependent manner, and preserve pancreatic β-cell function and mass in preclinical studies (reviewed in (380)). A number of extra-pancreatic effects have also been demonstrated following exogenous administration of these two hormones. GLP-1 slows gastric emptying and induces anorectic effects (381), while GIP has anabolic effects on adipose tissue (382) and regulates adipokine secretion (383). However, both GLP-1 and GIP are rapidly inactivated by DPP-4-mediated cleavage, thus limiting the duration of action. Insulin resistance is associated with impaired stimulated GLP release. In high fat diet-fed mice, ileal and colonic content of GLP-1 have been reported to be higher (226; 384), although the L-cells exhibit a blunted GLP secretory response to glucose stimulation (226). Insulin-resistant MKR mice have higher basal GLP-1 levels, but likewise also exhibit impaired glucose-stimulated GLP secretion (225). Inducing insulin resistance in murine GLUTag cells in vitro also compromised GLP-1 secretion in response to insulin (225). While some clinical studies agree with the findings in these experimental models (223; 385; 386), some studies have reported no diminution in GLP-1 secretion in type 2 diabetic patients (387). Therefore, there is considerable interest in enhancing incretin action for the treatment of type 2 diabetes.
DPP-4 inhibitors such as sitagliptin or vildagliptin protect endogenous GLP-1 and GIP from degradation-terminal enzymatic inactivation, thereby prolonging their bioactivity. DPP-4 inhibition effectively lowers fasting and postprandial glycemia in patients with T2DM (388; 389). Given the increasing use of sitagliptin and vildagliptin for the treatment of T2DM, there is considerable interest in understanding the effects of incretins and DPP-4 inhibitors on plasma lipid profiles (390). Acute administration of GIP reduces circulating chylomicrons, likely through promoting TG catabolism by adipose tissue (391; 392) and GLP-1 also attenuates postprandial triglyceride secretion (393; 394), although the exact mechanisms underlying these observations are not clear.

In the present study we studied the role of incretin action in controlling intestinal lipid and lipoprotein metabolism using wildtype mice and a hamster model of insulin resistance and postprandial dyslipidemia (146). The fructose-fed hamster exhibits intestinal overproduction of TRL (71), along with aberrant insulin signalling in the absorptive enterocyte (154). Mice and hamsters were treated with sitagliptin or exendin-4 to potentiate incretin action or with or without exendin 9-39 to assess the importance of exogenous GLP-1R activation vs. endogenous basal GLP-1R signaling for regulation of intestinal lipoprotein metabolism.
2.3 Materials and Methods

2.3.1 Animals

Male Syrian golden hamsters (*Mesocricetus auratus*) weighing 130-150 g were purchased from Charles River (Montreal, PQ) and housed individually with room lighting set for a 12:12-h light-dark cycle with ad libitum access to food and water. Animals were acclimatized for 1 week prior to being given either a standard chow diet or a fructose-enriched diet (pelleted hamster diet containing 60% fructose and 20% casein, Dyets Inc., Bethlehem, PA) for 10 day to induce insulin resistance (145). The hamsters were then randomized to receive either sitagliptin phosphate monohydrate (Merck, Whitehouse Station, NJ) (5 mg/kg) or water by oral gavage QD in the afternoon under light inhalant anaesthesia. Chow-fed hamsters were given sitagliptin for two weeks while fructose-fed hamsters were dosed for three weeks. Blood was collected by retro-orbital bleeding at the baseline, endpoint, and midpoint of the dosing regimen to assess ongoing effect of the treatment, in both the morning for ambient (fed) measurements and after a 6 hr fast. The hamsters were then sacrificed for the *ex vivo* protocol or underwent the *in vivo* protocol. All animal protocols were approved by the animal ethics committee of the Hospital for Sick Children, University of Toronto. Wild type C57BL/6J mice (Jackson laboratory), *Glp1r*−/− and age and sex-matched *Glp1r*+/+ littermate controls fed a normal chow were used at the age of 10-12 weeks of age and maintained under a 12 h light/12 hr dark cycle. Treatments performed in mice utilized a single dose of sitagliptin (10 mg/kg BW) or exendin-4 (24 nmol/kg) administered as described in figure legends.

2.3.2 Assessment of intestinal lipoprotein production by *in vivo* Triton-WR1339 infusion

Hamsters were anesthetized with isoflurane administered through a vaporizer. A cannula was inserted into the right jugular vein, exteriorized at the back of the neck, filled with heparinized saline (40 IU/mL) and sealed. The hamsters were allowed to recover and fasted overnight. Conscious hamsters were then given a 200 µL olive oil load via oral gavage. 20 min later, vehicle (saline), exendin-4 (5 nmol/kg) (Bachem, Torrance, CA), exendin(9-39) (50 nmol/kg) (Bachem), or both were administered via an intraperitoneal injection plus a separate
bolus of Triton WR-1339 diluted 20% (w/v) (0.5 g/kg) injected into the jugular vein to inhibit lipoprotein catabolism and uptake (395; 396). Triton WR-1339 coats TRL particles to prevent hydrolysis of the TG content by LpL, allowing for assessment of secretion without taking into account any changes in peripheral catabolism. 300 µL of blood was collected from the jugular cannula into lithium heparin-coated tubes (BD Biosciences, Franklin Lakes, NJ) at baseline and at 30, 60, and 120 min. The oral gavage, peptide administration, Triton infusion, and blood collection were all performed on conscious animals in the absence of anesthetics. The jejunum was excised under isoflurane anesthesia at the endpoint.

Mice fasted for 5 or 16 h were given 200 µL olive oil orally. 20 min after gavage, a blood sample was collected via the tail vein (time 0). Acute sitagliptin administration was given orally to conscious mice 20 min prior to olive oil gavage. 20 min after fat load, Triton WR-1339 (0.5 g/kg BW of a 15% solution prepared in PBS) was injected via the tail vein with or without exendin-4 (24 nmol/kg). Blood samples were collected via tail bleed without anesthetics for plasma triglyceride and cholesterol assay and apoB48 immunoblotting. 90 min after Triton WR-1339 injection, mice were euthanized and cardiac puncture was performed for plasma triglyceride and cholesterol assay, apoB48 western blot analysis, and TRL purification as described below.

2.3.3 Isolation of triglyceride-rich lipoproteins (TRL)

To isolate the TRL fraction of the plasma, blood samples were first centrifuged at 4°C for 15 min at 5000 rpm to separate the plasma layer. 150 µL of plasma was overlayed with 4 mL of potassium bromide solution (density = 1.006g/mL) in a 5 mL ultracentrifuge tube and centrifuged at 35,000 rpm for 70 min at 15°C using a SW 55 Ti rotor (Beckman Coulter, Mississauga, ON). The TRL fraction (Sf >400) was collected as the top 300 µL of the tube.

2.3.4 Chemiluminescent immunoblotting

ApoB48 immunoblotting was performed on TRL fractions and diluted plasma (1:200) by SDS-PAGE analysis as previously described (145). Following SDS-PAGE, the proteins were transferred electrophoretically overnight at 4 °C onto PVDF membranes using a Bio-Rad wet transfer system. The membranes were blocked with 5% fat-free milk solution and then incubated
with rabbit anti-hamster apoB antibody (1:1000) for 1-24 h. After several washes with TBST, the membranes were incubated with peroxidase-conjugated secondary antibody (1:10,000 dilution) for 1-2 hr. Membranes were then incubated in ECL detection reagents and exposed to Hyperfilm. Quantitative analysis was performed using an imaging densitometer.

2.3.5  *Ex vivo* metabolic labeling of intact primary enterocytes

Primary enterocytes were isolated from hamster intestinal tissue as described previously, which allows for over 90% viability for 4 h (71). This method developed by Perreault and Beaulieu gives fully differentiated intestinal epithelial cells free of mesenchymal contamination (397). Briefly, the jejunum was harvested from chow-fed hamsters, flushed with PBS to remove luminal contents, and then cut longitudinally and into 5 mm strips. The jejunal pieces were treated with Cell Recovery Solution (BD Biosciences) at 4°C for 45 minutes with agitation, washed with ice-cold PBS, and then viable villi were collected. The villi suspension was washed twice in ice-cold phosphate-buffered saline (180 × g, 5 min). After the final spin, villi were resuspended in Dulbecco's modified Eagle's medium (DMEM, Wisent) supplemented with 1% fetal bovine serum and placed in an incubator (37 °C, 5% CO₂, 95% air, 100% humidity). The experiment was performed with 8 mM glucose and 100 pM exendin-4 added to the media.

Primary hamster enterocytes were preincubated in methionine-free MEM at 37 °C for 45 min and pulsed with 30-50 µCi/ml of [³⁵S]methionine for 30 min. The cells were then chased with unlabelled 40 mM methionine-enriched DMEM.

2.3.6  Immunoprecipitation, SDS-PAGE, and fluorography

ApoB48 immunoprecipitation was performed as described previously (71). Immunoprecipitates were washed and prepared for SDS-PAGE. Gels were then fixed, incubated in Amplify (Amersham), dried and exposed to film at -80 °C for 1-4 days. The apoB48 bands were excised and quantitative analysis was performed using a liquid scintillation counter. No major differences were noticed in the trichloroacetic acid (TCA) protein precipitation counts between the control and experimental groups. Media counts were normalized to initial cellular TCA counts. Radioactivity incorporation into apoB48 was also visualized with a Phosphor Imager.
2.3.7 **Plasma measurements**

Plasma TG and cholesterol was determined by an enzymatic-based colorimetric assay (Randox, Crumlin, UK).

2.3.8 **Fast protein liquid chromatography of plasma lipoproteins**

200 μL of hamster plasma was first filtered through a 0.45 μM micro-spin polysulphone filter (Alltech, Mandel Scientific, Lachine, PQ) to remove macroparticles. The plasma was then subjected to gel filtration through a HR 10/300 GL Superose 6 column (Pharmacia, Uppsala, Sweden) with a solution of 10 mM Tris, 150 mM NaCl, 2 mM CaCl₂, 100 μM DTPA, 0.02% NaN₃, pH 7.4 was pumped through at a flow rate of 0.5 mL/min. Fractions were collected every min.

2.3.9 **Statistical analysis**

All results are presented as mean ± SEM. Statistical comparisons were performed using Student's t-test when comparing two groups and two-way ANOVA with Bonferroni’s post-test as indicated in the text and figure legends.
2.4 Results

2.4.1 A DPP-4 inhibitor attenuates dyslipidemia in fructose-fed hamsters

As increased incretin action can attenuate postprandial plasma lipid accumulation (393; 394; 398), we first examined the consequences of DPP-4 inhibition on accumulation of TG and cholesterol in hamsters fed a high fructose diet (60%), a regimen previously shown to result in dyslipidemia and mild insulin resistance (399). Baseline values after a 5 h fast, obtained 10 days after the beginning of high fructose diet, and prior to start of sitagliptin treatment, show a significant increase in plasma of TG and cholesterol (Figure 2-1A,B; p = 0.0002 and 0.006 respectively), in the absence of changes in blood glucose or body weight (summarized in Table 2-1). After three weeks of sitagliptin treatment, plasma TG levels did not continue to rise (Figure 2-1A).

Fast protein liquid chromatography (FPLC) was performed to separate the various densities of lipid particles. In fructose-fed hamsters, levels of VLDL and LDL TG and cholesterol were significantly higher than in control animals (data not shown). Sitagliptin significantly attenuated the accumulation of VLDL-TG by 3-fold (Figure 2-1C). Although LDL and HDL TG levels in fructose-fed hamsters were slightly decreased by sitagliptin treatment, this effect was not statistically significant (Figure 2-1C). LDL and HDL TG levels were slightly but not significantly decreased by sitagliptin treatment (Figure 2-1C). Whereas total plasma cholesterol was not altered by sitagliptin, LDL-cholesterol trended towards reduction and a significant decrease in VLDL- and HDL-cholesterol (Fig 2-1D) was observed in sitagliptin-treated vs. control animals (p < 0.05).
Figure 2-1. Changes in plasma lipids following administration of sitagliptin

Blood samples were drawn from hamsters fed either regular chow or a high fructose diet supplemented with vehicle or sitagliptin for 2 to 3 weeks. Plasma TG (A) and cholesterol (B) levels were determined both prior to and after each dosing period. Lipoproteins were separated from plasma by FPLC fractionation into VLDL/chylomicron remnants, LDL and HDL and levels of TG (C) and cholesterol (D) were determined. n = 9-16 for plasma lipid parameters analyzed by paired t-test; n= 4 for FPLC parameters analyzed by Student’s t-test, *p<0.05 fructose-fed sitagliptin vs. fructose-fed vehicle, ***p<0.001 fructose-fed vehicle vs. chow-fed vehicle or fructose-fed sitagliptin vs. chow-fed sitagliptin.
<table>
<thead>
<tr>
<th></th>
<th>Chow fed vehicle</th>
<th>Chow fed sitagliptin</th>
<th>Fructose fed vehicle</th>
<th>Fructose fed sitagliptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline body weight (g)</td>
<td>130.0 ± 3.1</td>
<td>129.3 ± 3.5</td>
<td>123.7 ± 1.9</td>
<td>124.6 ± 2.1</td>
</tr>
<tr>
<td>Endpoint body weight (g)</td>
<td>141.4 ± 3.2</td>
<td>139.4 ± 3.7</td>
<td>129.0 ± 1.8</td>
<td>129.1 ± 1.0</td>
</tr>
<tr>
<td>Baseline fasting glucose (mM)</td>
<td>4.7 ± 0.3</td>
<td>4.5 ± 0.1</td>
<td>5.3 ± 0.3</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Endpoint fasting glucose (mM)</td>
<td>4.7 ± 0.5</td>
<td>4.1 ± 0.2</td>
<td>5.5 ± 0.4</td>
<td>5.1 ± 0.3</td>
</tr>
</tbody>
</table>

Table 2-1. **Body mass and blood glucose following sitagliptin treatment.**

Body mass and blood glucose in chow-fed hamsters and fructose-fed hamsters chronically treated with sitagliptin as described in methods. Values shown are mean ± SE, n = 5 – 10 per group. None of the values were statistically significant as determined by two-way ANOVA.
2.4.2 A DPP-4 inhibitor decreases intestinal production of TRL-TG and TRL-cholesterol

To understand how sitagliptin modulates plasma lipoprotein levels, we assessed intestinal lipoprotein production and secretion after treatment of chow fed hamsters with sitagliptin for 2 weeks. In chow-fed hamsters, there was a trend for sitagliptin to reduce TRL apoB48 (Figure 2-2C, p = 0.16) in TRL fraction after an acute fat load, while the accumulation of TRL-TG and TRL-cholesterol was unaffected (Fig 2-2A and Fig 2-2B). In contrast, sitagliptin significantly reduced the amount of TG in TRL fraction 90 min after the fat load (Figure 2-2E) in fructose-fed hamsters and there was a trend for lowered TRL cholesterol (Figure 2-2D, p = 0.19) compared to vehicle-treated high fructose fed animals. Slope calculations for TRL-cholesterol and TG secretion in fructose-fed hamsters (for cholesterol, 0.280 ± 0.060 mg/dL min\(^{-1}\) vs. 0.428 ± 0.104 mg/dL min\(^{-1}\), sitagliptin vs. control, p = 0.23 for the slope difference, and p = 0.037 for elevation difference; for TG, 1.55 ± 0.30 mg/dL min\(^{-1}\) vs. 2.23 ± 0.33 mg/dL min\(^{-1}\), sitagliptin vs. control, p = 0.13 for the slope difference, while p = 0.012 for the elevation difference) suggested that sitagliptin retards intestinal postprandial TG secretion. Because of normalization to very high baseline TRL-apoB48 levels, the quantified postprandial rise in apoB48 in fructose-fed hamsters appeared to be less pronounced compared to chow-fed hamsters (Figure 2-2F). While decreased postprandial production of lipoproteins may play a role in reduced fasting lipoprotein levels observed in Figure 2-1, the effect of hepatic production at this point cannot be excluded.

To determine if the effects of sitagliptin on plasma levels of cholesterol and TG were restricted to hamsters, the effect of an acute dose of sitagliptin on chylomicron production was assessed in chow-fed mice. Sitagliptin significantly decreased cholesterol in plasma (Figure 2-3A) and in the TRL enriched fraction (Table 2-2) after an acute fat load; furthermore, plasma TG (Figure 2-3B) and TRL-TG (Table 2-2) were significantly reduced 90 min after Triton injection in sitagliptin-treated mice. The accumulation of ApoB48 in plasma was also decreased by sitagliptin administration (Figure 2-3C).
Figure 2-2. Changes in TRL lipid mass following chronic sitagliptin administration

Hamsters were fed regular chow (A-C) or a high fructose diet (D-F) and administered either vehicle or sitagliptin for 2 to 3 weeks following which hamsters were fat-loaded, administered Triton WR-1339 and blood was drawn at 0, 30, 60, 90 and 120 minutes. Plasma was spun to isolate TRL fraction and levels of cholesterol, TG and ApoB 48 were determined. Data shown illustrates TRL levels of (A) cholesterol, (B) TG and (C) ApoB48 in chow fed hamsters with representative apoB48 blots shown above. (D) Cholesterol (E) TG and (F) ApoB48 content of the TRL fractions from fructose fed hamsters with representative apoB48 blots shown above. (n = 4 for each group * p < 0.05 as analyzed by two-way ANOVA with Bonferroni’s post-test).
<table>
<thead>
<tr>
<th></th>
<th>TRL TG (mmol/L)</th>
<th>TRL-cholesterol (mmol/L)</th>
<th>TRL-apoB48 mass (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>4.52 ± 1.04</td>
<td>1.01 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>Sitagliptin</td>
<td>2.10 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.73 ± 0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100.00 ± 6.41</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1.61 ± 0.30</td>
<td>0.55 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>Insulin</td>
<td>1.98 ± 0.67</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>D-Ala&lt;sup&gt;2&lt;/sup&gt;-GIP</td>
<td>3.57 ± 1.13</td>
<td>0.95 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>Exendin-4</td>
<td>0.53 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.19 ± 0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>173.13 ± 26.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>59.78 ± 10.25</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>3.06 ± 0.64</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Exendin-4</td>
<td>1.13 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100.00 ± 10.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>71.61 ± 17.41</td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wild type</td>
<td>1.78 ± 0.16</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Glp1r&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>2.84 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>121.32 ± 2.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Table 2-2. TRL fraction measurements in chow-fed mouse studies**

TRL fraction lipid masses determined by colorimetric assays and TRL-apoB48 mass determined by immunoblotting in chow-fed mouse studies, as described in methods and corresponding figure legends. **A:** Mice were given sitagliptin 20 min prior fat load. Methods and plasma levels of triglycerides, cholesterol and ApoB48 are reported in Figure 3. **B:** Mice were given insulin, D-[Ala<sup>2</sup>]GIP or exendin-4 20 min after fat load. Plasma levels of triglycerides, cholesterol and ApoB48 are reported in Figure 2A-E. **C:** Mice were given exendin-4 1h after fat load. Plasma levels of TG and ApoB48 are reported in Figure 2-4F and 2-4G. **D:** Glp1r<sup>-/-</sup> mice and littermate controls were given an oral fat load to monitor intestinal lipid absorption. TG and ApoB48 plasma levels are reported in figure 5C and D. Values shown are mean ± SEM, <sup>a</sup> p < 0.05 vs. control of respective group as determined by Student’s t-test, n.d. not determined.
Figure 2-3. Changes in postprandial lipid secretion following a single acute administration of sitagliptin in chow-fed mice

Chow-fed wild type mice fasted for 5 h were given 10 mg/kg of sitagliptin prepared in water by oral gavage. 20 min after sitagliptin treatment, mice were administered 200 μL of olive oil and mice were injected intravenously 20 minutes later with Triton WR1339 (0.5 g/kg). Blood samples were collected prior to IV injection (time 0), as well as 30, 60 and 90 min after injection for (C) cholesterol, (D) TG and (B) ApoB48 measurements as described in methods, n = 4 per group. * p < 0.05, *** p < 0.001 as determined by two-way ANOVA with Bonferroni’s post-test analysis.
2.4.3 Pharmacological activation of the GLP1R mimics the effects of sitagliptin on intestinal lipid absorption

As both GLP-1 and GIP have been involved in the regulation of plasma lipid levels and are DPP-4 substrates, we measured intestinal lipid absorption in mice after acute administration of the DPP-4 resistant analogues of GIP (D-Ala²-GIP) and GLP-1 (exendin-4). As shown in figure 4, D-Ala² GIP significantly increased levels of plasma triglyceride and ApoB48 after an acute fat load (Figure 2-4A and 2-4B), and therefore could not have been responsible for the lowering actions of sitagliptin on intestinal lipid absorption. In contrast, the GLP-1R agonist exendin-4 reduced plasma and TRL-TG and ApoB48 accumulation (Figure 2-4D and 2-4E, Table 2-2), and no effect was noted on plasma and TRL cholesterol (Figure 2-4C, Table 2-2). Hence, activation of GLP1R, but not GIPR signaling, mimics the actions of sitagliptin on intestinal lipid absorption.

Both insulin and glucagon have been involved in the regulation of lipemia and activation of GLP1R signalling has been shown to stimulate insulin secretion and inhibit glucagon secretion. Accordingly we measured plasma insulin and glucagon levels after an oral fat load, with or without sitagliptin or exendin-4 treatment. Both sitagliptin and exendin-4 significantly increased plasma insulin levels compared to controls (Figure 2-5A), but this increase was much more rapid after exendin-4 administration. However, this transient difference in plasma insulin levels does not likely account for the effect of sitagliptin or exendin-4 on intestinal lipid absorption or for the difference in magnitude of the effect of those 2 treatments, as plasma insulin levels are no longer significantly different between the groups 20 min after the fat load (Figure 2-5A). More importantly, exogenous insulin administration had no effect on TG accumulation (Figure 2-5B). Furthermore, neither sitagliptin nor exendin-4 had a significant effect on plasma glucagon levels (Figure 2-5C). As expected, there was a trend for increased plasma GLP-1 levels, as soon as 5 min following sitagliptin administration (Figure 2-5D).

As exendin-4 is a rapid and potent inhibitor of gastric emptying, which in turn might contribute to decreased intestinal lipid absorption, we monitored intestinal lipid absorption when exendin-4 was administered 1 h after the oral fat load, to facilitate entry of the olive oil into the mouse...
small bowel. Under these conditions, exendin-4 still significantly decreased triglyceride and ApoB48 accumulation in plasma (Figure 2-4F and 2-4G) and TRL fraction (Table 2-2).
Figure 2-4. Pharmacological activation of the GLP1R mimics the effect of sitagliptin on post-prandial lipid excursion

Chow-fed wild type mice fasted for 5h were gavaged with olive oil, and injected with Triton WR-1339 (0.5 g/kg body weight) and D[Ala²]-GIP (24 nmol/kg) (A, B) or exendin-4 (24 nmol/kg) (C-G) 20 min (A-E) or 1h (F, G) after fat load. Blood samples were collected prior to intravenous injection (time 0), as well as 30, 60 and 90 min after injection. (A, D, F) TG, (C) cholesterol and (B, E, G) apoB48 secretion were measured, n = 3-6 per group, * p < 0.05 as determined by two-way ANOVA with Bonferroni post-test.
Figure 2-5. Role of glucose-modulating hormones in mice

(A, C-E) Mice fasted for 5h were administered a single dose of sitagliptin at 10 mg/kg (A, C, D) or exendin-4 24 nmol/kg (A, C, E), and 20 min later were challenged with an oral fat load. Blood was collected by cardiac puncture 5 and 20 min after the olive oil gavage (25 and 40 min after the initial sitagliptin dose). Hormones were measured at the indicated time after the olive oil gavage. (A) Insulin (Mouse insulin ultrasensitive ELISA – Alpco Diagnostic) (C) Glucagon (Mouse endocrine Lincoplex – Linco research) (D) Total GLP-1 (Mouse total GLP-1 ELISA – Alpco Diagnostic) (E) Exendin-4 (Exendin-4 EIA kit – Phoenix Pharmaceuticals). n = 3 per group, * p < 0.05 control vs. sitagliptin, ** p < 0.01 control vs. exendin-4 as determined by two-way ANOVA. (B) Mice were challenged with an oral fat load, and then injected with insulin (1.2 mU/kg BW) 20 min later concomitant with intravenous Triton WR-1339 (0.5 mg/kg BW) administration to measure TG secretion, n = 6 per group, p > 0.05 as determined by two-way ANOVA.
2.4.4 **Endogenous GLP-1R signalling is required for control of postprandial lipemia**

We next investigated the role of physiological levels of GLP-1 in the regulation of intestinal lipid absorption in both mice and hamsters after an acute fat load. Exendin-4 decreased TG in the VLDL/chylomicron remnant-sized lipoproteins in chow-fed hamsters not given a bolus of Triton WR-1339 (Figure 2-6A), and significantly reduced the levels of apoB48 in the TRL fraction 90 min after a fat load (Figure 2-6B). The antagonist exendin9-39 blocked the exendin-4-mediated reduction of TRL-apoB48, and exendin9-39 alone augmented levels of TRL-apoB48 120 minutes after fat load (Figure 2-6B), indicating that endogenous basal GLP-1R signalling modulates postprandial lipemia.

Consistent with data obtained using the GLP1-R antagonist exendin9-39 in hamsters, TG accumulation in plasma (Figure 2-6C) and TRL fraction (Table 2-2) was significantly enhanced in Glp1r−/− mice compared to Glp1r+/+ littermate controls despite the fact that Glp1r−/− mice have a gastric emptying rate similar to Glp1r+/+ littermate controls (400). There was also significantly greater TRL apoB48 mass in GLP-1R-deficient mice (Table 2-2). Therefore, the modulatory actions of basal GLP-1R signaling on intestinal lipid absorption are not due to regulation of gastric emptying, and implicate the endogenous GLP-1 receptor in the control of intestinal lipid absorption.

2.4.5 **Intact GLP-1R signalling is required for the hypolipidemic action of sitagliptin**

To determine whether the actions of sitagliptin on postprandial lipid secretion required GLP-1R signalling, the GLP-1R antagonist exendin9-39 was co-administered with sitagliptin 20 min before the fat load. Sitagliptin attenuated postprandial TG excursion in mice (Figure 2-6F) but did not significantly affect plasma cholesterol levels (Figure 2-6E). Furthermore, the GLP-1R antagonist exendin9-39 eliminated the hypolipidemic actions of sitagliptin on plasma TG levels (Figure 2-6F). Unlike in hamsters (Figure 2-6B), exendin9-39 alone did not induce hyperlipidemia in mice, which may have been attributable to a species-specific response.
Figure 2-6. Role of GLP-1 in postprandial lipemia and sitagliptin action

(A and B): Acute effect of the GLP-1R agonist exendin-4 and antagonist exendin9-39 on apoB48 metabolism in chow-fed hamsters. (A) Representative FPLC profile of plasma from chow-fed hamsters (n = 3) injected intraperitoneally with exendin-4 without intravenous Triton WR-1339, 2 h following an oral gavage of olive oil. (B) Hamsters were challenged with a fat load with Triton WR-1339 administration. TRL apoB48 mass was measured by immunoblotting. Data is presented normalized to baseline values for each treatment. n = 3 per group, **p<0.01, exendin 9-39 or exendin 9-39 + exendin-4 vs. control, *** p <0.001 exendin-4 vs. control as determined by two-way ANOVA with Bonferroni post-test. (C and D): Glp1r−/− mice or littermate control mice fasted for 16 h were given orally 200 μL of olive oil. 20 min after gavage, mice were injected IV with Triton WR1339 (0.5 g/kg). Blood samples were collected prior to IV injection (time 0), as well as 30, 60 and 90 min after injection for triglyceride (C) and ApoB48 (D).
measurement as described in methods, n = 6 per group, * p<0.05, ** p<0.01 as determined by two-way ANOVA with Bonferroni post-test. (E and F): Mice were fasted for 5 h and administered sitagliptin (10 mg/kg) by oral gavage. GLP-1R signalling was blocked by co-administering the GLP-1R antagonist exendin9-39 by intraperitoneal injection. 20 min later, the mice were challenged with an oral fat load and given Triton WR-1339 by intravenous injection. (E) Cholesterol and (F) TG secretion as measured as described in methods. n = 5-6 per group, * p<0.05, ** p<0.01 sitagliptin vs. control or sitagliptin + exendin9-39 as determined by two-way ANOVA with Bonferroni post-test.
2.4.6 Exendin-4 directly reduces enterocyte ApoB48 secretion

We next evaluated the effect of exendin-4 treatment on enterocytes isolated from chow-fed hamsters. Pulse chase studies in primary enterocytes showed that cellular apoB48 was not changed in exendin-4-treated hamsters (Figure 2-7A). In contrast, exendin-4 decreased the amount of secreted apoB48 from enterocytes of fructose-fed hamsters (Figure 2-7B) (p < 0.01 at 60 min, p < 0.001 at 90 min). Calculations of total [\(^{35}\)S]-apoB48 indicate there was significantly less apoB48 production overall with exendin-4 (not shown). However, the total amount of [\(^{35}\)S]-apoB48 at 90 min relative to the starting amount (at time 0) was not different between control and exendin-4-treated cells (Figure 2-7C). Therefore, the decreased apoB48 secretion appeared to be due to decreased overall apoB48 production, as opposed to enhanced apoB48 degradation. Based on [\(^{35}\)S]methionine incorporation, viability and global protein synthesis was not different between exendin-4 and control groups. Hence GLP-1R activation directly regulates intestinal apoB48 secretion \textit{ex vivo}.
Figure 2-7. Direct effects of exendin-4 on intestinal apoB48 metabolism *ex vivo*

Enterocytes from chow-fed hamsters were analyzed. Cellular (A) and secreted (B) levels of apoB 48 were determined in primary enterocytes metabolically labelled with $[^{35}\text{S}]$methionine in media containing 100 pM exendin-4. Direct treatment with exendin-4 significantly decreased secretion in newly-synthesized apoB48. (C) Total $[^{34}\text{S}]$-apoB48 expressed relative to the amount at the beginning of the chase. Open circles = control, closed circles = exendin-4, n = 3 per group, **p<0.01, ***p<0.001 as determined by two-way ANOVA with Bonferroni post-test.
2.5 Discussion

DPP-4 inhibitors exert their actions in part via augmentation of GLP-1 action, which leads to reduction of glucagon, increases in insulin, and reduced glycemia in human subjects (401; 402). Moreover, GLP-1 administration reduced postprandial circulating lipid levels in human subjects (393), although the underlying mechanisms remain uncertain. In the current study, we found that sitagliptin decreased TG accumulation, specifically in the VLDL fraction, in both hamsters and mice. Sitagliptin treatment also decreased apoB48 accumulation in the plasma, specifically in the intestinally produced TRL fraction. Treatment with DPP-4 inhibitors can significantly decrease postprandial TG, cholesterol and apoB48 levels in humans (403; 404) however, DPP-4 inhibition has minimal effects on fasting lipid levels (403). Our data indicate that a prominent aspect of sitagliptin’s effects on fasting plasma TG levels is the decrease in levels of VLDL. This suggests that the major effect of augmenting GLP-1 action is the reduction in the number and/or size of large TRL particles.

Our observations demonstrate that both sitagliptin and exendin-4 exert similar effects on postprandial lipid profiles, consistent with the notion that increased GLP-1 action is likely responsible for the reduced circulating lipid levels and reduced intestinally-derived TRL observed following sitagliptin administration. Furthermore, although increased levels of insulin may also reduce plasma TG levels and apoB 48 secretion from the intestine (405) administration of exogenous insulin did not affect intestinal lipid secretion in mice (Figure 2-5B), excluding insulin as a mediator for sitagliptin or exendin-4 effects on post-prandial lipemia. Modulated insulin sensitivity and glycemia was also unlikely to play a role in sitagliptin’s effects as insulin sensitivity was not noted to change following 3 weeks of treatment. While fructose-fed hamsters exhibit glucose intolerance, fasting hyperinsulinemia, and fasting hyperlipidemia, they have not been observed to have fasting hyperglycemia (145). Though sitagliptin does augment bioactive GLP-1 levels, GLP-1 stimulates insulin secretion in a glucose-dependent manner. As glucose levels were not different between control and sitagliptin-treated groups, insulin and therefore glucose were not expected to be significantly different in the fasting state. Moreover, pharmacological levels of GLP-1R agonism can result in improved insulin sensitivity (339; 406), disrupting endogenous GLP-1R signalling did not adversely affect glucose utilization (407).
However, the sitagliptin apoB48 effect appeared to be attenuated in fructose-fed hamsters (Figure 2-4) probably because hamsters are still insulin-resistant and oversecrete apoB48. As sitagliptin did not improve insulin sensitivity, apoB48 secretion was still refractory to the insulin arm of GLP-1 action (154). Therefore, extending the bioactivity of endogenous GLP-1 with sitagliptin was unlikely to yield great improvements in insulin sensitivity.

We also present data clarifying the putative role of GIP or glucagon in the actions of sitagliptin on intestinal lipid secretion. Although glucagon has been reported to inhibit lipid secretion from hepatocytes (408), circulating levels of glucagon were reduced following sitagliptin administration to mice. Furthermore D-Ala²-GIP did not reduce but actually promoted postprandial TG and apoB48 secretion. These experiments, taken together with data using exendin9-39 and Glp1r⁻/⁻ mice implicate GLP-1 as the predominant mediator of sitagliptin action on intestinal apoB48 secretion.

Our data also demonstrated overlapping effects of sitagliptin and exendin-4 treatment on plasma and TRL lipoprotein levels. Sitagliptin decreased TG levels in the TRL fraction of fructose-fed hamsters. Similarly, exendin-4 decreased apoB48 secretion in chow-fed hamsters and in freshly-isolated primary enterocyte cultures. A discrepancy in plasma cholesterol was noted in unchanged total cholesterol Figure 2-1B and the decreased cholesterol across almost all lipoprotein fractions in Figure 2-1D measured in sitagliptin-treated fructose-fed hamsters. This discrepancy in fasting cholesterol levels needs to be verified, possibly by assessing hepatic cholesterol secretion. These observations suggest for the first time that GLP-1 directly regulates lipoprotein assembly and/or secretory machinery in the enterocyte. However, additional work is required to understand the molecular mechanism underlying the suppressive effect of GLP-1 on apoB48-TRL production.

An important aspect of our studies is the demonstration that reduction or elimination of GLP-1 receptor signalling in the absence of exogenous administration of DPP-4 inhibitors or GLP-1R agonists results in detectable changes in postprandial lipoprotein profiles. Specifically, administration of exendin9-39 alone resulted in increased levels of TRL apoB48 in hamsters, whereas levels of apoB48 and triglyceride mass were increased in Glp1r⁻/⁻ compared to Glp1r⁺/+.
mice. Hence, these findings establish an essential role for basal levels of GLP-1R signaling in the control of intestinal lipoprotein synthesis/secretion in vivo.

In summary, our data supports an important role for the GLP-1 receptor signaling system in regulating intestinal lipid and lipoprotein metabolism. Augmentation of GLP-1R signaling lowers postprandial circulating levels of TG and cholesterol-rich TRL, and apoB48-containing chylomicrons. The current study suggests that enhanced GLP-1 action, achieved via DPP-4 inhibition or use of GLP-1R agonists, may contribute to control of postprandial lipid excursion through control of intestinal lipoprotein synthesis and secretion.
Chapter 3: GLP -2 INCREASES INTESTINAL LIPID ABSORPTION AND CHYLOMICRON PRODUCTION VIA CD36

Text and figures in this chapter were reproduced from:


Author contributions:

J. Hsieh generated all of the hamster and Cd36−/− mice data with assistance. C. Longuet provided the data presented in Figure 3-6. Data analysis and writing was completed by J. Hsieh.
3.1 Summary

Background: Excessive postprandial lipemia is a prevalent condition that results from intestinal oversecretion of apolipoprotein B48 (apoB48)-containing lipoproteins. Glucagon-like peptide-2 (GLP-2) is a gastrointestinal-derived intestinotropic hormone that links nutrient absorption to intestinal structure and function. We investigated the effects of GLP-2 on intestinal lipid absorption and lipoprotein production.

Methods: Intestinal lipid absorption and chylomicron production were quantified in hamsters, wild-type (WT) mice, and Cd36−/− mice infused with exogenous GLP-2. Newly synthesized apoB48 was metabolically labelled in primary hamster jejunal fragments. Fatty acid absorption was measured and putative fatty acid transporters were assessed by immunoblotting.

Results: Human GLP-2 increased secretion of the triglyceride (TG)-rich lipoprotein (TRL)-apoB48 following oral administration of olive oil to hamsters; TRL-TG and cholesterol mass each increased 3-fold. Fast protein liquid chromatography (FPLC) profiling indicated that GLP-2 stimulated secretion of chylomicron/very low density lipoprotein (VLDL)-sized particles. Moreover, GLP-2 directly stimulated apoB48 secretion in jejunal fragments cultured ex vivo, increased expression of fully glycosylated CD36, and induced intestinal absorption of [3H]triolein. The ability of GLP-2 to increase intestinal lipoprotein production was lost in Cd36−/− mice.

Conclusions: GLP-2 stimulates intestinal apoB48-containing lipoprotein secretion, possibly through increased lipid uptake, through a pathway that requires CD36. These findings suggest that GLP-2 represents a nutrient-dependent signal that regulates intestinal lipid absorption and the assembly and secretion of TRLs from intestinal enterocytes.
3.2 Introduction

Postprandial lipemia, particularly excessive accumulation of intestinally-derived lipoproteins, is gaining recognition as a cardiovascular risk factor since the magnitude of postprandial lipemia correlates with intima media thickness (3). ApoB48 serves as the main structural apolipoprotein of the buoyant, TG-rich chylomicrons that deliver dietary lipids to tissues. Interestingly, chylomicron remnants and apoB48 particles have been detected in atherosclerotic plaques (409). In addition to augmenting the risk of atherosclerosis, postprandial hypertriglyceridemia is also an important facet of the metabolic dyslipidemia observed in insulin resistance (410), and there is evidence to suggest that intestinal cholesterol absorption is upregulated in type 1 diabetes (411).

Despite increasing awareness that intestinally-derived lipoproteins may contribute to the pathophysiology of atherosclerosis, our understanding of the cellular pathways involved in chylomicron production and secretion remains limited. Chylomicron assembly is proposed to occur in a three step manner: a) assembly of the primordial lipoprotein during the translocation of apoB48 into the ER, b) accumulation of TG-rich lipid droplets in the lumen of smooth ER, and c) core expansion achieved through additional lipidation of the primordial particle as it proceeds through the secretory pathway to generate a large TRL. As the nascent apoB48 polypeptide traverses the secretory system, its lipidation is achieved through the action of MTP (68). The intestine is capable of secreting small, dense, lipid-poor apoB48-containing lipoproteins (76), but the secretion of TRL is dependent on TG availability, which can be derived from the diet or intracellular de novo lipogenesis.

The observation that insulin can acutely inhibit intestinal apoB48 secretion (154), and that insulin resistance is associated with intestinal apoB48 overproduction (71; 72), suggests that intestinal lipid metabolism is sensitive to endocrine signals. GLP-2 is a 33 amino acid peptide co-secreted with GLP-1 from enteroendocrine L cells in response to carbohydrate and fat ingestion, is an attractive candidate for such an endocrine signal. GLP-2 is highly intestinotrophic and prevents the intestinal mucosal hypoplasia observed with parenteral nutrition (reviewed in (412)). GLP-2 exerts its actions through the GLP-2R, a G-protein-coupled receptor that exhibits a highly restricted specific pattern of expression in enteroendocrine cells (355),
enteric neurons (304), ISEMFs (354) and the central nervous system (282). GLP-2R activation results in cAMP accumulation and protein kinase A activation (366). Notably, GLP-2R mRNA or protein has not been detected in absorptive enterocytes. Thus, GLP-2’s proliferative and cytoprotective actions on the intestinal epithelium must occur indirectly likely involving GLP-2R-dependent stimulation of downstream mediators such as KGF (354) and IGF-1 (327).

GLP-2 also exerts rapid actions converging on stimulation of nutrient absorption in the gut. GLP-2 enhances hexose transport through upregulation of SGLT-1 (312) and GLUT-2 (311) in the BBM. Intriguingly, long chain fatty acids stimulate secretion of proglucagon-derived peptides from the gut (212) and acute GLP-2 administration enhances lipid absorption in normal human subjects (284). Nevertheless, the mechanism(s) through which GLP-2 modulates intestinal lipid secretion and/or metabolism remain poorly understood. In this study, we utilized both Syrian Golden hamster and mouse models to characterize the effect of GLP-2 on intestinal lipid metabolism. As hamsters secrete apoB48 exclusively from the intestine, this facilitates delineation of intestinal contributions to circulating lipoprotein levels. We present new evidence implicating GLP-2 as a potent stimulator of apoB48-containing TRL secretion from the intestine, and describe potential mechanisms via which GLP-2 augments the TG pool for secretion.
3.3 Materials and Methods

3.3.1 Antibodies and chemicals

Rabbit polyclonal anti-human CD36 antibody (Cayman Chemical, Ann Arbor, MI), rabbit polyclonal anti-human FATP4 (kind gift from Dr. Paul A. Watkins, John Hopkins University), and rabbit anti-MTP antibody (kind gift of Dr. André Theriault, University of Hawaii), were used for immunoblots of protein from isolated hamster enterocytes.

3.3.2 Animals

Male Syrian Golden hamsters (*Mesocricetus auratus*) from 130 to 150 g (Charles River, Montreal, Quebec, Canada) were housed individually in a 12:12 h light:dark cycle. Animals were fed *ad libitum* with a standard chow diet. Male *Cd36*−/− mice bred on the C57BL/6 background from 12 to 15 weeks of age were obtained from Dr. Maria Febbraio, Cleveland Clinic via Dr. Kevin Kain, University of Toronto. Following at least a 1 week acclimatization period, animals underwent the *in vivo* protocols described below. All procedures were approved by the Hospital for Sick Children and Toronto General Hospital Animal Care Committees. C57BL/6 male mice 8-12 weeks old (Charles River, Montreal, Quebec, Canada) were maintained on standard rodent chow under a normal 12:12 h light:dark cycle.

3.3.3 Determination of triglyceride-rich lipoprotein apoB48 secretion *in vivo* in hamsters

The *in vivo* protocol for Syrian Golden hamsters was performed as previously described (165). Briefly, the right jugular vein was cannulated with a silastic catheter (VWR), filled with heparinized saline (40 IU/mL in 0.9% NaCl), and exteriorized at back of neck under isoflurane anesthesia. Animals were allowed to recover overnight and henceforth experiments were performed on conscious hamsters. Following a 16 h overnight fast, a 400 μL blood sample was collected into heparinized tubes (Microtainer® PST tubes with lithium heparin, BD, Franklin Lakes, NJ) from the jugular catheter as the baseline reading and succeeded by an oral gavage of 200 μL olive oil. 20 min following the gavage, the hamster was administered human GLP-2<sup>(1-33)</sup> (0.25 mg/kg) (Bachem Bioscience Inc, King of Prussia, PA) by intraperitoneal injection. This dose of GLP-2 was a pharmacological dose. A subcutaneous injection of 5 μg GLP-2 to mice
(approximately 0.19 mg/kg) induced jejunal crypt proliferation within 2 h of administration (288), but the dose was increased as subcutaneous injections were more efficient at stimulating an intestinotrophic response than an intraperitoneal injection (413). Following immediately, an intravenous bolus of Triton WR-1339 (20% w/v in saline, 0.5 g/kg) (Tyloxapol, Sigma-Aldrich Co., St. Louis, MO) was given. 400 μL of blood was sampled at 30 min intervals until 120 min post-gavage, at which point the intestine was excised under isoflurane anaesthesia and the hamster was sacrificed.

3.3.4 Determination of TG-rich lipoprotein apoB48 secretion in vivo in mice

Analysis of intestinal triglyceride and ApoB48 secretion in mice was performed following a similar procedure. After a 5 h fast, mice were administered 200 μL olive oil by gavage, and 20 min later Triton WR-1339 (15% in saline, 0.5 g/kg) with or without the GLP2 analogue hGly²-GLP-2 (0.25 mg/kg body weight) was injected intravenously (IV). 50 μL blood samples were collected via the tail vein prior to (time 0), 30 and 60 min after IV injection for determination of plasma triglyceride and ApoB48. 90 min after IV injection, mice were sacrificed and blood was collected by cardiac puncture.

3.3.5 Isolation of TRL

The plasma layer was separated by centrifuging the blood for 15 min in 4°C at 5,000 rpm. The plasma was supplemented with sodium azide and a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany), and 150 μL was layered under 4 mL of 1.006 g/mL potassium bromide solution. The layered plasma was centrifuged at 10°C for 70 min at 35,000 rpm using a SW55Ti rotor (Beckmann Coulter, Mississauga, ON, Canada). The top 300 μL was collected as the triglyceride-rich lipoprotein (TRL) fraction.

3.3.6 FPLC of plasma lipoproteins

Blood was collected by cardiac puncture 2 hr following a gavage of 200 μL olive oil. 200 μL of plasma was filtered through a 0.45 μM micro-spin polysulphone filter (Alltech, Mandel Scientific, Lachine, PQ) and separated according to size through a HR 10/300 GL Superose 6 column (Pharmacia, Uppsala, Sweden). A solution of 10 mM Tris, 150 mM NaCl, 2 mM CaCl₂, 100 μM DTPA, 0.02% NaN₃, pH 7.4 was pumped through at a flow rate of 0.5
mL/min and 0.5 mL fractions were collected. TG and cholesterol concentration of the fractions were ascertained with one-step colorimetric assays (Roche Diagnostics).

3.3.7 Density ultracentrifugation of plasma lipoproteins

150 μL plasma obtained from hamsters 40 min after fat loading was subject to density ultracentrifugation over a KBr gradient as previously described for microsome luminal contents (414). Concentrations of TG and total cholesterol (Roche Diagnostics) and free cholesterol and phospholipids (Wako Diagnostics, Osaka, Japan) were assayed in each fraction using enzymatic-based colorimetric assays.

3.3.8 Metabolic labelling of primary jejunal fragments ex vivo

Hamsters were given an oral gavage of 200 μL olive oil. 60 min later, the jejunum was excised under isoflurane anesthesia and the hamster was sacrificed. The jejunum was flushed with ice-cold phosphate buffered saline (PBS, pH 7.4) and sliced longitudinally to reveal the mucosa. 0.5 mm long fragments of jejunum were divided randomly among wells, with the mucosal side facing up. The fragments were pre-pulsed for 45 min in DMEM lacking methionine and cysteine and supplemented with 1% L-glutamine, 10% FBS, 1% penicillin/streptomycin, and with/without the addition of 100 nM human GLP-2(1-33). The fragments were then radioactively labeled with 225 μCi of [35S]methionine for 30 min. After washing with PBS, the fragments were chased in 40 mM methionine-enriched DMEM for 30, 60 and 90 min, following which tissues were homogenized with a Polytron homogenizer and media were collected. Immunoprecipitation and SDS-PAGE was performed as previously described (71). ATP binding cassette transporter G5 (ABCG5) was immunoprecipitated with a rabbit anti-ABCG5 antibody (Santa Cruz Biotechnology, CA). Following SDS-PAGE, the gel was dried and exposed to a phosphor imaging screen (Molecular Diagnostics). The screen was then read with the Storm 840 Phosphorimager (Amersham).

3.3.9 Monitoring labelled triolein secretion in vivo

Hamsters with a jugular catheter were gavaged with 3 μCi [9,10-3H(N)] triolein mixed in with 200 μL olive oil and then injected intraperitoneally with 0.25 mg/kg GLP-2 20 min later. Labelled triolein was used to ensure efficient absorption and reproducibility (415). 200 μL of
blood was sampled from the jugular catheter into heparinized tubes at half hour intervals. The activity of tritium in 20 μL of plasma was determined by scintillation counting in triplicate. After an overnight fast, mice were administered 3 μCi [9,10-\(^{3}\)H(N)]triolein mixed in with 200 μL Intralipid (10%) and 50 μL blood samples were collected via the tail vein.

3.3.10 In situ apical membrane protein biotinylation

To specifically label enterocyte brush border membrane proteins with biotin, intact proximal jejunum was filled with a solution of 1.5 mg/mL sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) as described in (311). Enterocytes were isolated as previously described (71), and lysed in 1% Triton X-100, 150 mmol/L NaCl, 50 mmol/L EDTA, 50 mmol/L Tris, pH 7.5. Biotinylated proteins were pulled down by incubating 0.75 mg of total protein with 50 μL streptavidin agarose beads (Pierce, Rockford, IL) at 4°C overnight. The beads were washed, and the biotin cleaved from the protein by boiling the sample in 8% SDS containing β-mercaptoethanol and dithiothreitol.

3.3.11 Statistical analysis

Two-way repeated measures ANOVA with the Bonferroni post-hoc analysis was performed on time course experiments. For enterocyte expression of proteins and lipid synthesis assays, a paired t-test was used. Results are expressed as mean ± SEM.
3.4 Results

3.4.1 GLP-2 acutely increases circulating levels of apoB48-containing TRL in hamsters

Hamsters were administered human GLP-2(1-33) (0.25 mg/kg) by intraperitoneal injection, challenged with a fat load and the apoB48 mass in the TRL fraction of the plasma was monitored by immunoblotting. An intravenous bolus of Triton WR-1339 was also introduced to inhibit lipoprotein catabolism, thus maintaining the buoyancy of newly-secreted chylomicrons. As shown in the photograph in Figure 3-1A, treatment with GLP-2 resulted in a marked lipemia that was visibly noticeable, especially by 120 min post-gavage. Quantification of the apoB48 mass by immunoblotting demonstrated that GLP-2 increased the mass of circulating apoB48-containing TRL by 60 minutes (Figure 3-1B). The slopes were 4.36 ± 0.75 %·min⁻¹ and 2.57 ± 0.9 %·min⁻¹ for GLP-2 vs. vehicle-treated hamsters, p = 0.11 for slope difference, but p = 0.007 for elevation difference.

We next assessed whether GLP-2 produces changes in intestinal lipoproteins by both FPLC analysis and salt-density sedimentation of plasma from hamsters challenged with a fat load, but not given a bolus of Triton WR-1339. FPLC separates lipoproteins according to size via gel filtration. 120 min after the gavage, levels of TG and cholesterol were higher in the VLDL/chylomicron remnant-sized fractions in plasma from GLP-2 treated hamsters (Figure 3-1C). No appreciable differences were observed in the LDL and HDL fractions, suggesting that GLP-2’s stimulatory effect on circulating lipoprotein levels is limited largely to the TRL.

Complete profiling of plasma lipoproteins by sedimentation yielded similar observations, with higher TG and free cholesterol concentrations in the large chylomicron, small chylomicron, and large VLDL fractions isolated from GLP-2 treated hamster plasma (Figure 3-2). Free cholesterol concentrations appeared higher than total cholesterol concentrations due to detection limit of the assay kit. The concentration of TG in the TRL fraction of GLP-2-treated hamsters was 3-fold higher than in control hamsters (Figure 3-1D). TRL cholesterol was also 3-fold higher in GLP-2-treated hamsters (Figure 3-1E).

Metabolic labelling of primary hamster jejunal segments with [³⁵S]methionine was performed to see if these in vivo observations could be recapitulated ex vivo. To rule out the
possibility that the effect of GLP2 on specific protein synthesis may be a result of GLP-2-enhanced enterocyte survival, ApoB48 and ABCG5 synthesis data were normalized to total protein synthesis. Addition of GLP-2 to the incubation media did not affect cellular levels of apoB48 (Figure 3-3A), but resulted in significantly enhanced secretion of newly-synthesized apoB48 (Figure 3-3B) that is reflected in significantly greater total levels of $[^{35}\text{S}]-$labelled apoB48 (Figure 3-3C). This increase in apoB48 synthesis was specific, as GLP-2 did not significantly affect ATP-binding cassette transporter G5 (ABCG5) synthesis (Figure 3-3D). Percent viability of the absorptive enterocytes themselves could not be provided but based on $[^{35}\text{S}]$methionine incorporation into proteins, control-treated fragments had $3.68 \pm 0.14 \times 10^5$ dpm/mg fragment while GLP-2-treated fragments had $5.25 \pm 1.39 \times 10^5$ dpm/mg.
Figure 3-1. In vivo effects of GLP-2 on apoB48-containing TRL secretion

(A) Plasma collected from hamsters challenged with an oral gavage of olive oil coupled with an intravenous bolus of Triton is shown. The time points represent the minutes after the initial fat load. (B) Time-dependent increase of apoB48 mass in the plasma TRL fraction from chow-fed hamsters given an oral olive oil gavage and Triton infusion, as determined by immunoblotting. ApoB48 mass was normalized to baseline fasting levels determined immediately prior to the oil gavage (time 0) and before GLP-2 injection (time 20 min). Representative apoB48 blots are shown above the graphical representation. n = 7 per group, * p < 0.05. (C) Representative FPLC lipoprotein profile of plasma from fat-loaded hamsters not given an intravenous bolus of Triton WR-1339. Profiles are representative of 3 FPLC runs per group. (D) TG and (E) cholesterol concentrations of the plasma TRL fraction, 120 min following fat load and without Triton infusion. n = 4 per group, * p < 0.05.
Figure 3-2. Plasma lipoprotein profiling by salt-density sedimentation in hamsters

Hamsters were given an oral gavage of olive oil and followed by injection with GLP-2 or PBS 20 min later, and no Triton WR-1339 was used in this experiment. Profiling was performed on plasma collected 20 min after peptide injection. Representative of 3 density profiles depicting TG (upper left), total cholesterol (lower left), free cholesterol (upper right), and phospholipids (lower right) content of plasma lipoproteins separated by ultracentrifugation on a discontinuous KBr gradient. Fractions are arranged as numbers 1 to 10 in order of increasing density.
Figure 3-3. Ex vivo effects of GLP-2 on apoB48-containing lipoprotein secretion

$^{[35}S]$-labeled apoB48 was measured during the cold methionine-enriched chase after a 30 min pulse in jejunal fragments freshly isolated from chow-fed hamsters and treated with or without GLP-2 ex vivo, and normalized to total protein synthesis. Time ‘0’ refers to the end of $^{[35}S]$methionine labeling and beginning of chase. (A) Intracellular levels of $^{35}S$-apoB48 (B) Secreted $^{35}S$-apoB48 (C) Total levels of $^{35}S$-apoB48. n=3 per group; * p < 0.05, *** p < 0.001. (D) Cellular $^{35}S$-ABCG5. n = 4 per group.
3.4.2 GLP-2 accelerates secretion of luminal fatty acids

To understand the mechanism by which GLP-2 induces apoB48-lipoprotein secretion, we next examined the handling of luminally-derived FA. As a surrogate measure of dietary FA transport, hamsters were challenged with a fat load that contained 3 μCi of [9,10-3H(N)]triolein, but without Triton WR-1339 injection. By 60 min after the gavage, there was a 2-fold increase in 3H radioactivity in the plasma of GLP-2-treated hamsters (Figure 3-4A). The majority of the tritium label was incorporated into secreted TG, which accounted for the GLP-2-induced increase in plasma 3H radioactivity at both 60 (Figure 3-4B) and 90 min (Figure 3-4C). No difference in total intestinal 3H counts was detected (data not shown).

The expression of specific protein transporters implicated in intestinal fatty acid absorption was examined by Western blot analyses. No difference was noted in the protein levels of fatty acid transporter 4 (FATP4) (Figure 3-5A); similarly, intestinal MTP protein expression was found not to change with acute GLP-2 treatment (Figure 3-5B). However, GLP-2 significantly increased the expression of glycosylated CD36 (Figure 3-5C). Immunohistochemical visualization of CD36 suggests this protein is expressed on hamster intestinal villi tips and appears to be present on the apical membrane and intracellularly (Figure 3-5D), poised for luminal fatty acid uptake. To verify that high molecular weight CD36 is the isoform that is expressed on the enterocyte apical membrane, a cell membrane-impermeable biotinyllating agent (sulfo-NHS-SS-biotin) was introduced into the intestinal lumens of both control and GLP-2-treated hamsters in situ to specifically label apically expressed proteins of the epithelium. As shown in the “PD” lane of Figure 3-5E, streptavidin affinity pulldown of biotinylated proteins revealed only a higher molecular weight protein slightly larger than 88kDa in enterocytes from both control and GLP-2-treated hamsters. However, this isoform of CD36 was not prominent in the supernatant and whole cell lysate samples under both conditions. Such glycosylation behaviour of CD36 was observed in 3 animals per treatment group. Treatment with PNGase F to remove all N-linked oligosaccharides yielded only the lower molecular weight band across all lanes. Thus, a potential mechanism by which GLP-2 may promote fat absorption is through increasing apical CD36 by means of advanced glycosylation.
Figure 3-4. GLP-2 accelerates secretion of luminal fatty acids in hamsters

(A) Rate of appearance of radioactivity in plasma following an oral gavage of 3 μCi [9,10-\(^3\)H(N)]triolein in 200μL olive oil without an intravenous bolus of Triton WR-1339. n = 7 per group, ** p < 0.01. Thin layer chromatography was performed on the hexane-soluble phase of plasma to characterize the incorporation of the \(^3\)H label into secreted lipids at (B) 60 min and (C) 90 min. n = 4 per group, ** p < 0.01, *** p < 0.001.
Figure 3-5. GLP-2 enhances intestinal CD36 action in hamsters

Hamsters were given an oral gavage of olive oil and then injected with GLP-2. Enterocytes were collected 60 min later for immunoblots. (A) FATP4 expression in hamster enterocytes. FATP4 immunoblot is shown above graphical representation. FATP4 expression was normalized to cellular levels of β-actin. n = 4 per group. (B) Immunoblot analysis of MTP protein in enterocytes from hamsters treated with GLP-2 in vivo in comparison to control, normalized to β-actin. Immunoblots of individual animals in duplicate shown above. n = 3 per group. (C) Ratio of fully glycosylated CD36 to unglycosylated CD36 as determined by immunoblotting. The top band was visualized after a longer exposure than the bottom band. n = 4 per group, * p < 0.05. (D) Immunohistochemistry of hamster intestinal villi CD36 expression with an anti-CD36 antibody, visualized with diaminobenzidine (DAB) deposition (brown) and counter-stained with hematoxylin and eosin. (E) Intestinal apical membrane proteins were biotinylated in situ, subject to streptavidin pulldown, and probed with anti-CD36 antibody by immunoblotting. The biotinylated proteins were also treated with PNGase F for 120 min. PD, biotinylated intestinal apical proteins pulled down with streptavidin; Sup, supernatant after streptavidin pulldown; WCL, whole cell lysate previous to addition of streptavidin-agarose beads. Molecular weight markers are indicated on the left.
3.4.3 Acute GLP-2 treatment increases apoB48-containing TRL production in mice

To determine whether GLP-2 exerts similar actions on intestinal lipid absorption and lipoprotein production in mice, experiments were performed in wild type mice with hGly<sup>2</sup>-GLP-2, a human DPP-4-resistant GLP-2 analogue. hGly<sup>2</sup>-GLP-2 rapidly increased the accumulation of plasma TG in the presence of Triton WR-1339 (Figure 3-6A) in a linear manner (R<sup>2</sup> = 0.9918 for control mice and 0.9966 for GLP-2-treated mice), as well as the levels of TG and apoB48 in TRL fraction (Figures 3-6B and 3-6C) in wild type mice after an oral gavage of olive oil in the presence of Triton WR-1339. Furthermore, FPLC profiling showed that exogenous GLP-2 increased TG levels in the VLDL/chylomicron remnant fractions when lipoprotein clearance was not inhibited (Figure 3-6D). These experiments demonstrate that the stimulatory effects of GLP-2 on intestinal lipid absorption and chylomicron secretion are conserved in different species.
Figure 3-6. Stimulatory effects of GLP-2 on intestinal lipid absorption and apoB48-chylomicron production in wild type mice

(A) Plasma TG accumulation after a fat load (200 µL olive oil) and IV Triton WR-1339 injection in wild type mice fasted for 5 h with or without Gly²-GLP-2 as described in methods. n = 4-6, ** p < 0.01. (B) TG and (C) apoB48 accumulation in TRL fraction purified 90 minutes after triton WR-1339 injection as described in methods. n = 4-6 per group, ** p < 0.01. (D) Lipoprotein profile of plasma pooled from 6 mice per group 90 min after the fat load.
3.4.4 GLP-2-stimulated chylomicron secretion requires CD36

The immunoblots shown in Figure 3-5 suggested that GLP-2 rapidly promotes intestinal fat uptake by regulating the trafficking of CD36. To determine whether CD36 is required for GLP-2-stimulated intestinal lipid absorption, we assessed GLP-2 action in \textit{Cd36}^{+/−} mice. Exogenous GLP-2 had no effect on levels of plasma triglycerides after an oral fat load in \textit{Cd36}^{+/−} mice (Figure 3-7A). The time-dependent increase in plasma apoB48 following Triton WR-1339 injection indicate that \textit{Cd36}^{+/−} mice are indeed competent in secreting postprandial apoB48-containing lipoproteins (Figure 3-7A). Consistent with the lack of GLP-2 action on TG absorption in \textit{Cd36}^{+/−} mice, no significant differences were noticed among TG (Figure 3-7B), cholesterol (Figure 3-7C), and apoB48 mass (Figure 3-7D) measurements on the TRL fraction, even though there were significant increases in TRL-apoB48 in their littermate controls. Hence, the actions of GLP-2 on the stimulation of chylomicron secretion require CD36. Additionally, Gly²-GLP-2 failed to augment luminal [³H]triolein uptake in \textit{Cd36}^{+/−} mice, (Figure 3-7E).

As quantified by scoring ten different histological sections of the small intestine, there was negligible oil red O staining in sections of proximal intestine from \textit{Cd36}^{+/−} mice (Figure 3-7F), consistent with observations of Nassir \textit{et al} (19). There was however abundant oil red O staining in the distal jejunum of the \textit{Cd36}^{+/−} mice (data not shown), which could explain why these mice were competent in TG secretion despite the lack of neutral lipid detected in their proximal intestine. Finally, we observed increased lipid accumulation in the enterocytes of GLP-2-treated wild type but not \textit{Cd36}^{+/−} mice (Figure 3-7F).
Figure 3-7. Lack of GLP-2 mediated stimulation of intestinal lipoprotein secretion in Cd36⁻/⁻ mice

Cd36⁻/⁻ mice bred on the C57BL/6 background were challenged with an oral fat load, injected intraperitoneally with hGLP-2(1-33) and given an intravenous bolus of Triton WR-1339 to inhibit lipoprotein catabolism. (A) Plasma TG following an oral fat load. n = 3-4 per group, * p < 0.05, wild type GLP-2 vs. wild type control, # p < 0.05, wild type GLP-2 vs. Cd36⁻/⁻ control. (B) TG mass, (C) Cholesterol mass and (D) apoB48 mass in the TRL fraction; n = 3-4 per group, * p < 0.05, wild type GLP-2 vs. wild type control, ** p < 0.01, wild type GLP-2 vs. Cd36⁻/⁻ groups. (E) Plasma tritium levels in response to an oral gavage of 3 μCi [9,10⁻³H(N)]triolein in 200 μL olive oil, Gly²-GLP-2 injection, and no Triton WR-1339; n = 5 per group. (F) Representative
oil red O stains of the proximal intestine 90 min following an oral gavage, with quantification of average area stained by oil red O, based on 10 separate fields of view per animal. n = 3 per group, *** p < 0.001, Cd36−/− mice vs. wild type mice.
3.5 Discussion

There is growing evidence that, in addition to its intestinotrophic effects, GLP-2 may act as an endocrine signal controlling intestinal nutrient absorption. Although it has been previously demonstrated that chronic GLP-2 administration enhanced $[^3]$Htriolein uptake into the circulation (310), there is limited data available as to the role of GLP-2 in regulating acute intestinal absorption, packaging, and secretion of chylomicron lipids. Long chain fatty acids have been shown to regulate proglucagon-derived peptide secretion from ileal enteroendocrine L cells (212), suggesting that these cells may act as important postprandial sensors for the presence of luminal fatty acids and secrete factors such as GLP-1/GLP-2 to regulate intestinal lipid absorption and/or metabolism. Data presented in this thesis establish a role for GLP-2 in the regulation of intestinal lipid and lipoprotein metabolism. GLP-2 was found to acutely and rapidly bring about changes in the intestine that ultimately result in an exaggerated postprandial lipemia. The most pronounced changes in plasma lipids were noticed in the TRL fraction of circulating lipoproteins, both in apoB48 mass and lipid content, which indicates that GLP-2 promotes the lipidation of nascent apoB48 polypeptides. These data provide a mechanistic explanation for the rise in postprandial TG observed in human subjects infused with GLP-2 (284). Since no change was noted in MTP mass of GLP-2-treated enterocytes, GLP-2 likely acts to stimulate chylomicron assembly and secretion by providing more lipid substrate for lipidation of the growing chylomicron particle.

Increased GLP-2-stimulated packaging and secretion of diet-derived fatty acids as chylomicrons was observed, as indicated by results from the $[^3]$Htriolein experiment. Since GLP-2 has not been shown to affect pancreatic exocrine function (416), changes in pancreatic lipase levels to accelerate the hydrolysis of luminal TG is likely not a contributing factor to GLP-2-enhanced dietary lipid transport. Though Triton WR-1339 was not included in this experiment, the elevation in plasma tritium was unlikely due to inhibited catabolism. GLP-2 infusion in humans has been reported to modestly elevate postprandial TG following a low calorie test meal without a change in glycerol levels (284), signifying that changes in lipolysis did not occur. Evidence is amassing that long chain fatty acid transport across the intestinal BBM is protein-mediated. The role of FATP4 was considered, but no difference in enterocyte
FATP4 protein expression was noted between control and GLP-2-treated hamsters. Although FATP4 has been proposed as an important mediator of intestinal fatty absorption (36), recent evidence suggests that FATP4’s role lies within its acyl CoA-synthetase activity at the ER rather than as a solute carrier at the BBM (37).

Our data suggest that GLP-2-accelerated secretion of dietary lipid can be attributed at least in part to increased expression of fully glycosylated CD36. The highly glycosylated CD36 appears to be the isoform exclusively expressed on the enterocyte apical membrane as determined by in situ biotinylation. CD36/fatty acid translocase, is essential for chylomicron secretion (23), and appears to contribute significantly to intestinal cholesterol absorption (21). Studies in CD36-deficient mice also established this scavenger receptor as an intestinal long chain fatty acid transporter (22). It has been suggested that the carbohydrate moieties of CD36 are important for its subcellular localization. Deletions in the C-terminus of CD36 downregulates its expression on the cell surface, and this was accompanied by reduced glycosylation (417). The effect of GLP-2 on CD36 was rapid, detected within 40 minutes of peptide injection. Interestingly, CD36 translocation to the cell surface from intracellular membranes is a process sensitive to metabolic signals in cardiac myocytes (418). GLP-2 has previously been documented to influence the trafficking of another nutrient transporter, SGLT-1 from intracellular stores to the BBM of enterocytes (312).

Since Western blot analysis of enterocytes suggests that GLP-2 modifies CD36 glycosylation, studies in Cd36−/− mice were performed to confirm the transporter’s role. In contrast to the stimulatory effects of GLP-2 observed in wild type mice, the actions of GLP-2 on intestinal lipid and lipoprotein secretion were absent in Cd36−/− mice. The rate of TG, cholesterol, and apoB48 secretion in GLP-2 and control-treated Cd36−/− mice were all superimposable. Since secretion rates were so similar to control-treated wild type mice, it appears that CD36 is required for hormone-stimulated chylomicron secretion as opposed to being essential in the basal uptake and secretion of dietary lipid. There is no evidence of fat malabsorption in CD36-deficient mice based on fecal analysis (21), indicating that these mice are competent in dietary FA absorption. TRL TG and apoB48 was higher in Cd36−/− mice compared to wild type (Figure 3-7), but this may be attributable to this mouse model’s elevated fasting VLDL (93), due to impaired clearance of TG-rich VLDL-sized (96) and chylomicron-sized (419)
particles. Impairment in lipoprotein lipase-independent clearance pathways could also explain the elevated TRL TG and apoB48 observed in Cd36<sup>-/-</sup> mice (420). Enteroendocrine-regulated post-translational modification of CD36 in the intestine may contribute to mechanisms controlling lipid absorption. However, the pathways regulating CD36 movement to the apical membrane in enterocytes have yet to be elucidated, and the intracellular compartments in which CD36 resides are still unidentified. Interestingly, a large portion of the enterocyte’s CD36 is not found at the plasma membrane, as shown by the strong signal of the 43-55 kDa-sized band in the supernatant and whole cell lysate lanes of Figure 3-5E. CD36 has also been localized to the Golgi apparatus in adipocytes (421), like MTP (422), so it may play a role in further lipidation of the apoB48-containing particle in that compartment. Cd36<sup>-/-</sup> mice have been reported to accumulate neutral lipids in enterocytes during a high fat load (23), hinting at the importance of intracellular movement of CD36 to target fatty acids for assembly and secretion as chylomicrons.

Figure 3-1B suggests there is an inflection point in increased TRL-apoB48 secretion in GLP-2-treated hamsters at 60 min, after which the difference between the two groups diminished, unlike the observations made in wild type mice in Figure 3-6. Since DPP-4-sensitive native GLP-2 was administered to the hamsters, while DPP-4-resistant Gly<sup>2</sup>-GLP-2 was used in mice in Figure 3-6, it can be inferred that sustained GLP-2 action is necessary to maintain upregulated chylomicron secretion. In addition, given that GLP-2 resulted in an inflection in TG secretion between 40 min and 60 min in both wild type and Cd36<sup>-/-</sup> mice (Figure 3-7A), and the results from hepatic secretion studies (data not shown) refute differential hepatic TG secretion, it suggests these kinetics of lipoprotein secretion may be a CD36-independent actions of GLP-2 that can modulate fat absorption <em>in vivo</em>, perhaps due to alterations in gastric (372) and intestinal (338) motility.

The striking absence of lipid accumulation in the proximal intestine following a fat load is consistent with the observations of Nassir <i>et al</i>, who observed a 50% reduction in triolein-derived lipid accumulation in the proximal mucosa 90 min after a fat load (19). On the other hand, the lipid accumulation in the proximal intestine noted by Drover <i>et al</i> was observed following administration of a FA analog, [<sup>125</sup>I]<sup>15-(p-iodophenyl)-3-(R,S)-methyl pentadecanoic acid (BMIPP), that is slowly oxidized (23). Moreover, CD36’s essentiality in uptake was concluded for cholesterol and very long chain FA, two lipids that typically have less efficient
rates of absorption (21; 22). The role of CD36 therefore appears to differ with certain lipid species, but this data implicates a role for CD36 in handling FA that are more abundant in the diet in response to GLP-2. Since \(^{3}H\)triolein was administered \textit{in vivo}, a number of steps from apical FA uptake to chylomicron assembly could contribute to the modulated appearance of tritium in the plasma. Therefore, additional experiments are necessary to definitively test apical FA uptake. One experiment could involve assessing the accumulation of labeled FA in isolated brush border membrane vesicles. Another approach could involve gavaging \(^{3}H\)triolein \textit{in vivo} while blocking chylomicron secretion with Pluronic L-81 administration (423), followed by measuring intestinal tritium accumulation.

In conclusion, our data demonstrates that GLP-2 promotes assembly and secretion of intestinally-derived apoB48-containing TRL through accelerated dietary fat absorption and increased lipidation and secretion of apoB48-containing chylomicron particles. This suggests that GLP-2 may facilitate the efficient absorption of dietary fats. The potent stimulatory role of GLP-2 raises the intriguing possibility of a link between GLP-2 function and postprandial dyslipidemia observed in conditions such as type 1 diabetes. Elevated levels of GLP-2 have been reported and implicated in mediating the intestinal hyperplasia in the STZ-induced diabetic rat model (230). This same animal model also exhibits excessive postprandial lipemia (424). GLP-2 may thus provide a possible endocrine explanation for the intestinal apoB48-containing lipoprotein oversecretion observed in pathological conditions.
Chapter 4: INTERCELLULAR COMMUNICATIONS MEDIATING GLP-2-STIMULATED CHYLOMICRON SECRETION

Author contributions:

With the exception of ISEM treatment and conditioned media collection, all data was generated by J. Hsieh. ISEM treatment and conditioned media collection was performed by A. Izzo in Dr. P. Brubaker’s lab.
4.1 Summary

Background: Glucagon-like peptide-2 (GLP-2) has been shown to stimulate TRL-apoB48 output by promoting the uptake of dietary fatty acids (FA) via the action of CD36. However, the GLP-2 receptor (GLP-2R) is not expressed on the enterocyte, indicating that the hormone signals to the intestinal epithelium indirectly via a paracrine mediator. We postulated that nitric oxide and vascular endothelial growth factor (VEGF) may mediate the GLP-2 effects on enterocyte function.

Methods: Syrian golden hamsters were pre-treated with L-N^G^-nitroarginine methyl ester (L-NAME) and challenged to an oral fat load along with a GLP-2 injection to assess postprandial TRL secretion. Similar studies were performed on endothelial nitric oxide synthase (eNOS) knockout mice. Primary murine enterocytes were cultured in GLP-2-stimulated murine ISEMF-conditioned media and apoB48 synthesis was steady-state labelled with [35S]methionine. Primary rodent enterocytes were also subjected to a pulse chase study in the presence of S-nitroso-L-glutathione (GSNO) or vascular endothelial growth factor (VEGF).

Results: L-NAME pre-treatment blocked the stimulatory effect of GLP-2 on TRL-apoB48 particle number and dietary FA uptake, and significantly reduced total lipid mass in the TRL fraction. eNOS-deficient mice were also resistant to the stimulatory effects of GLP-2 and secreted smaller apoB48-containing TRL particles. Both GSNO and VEGF promoted apoB48 synthesis and secretion in primary enterocytes. Primary enterocytes also upregulated apoB48 production when cultured in GLP-2-stimulated ISEMF-conditioned media, a change that diminished when a VEGF-neutralizing antibody was included in the media.

Conclusions: GLP-2 produced by enteroendocrine L-cells agonizes its receptor on ISEMFs, which secrete VEGF as a paracrine factor that participates in stimulating enterocytes to secrete chylomicrons. The generation of nitric oxide is important in this relay of intercellular communication to fully lipidate apoB48 and stimulate TG-rich chylomicron particles.
4.2 Introduction

Postprandial TG-rich apoB48-containing lipoproteins are increasingly acknowledged for their atherogenic role (8; 425-427), which renders their elevation a cause for concern. Intestinal chylomicron overproduction has been recognized as a contributor to postprandial dyslipidemia in insulin resistance and diabetes in humans and multiple animal models (71; 72; 152; 158; 428). However, intestinal lipoprotein secretion has historically been regarded as a passive process and consequently our understanding of the regulation of apoB48 secretion has lagged behind that of hepatic apoB100 metabolism. Recently, it has become clear that intestinal apoB48 production is acutely sensitive to endocrine signals including insulin (154; 163), GLP-1 (394; 429), and GLP-2 (430), but the mechanisms behind these observations are largely unknown. GLP-2 has been noted to accelerate the uptake of dietary FA through upregulating the posttranslational glycosylation of CD36 in the enterocyte, but the sequence of cellular events leading up to the modification of CD36 have yet to be elucidated (430).

In addition to its well-characterized intestinotrophic actions (431), GLP-2 stimulates the uptake of lipids, as noted above, and sugars in the gut (312; 314). However, the G protein-coupled receptor for GLP-2 has a conspicuous general absence in the intestinal epithelium, the site of most of its observed biological actions to date, but rather has been identified on ISEMFs (354), specific enteroendocrine cells (304; 355), enteric neurons (304; 357), and the vagal afferent nerve (432). Therefore, GLP-2 must bind its receptor on a certain intermediary cell, stimulating it to secrete a mediating signal that would act directly on the enterocyte to bring about chylomicron overproduction. Among others, IGF-1 (433), KGF (354), VEGF (434), and EGF (328) have been suggested as mediators of the proliferative and protective effects of GLP-2 on the intestinal epithelium. We have demonstrated that intact fragments of jejunum treated with GLP-2 exhibited increased newly-synthesized apoB48 secretion ex vivo (430), so ostensibly such an intermediary cell would lie in close apposition to the absorptive enterocyte.

One biological action of GLP-2 is stimulating mesenteric blood flow in a NO-dependent mechanism (303; 306). GLP-2’s ability to increase blood flow may have implications for intestinal lipoprotein metabolism, given that apoB48 secretion can be induced by circulating FFA (164; 435), and increased blood volume at the basolateral side of the epithelium may serve
to increase FA delivery to the enterocyte. However, it has been noted that GLP-2-stimulated intestinal glucose uptake can also be blocked by the pan-specific NOS inhibitor L-NAME to an extent that cannot be completely explained by changes in circulation, suggesting a direct role for NO in enterocyte nutrient handling (303). GLP-2 has been shown to acutely stimulate intestinal eNOS (304), and there is evidence that eNOS is a NOS isoform expressed in the intestinal mucosa (436). A hormone that can stimulate NO production is VEGF, which has been shown to be secreted by intestinal subepithelial myofibroblasts (434). VEGF is best known for its vascular angiogenic effects, but has also been shown to play a role in lymphoangiogenesis (437). There are multiple members in the VEGF family, with VEGF-A being ubiquitously expressed. VEGF-A binds both VEGF receptor 1 (VEGFR1) and VEGFR2 as a homodimer, and exists in multiple isoforms in which C-terminal extensions confer the ability to bind heparan sulfate proteoglycans in the extracellular matrix (438). VEGFR2 is a receptor tyrosine kinase that autophosphorylates upon ligand binding and has a well-documented ability to activate eNOS via either Akt or PKCε (439; 440). In this study we investigated the role of NO generation in GLP-2-stimulated dietary fat absorption and TRL secretion. We employed the Syrian golden hamster and various mouse models in in vivo studies along with intestinal cell cultures in ex vivo, and propose a relay of intercellular communication that can regulate postprandial lipid metabolism in the gut.
4.3 Materials and Methods

4.3.1 Animals

Male Syrian golden hamsters (Mesocricetus auratus; Charles River, Montreal, QC) weighing 110g, male C57Bl/6J mice at 12 weeks of age (Jackson Laboratory, Bar Harbor, ME), and male Nos3−/− mice at 12 weeks of age (B6.129P2-Nos3tm1Unc/J strain; Jackson Laboratory) were housed in 12:12 h light:dark cycle and fed standard rodent chow. For in vivo experiments, hamsters were fasted overnight and mice were fasted for 5 h in the morning. Animals were not fasted when intestine was being collected for ex vivo experiments. Upon sacrifice, all animals were anaesthetized with 3% isoflurane and the small intestine was excised and flash frozen in liquid nitrogen. All procedures were carried out in compliance with the guidelines of The Hospital for Sick Children Animal Ethics Committee.

4.3.2 Determination of dietary fat secretion in vivo

In hamsters under isoflurane anaesthesia, a silastic catheter (VWR) was implanted into the right jugular vein, exteriorized at the back of the neck, and filled with heparinized saline (40 IU/mL). After overnight recovery, the remainder of the experiment was performed on conscious hamsters. A bolus of L-N(−)-nitroarginine methyl ester (L-NAME, Cayman Chemical, Ann Arbor, MI) was infused via the jugular vein at a dose of 75 μmol kg−1 to inhibit NOS. 10 min later, 200 μL blood was withdrawn from the jugular catheter into lithium heparin-coated tubes (BD, Franklin Lakes, NJ) as the baseline (time ‘0 min’) sample followed by an oralgastric gavage of 3 μCi [9,10-3H(N)]triolein (PerkinElmer, Boston, MA) in 200 μL olive oil (Fluka, Buchs, Switzerland). At 20 min, the hamsters received an intraperitoneal injection of PBS vehicle or human GLP-21−33 (Bachem, King of Prussia, PA) at a dose of 0.25 mg kg−1. 200 μL blood samples were collected every half hour until sacrifice at 2 h. In mice, 50 μL blood was collected from the tail vein with EDTA-coated Microvette tubes (Sarstedt, Newton, NC) every 30 min until sacrifice at 90 min, and Gly2−GLP-2 (Pepceuticals, Leicester, UK) was administered 20 min after the oral fat load. Blood was centrifuged at 6,000 rpm for 10 min in 4°C to isolate plasma. 4 mL of Ready Safe scintillation fluid (Beckman Coulter, Fullerton, CA) was added to 20 μL
plasma and counted for 1 min in a Beckman LS6500 IC scintillation counter to measure tritium radioactivity.

4.3.3 Determination of postprandial TRL production in vivo

Hamsters received 20% Pluronic F-127 (Poloxamer 407, Sigma-Aldrich, St. Louis, MO) at a dose of 1000 mg kg\(^{-1}\) by intraperitoneal injection to inhibit peripheral TRL catabolism. Hamsters were pretreated with L-NAME as described above. 20 min later, conscious hamsters were administered 200 \(\mu\)L olive oil by oralgastric gavage and the sample collected immediately prior to the gavage was considered the ‘0 min’ time point. 20 min following the gavage, GLP-2 or PBS vehicle was administered by intraperitoneal injection. 400 \(\mu\)L blood was collected every half hour until sacrifice at 2 h. In mice, 50 \(\mu\)L blood was collected from the tail vein every 30 min until sacrifice at 90 min. 100 \(\mu\)L plasma was supplemented with preservatives, layered under 1.006 g mL\(^{-1}\) KBr solution in polyallomer Microfuge tubes (Beckman Coulter, Palo Alto, CA), and centrifuged at 45,000 rpm in 15\(^\circ\)C for 15 min in a TLA-45 rotor using an Optima TL Ultracentrifuge (Beckman Coulter) to isolate the Sf > 400 fraction, followed by another 45 min centrifugation to isolate the Sf 100-400 fraction. ApoB48 immunoblotting was performed as previously described (430). Total lipid concentration was determined by Intralipid levels measured with the Roche Cobas Integra 400 Plus Analyzer. This method measures lipaemia according to the optical properties of the lipid emulsion Intralipid, and based on the ratio of absorbance at 800 nm to 659 nm.

4.3.4 Determination of intestinal apoB48 secretion ex vivo

Primary enterocytes were isolated from murine small intestine and a pulse chase experiment was performed as previously described (71; 430). Briefly, 0.5 cm fragments of intestine were bathed in Cell Recovery Solution (BD Biosciences, Bedford, MA) at 4\(^\circ\)C for 1 h to loosen epithelial cells from the basement membrane. Enterocytes were pulsed in methionine- and cystine-free DMEM (Wisent, St-Bruno, QC) containing 100 \(\mu\)M S-nitroso-L-glutathione (GSNO, Cayman Chemical), 100 ng mL\(^{-1}\) recombinant mouse VEGF-120 (R&D Systems, Minneapolis, MN), or vehicle for 45 min. The enterocytes were then labelled with \(^{35}\)S\)methionine (PerkinElmer) for 30 min, followed by a chase in DMEM supplemented with
0.5% BSA and bile salt lipid micelles prepared as previously described (441). ApoB48 in cell lysates and media was immunoprecipitated with goat anti-human apoB antibody (Midland, Boone, IA) and protein A from *Staphylococcus aureus* (Sigma-Aldrich, St. Louis, MO), and resolved by SDS-PAGE. \[^{35}S\]methionine-labelled apoB48 was visualized by exposing dried gels to a phosphor screen, visualized with Storm 840 Optical Scanner and quantified using ImageQuant software (Molecular Dynamics, GE Healthcare Life Sciences, Piscataway, NJ).

### 4.3.5 ISEMF-conditioned media

Murine small ISEMFs were isolated and cultured as previously described (359). ISEMFs were serum-starved in 0.5% BSA-containing DMEM for 24 h, and then stimulated with 10 nM GLP-2 for 30 min. The media was passed through a 0.2-μm filter to remove cellular debris immediately prior to its use in the *ex vivo* steady state labelling experiment.

### 4.3.6 Steady state labelling of apoB48 production *ex vivo*

Primary murine enterocytes were suspended in ISEMF-conditioned media, supplemented with bile salt lipid micelles, and labelled with 150 μCi \[^{35}S\]methionine. For neutralization experiments, goat monoclonal anti-mouse VEGF (R&D Biosystems, Minneapolis, MN) or goat IgG control was added for a final concentration of 0.2 ng mL\(^{-1}\). Media and cell samples were collected every 45 min for 135 min and newly-synthesized apoB48 production was quantified as described above.

### 4.3.7 Other biochemical measurements

Intact jejunum was crushed to a powder using a mortar pestle and liquid nitrogen. Lipids were extracted using a 2:1 mixture of chloroform:methanol, and TG mass was determined using an enzymatic-based colourimetric kit (Randox, Crumlin, UK). Nitrate content of jejunum homogenate was assessed by an assay that involved reducing with nitrate reductase and visualizing nitrite colourimetrically with the Griess reagent (Cayman Chemical). ApoAIV levels were determined by immunoblotting using a goat anti-apo AIV antibody (kind gift of Dr. Patrick Tso, University of Cincinnati, OH). VEGF levels in ISEMF-conditioned media were determined by ELISA (R&D Biosystems).
4.3.8 Kinex phosphoprotein screen

C57BL/6 mice were given 200 µL olive oil by oralgastric gavage and then given an injection of GLP-2 or PBS as described in experiments above. After 30 min, the jejunum was excised and the mucosa was scraped off on ice with a glass microscope slide. Mucosa was homogenized in lysis buffer (20 mM MOPS, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, and 1 mM DDT) supplemented with phosphatase and protease inhibitors. The homogenate was cleared by centrifuging at 45,000 rpm in the Beckman Optima TL Ultracentrifuge. The cleared homogenate was sent to Kinexus (Vancouver, BC) and analyzed by the Kinex Antibody Microarray service.

4.3.9 Statistical analysis

Results are expressed and depicted as mean ± SEM. Secretion studies were analyzed by two-way ANOVA with Bonferroni’s post-test to analyze individual time points. Other measurements were analyzed either by two-tailed Student’s t-test or a one-way ANOVA.
4.4 Results

4.4.1 In vivo role of NO in GLP-2-stimulated postprandial TRL secretion

To determine whether GLP-2 can activate intestinal NOS in the hamster model in the context of a fat load, nitrate, jejunal levels of a downstream metabolite of NO that occurs in normoxic conditions were measured. There appeared to be an increase in jejunal nitrate in association with GLP-2 treatment (Figure 4-1A). Next, functional studies were performed using the pan-specific NOS inhibitor L-NAME. Hamsters were pretreated with L-NAME at a dose sufficient to block GLP-2-induced circulation changes in rats (306) and induce hypertension in Syrian golden hamsters (442), challenged with an oral fat load of olive oil and given an intraperitoneal injection of Poloxamer 407 to inhibit peripheral TRL catabolism. GLP-2 increased apoB48 accumulation in the TRL fraction as previously described (Figure 4-1B), and this increase was blocked by L-NAME pretreatment. Neither the L-NAME nor the GLP-2 + L-NAME group had a difference TRL-apoB48 secretion relative to control. There was an increase in postprandial TRL-lipids with an intraperitoneal injection of GLP-2 (slope $3.65 \pm 0.73 \times 10^{-3}$ g L$^{-1}$ min$^{-1}$ vs. $1.63 \pm 0.28 \times 10^{-3}$ g L$^{-1}$ min$^{-1}$, GLP-2 vs. control) and this was mitigated with L-NAME pretreatment (slope $3.67 \pm 0.15 \times 10^{-4}$ g L$^{-1}$ min$^{-1}$, p < 0.01 vs. GLP-2) (Figure 4-1C). L-NAME alone also suppressed TRL-lipid mass relative to the control group (slope $3.60 \pm 0.17 \times 10^{-4}$ g L$^{-1}$ min$^{-1}$, p < 0.01 vs. control), altogether suggesting a basal role of NOS in lipidating apoB48 particles. Because the source of fatty acid substrate could have been derived from the circulation or the gavage sources in the previous experiment, a $[^3$H]triolein label was included in the oral gavage to label exogenously-derived fatty acids. As noted before, GLP-2 acutely increased the appearance of tritium in the circulation, signifying increased uptake of luminal fatty acids (slope $1.66 \pm 0.25 \times 10^2$ dpm mL$^{-1}$ min$^{-1}$ vs. $1.11 \pm 0.17 \times 10^2$ dpm mL$^{-1}$ min$^{-1}$, GLP-2 vs. control) (Figure 4-1D). As with TRL-apoB48, L-NAME pretreatment abrogated the stimulatory effect of GLP-2 on fatty acid uptake (slope $1.02 \pm 0.13 \times 10^2$ dpm mL$^{-1}$ min$^{-1}$), but alone did not have any effect relative to control (slope $1.38 \pm 0.13 \times 10^2$ dpm mL$^{-1}$ min$^{-1}$). Similar observations were made in the TRL tritium content (Figure 4-1E). The data suggests that NOS is necessary for GLP-2-stimulated luminal FA uptake, but inhibition of all NOS isoforms does not affect basal levels of uptake.
Figure 4-1. The role of nitric oxide in apoB48 production in hamsters in vivo

Hamsters were challenged with an oral fat load, and given an intraperitoneal injection of Poloxamer 407 (1000 mg kg$^{-1}$) and GLP-2 (0.25 mg kg$^{-1}$). (A) Jejunal nitrate levels 2 h after the fat load as determined using the Greiss reagent. n = 5-10 per group. (B) Increase in TRL-apoB48 mass following the fat load. Representative apoB48 immunoblots are shown to the left. n = 4 per group, * p < 0.05 GLP-2 vs. control, # p < 0.05 GLP-2 vs. GLP-2 + L-NAME, $ GLP-2 vs. L-NAME. (C) Total TRL-lipid mass following the fat load. n = 5 per group, % p < 0.05 L-NAME vs. control, ** p < 0.01 GLP-2 vs. control, $$$ p < 0.001 GLP-2 vs. L-NAME, ### p < 0.001 GLP-2 vs. L-NAME + GLP-2. (D) Hamsters were challenged with an oral fat load of olive oil containing 3 mCi [$^3$H]triolein label. Circulating tritium levels was quantified as a measure of luminal fatty acid uptake. n = 5 per group, # p < 0.05 GLP-2 vs. GLP-2 + L-NAME, $$$ p < 0.001 GLP-2 vs. GLP-2 + L-NAME, * p < 0.05 GLP-2 vs. control, ** p < 0.01 GLP-2 vs. L-NAME. (E) TRL tritium levels following the oral fat load containing the [$^3$H]triolein label. n = 5 per group.
4.4.2  *Ex vivo role of NO in apoB48 secretion*

Primary hamster enterocytes were isolated and exposed to a physiologically occurring NO donor in the gut, GSNO at a dose sufficient to improve barrier function (443), which is a described function of GLP-2. The enterocytes were also cultured in methionine- and cystine-free media, followed by labelling with $[^{35}\text{S}]$methionine in a pulse chase study. GSNO trended to increase intracellular amounts of $[^{35}\text{S}]$-apoB48 (Figure 4-2B) and significantly upregulated the secretion of newly-synthesized apoB48 within 90 min of the chase (Figure 4-2C). Similar observations were made with a chemically distinct NO donor, DEANONate (data not shown), thereby suggesting that NO has a direct role in modulating enterocyte apoB48 synthesis and secretion.
Figure 4-2. The effect of an NO donor on apoB48 production in hamster enterocytes ex vivo

Primary hamster enterocytes were treated with the NO donor GSNO or reduced glutathione control, starved in methionine, cysteine, and serum-free DMEM supplemented in bile salt lipid micelles, and then given $[^{35}S]$methionine to label protein synthesis. Samples were subject to immunoprecipitation with a hamster anti-apoB antibody. (A) Representative SDS-PAGE of immunoprecipitated $[^{35}S]$-apoB48 from cell and media. (B) Quantification of intracellular amounts of newly-synthesized apoB48. (C) Quantification of secreted amounts of newly-synthesized apoB48. n = 3 per group, * p < 0.05.
4.4.3 Role of eNOS in GLP-2-stimulated chylomicron secretion

Since NO can be generated by one of three NOS isoforms in the mammalian system, we sought to identify the isoform involved in GLP-2’s action on chylomicron secretion. We assessed the ability of GLP-2 to promote dietary fat absorption in the eNOS KO mouse model. When challenged with an oral fat load containing $[^3]$Htriolein, GLP-2-treated eNOS KO mice exhibited significantly less tritium secretion relative to their wild type counterparts (slope $3.43 \pm 0.65 \times 10^3$ dpm mL$^{-1}$ min$^{-1}$ vs. $2.16 \pm 0.31 \times 10^3$ dpm mL$^{-1}$ min$^{-1}$, wild type + GLP-2 vs. eNOS KO + GLP-2) (Figure 4-3A), suggesting that eNOS is required to some degree to mediate the lipid effect of GLP-2. ApoB48 mass in the TRL fraction was examined, and there was a noticeable decrease in the largest lipoprotein fraction (Sf > 400) when mice were eNOS-deficient (Figure 4-3B). No discernible difference was noted in the apoB48 content of the smaller Sf 100-400 lipoprotein fraction (Figure 4-3C), suggesting that eNOS is required to maximize the amount of lipid associated with each apoB48 polypeptide. The inability to fully lipidate apoB48 conferred by eNOS deficiency resulted in significantly more TG accumulation in the jejunum 90 min after the oral fat load ($4.47 \pm 0.20$ mg TG/mg intestine vs. $2.77 \pm 0.75$ mg TG/mg intestine, eNOS KO vs. wild type) (Figure 4-3D), indicating an inefficiency in exporting dietary fat out as chylomicrons. The mechanism for this observation remains unexplained, as it was noted that secretion of apoAIV, an intestine-specific apolipoprotein whose putative role is to expand the chylomicron lipid core (77), was unchanged (Figure 4-3E). In summary, it appears that eNOS is necessary for both GLP-2-stimulated TRL secretion and basally for proper lipidation of apoB48 in the intestine.
Figure 4-3. Effect of GLP-2 on dietary fat absorption and postprandial lipoprotein secretion in C57Bl/6J and eNOS KO mice

Mice were challenged with an oral fat load containing 3 mCi [³H]triolein along with an intraperitoneal injection of Poloxamer 407 (1000 mg kg⁻¹) and Gly²-GLP2 (0.25 mg kg⁻¹). (A) Circulating tritium levels as an indicator of dietary fat absorption. n = 7 per group, * p < 0.05 wild type GLP-2 vs. eNOS KO GLP-2. (B) Sf > 400 TRL-apoB48 and (C) Sf 100-400 TRL-apoB48 levels as assessed by immunoblot. n = 4 per group, *** p < 0.01 inter-genotype. (D) TG accumulated in jejunum of mice 90 min following an oral fat load. (E) Sf > 400 TRL-apoAIV and (F) Sf 100-400 TRL-apoAIV levels as determined by immunoblot.
4.4.4 Role of ISEMFs in GLP-2-stimulated apoB48 secretion

Next we sought to identify the GLP-2R-positive cell that could be secreting a paracrine factor that stimulates chylomicron oversecretion in absorptive enterocytes. Cultured ISEMFs were used as they are known to express the GLP-2R (359) and have been demonstrated to increase Igf1 mRNA levels acutely in response to GLP-2. Conditioned media were obtained from cultured murine intestinal SEMFs stimulated following treatment with GLP-2 (10 nM) for 30 min. Primary murine enterocytes were cultured in this conditioned media in the presence of bile salt lipid micelles to mimic the lipid-rich environment of the postprandial lumen, and apoB48 synthesis and secretion was steady-state labelled with [35S]-methionine. There was a trend for increased intracellular [35S]-apoB48 levels (Figure 4A) and secreted [35S]-apoB48 (Figure 4B) with significantly more total [35S]-apoB48 in enterocytes that were incubated in conditioned media collected from GLP-2-stimulated ISEMFs (p < 0.05, GLP-2 conditioned media vs. control conditioned media) (Figure 4C). Enterocytes exposed to GLP-2 conditioned media also had increased levels of glycosylated CD36 (Figure 4D). This would suggest that ISEMF is at least one GLP-2R-positive gastrointestinal cell type that can release a mediator capable of eliciting more chylomicron secretion in enterocytes.
Figure 4-4. Effect of ISEMF conditioned media on apoB48 production in primary hamster enterocytes

Primary murine enterocytes were exposed to conditioned media collected from cultured murine jejunal subepithelial myofibroblasts stimulated with 10 nM GLP-2 for 30 min. ApoB48 synthesis and secretion was steady-stated labelled with 150 mCi $^{35}$S-methionine and immunoprecipitated with an anti-apoB antibody. Representative SDS-PAGE of immunoprecipitated $^{35}$S-labelled apoB48 shown above. (A) Amount of $^{35}$S-apoB48 within the cell normalized to total protein synthesis as assessed by TCA precipitation. (B) Amount of $^{35}$S-apoB48 secreted from enterocytes. (C) Total amounts of $^{35}$S-apoB48. n = 4 per group, * p < 0.05 by two-way ANOVA. (D) Ratio of glycosylated CD36 to unglycosylated CD36 in enterocytes incubated in conditioned media. n = 3 per group, * p < 0.05.
4.4.5 VEGF action in enterocyte apoB48 secretion

ISEMFs have been shown to secrete myriad peptide factors, including KGF and IGF-1 (354; 359). Both KGF and IGF-1 were tested and found not to promote apoB48 secretion in primary hamster enterocytes \textit{ex vivo} (data not shown). Given the observations with L-NAME and GSNO, we searched for a factor that could activate NOS, or particularly, eNOS. VEGF has a well-documented ability to activate eNOS (444). In the Kinex phosphoprotein screen of mucosal scraping lysates, it was noted that there was significantly increased Tyr1054 phosphorylation on VEGFR2 (VEGFR2) with GLP-2 administration in C57Bl/6 mice (Figure 4-5A), indicating the receptor’s activation. However, such magnitude of increase in phosphorylation may have been attributable to receptor activation in other contaminating intestinal cell types that were removed in mucosal scraping. Primary murine enterocytes were then isolated and exposed to 100 ng/mL VEGF-120 in a pulse chase study, while $[^{35}S]$-labelled apoB48 was immunoprecipitated (Figure 4-5B). The inclusion of VEGF in the pulse media trended towards an increase in the total accumulation of $[^{35}S]$-apoB48 over time, although this effect was not significant for this sample size (Figure 4-5C).
Figure 4-5. The role of VEGF in intestinal apoB48 secretion in mice

(A) VEGFR2 Tyr1054 phosphorylation of intestinal mucosa scrapings of C57Bl/6 mice 1 h after an oral fat load and GLP-2 administration. Phosphorylation was determined by Kinexus Kinex phosphoprotein microarray analysis. n = 3 per group, * p < 0.05. (B) Primary murine enterocytes were labelled with [35S]methionine in a pulse chase experiment while cultured with 100 ng mL⁻¹ VEGF-120. Representative SDS-PAGE of immunoprecipitated [35S]-apoB48 from three experiments. (C) Total amounts of [35S]-apoB48, n = 3 per group.
4.4.6 VEGF as a mediator linking ISEMFs and enterocytes

The data presented in Figure 4-5 suggests that GLP-2 is capable of eliciting VEGF secretion from this GLP-2R-positive intestinal cell, and may be a peptide communicator to enterocytes. Therefore, a goat monoclonal antibody against murine VEGF was added to the GLP-2-conditioned media to neutralize the peptide. Primary murine enterocytes were again steady-state labelled and we assessed the anti-VEGF antibody’s ability to suppress the GLP-2-conditioned media’s augmenting action on $^{35}$S-apoB48. Relative to the IgG control, VEGF neutralization decreased intracellular $^{35}$S-apoB48 (Figure 5-6B), had a less pronounced effect on secreted $^{35}$S-apoB48 (Figure 5-6C), but had very similar values to the control conditioned media curve with regards to total $^{35}$S-apoB48 (Figure 5-6D).
Figure 4-6. The role of VEGF in GLP-2-stimulated ISEMF-mediated intestinal apoB48 secretion in enterocytes

(A) Primary murine enterocytes were steady-state labelled with [35S]methionine in conditioned media collected from jejunal SEMFs treated for GLP-2 for 30 min. VEGF in the media was neutralized with goat anti-mouse VEGF antibody and compared to goat IgG control. Representative SDS-PAGE of immunoprecipitated [35S]-apoB48 from 3 experiments. (C) Cellular amounts of [35S]-apoB48. (D) Secreted amounts of [35S]-apoB48. (E) Total amounts of [35S]-apoB48. n = 3 per group, * p < 0.05.
4.5 Discussion

Aside from the observation that GLP-2 acutely promotes CD36 glycosylation to facilitate its expression at the apical brush border membrane, little is known as to how this postprandial hormone promotes the secretion of apoB48-containing TRL secretion from the intestine. The difficulty in determining the physiological mechanism lies in the absence of the GLP-2R on the absorptive enterocyte. In the present study we found at least one intestinal cell type, the ISEMF that can stimulate enterocytes with a paracrine mediator to promote dietary fatty acid uptake. Based on studies using a NOS inhibitor, it is clear that NO generation in the intestine is important to GLP-2-stimulated and basal chylomicron secretion. A search for a peptide that can provoke NO production yielded VEGF, which was shown to induce newly-synthesized apoB48 production in enterocytes. We therefore propose that the schematic illustrated in Figure 4-7 is one paracrine pathway that GLP-2 may use to promote chylomicron secretion in enterocytes. The involvement of other cytokines and peptide hormones, however, cannot be excluded.
Figure 4-7. Intercellular interactions resulting in GLP-2-stimulated chylomicron secretion

We postulate that GLP-2 secreted by the enteroendocrine L-cell agonizes its G protein-coupled receptor on SEMFs in the intestine. This induces SEMFs to secrete a number of cytokines, including VEGF, which acts on its receptor VEGFR2 on the absorptive enterocyte. Stimulation of VEGFR2 activates eNOS in the enterocyte, which leads to increases in dietary FA uptake and subsequently chylomicron output. There is likely the involvement of additional factors secreted by ISEMFs to bring about an increase in chylomicron production.
The decreased TRL-lipid associated with L-NAME pretreatment alone (Figure 4-1C) suggests that NO that is constitutively produced, for example by neuronal NOS in the intestine, has a role in regulating chylomicron metabolism. There is existing evidence that NO generation, particularly by the eNOS isoform, is important to lipid metabolism in the liver. The eNOS-deficient mice have abnormally high hepatic TG accumulation, but the authors propose the observation was due to relief of NO-inhibited FA synthesis (445). Similarly, analysis of the S-nitrosylated proteome in the liver reveals that the covalent modification occurs on proteins involved in lipid metabolism, such as very long chain acyl-CoA dehydrogenase (446). A NO donor has been implicated in promoting hepatic lipoprotein secretion by upregulating MTP mRNA (447). While inhibiting NO synthesis could block the stimulatory effects of GLP-2 on TRL secretion, the L-NAME data would suggest that NO has a basic role in intestinal lipoprotein output. Though L-NAME pretreatment alone did not affect the number of apoB48 particles in the TRL fraction (Figure 1B), there was a dramatic reduction in the lipid mass of the fraction compared to control-treated hamsters (Figure 4-1C), while having no effect on the secretion of exogenous labeled lipid (Figure 4-1D). Data from eNOS-deficient mice also support this notion, as these mice secreted significantly fewer particles of Sf > 400 size (Figure 4-3B). Altogether, this may suggest that NO is essential to secreting fully lipidated apoB48 particles but the lipid is not sourced exogenously. The FA substrates for NO-mediated apoB48 assembly may come from the circulation. FFA have been demonstrated to induce apoB48 secretion from isolated enterocytes (76), and plasma FFA have been shown to promote apoB48 oversecretion in hamsters (435) and humans (164). However, FFA derived from the circulation are unlikely to account for the core size differences of apoB48 particles secreted by L-NAME-treated hamsters and eNOS-deficient mice, as FA taken up at the basolateral surface are preferentially incorporated into phospholipids, not TG, and catabolized by β-oxidation (448). Another possible source of lipid could come from mobilization of intestinal intracellular stores, as there is evidence that FA from a meal is retained until release with Sf > 400 particles in the next meal (174).

In Figure 4-3A, eNOS-deficient mice did not have altered postprandial appearance of tritium in the plasma relative to control. While eNOS appears to be necessary for GLP-2-increased chylomicron secretion, it may not be essential for dietary lipid transport per se. L-
NAME pre-treatment had no effect on the appearance of tritium in plasma relative to control (Figure 4-1D), so constitutional nitric oxide generation was evidently not essential to this process in the basal state. Following a fat load, eNOS-deficient mice accumulated TG in the jejunum regardless of treatment (Figure 4-3D). Therefore, while eNOS possibly mediates the effect of GLP-2-stimulated CD36 action, basal eNOS activity also plays an additional role later on in chylomicron secretion pathway.

The VEGF data is still preliminary and requires confirmation. While VEGF neutralization inhibited an increase in intracellular amounts of $[^{35}S]$-apoB48 (Figure 4-6B), there was little effect on secretion. Therefore, VEGF may contribute to GLP-2-stimulated apoB48 biogenesis, but the secretion of chylomicrons involves another factor. To clarify the role of VEGF in apoB48 production, basal and GLP-2-stimulated intestinal apoB48 levels can be assessed in Vegf$^{+/−}$ mice. The role of NO in VEGF’s actions on apoB48 can be confirmed by administering eNOS knockout mice with VEGF-A in vivo, and assessing the effect of VEGF on eNOS-deficient enterocytes ex vivo.

Though the ex vivo experiments with GSNO would suggest that NO can promote apoB48 secretion at the level of the enterocyte, NO generation in other cell types may contribute to the mechanism of GLP-2 action. With low doses of GLP-1, NO has been implicated in the inhibition of gastrointestinal motility (449). GLP-2 has been shown to potentiate the inhibitory effect of GLP-1 (338), but GLP-2-suppressed gastric motility has been shown to occur via VIP and independently of NOS activity (372). Given that GLP-2 has been co-localized to eNOS-positive enteric neurons (304), and enteric neurons can secrete GSNO (443), it is possible that the enteric nervous system plays a role in GLP-2-stimulated apoB48 secretion. Interestingly, GLP-2’s intestinal epithelial restitution effects have been ascribed to VEGF secreted by ISEM, and such an intestinal epithelial wound healing effect is a biological function of GSNO (443), so this functional overlap suggests that VEGF and NO act in the same pathway. Though the anti-inflammatory effects described for GLP-2 include downregulation of inducible NOS (iNOS) (300), inflammatory signalling has been shown to result in chylomicron oversecretion (165). It may be worthwhile to investigate the specific role of other NOS isoforms in regulating intestinal lipoprotein production. VEGFR2 signalling in the enterocyte itself also has the potential to stimulate chylomicron secretion. Phospholipase Cγ (PLCγ) directly binds a
phosphotyrosine residue on VEGFR2 to activate the MEK/ERK cascade (450), and ERK activation has been associated with chylomicron overproduction in insulin-resistant enterocytes (154). On the other hand, the adaptor protein Shb binds another phosphotyrosine residue on VEGFR2 that leads to PI3-K activation (451), with PI3-K being instrumental in lipid transporter trafficking in Caco-2 cells (441).

The identification of a paracrine factor that can directly stimulate the enterocyte to increase apoB48 output could serve as an important tool in understanding intestinal lipid metabolism. In future studies, VEGF can be used in enterocyte cell models to elucidate the molecular mechanisms behind the hyperlipidemia observed in vivo at the whole body level.
Chapter 5: DISCUSSION AND CONCLUSIONS

Author contribution: J. Hsieh produced all text and figures in this chapter.
5.1 Summary of Results

The present studies on GLP-1 and GLP-2 indicate that pharmacological levels of these hormones are capable of acutely bringing about considerable changes in intestinal apoB48 output in multiple rodent models. Though co-secreted in equimolar amounts, these gut peptides exact complete opposite effects on intestinal lipoprotein secretion. Raising endogenous levels of GLP-1 with a DPP-4 inhibitor or administrating pharmacological levels of a long-acting GLP-1R agonist suppressed TRL-TG and TRL-apoB48 secretion in chow-fed hamsters, fructose-fed hamsters, and mice challenged with an oral fat load within a 2 h period. The hypolipidemic effect of the DPP-IV inhibitor sitagliptin was dependent on intact GLP-1R signalling, and was independent of insulin secretion and prolonged GIP bioactivity. In contrast, pharmacological levels of GLP-2 rapidly increased uptake of luminal FA to promote chylomicron secretion in vivo in hamsters and mice, but not CD36-deficient mice. Increased dietary FA uptake was achieved by GLP2-induced glycosylation of CD36 facilitating its insertion into the brush border membrane of enterocytes, where it can perform its lipid transport function. NOS activity was also found to be important in chylomicron secretion, as it was essential for basal levels of apoB48 particle lipidation GLP-2-accelerated dietary FA uptake in vivo. GLP-2 appeared to indirectly regulate the absorptive enterocyte through VEGF secreted by ISEMFs, thereby establishing the involvement of paracrine communication in the physiological action of GLP-2.
5.2 GLP-1 as a Direct Regulator of Intestinal Lipoprotein Metabolism

An exogenous GLP-1 agonist, exendin-4 acted to acutely suppress apoB48-containing TRL secretion from the intestine in both hamsters and mice, thereby favourably moderating postprandial lipemia. Our data suggested that endogenous levels of GLP-1 were also capable of suppressing secretion, as long as its bioactivity was maintained by inhibiting DPP-4 activity. The apoB48- and TG-lowering effect observed with exendin-4 is consistent with studies in which GLP-1 was intravenously infused in rats (394) and humans (393). However, these studies attributed the postprandial lipid lowering to decreased lymphatic flow rate (394), or speculated that it was a result of increased peripheral clearance of TRL-TG (393). Moreover, delayed gastric emptying could explain the blunted rise in TG in the study performed by Meier et al (393). However the hypolipidemic effect was still observed when exendin-4 was injected 1 h after the oral gastric gavage, which obviated any gastric emptying effects. Additionally, both fructose-fed hamsters and mice secreted less TG following sitagliptin administration, and DPP-4 inhibition has been shown to have no effect on gastric emptying (452; 453). Though insulin likely plays a role in the hypolipidemic action of GLP-1, there is evidence that GLP-1 can directly modulate lipoprotein metabolism in the intestine. The decrease in apoB48 and TG secretion persisted after differences in insulin levels diminished between sitagliptin-treated and control mice, and insulin did not appear to have the same acute hypolipidemic effect in mice as it does in hamsters (154) and humans (163). A single subcutaneous injection of the GLP-1R agonist therapeutic exenatide has been shown to decrease serum TG and apoB48 in individuals with impaired glucose tolerance challenged with a fat-enriched meal. The lipid lowering occurred despite defective insulin action in these individuals, and was observed concurrently with an unexpected drop in insulin and minimal alterations in circulating non-esterified FA (454). The glucagonostatic activity of GLP-1 is unlikely to explain the changes in intestinal lipoprotein secretion, as glucagon does not have an effect on VLDL1-apoB48 and VLDL2-apoB48 production rates in healthy men (455). Last but not least, in pulse-chase experiments exendin-4 inhibited newly-synthesized apoB48 secretion in primary enterocyte cultures. The data presented herein indicate that GLP-1R signalling has a direct role in affecting enterocyte lipid metabolism. This direct effect of GLP-1 may explain the improvement in postprandial...
serum TG and apoB48 seen in exenatide-treated patients but not insulin glargine-treated patients, especially since body weights were similar between the two groups (456). An interesting observation was that exendin-4 could acutely raise HDL cholesterol levels which, given that hamsters are cholesteryl ester transfer protein-expressing animals (457), could be a result of the lower TRL TG allowing more cholesteryl ester to be retained in HDL particles.

### 5.3 CD36 as a Hormone-Sensitive FA Transporter in the Gut

The observation that GLP-2 can acutely affect the subcellular distribution of CD36 adds the intestinal epithelium to the growing list of tissues in which the trafficking of CD36 can be regulated by hormonal signals. Insulin has been recognized as a hormone that can direct CD36 to the plasma membrane in skeletal muscle (458), cardiomyocytes (418), and adipocytes (459). In skeletal muscle and cardiomyocytes, the insulin-stimulated translocation of CD36 is PI3-K-dependent, as it can be blocked by wortmannin or LY294002 (418; 458). Insulin-stimulated CD36 translocation from subcellular compartments requires vesicle-associated membrane proteins (VAMP). In addition to VAMP2 and VAMP5, VAMP4 appears to be specific to insulin-stimulated CD36 trafficking (460). Notably, GLP-2-induced translocation of the glucose transporter SGLT-1 is also a PI3-K-dependent process (312). It is also worth noting that the PI3-K/Akt pathway is activated by VEGFR2 signalling (438), suggesting a signalling mechanism by which the VEGF secreted by ISEMFs can direct CD36 insertion into the BBM.

Though the functional significance of greater CD36 surface expression is expected to be the increased facilitated transport of dietary FA, apically-expressed CD36 may also serve as a lipid sensor. Lingual CD36 is involved in the gustatory perception of fat and is responsible for preference of lipid-rich foods (24). It has been noted that CD36 in rat enterocytes disappeared from the apical membrane starting at 1 h after exposure to luminal lipids, coupled with protein polyubiquitination and degradation. Moreover, in the same study, FA-induced ERK1/2 activation in the enterocyte paralleled CD36 levels, and coincided with increased apoB48 and MTP levels (461). Since ERK1/2 activation is associated with apoB48-containing lipoprotein overproduction in intestinal insulin-resistance (154), the result of GLP-2-stimulated apical CD36 expression may be the initiation of cellular signalling to promote chylomicron secretion. A similar role has been proposed for a structurally-related scavenger receptor in the enterocyte, SR-
BI, which drives the trafficking of apoB48 towards the basolateral membrane for secretion following micelle-stimulated MAPK signalling (462). In a corroborative study, SR-BI knockdown by RNAi was shown to decrease apoB secretion from Caco-2 cells (441).

The biotinylation experiment demonstrated that the fully glycosylated form of CD36 is specific to the BBM, and also indicated that the majority of enterocyte CD36 is unglycosylated and intracellular. The intracellular compartment from which CD36 translocates is currently unidentified. In 3T3-L1 adipocytes, surface CD36 is found specifically in cholesterol-enriched detergent-resistant membranes, where it is important to its FA transport function (15). It has been reported that CD36 functionally interacts with caveolin-1 in caveolae (463), where the presence of sphingomyelin synthase 2 is also necessary for oleic acid uptake and lipid droplet formation in hepatocytes (464). Interesting, the localization of CD36 in caveolae has implications for eNOS regulation. The binding of LDL to CD36 lead to caveolae cholesterol depletion, which in turn relocalized eNOS to intracellular compartments, rather than keeping eNOS in close proximity to the signalling components such as receptors and Gq proteins that are enriched in caveolae (465). However, CD36’s association with FA, in particular myristic acid, has been linked with eNOS activation in a PI3-K-independent pathway (466). Therefore, it is possible there is reciprocal regulation between CD36 and eNOS, and this can occur in a substrate-specific manner. Endosomal alkalinization sustained CD36 expression at the plasma membrane, thus indicating that CD36 is internalized into endosomal compartments (467). The endosomal structure though, at least when CD36 is internalized upon binding oxidized LDL, is transferrin- and caveolin-free, but colocalizes with glycosyl-phosphatidylinositol-anchored protein decay factor (468). In hepatocytes however, CD36 colocalizes with caveolin-1 in intracellular vesicles (464). Transfection of primary cultured human muscle cells with a CD36-EGFP construct showed that CD36 was not coincident with the Golgi marker GM-130, suggesting that unlike FATP4, CD36 is not found in the Golgi (469). CD36 has been detected in the ER, where it appears to be essential for PCTV budding (83). The role of ER-localized CD36 may confer a functional importance to the large amount of unglycosylated intracellular CD36 detected in primary hamster enterocytes.

Mutation of several of the consensus sequence N-X-S/T sites indicated that N-linked glycosylation is essential in targeting CD36 to the plasma membrane in HEK293T cells (470).
While our study confirms that glycosylation is important to CD36 trafficking in the intestine, other posttranslational modifications contribute to CD36 processing and eventual localization in the plasma membrane. CD36 contains two –SS- sites at both termini of the molecule, and palmitoylation of these sites is necessary for CD36 to proceed from the ER to Golgi for further maturation and for greater stability (471). Insulin has also been shown to affect covalent modification of CD36 by suppressing polyubiquitination on lysine residues in the carboxy terminus, thereby maintaining CD36 levels at the plasma membrane (472). There is also emerging evidence that CD36 can be modulated according to its phosphorylation state. In the intestine, there is evidence alkaline phosphatase is an enzyme that dephosphorylates CD36, which could allow regulation of CD36 activity at the brush border membrane (473).

5.4 VEGF and NO as Modulators of Intestinal Function

Our studies show that VEGF is a paracrine factor secreted by subepithelial myofibroblasts in response to GLP-2 that can enhance lipid transport in intestinal epithelial cells. Though VEGF is more known for its angiogenic effects, there is abundant information that it has trophic functions in the gastrointestinal tract. Upon an injury in the gastrointestinal tract such as gastric ulceration, VEGF production is induced and can be detected in regenerated epithelial cells (474). Gastric mucosal protection against ethanol is conferred after only a single oral dose of VEGF, with the onset of angiogenesis occurring after three weeks of treatment (475). Following small bowel resection, it was found that VEGF derived from salivary glands mediated the adaptive villus heightening in the gut (476). Moreover, encapsulating VEGF in biodegradable microspheres for sustained, controlled release increased villus height and crypt depth 2 weeks following extensive bowel resection (477). Transgenic mice with VEGF expression driven by the villin promoter have intestinal epithelial hyperproliferation and even cyst formation in crypts when the mice were crossed with Multiple intestinal neoplasm (Min) mice. Interestingly, these tumours were not highly vascularized, suggesting that VEGF has neoplastic effects independent of angiogenesis (478). In addition, non-malignant human colonocytes exhibited increased proliferation when co-cultured with fibroblasts and endothelial cells, suggesting that communication between these cells types results in mucosal proliferation independent of blood flow (478).
Like VEGF, NO has been implicated in improving intestinal function. Inhibiting all NOS isoforms with N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) exacerbates jejunal injury induced by endotoxin, but the injury was improved with infusion of the NO donor S-nitroso-N-acetyl penicillamine in rats (479). Very high levels of NO have been shown to reduce intestinal epithelial cell viability, but such levels are achieved by the activity of iNOS, as opposed to the constitutive, calcium-sensitive isoforms that include eNOS and neuronal NOS (480). Since GLP-2 is generally considered anti-inflammatory and has been shown to lower iNOS expression (302; 481), it is likely that the NO arising from GLP-2 action is generated by the constitutive isoforms of NOS. Studies looking at the effect of dietary L-arginine on gut function may also provide clues about the role of NO, since L-arginine is the substrate of NOS. In weaned pigs, dietary L-arginine protected the intestinal epithelium from apoptosis and maintained cell proliferation in response to lipopolysaccharide (482). In a neonatal pig model of necrotizing enterocolitis, an intravenous infusion of L-arginine attenuated tissue damage while L-NAME infusion compounded the problem with hemorrhagic congestion (483). Interestingly, L-arginine supplementation was noted to stimulate mucosal growth independent of mesenteric arterial blood flow, but this effect still proceeded in the presence of L-NAME (484). Therefore, it appears that NO is more instrumental in intestinal repair and healing as opposed to basal levels of growth. NO has also been implicated as a regulator of intestinal absorption. While the opposite has been shown for high pathophysiological levels, physiological levels of NO appears to promote fluid absorption in the gut and directly regulate ion secretion in the enterocyte by activating soluble guanylate cyclase (485). Such a proabsorptive function has also been described for GLP-2, which suppresses chloride secretion in the epithelium (486).

NO can regulate membrane nutrient transporters, but as is the case with intestinal growth and absorption, there are differential effects according to its concentration. Inhibiting constitutive NOS activity with L-NAME actually decreased SGLT-1’s affinity for glucose in IEC-18 cells, while increasing Na/H exchange (NHE3) (487). Interestingly, inhibiting NOS activity has been shown to inhibit SGLT-1 glycosylation, which may have implications for its trafficking and activity (488). In muscle cells, high levels of NO generated by iNOS blunts insulin-stimulated glucose uptake (489). However, lower levels of NO arising from eNOS action have been implicated in stimulating nutrient transport, particularly in insulin-independent
pathways. Exercise training increases eNOS protein levels in skeletal muscle (490), and inhibiting NOS with L-NAME completely prevented exercise-increased sarcolemma GLUT4 content and glucose transport in rats (491). Moreover, L-NMMA at a dose of 0.2 mg kg$^{-1}$ min$^{-1}$ decreased leg glucose uptake during exercise in humans, without any measurable difference in blood flow (492). The NO donor sodium nitroprusside has been shown to increase glucose uptake in 3T3-L1 adipocytes in a guanylate cyclase-dependent manner and without Akt activation (493). In endothelial cells, peroxynitrite, which is generated by NO reacting with superoxide anion, in low levels activates AMPK in a c-Src and PI3-K-dependent and [AMP]/[ATP]-independent pathway (494). The ability of metformin, but not AICAR, to activate AMPK is actually lost in the tissues of eNOS-knockout mice (494). Given the ability of AMPK to promote CD36 trafficking to the sarcolemma in muscle cells (460), it may have a similar effect in enterocytes. Also, considering that AMPK action in the liver leads to bile acid secretion and subsequently dietary lipid emulsification (495), the possibility of AMPK-stimulated CD36 translocation in enterocytes could help to transport the emulsified lipids and thus complement the activity of the liver to maximize calorie absorption during energy-depleted states.

5.5 Concerted GLP-1 and GLP-2 Action

Our findings indicate that two gut peptides that are co-secreted in equimolar amounts paradoxically have completely opposite acute effects on intestinal apoB48 secretion: GLP-2 increased both apoB48 particle and size, while GLP-1 appears to completely blunt apoB48 secretion. It is not unlikely that GLP-1 and GLP-2 act via distinct pathways in modulating intestinal lipoprotein metabolism. In GLP-1-treated rats, there was significantly greater [$^{3}$H]triolein-derived tritium accumulation in the mucosa of the proximal small intestine, suggesting that there was no deficiency in the uptake of luminal FA (394). GLP-2 did not affect MTP levels, while it is possible that the GLP-1-stimulated insulin may indirectly suppress MTP gene expression possibly through forkhead box O1 (FoxO1) (496), thus inhibiting chylomicron assembly in vivo. Labelling studies with [$^{3}$H]oleic acid and [$^{3}$H]acetate indicated that GLP-2 had no effect on de novo lipogenesis and cholesterogenesis (data not shown). There are multiple reports that GLP-1 directly inhibits lipogenic genes in hepatocytes (279; 497), and since apoB48 overproduction has been coupled to SREBP-1c activation in enterocytes (154), the modulation of lipid synthesis may be one pathway through which GLP-1 acts in the intestine.
Given that GLP-1 and GLP-2 are secreted in equimolar amounts, it may be expected that the net physiological effect of GLP-1 and GLP-2 *in vivo* would be no change in postprandial lipemia. However, the indirect mechanism of GLP-2’s hyperlipidemic action, in which GLP-2 stimulates intestinal subepithelial myofibroblasts (and possibly other GLP-2R-positive cell types) to secrete factors that regulate the absorptive enterocyte, would provide an opportunity for amplification of the GLP-2 signal in the intestine. In contrast, such biological amplification is not operative when GLP-1 can directly agonize its receptor in the intestine. Therefore, it would not be surprising if the net outcome of postprandial GLP secretion is enhanced dietary lipid transport. Recent work in our lab has indicated that co-infusion of GLP-1 and GLP-2 at physiological levels in chow-fed hamsters for 120 min results in postprandial hyperlipidemia, indicating a predominantly GLP-2 effect. GLP-2-induced hyperlipidemia was also more dominant in insulin-resistant fructose-fed hamsters, but was attenuated in the context of a mixed meal challenge in which the oral fat load was given in conjunction with glucose. However, in the presence of the DPP-4 inhibitor sitagliptin, the hypolipidemic effect of GLP-1 was predominant (498). The diminution of the hyperlipidemic effect with the inclusion of glucose in the oral load suggests that glucose-dependent insulinotropism, at least in hamsters, is one pathway that mediates GLP-1’s hypolipidemic function. The involvement of insulin may also explain the delayed peak postprandial TG concentration observed with glucose ingestion in humans during a fat tolerance test where ostensibly both GLP-1 and GLP-2 were secreted (499). This would have pathophysiological implications in states of defective insulin secretion or compromised insulin sensitivity, where one arm of GLP-1 influence is unavailable and would thus allow the GLP-2 effect to outweigh that of GLP-1. Hence in STZ-treated rats that are hyperphagic and oversecrete GLP and cannot secrete insulin, the elevated plasma apoB48 levels (500) could be attributed to a dominance of GLP-2. In B6D2F1 mice fed a HFD for 3 weeks to induce insulin resistance, there is greater transport capacity of linoleic acid, along with elevated CD36 expression and higher intestinal mitotic index (161), thereby consistent with a preponderance of GLP-2 action. Moreover, in diseases with dampened GLP secretion such as type 2 diabetes, decreased insulin sensitivity in these patients implies that the reduced GLP-1 levels would have an additionally compromised acute hypolipidemic effect. Meanwhile the GLP-2 molecules, although also reduced in number, could stimulate chylomicron production to...
their full extent. The differing magnitudes of effect may possibly explain the exacerbated postprandial apoB48 secretion in type 2 diabetic individuals (501).

A major contribution of DPP-4-mediated GLP truncation to their net physiological effect on apoB48 secretion is consistent with the conclusions that can be inferred from the sitagliptin-treated fructose-fed hamsters and mice. Sitagliptin administration alone to hamsters and mice attenuated postprandial chylomicron secretion, suggesting that protection of endogenously-produced peptides from DPP-IV yielded a net hypolipidemic response. Though both GLP-1 and GLP-2 are substrates for DPP-4 with similar $K_m$ values, DPP-4’s $k_{cat}$ for GLP-1 is 7.1 s$^{-1}$ as opposed to 0.87 s$^{-1}$ for GLP-2, so the former is degraded 9 times faster (502). Infusing a DPP-4 inhibitor valine-pyrrolidide extended GLP-2’s plasma half-life only by about 3 min in pigs (503), and one study showed that extending the bioactivity of endogenous GLP-2 with valine-pyrrolidide did not affect intestinal morphology in mice or rats (504), so it is not unlikely that there are physiological GLP-2 functions that do not intensify with protection from DPP-4-mediated degradation. Moreover, the site of fat absorption along the longitudinal axis of the intestine may explain the net physiological effect of the GLPs. Within the first half hour of meal ingestion, bile salts can be detected in the duodenum and proximal jejunum, signifying that nutrients have entered the small intestine and the gall bladder has emptied (505). The first phase of GLP secretion also occurs within this time frame (198; 202). Given the absorption of ingested glucose is almost complete within the first half hour, indicating that glucose is transported in more proximal portions of the intestine (506), the insulinotropic effect of GLP-1 would therefore be necessary to combat the ensuing hyperglycemia. On the other hand, dietary lipid is absorbed more distally (507), likely to allow time for fat emulsification to smaller lipid droplets and hydrolytic attack by pancreatic lipase in the intestinal lumen (508). The extended bioactivity of GLP-2 relative to GLP-1 may therefore be more instrumental in intestinal lipid transport. Therefore, in the physiological setting, the hypolipidemic effect of GLP-1 is limited largely by its susceptibility to DPP-4, and this presents implications for therapeutic strategies to address aberrant postprandial lipemia. It appears the hypolipidemic effect of GLP-1 benefits more from protection from DPP-IV, so the DPP-4 inhibitor sitagliptin (marketed as Januvia) or the long-acting GLP-1R agonists exenatide and liraglutide present therapies that can improve diabetic
dyslipidemia. A schematic of the physiological contributions of GLP-1 and GLP-2 to postprandial chylomicron secretion is shown in Figure 5-1.
Figure 5-1. Physiological contributions of GLP-1 and GLP-2 to chylomicron secretion

The enteroendocrine L-cell cosecretes GLP-1 and GLP-2 in equimolar amounts. GLP-1 can either directly inhibit TRL production in enterocytes. In hyperglycemia and intact insulin signalling, GLP-1 stimulates insulin secretion from the pancreatic β-cell, which can also attenuate apoB48 output. The magnitude of GLP-1’s hypolipidemic effect is sensitive to DPP-4. On the other hand, GLP-2 needs to proceed indirectly by first binding its receptor on ISEMFs, and this elicits, among other factors, VEGF secretion. VEGF activates the VEGFR2 receptor tyrosine kinase, which leads to NO activation in the enterocyte and subsequently CD36 activation and dietary FA uptake. The magnitude of GLP-2’s hyperlipidemic effect may depend on DPP-4 activity as well, but likely to less extent.
Despite the complete opposite effects on dietary lipid transport, the biological functions of GLP-1 and GLP-2 may in fact be complementary. The ileal brake is a primary function ascribed to GLP-1 and GLP-2 (509), so that when luminal lipids are detected by enteroendocrine L cells in the distal intestine, thus indicating inefficient proximal nutrient absorption, GLP-1 and GLP-2 is secreted to slow gastric emptying and prevent duodenal delivery of nutrients. This phenomenon is evident in MGAT2-deficient mice, in which the delayed kinetics of dietary FA absorption is accompanied with a two-fold increase in GLP-1 on a high fat diet (54). Similarly, increases in GLP-1 secretion have also been observed with rats fed a 35% fat diet and the intestine-specific MTP inhibitor JTT-130 (510). The bulk of dietary FA is normally absorbed by passive diffusion across the BBM, but by the time dietary lipids could be detected in the distal intestine, the concentration of lipids in the lumen of the proximal intestine would be considerably lower. In this case, it would be advantageous to have GLP-2 increase the expression of CD36 at the apical membrane to provide facilitated transport of luminal FA and stimulate chylomicron assembly. The stimulatory effect of GLP-2 on lipid transport may also serve to coordinate with GLP-1 in mediating satiety. After all, central administration of GLP-2 has been shown to inhibit feeding in rodents (282), and GLP-2 reduces the secretion of the orexigenic hormone ghrelin by about 10% in humans (297). The GLP-2-increased presence of CD36 at the apical membrane may be instrumental in transporting oleic acid as a substrate for the synthesis of oleoylethanolamide (OEA), a satiety factor. Lastly, GLP-2-stimulated VEGF secretion may have implications for the fate of the chylomicrons. VEGF-B has been shown to promote CD36 expression and lipid uptake in vascular endothelial cells, and these lipids are preferentially targeted to muscle, heart, and brown adipose tissue where there are high levels of β-oxidation (511). Such a pathway helps to accelerate peripheral clearance of TRL (512), which in the case of GLP-2-stimulated chylomicron secretion, may help to alleviate the consequences of postprandial lipemia more rapidly. Moreover, insulin, which is secreted in response to GLP-1, is well-known to stimulate lipoprotein lipase activity in adipocytes (513), which would help to catabolize the lipids transported by chylomicrons. Therefore, while the net effect of GLP secretion may enhance intestinal TRL secretion, the persistence of these lipoproteins in circulation may not be of major concern.
5.6 Future Directions

There are still a lot of questions regarding the modulation of postprandial lipemia by GLP-1 and GLP-2. For GLP-1, it remains to be determined the signal that directs dietary FA to be stored in the mucosa as TG (394) rather than for secretion as chylomicrons. The signal could be a result of direct GLP-1R signalling in the enterocyte or arise from other cell types such as enteric neurons. Also of fascination is the fate of the dietary lipid that was not acutely secreted. The possibilities include the FA was oxidized to produce a satiety signal, or mobilized later for secretion as TRL in the early postprandial period of the next meal – the ‘second meal’ effect (514). Therefore, it would be interesting to examine oxidative gene expression, mitochondrial activity, and lipid droplet biology in exendin-4-treated enterocytes. To further explore the mechanism of GLP-1-inhibited chylomicron secretion, it may be worthwhile to delineate the contributions of insulin and direct GLP-1 action in enterocytes. Defining these contributions may have implications for the use of existing therapies in ameliorating diabetic dyslipidemia.

As for GLP-2-stimulated chylomicron secretion, the involvements of other intestinal cell types have not yet been thoroughly investigated. Enteric neurons are a rich physiological source of NO donors and have stimulatory effects on intestinal epithelial cells (357), so this is one intestinal cell type, in addition to subepithelial myofibroblasts, that could potentially signal the enterocyte to increase chylomicron secretion. The role of enteric glia in GLP-2 action could be considered using GFAP-HSVtk transgenic mice, which will have ablation of enteric glial cells following a 14-day administration of ganciclovir (443). In addition, the molecular target of NO in enterocytes can be identified by the trapping of S-nitrosylated and tyrosine nitrated proteins and identification by mass spectrometric methods (515; 516). Lastly, mapping out the pertinent arms of VEGFR2 signalling that drive chylomicron secretion could provide invaluable insight into the basic regulation of lipoprotein metabolism in enterocytes, be it via the PI3-K/Akt pathway, through activation of the MAPK cascade, or as a result of direct stimulation of eNOS itself. Therefore, probing deeper in the mechanism of GLP-1 and GLP-2-regulated postprandial lipemia could contribute greatly to the understanding of intestinal lipoprotein production.
5.7 Conclusions

GLP-1 and GLP-2 are important acute modulators of intestinal lipoprotein secretion and the interplay of these two co-secreted hormones is important to appropriate handling of dietary lipids to maximize absorption and to elicit signalling an appropriate postprandial response in other tissues. While the net physiological and pathophysiological effect may be enhanced chylomicron secretion due to GLP-2, the hypolipidemic action of GLP-1 presents a therapeutic strategy to improve postprandial dyslipidemia.
PERMISSION TO PUBLISH COPYRIGHTED MATERIAL
This is a License Agreement between Joanne Hsieh ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

<table>
<thead>
<tr>
<th></th>
<th>License Number</th>
<th>2724810663588</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>License date</td>
<td>Aug 09, 2011</td>
</tr>
<tr>
<td>3</td>
<td>Licensed content publisher</td>
<td>Springer</td>
</tr>
<tr>
<td>4</td>
<td>Licensed content publication</td>
<td>Diabetologia</td>
</tr>
<tr>
<td>5</td>
<td>Licensed content title</td>
<td>The glucagon-like peptide 1 receptor is essential for postprandial lipoprotein synthesis and secretion in hamsters and mice</td>
</tr>
<tr>
<td>6</td>
<td>Licensed content author</td>
<td>J. Hsieh</td>
</tr>
<tr>
<td>7</td>
<td>Licensed content date</td>
<td>Jan 1, 2009</td>
</tr>
<tr>
<td>8</td>
<td>Volume number</td>
<td>53</td>
</tr>
<tr>
<td>9</td>
<td>Issue number</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>Type of Use</td>
<td>Thesis/Dissertation</td>
</tr>
<tr>
<td>11</td>
<td>Portion</td>
<td>Full text</td>
</tr>
<tr>
<td>12</td>
<td>Number of copies</td>
<td>1</td>
</tr>
</tbody>
</table>

13. Author of this Springer article | Yes and you are a contributor of the new work |
14. Order reference number

15. Title of your thesis / dissertation
   The Acute Regulation of Intestinal Chylomicron Secretion by Glucagon-Like Peptides

16. Expected completion date
   Nov 2011

17. Estimated size(pages)
   200

18. Total
   0.00 USD

19. Terms and Conditions

Introduction
The publisher for this copyrighted material is Springer Science + Business Media. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

Limited License
With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry. Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided it is password protected or on the university's intranet, destined to microfilming by UMI and University repository. For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

The material can only be used for the purpose of defending your thesis, and with a maximum of 100 extra copies in paper.

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, provided permission is also obtained from the (co) author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well). Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted
However figures and illustrations may be altered minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights
Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

Copyright Notice:
Please include the following copyright citation referencing the publication in which the material was originally published. Where wording is within brackets, please include verbatim.
"With kind permission from Springer Science+Business Media: <book/journal title, chapter/article title, volume, year of publication, page, name(s) of author(s), figure number(s), and any original (first) copyright notice displayed with material>.

Warranties: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Indemnity
You hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License
This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing
This license may not be amended except in a writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms
Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions
and those established by CCC’s Billing and Payment terms and conditions, these terms and conditions shall control.

**Jurisdiction**
All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by the country's law in which the work was originally published.
This is a License Agreement between Joanne Hsieh ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
</table>
| 20. Supplier | Elsevier Limited  
The Boulevard, Langford Lane  
Kidlington, Oxford, OX5 1GB, UK |
<p>| 21. Registered Company Number | 1982084 |
| 22. Customer name | Joanne Hsieh |
| 23. Customer address | The Hospital for Sick Children |
| 24. | Toronto, ON M5G 1X8 |
| 25. License number | 2724820175995 |
| 26. License date | Aug 09, 2011 |
| 27. Licensed content publisher | Elsevier |
| 28. Licensed content publication | Atherosclerosis Supplements |
| 29. Licensed content title | Postprandial dyslipidemia in insulin resistant Mechanisms and role of intestinal insulin sensitivity |
| 30. Licensed content author | Joanne Hsieh, Amanda A. Hayashi, Jennifer Webb, Khosrow Adeli |
| 31. Licensed content date | September 2008 |
| 32. Licensed content volume | 9 |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>33.</td>
<td>Licensed content issue number</td>
<td>2</td>
</tr>
<tr>
<td>34.</td>
<td>Number of pages</td>
<td>7</td>
</tr>
<tr>
<td>35.</td>
<td>Start Page</td>
<td>7</td>
</tr>
<tr>
<td>36.</td>
<td>End Page</td>
<td>13</td>
</tr>
<tr>
<td>37.</td>
<td>Type of Use</td>
<td>reuse in a thesis/dissertation</td>
</tr>
<tr>
<td>38.</td>
<td>Intended publisher of new work</td>
<td>other</td>
</tr>
<tr>
<td>39.</td>
<td>Portion</td>
<td>figures/tables/illustrations</td>
</tr>
<tr>
<td>40.</td>
<td>Number of figures/tables/illustrations</td>
<td>1</td>
</tr>
<tr>
<td>41.</td>
<td>Format</td>
<td>both print and electronic</td>
</tr>
<tr>
<td>42.</td>
<td>Are you the author of this Elsevier article?</td>
<td>Yes</td>
</tr>
<tr>
<td>43.</td>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>44.</td>
<td>Order reference number</td>
<td></td>
</tr>
<tr>
<td>45.</td>
<td>Title of your thesis/dissertation</td>
<td>The Acute Regulation of Intestinal Chylomicron Secretion by Glucagon-Like Peptides</td>
</tr>
<tr>
<td>46.</td>
<td>Expected completion date</td>
<td>Nov 2011</td>
</tr>
<tr>
<td>47.</td>
<td>Estimated size (number of pages)</td>
<td>200</td>
</tr>
<tr>
<td>48.</td>
<td>Elsevier VAT number</td>
<td>GB 494 6272 12</td>
</tr>
<tr>
<td>49.</td>
<td>Permissions price</td>
<td>0.00 USD</td>
</tr>
</tbody>
</table>
INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

   “Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER].” Also Lancet special credit - “Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier.”

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the
combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In
no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

**LIMITED LICENSE**

The following terms and conditions apply only to specific license types:

15. **Translation:** This permission is granted for non-exclusive world English rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website:** The following terms and conditions apply to electronic reserve and author websites:

- **Electronic reserve:** If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
  - This license was made in connection with a course,
  - This permission is granted for 1 year only. You may obtain a license for future website posting,
  - All content posted to the web site must maintain the copyright information line on the bottom of each image,
  - A hyperlink must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx) or the Elsevier homepage for books at [http://www.elsevier.com](http://www.elsevier.com), and
  - Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

- All content posted to the web site must maintain the copyright information line on the bottom of each image, and
- The permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,
- A hyper-text must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx). As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier’s online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article’s Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal...
version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com

All content posted to the web site must maintain the copyright information line on the bottom of each image.
You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx), or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.
This is a License Agreement between Joanne Hsieh ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Elsevier Limited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Registered Company Number</td>
<td>1982084</td>
</tr>
<tr>
<td>Customer name</td>
<td>Joanne Hsieh</td>
</tr>
<tr>
<td>Customer address</td>
<td>The Hospital for Sick Children</td>
</tr>
<tr>
<td></td>
<td>Toronto, ON M5G 1X8</td>
</tr>
<tr>
<td>License number</td>
<td>2736901210242</td>
</tr>
<tr>
<td>License date</td>
<td>Aug 27, 2011</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>Elsevier</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>Gastroenterology</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Glucagon-Like Peptide-2 Increases Intestinal Lipid Absorption and Chylomicron Production via CD36</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Joanne Hsieh, Christine Longuet, Adriano Maida, Jasmine Bahrani, Elaine Xu, Christopher L. Baker, Patricia L. Brubaker, Daniel J. Drucker, Khosrow Adeli</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>September 2009</td>
</tr>
<tr>
<td>Licensed content volume number</td>
<td>137</td>
</tr>
<tr>
<td>Licensed content issue number</td>
<td>3</td>
</tr>
<tr>
<td>Number of pages</td>
<td>13</td>
</tr>
<tr>
<td>Start Page</td>
<td>997</td>
</tr>
</tbody>
</table>
INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material
may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

“Reprinted from Publication title, Vol / edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER].” Also Lancet special credit - “Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier.”

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never
granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full
refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

**LIMITED LICENSE**

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world *English* rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:

   **Electronic reserve**: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
   
   This license was made in connection with a course,
   
   This permission is granted for 1 year only. You may obtain a license for future website posting,
   
   All content posted to the web site must maintain the copyright information line on the bottom of each image,
   
   A hyper-text must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx) or the Elsevier homepage for books at [http://www.elsevier.com](http://www.elsevier.com), and
   
   Central Storage: This license does not include permission for a scanned version of the
material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and
he permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,
A hyper-text must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx). As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier’s online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article’s Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at [http://www.elsevier.com](http://www.elsevier.com)
All content posted to the web site must maintain the copyright information line on the bottom of each image
You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.
Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.
19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at [http://www.sciencedirect.com/science/journal/xxxxx](http://www.sciencedirect.com/science/journal/xxxxx). or for books to the Elsevier homepage at [http://www.elsevier.com](http://www.elsevier.com)

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

---

v1.6

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLINK11045309.

Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support: [customercare@copyright.com](mailto:customercare@copyright.com) or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.
REFERENCES


63. Sparks JD, Cianci J, Jokinen J, Chen LS, Sparks CE. Interleukin-6 mediates hepatic hypersecretion of apolipoprotein B. Am J Physiol Gastrointest Liver Physiol 2010;299:G980-G989.


66. Sidiropoulos KG, Pontrelli L, Adeli K. Insulin-mediated suppression of apolipoprotein B mRNA translation requires the 5' UTR and is characterized by decreased binding of an insulin-sensitive 110-kDa 5' UTR RNA-binding protein. Biochemistry 2005;44:12572-12581.


103. Gao N, Qu X, Yan J, Huang Q, Yuan HY, Ouyang DS. L-FABP T94A decreased fatty acid uptake and altered hepatic triglyceride and cholesterol accumulation in Chang liver cells stably transfected with L-FABP. Mol Cell Biochem 2010;345:207-214.


fatty acid-binding protein 2 gene is associated with a change in insulin sensitivity after a change in the type of dietary fat. Am J Clin Nutr 2005;82:196-200.


115. Kim CH, Yun SK, Byun DW, Yoo MH, Lee KU, Suh KI. Codon 54 polymorphism of the fatty acid binding protein 2 gene is associated with increased fat oxidation and hyperinsulinemia, but not with intestinal fatty acid absorption in Korean men. Metabolism 2001;50:473-476.


selective release of GLP-1 in the lymph system. Am J Physiol Regul Integr Comp Physiol 2007;293:R2163-R2169.


446. Doulias PT, Greene JL, Greco TM, Tenopoulou M, Seeholzer SH, Dunbrack RL, Ischiropoulos H. Structural profiling of endogenous S-nitrosocysteine residues reveals


