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Mechanisms of glucagon-like peptide-1 in non-alcoholic fatty liver disease

By

Jamie E. Mells

Doctor of Philosophy, 2011

Graduate Division of Biological and Biomedical Sciences

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A dissertation submitted to the Faculty of the

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Non-alcoholic fatty liver disease (NAFLD) in developed nations is a significant disease burden, but safe and effective medical therapy is still lacking. Glucagon-like peptide (GLP-1) is a hormone secreted from L-cells in the distal ileum and colon. GLP-1 functions as an incretin stimulating glucose-mediated insulin release from pancreatic β-cells. GLP-1 has additional pleotropic functions in mammals including reduction in gastric emptying, inhibiting glucagon secretion, increase satiety and induction of insulin mediated glucose uptake. At present, GLP-1 analogues, including Exendin-4 and Liraglutide, are currently being used in conjunction with other pharmacotherapies for the treatment of type 2 diabetes mellitus. Previous studies performed in our lab have shown that GLP-1 and Exendin-4 significantly reduced steatosis in the non-alcoholic fatty livers of *ob/ob* mice and improve systemic insulin sensitivity. In addition we also demonstrated that both GLP-1 and Exendin-4, increased cAMP production in rat hepatocytes, and reduced mRNA expression of stearoyl-CoA desaturase 1 an enzyme involved in hepatic *de novo* lipogenesis. Nonetheless, there is debate about whether GLP-1R receptors are present and functional in human hepatocytes. The aims of this study were to 1) To characterize the cellular interactions of GLP-1 in hepatocytes; 2) To characterize the normal and abnormal pathways of hepatic lipid metabolism. The results from my study indicate that GLP-1R is present in human hepatocytes, and is able to signal through key elements of the signaling pathway including the phosphorylation of Akt, PDK-1 and PKC-ζ. In addition we found that administering a Western diet causes hepatic insulin resistance that precedes systemic insulin resistance in mice. In addition the diet caused hepatic steatosis, high blood pressure and cardiac hypertrophy. GLP-1 analogues reduce lipid accumulation in hepatocytes, by improving insulin sensitivity, and increasing the expression of critical proteins involved in lipid transport, secretion and β-oxidation; which we hypothesize reduce fat accumulation in the liver. GLP-1 analogues may provide an attractive alternative for use as a medical therapy for NAFLD, particularly in diabetic patients or earlier forms of insulin resistance.

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Acknowledgements

This dissertation is the culmination of lot of tears, prayers, triumphs and setbacks. I certainly could not have finished it without the love and support of my family, friends and my lab.

I would like to first thank my mom for all of her advice and encouragement, and for teaching me how to pray. This journey has increased my faith in God, and you have truly been a blessing in my life. You have always been my greatest champion and you always believed in me. You taught me the importance of persistence, helped me learn what having faith *really* means in my daily life. Words cannot express how I grateful I am to be your son.

I would also like to thank Roger Dickson for being the kind of partner and friend that people dream about. You kept me sane during this long arduous process. You pushed when I needed pushing, and supported me when I could not stand on my own. I cannot imagine my life without you in it- you are truly a light in my world.

I would like give a special thank you to my mentor Frank Anania. You took a chance on me, when no one else would. You have guided me and taught me how to not only be a scientist, but how to survive in academia. Since day one you have always listened to my input. It was the reason I wanted to work in your lab, because I always felt like my input was valued and appreciated. You have been my ally and staunchest defender, and I will be forever grateful.

Next, I would like to thank Neeraj Saxena and Jeffrey Handy. Who hazed me constantly and always made me do more work than I really wanted to do. You taught me the "nuts and bolts" about how to conduct solid research. I am a product of your creation- I hope I will make you proud. Every graduate student should have mentors like you.

Finally I would like to thank Andrew Brown, the Brain Trust, and all of my friends here in Atlanta. I certainly would not have made it without your teasing, laughter, and occasional drunken episode. Thanks for helping make this time wonderful- I am truly blessed.

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List of Abbreviations

4-hydroxy-2-nonenal	HNE
Activated protein kinase	AMPK
Acyl-CoA carboxylase-α	ACCα
Acyl-CoA oxidase II	ACOX2
Adjustable gastric band	AB
Alanine aminotransferase	ALT
American association for the study of liver diseases	AASLD
American lifestyle induced obesity syndrome	ALIOS
Aminotransferase	AST
Apolipoprotein B100	ApoB
Area under the curve	AUC
Body mass index	BMI
Carbon tetra-chloride	CCL4
Cardiovascular disease	CVD
Carnitine palmitoyl transferase-1a	CPT-1A
Centers for disease control	CDC
C-jun n-terminal kinase	JNK
Cyclic AMP	cAMP
Dipeptidyl peptidase IV	DPPIV
Docosahexaenoic acid	DHA
Double incretin knockout mice	DIRKO

Eicosapentaenoic	EPA
Endoplasmic reticular	ER
Fatty acid binding protein	LFABP
Fatty acyl synthase	FASN
Fatty Liver Shionogi Mice	FLS
Free fatty acids	FFA
Glucagon-like peptide 1	GLP-1
G-protein coupled receptor	GPCR
Hematoxylin and eosin stain	H&E
Hepatitis C	HCV
High density lipoproteins	HDL
High fructose corn syrup	HFCS
Immunohistochemistry	IHC
Insulin receptor substrate-1	IRS-1
Insulin receptor substrate-2	IRS-2
Insulin resistance	IR
Intraperitoneally	IP
Knockout	KO
Liraglutide	LG
Liver-specific insulin receptor knockout	LIRKO
Long-chain acyl-CoA dehydrogenase	LCAD
Low-density lipoprotein receptor-deficient mice	$LDL_r^{-/-}$)
Medium-chain acyl-CoA dehydrogenase	MCAD

Methionine choline deficient diet	MCD
Microsomal triglyceride transfer protein	MTTP
Monochemoattractant protein 1	MCP-1
Monounsaturated fatty acids	MUFAS
Muscle-specific insulin receptor knockout	MIRKO
National Health and Nutrition Examination Survey	NHANES
Non-alcoholic fatty liver disease	NAFLD
Non-alcoholic steatohepatitis	NASH
Peroxisome proliferator-activated receptor-α	PPARα
Peroxisome-proliferator activator receptor-gamma	PPAR-γ
Phosphoinositide 3-kinase	PI3K
Polyunsaturated fats	PUFA
Protein kinase C-zeta	PKC-ζ.
Pyruvate dehydrogenase kinase 1,	PDK-1
Quantitative real-time polymerase chain reaction	qRT-PCR
Real-time polymerase chain reaction	RT-PCR
Roux-en-Y gastric bypass	RYGB
Saturated fatty acid	SFA
Stearoyl-CoA desaturase-1	SCD1
Sterol regulatory element-binding protein-1c	SREBP-1C
Thiazolidenediones	TZDs
Toll-like receptor 4	TLR4
Triglyceride	TG

Tumor necrosis factor-alpha	TNF-α
Type 2 diabetes mellitus	T2DM.
Very-low-density lipoprotein	VLDL
Visceral adipose tissue	VAT

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Chapter 1: Introduction

Background and Significance

Obesity and Type II Diabetes

In the United States there has been a dramatic increase in the number of persons classified as obese (body mass index [BMI] ≥ 30 kg/m^2). According to the Centers for Disease Control (CDC) the U.S. has gone from a prevalence of 14-17% in 1994 to ≥ 26.0% in 2007 of the adult population being classified as obese. The data collected from the National Health and Nutrition Examination Survey (NHANES) shows that the number of Americans classified as obese (BMI ≥30) continues to increase, with the greatest increases occurring in adult men (30.4%). Compounding this problem is the disproportionate impact on women and minority groups. In women, 35.5% are classified as obese (*1*). African Americans have the highest prevalence of obesity (44.1%), followed by Hispanics (37.9 %) - Caucasians have the lowest prevalence level (32.8 %) (*1*).

The obesity epidemic also extends to the American youth. Obesity levels have significantly increased in children aged 6 through 19 years (16%) (*2*). Obesity for children and teens is defined as a BMI at or above the 95th percentile for children of the same age and sex (based on the CDC growth charts). Similar to the adult population African American and Hispanic children have significantly higher prevalence rates (22.3% and 20.9 % respectively) than their Caucasian counterparts (17.9%) (*3*).

Alarmingly the trend towards extreme obesity (BMI >40) has also increased within the last ten years; in adult males (2.9%) and females (6.9%) are classified as morbidly obese [4]. Morbid obesity in children aged 6 through 19 years (defined as being ≥ 97th percentile on the BMI-for age growth chart) has reached 11.9%. (*2-4*).

The total cost of medical expenses associated with overweight and obese Americans is estimated to be approximately 80-117 billion dollars (*5*). Additionally, obesity (BMI ≥30) is associated with >111,000 excess deaths as compared to persons of normal weight (*6, 7*). With these epidemiologic trends marking a substantial increase in obese and morbidly obese populations, there is a pressing need for the development of new therapies to combat obesity-induced co-morbidities. Obesity related effects including: increased circulating free fatty acids, hyperinsulinemia, dyslipidemia, hyperglycemia and poor glucose utilization; all of which have been shown to be hallmarks leading to pancreatic β -cell destruction resulting in T2DM.

Numerous studies have demonstrated that obesity is an independent risk factor for the development of T2DM. Epidemiological data supports a strong association between increasing obesity levels and the significant increase diabetes prevalence in the U.S.

Parallel to the obesity trend, the number of new persons diagnosed with diabetes has steadily increase over the last 20 years (*8*). In the year 2000, approximately 11 million Americans were diagnosed with diabetes (*9*), according to the 2011 National Diabetes Fact Sheet approximately18.8 million Americans have diagnosed diabetes (*10*). The CDC estimates that an additional 7 million Americans are undiagnosed, and a staggering 79 million Americans are classified as prediabetic (persons having elevated blood glucose or A1c levels) (*10*).

For those affected by diabetes and extreme obesity, dietary changes, bariatric surgery, and increased physical activity are among treatment options available for improving insulin sensitivity.

A prevailing theory links the induction of insulin resistance to an increasing inflammatory state (*11*). Current research indicates that adipose tissue is an active endocrine gland with high metabolic activity. A variety of inflammatory cytokines, referred to as adipokines, are secreted from adipose tissue. They include, leptin, adiponectin, tumor necrosis factor-alpha (TNF-α), IL-6, IL-8 and monochemoattractant protein 1 (MCP-1) (*12, 13*). This theory attributes the initiation of insulin resistance to changes in adipocyte-mediated adipokine secretion and the activation of the innate immune response (through Toll-like receptors), of particular importance is TNF -α (*14*).

TNF-α has been shown to induce insulin resistance by two mechanisms: (1) by promoting the phosphorylation of serine residues on insulin receptor substrate (IRS-1 and IRS-2) through the activation of serine kinases such as JNK-1, thereby diminishing the insulin-induced tyrosine phosphorylation of IRS-1 and IRS-2; and, (2) by reducing the cellular content of glucose transporter type 4 (GLUT4) mRNA and protein, in skeletal muscle, through the generation of ceramide (*15-18*).

In addition, TNF-α promotes lipolysis in adipose tissue, which leads to a condition that resembles the normal fasting state, with FFA (*19*). Elevated FFAs leads to impaired glucose utilization, because FFAs are preferentially oxidized over glucose. The higher FFA levels also increase the synthesis of hepatic-derived triglycerides (see Fig. 1) (*20*). Consequently, the initiation of TNF-α release results in a dangerous sequence of events. These abnormalities result in abnormal skeletal muscle inflammation, myocellular fat storage, and impaired muscle handling of glucose. Initial studies appeared to support this muscle centric hypothesis.

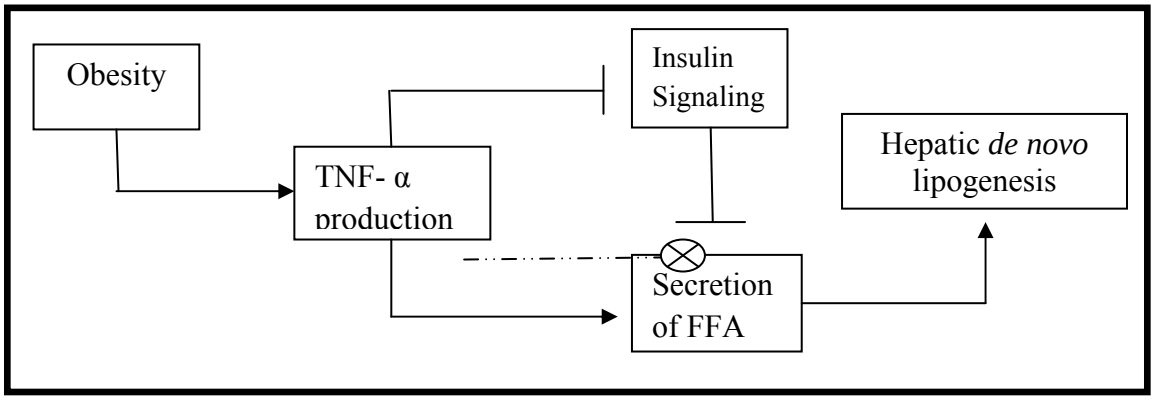


Figure 1.1: Schematic of TNF-α induced chronic inflammation

However, recent data from patients undergoing Roux-en-Y gastric bypass (RYGB) has raised questions about this hypothesis. A significant number of patients undergoing this procedure resolve their diabetes and insulin resistance days after the surgery, without significant changes in weight or adipokine secretion, indicating that insulin resistance may be independent of weight loss, and not related to dysfunction within the myocyte. (*21-23*).

There is a growing body of evidence linking hepatic dysfunction to the development of systemic insulin resistance. Hepatic insulin resistance is defined as the impaired insulin-mediated suppression of endogenous glucose production (*24*). Initial studies by Bruning *et al*, in muscle-specific insulin receptor knockout (MIRKO) mice, showed the mice had elevated plasma triglycerides and free fatty acids (FFA), but normal levels of insulin and glucose (*24-26*). By comparison, studies by Michael *et al.* demonstrated in liver-specific insulin receptor knockout (LIRKO) mice, the absence of insulin signaling ***in the liver*** resulted in severe insulin resistance, glucose intolerance with sustained glucose production (*24, 25*).

Taken together these data suggest that the etiology of T2DM does not appear to be primarily mediated by muscle and adipose tissue. It is clear that the liver plays a pivotal role in development of systemic insulin resistance.

A common feature in both theories (muscle and adipose centric vs. liver centric) is significant lipid accumulation. Hypertriglyceridemia and non-alcoholic fatty liver disease (NAFLD) have strong associations with hepatocyte and adipocyte insulin resistance and obesity. Indeed lipid accumulation, specifically hypertriglyceridemia is common in both T2DM and NAFLD. In addition, NAFLD is an independent predictor of cardiovascular morbidity and mortality (*27, 28*).

NAFLD: pathology—spectrum of disease

Non-alcoholic fatty liver disease (NAFLD) is a co-morbidity of growing concern. Diets high in fat and sugar increase the incidence of NAFLD within a population. NAFLD is characterized by fat accumulation in the liver in the absence of alcohol consumption.

Described as a diagnosis of exclusion: it is a diagnosis describing identical histological finding, liver fat exceeding 5-10% by weight, in persons with minimal alcohol consumption (*29*).

However simple lipid accumulation is not sufficient for NAFLD progression to more significant lesions. NAFL is characterized by fat infiltration (steatosis) that can progress to mixed lobular inflammation. Data from population based studies have consistently demonstrated that this progression is primarily benign (*30-32*). NAFLD can progress to non-alcoholic steatohepatitis (NASH) when accompanied by infiltration of inflammatory cells (*33, 34*). NASH is characterized by Mallory body formation,

ballooning degeneration and pericelluclar fibrosis which can develop into cirrhosis or hepatocellular carcinoma (*30, 35, 36*). A significantly greater proportion of patients with NASH can develop cirrhosis. NASH related cirrhosis is the third leading indication for liver transplantation in the US but it is likely to surpass chronic alcoholism and chronic hepatitis C (HCV) infection as the epidemic of obesity and insulin resistance increases.

NAFLD Epidemiology

NAFLD has become a major health concern in industrialized countries. According to NHANES III, it is estimated that approximately 30% of Americans may have NAFLD (*36*). While this estimate is staggering, it should be noted that NHANES III used elevated serum aminotransferases as a marker of NAFLD. Several studies have shown through the use of liver biopsy, ultrasonography, and magnetic resonance imaging, that a significant portion of patients have NAFLD, without elevated aminotransferase levels, hence there is a significant portion of the population that may be undiagnosed (*32*). NAFLD diagnosis as defined by the American Association for the Study of Liver Diseases (AASLD) as liver fat accumulation exceeding 5% by weight (*30, 32*). Among the obese population it is estimated that 60-70% has hepatic steatosis (*35*). Most alarming is the dramatic rise in NAFLD cases within the pediatric population with current estimates at 3-9% (*37*).

NAFLD--current hypotheses on development

As previously mentioned hepatic dysfunction has been implicated as an important pathophysiologic mediator in NAFLD. Current literature cites the two-hit hypothesis as a possible explanation for progression of NAFLD. The “two-hit” hypothesis implicates

insulin resistance as the so-called “first hit”, resulting in the accumulation of fatty acids that are esterified into stored hepatocyte triglycerides (*33, 36*). The “second hit” is a result of abnormal oxidation of these triglycerides resulting in free radical production, endoplasmic reticular (ER) stress, membrane lipid injury and peroxidation product formation (*36, 38*).

Hepatic lipid accumulation appears to be caused by four inter-related mechanisms (Fig. 2): increased uptake as result of diet and adipocyte lipolysis, increased *de novo* lipogenesis, decreased secretion (specifically VLDL secretion) and finally decreased β-oxidation (*27, 39, 40*). The aberrant storage of lipid in the hepatocyte, under the two-hit hypothesis results in cellular dysfunction (*27, 41*). While the two hit hypothesis appears to explain how steatosis occurs, it does not adequately account for the progression of NAFL to NASH – because as previously mentioned, in the majority of persons diagnosed with NAFL the condition is benign and does not progress to NASH.

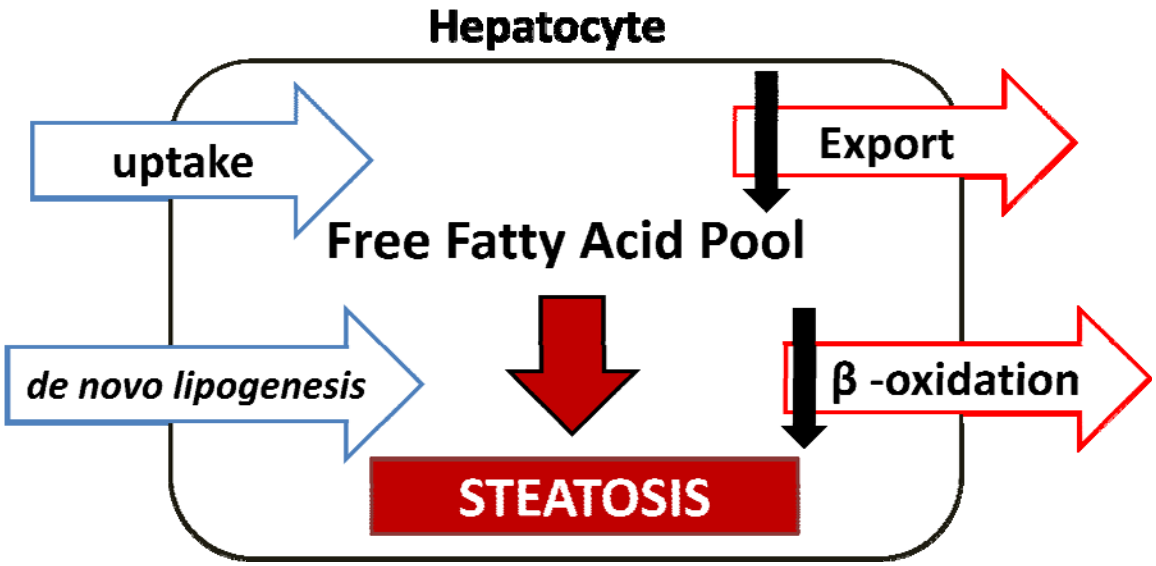


Figure 1.2: Mechanisms of Hepatic steatosis. Increases in uptake and/or *de novo* lipogenesis or decreases in export and/or β –oxidation cause the hepatocyte to store excess lipid creating a steatotic cell.

NAFLD—emerging concept of free fatty acids and the concept of lipotoxicity

There is growing body of data that supports an alternative mechanism for development of NAFLD and NASH. Lipotoxicity is defined as cellular toxicity that occurs in non-adipose tissue in the presence of free fatty acids (FFA) (*42-44*). There is growing support for the notion that triglyceride accumulation is actually a protective mechanism used to abate FFAs toxic effects (*43*). This is a significant departure from the two-hit hypothesis that implicated steatosis as the key factor in the development of NASH. The concept of lipotoxicity examines how cells respond to exposure FFA.

It is clear that simple triglyceride accumulation in hepatocytes is not sufficient to cause disease progression (*45*). Studies by Yamaguchi *et al*. demonstrated in *db/db* mice fed a methionine choline deficient diet (MCD), which induces steatosis, that liver injury and severity increased with ***decreasing*** hepatic triglyceride content (*43, 46*). Additional studies by Ricchi *et al.* in hepatic cell lines (HepG2, Huh7, and WRL68) revealed that oleic acid (unsaturated fatty acid) is more steatogenic than palmitic acid (saturated fatty acid [SFA]), but palmitic acid stimulated more apoptosis and decreased insulin signaling (*41*) . Experiments in our lab on primary human hepatocytes, demonstrated that exposure of hepatocytes to unsaturated free fatty acids resulted in steatosis without significant changes in cell viability or apoptosis (data unpublished). ***Indicating that simple steatosis was not indicative of pathogenesis.***

New data suggests that the type or "quality" of lipid is more important than the quantity when determining the lipotoxic effects (*41, 43, 45*). The ratio of monounsaturated fatty acids (MUFAS) to SFA determines the inflammatory and toxic effect on cells (*45, 47*). Several co-incubation studies have shown that MUFAS improve

SFA esterification processes, and enhance the incorporation of SFA into stable lipid droplets, thereby preventing them becoming lipotoxic and inflammatory (*41, 45, 47*).

Studies by Lee *et al.* demonstrated in a macrophage cell line (RAW264.7) that toll-like receptor 4 (TLR-4) is activated by SFA (*48-50*). This is significant because the activation of the innate immune system is accompanied by the release of TNF- α, creating a pro-inflammatory state (*51, 52*). Furthermore, polyunsaturated fats (PUFA) - docosahexaenoic acid (DHA) and eicosapentaenoic (EPA), preferentially inhibited NFκB activation consequently acting as "anti-inflammatory" agents (*53, 54*). Taken together, this data suggests that an individual's diet may play a critical role in determining a patient's susceptibility to development of NASH.

Changes in Food Manufacturing and it's relation to T2DM and NAFLD: Trans-fatty acids and High Fructose Corn Syrup

The rampant rise in cases of T2DM and NAFLD is associated with changes in the food supply particularly the increase use of trans-fat and high fructose corn syrup (HFCS) in food.

There is increasing evidence that trans-fatty acids are particularly harmful metabolically (*55-57*). Commercially trans-fats are generated from the partial hydrogenation of vegetable oils (*58, 59*). They are found in fried foods, cookies, and baked goods (*56, 60, 61*). Functionally, trans-fatty acids are unsaturated fatty acids with one double bond in the trans configuration (*61*). There is new evidence that suggests that trans configuration causes trans fatty acids to be metabolized differently (*62*). Yu *et al.* found that elaidic acid β-oxidation is slow, due in part to the accumulation of its intermediate metabolite, 5-

trans-tetradecenoyl-CoA (*63*). This accumulation occurs because elaidic's intermediate metabolite is a poor substrate for long-chain acyl-CoA dehydrogenase (LCAD); resulting in only partial degradation of the fatty acid, before it "leaks" from the mitochondria (*63*).

In humans trans fat ingestion is associated with decreases in the high density to low density lipoprotein (HDL/LDL) cholesterol ratio (*56*). Additional studies by Bassett *et al.* in low-density lipoprotein receptor-deficient mice ($LDL_r^{-/-}$) demonstrated that mice fed a diet enriched with trans-fat developed atherosclerotic lesions even in the absence of dietary cholesterol (*64*). Meta analysis studies have shown that intake as low as 2% (4g in 2000 calorie diet) is associated with a 23% increase in cardiovascular risk (*58, 59*). In Denmark, the banning of trans-fat in food has resulted in a 60% decrease CVD incidence (*65*).

The effect of HFCS is different from other carbohydrates because fructose does not stimulate insulin secretion from pancreatic β-cells. Studies using fructose or HFCS have demonstrated that high fructose consumption causes inflammation, leptin resistance, steatosis, and decrease catabolism of fatty acids, which would increase a cell's exposure to FFA (*66-69*).

Separately neither nutrient recapitulates all of the metabolic derangements associated with metabolic syndrome. HFCS studies by Collision *et al.* in mice revealed that although the mice significantly increase intrahepatic lipids and up-regulate several genes related to lipogenesis, the mice did not exhibit dyslipidemia and had normal fasting serum triglyceride levels (*70*). In contrast studies by Dorfman *et al.* showed that rats fed a diet low in fat but enriched with trans-fat had significant increases in visceral fat and

liver steatosis, however they found the diet had no impact on fasting glucose, insulin, and triglyceride levels (*55*). The combination of the trans-fats and HFCS produces insulin resistance and NASH in mice (*71*). This is significant because it indicates that ***insulin resistance is necessary to progress from NAFL to NASH.*** Consequently, pharmaceuticals aimed at ameliorating insulin resistance may also provide benefits to NAFLD patients.

Treatment Strategies for NAFLD: targeting insulin resistance

For those affected by T2DM, NAFLD or extreme obesity, dietary changes and increased physical activity remains the gold standard of treatment. Although lifestyle changes are the most effective treatment option for patients, it is not easily achieved by a large segment of the patient population, necessitating the need for pharmacologic treatment options.

Metformin is an oral hypoglycemic agent that belongs to a class of drugs called biguanides (*72, 73*). Metformin is a highly prescribed anti-diabetic drug. Metformin has been shown to decrease hepatic glucose output, lower serum fatty acid concentrations, while increasing glucose utilization in peripheral tissues [muscle] (*36, 72, 73*). Regarding NAFLD, several clinical studies have demonstrated improvements in serum aminotransferase levels, liver histology and improved insulin sensitivity, but these changes appear to be transient in nature (*36, 72, 73*). Consequently its usefulness in NAFLD treatment remains unclear.

Thiazolidenediones (TZDs) are a class of peroxisome-proliferator activator receptor-gamma (PPAR-γ) agonists also being used to treat T2DM. TZDs (rosiglitazone,

pioglitazone) have been shown to improve insulin sensitivity, decrease hepatic fat content, and decrease TNF-α release by adipose tissue (*35, 36, 72-74*). TZDs function by increasing adiponectin and inhibiting sterol regulatory element-binding protein-1c (SREBP-1C) effectively shutting down the expression of key genes related to *de novo* lipogenesis (*72, 73*). However, several studies have raised safety concerns about the use of TZDs specifically rosiglitazone (Avandia®). A meta-analysis study by Nissen and Wolski showed that patients prescribed rosiglitazone had a significantly higher risk of myocardial infarction, prompting the European Union to ban the use of rosiglitazone in the Europe; The US FDA has now placed significant restrictions on doctors prescribing Avandia® (*75, 76*).

The PIVENS (**Pi**oglitazone versus **V**itamin **E** versus Placebo for the treatment of non-diabetic patients with **N**onalcoholic **S**teatohepatitis) multi-center, phase 3 trial, recently concluded that vitamin E therapy was superior to pioglitazone in the treatment of NASH (*77*). There are several caveats worth mentioning about the PIVENS trial. First, it should be noted that the PIVENS trial excluded patients with diabetes or with a fasting plasma glucose ≥126 mg/dL, and as previously mentioned a significant number of patients with NAFLD and NASH have some form of insulin resistance (*77, 78*). Furthermore, although there were significant improvements in histological features and steatohepatitis-this occurred in less than 50% of study population. Moreover, either vitamin E or pioglitazone reduced fibrosis or portal inflammation during the study (*77*). Finally, TZDs have also been linked to excessive fluid retention, weight gain, and increased bone fracture rate in patients (*72*). Indicating that vitamin E therapy would help only a very small subset of the NASH population, and TZD therapy has considerable risks that do not

make it advantageous. Consequently, for patients with insulin resistance and NAFLD or NASH there remain very few treatment options for physicians.

Structure and Function of GLP-1

Because of limited available medical therapy specifically targeting NAFLD; and the results of post bariatric surgery clinical studies demonstrating (1) increased circulating glucagon-like peptide 1 (GLP-1) and (2) rapid elimination of hepatic steatosis our laboratory has been interested in GLP-1. GLP-1 is an incretin that has been shown to improve insulin secretion, by increasing insulin gene transcription, and stimulating glucose-dependent insulin release (*79, 80*).

GLP-1 is a 30 amino acid incretin released in response to nutrient ingestion potentiating the effect of glucose on insulin secretion (*81, 82*). GLP-1 is part of the pre-pro-glucagon molecule that is processed and released from **L**-cells in the distal ileum and proximal colon (*81, 83*). GLP-1 has numerous pleotropic effects including: increased satiety, lower energy intake, delayed gastric emptying and increased lower gastrointestinal motility (*83, 84*).

The two active forms of GLP-1 are GLP-1 (7-36) and GLP-1 (7-37) (*85*). Both forms are rapidly cleaved by dipeptidyl peptidase IV (DPPIV) rendering the peptide inactive; the half-life of GLP-1 is 1 to 1.5 min (*86*). Due to the rapid degradation of GLP-1, several DPPIV-resistant analogues with longer half-lives have been developed. GLP-1 and two of its long-acting synthetic analogues (exendin-4 and liraglutide) have been approved by the US FDA for treatment of T2DM.

Exendin-4 is a 39 amino acid GLP-1 analogue derived from the saliva of the Gila monster, *Heloderma suspectum* (*36, 83, 87*). Exendin-4 has 53% sequence homology with native human GLP-1 peptide, and similar affinity and potency (*86-88*). This twice a day analogue has a half-life of approximately 3.5-4 hours after injection, and peak action approximately 2-3 hours after subcutaneous injection (*87*). Liraglutide is a once daily GLP-1 analogue with a half-life of 11-13 hours (*86, 87*). Unlike exendin-4, liraglutide shares 97% sequence homology with human GLP-1 (*86, 87*). It has two modifications: an amino acid substitution at position 34 (arginine for lysine) and the attachment of a C-16 acyl chain (*86, 87*). Numerous studies have examined how GLP-1 and exendin-4 exert their physiologic effects through its cognate receptor glucagon like peptide-1 receptor (GLP-1R).

The role of GLP-1R: mechanisms of action

Cloned in 1992 by Dr. Thorens, the GLP-1R receptor is a seven transmembrane protein made up 463 amino acids (*81*). GLP-1 is a member of the B family of GPCRS (*89*). Known as the secretin-receptor family, receptors in this family have three extracellular loops, an amino terminal extracellular domain, and a intracellular carboxyl terminus (*89*). This family of receptors is characterized by their ability to regulate intracellular concentrations of cyclic AMP by coupling to adenylate cyclase, through the stimulatory G protein (G_s) (*82, 89*).

The GLP-1R receptor exhibits a high degree of sequence conservation between species (human GLP-1R shares 90% sequence homology with rat), and is highly specific for GLP-1 (*82*). However there appears to be some disparity in tissue distribution of the receptor. Studies by Sandu *et al.* in canines revealed that dogs have a vastly different

receptor distribution than mice and humans (*90*). Initial human studies revealed the receptor was distributed in the hypothalamus, pancreatic β-cells, lung and stomach in humans (*82*).

Rationale for Dissertation

In 2006 our lab demonstrated that both GLP-1 and exendin-4 were able to significantly reduce liver steatosis in mice (*36*). Initial studies in our lab in *ob/ob* mice demonstrated that treatment with exendin-4 for 60 days, resulted in significantly improvements in fasting glucose and serum ALT. Exendin-4 treated mice also had significant reduction in liver lipid accumulation and oxidative stress. Finally, we demonstrated that like other tissues, GLP-1R appeared to activate cyclic AMP (cAMP).

The original study raised several questions and observations from the scientific community. There were several criticisms levied against the study. The largest concern was that no lab had been able to demonstrate that there was a functional receptor present in human hepatocytes. As previously mentioned it had been demonstrated by others that there are wide species variations in mammals, consequently, the use of mouse model was deemed inappropriate because a receptor had not been found in human liver cells.

Coupled with the lack of evidence of liver specific receptor, many critics of the study believed the effects observed in our initial paper were a secondary effect caused by increased insulin production. Numerous studies have shown that GLP-1 and its analogues increase insulin secretion from β –cells, and prevented insulin resistance caused by high fat diets (*91-93*). Although this assertion was not supported by our data, (exendin-4 treated animals had significantly lower fasting insulin levels, than saline injected animals).

Finally, the last observation/criticism of the study was the effects observed in this initial study could be a secondary effect caused by the weight loss in the exendin-4 treated mice. GLP-1 and analogues are all known to cause transient reductions in feeding in mice (*94, 95*). Studies by Baggio *et al.* in transgenic mice with sustained elevations of exendin-4 revealed no significant change in feeding behavior (*79, 96*). At issue, in this initial study --was there an actual reduction in food intake in the exendin-4 treated mice or is the weight "loss" observed caused by other factors (i.e. increased β –oxidation). During the initial study the lab failed to provide caloric intake data, so there was no way to refute this argument.

At the heart of the GLP-1R controversy is the notion that GLP-1 can directly regulate glucose production and utilization independent of its effects on the pancreas (*97*).

Therefore in the first study of this project, we sought to: 1) characterize GLP-1 binding to its receptor, 2) determine if there was a functional receptor present in primary human hepatocytes, 3) and to determine if we could recapitulate the findings our initial study (i.e. reduction in lipid accumulation) in an *in vitro* human cell culture model, in the ***absence of insulin***.

Consequently, the first aims of my dissertation are:

Specific aim 1: To characterize the cellular interactions of GLP-1 in hepatocytes.

- The GLP-1/GLP-1R ligand-receptor activity can be used to better understand the pathophysiology of NAFLD.

- GLP-1 and its analogues (exendin-4 and liraglutide), have a novel function as an insulin sensitizing agent in hepatocytes, outside of its incretin effects.

The second part of my project focuses on the how nutrients- specifically, HFCS and trans-fat affect lipid metabolism, and the role GLP-1 and its analogue affect lipid metabolism. The second study in this project would return to an *in vivo* model with significantly more controls. Taking advantage of pair-feeding, hyperinsulinemic clamp techniques, and the publication of a new diet that induced hepatic fibrosis within 16 weeks we sought to: 1) determine when hepatic insulin resistance occurs, 2) determine if effects observed in our initial study were the result of changes in feeding behavior, 3) determine if a new analogue (liraglutide) could produce similar effects as seen in our initial paper.

Consequently, the second aim of my dissertation is:

Specific aim 2: To characterize the normal and abnormal pathways of hepatic lipid metabolism.

- GLP-1 has a beneficial consequence of eliminating the storage of hepatocyte triglycerides, a key feature in NAFLD.

Consequently the hypothesis for my dissertation is:

Hypothesis: GLP-1 and its synthetic analogues act directly on the hepatocyte to reduce net hepatic triglyceride stores via activation of its cognate receptor.

Chapter 2: Glucagon-like Peptide-1 Receptor (GLP-1R) is present on human hepatocytes and has a direct role in decreasing hepatic steatosis *in vitro* by modulating elements of the insulin signaling pathway

Abstract

BACKGROUND: Glucagon-like Peptide-1 (GLP-1) is a naturally occurring peptide secreted by the ***L***-cells of the small intestine. GLP-1 functions as an incretin and stimulates glucose-mediated insulin production by pancreatic β-cells. In this study, we demonstrate that Exendin-4/GLP-1 has a cognate receptor on human hepatocytes; and that Exendin-4 has a direct effect on the reduction of hepatic steatosis in the absence of insulin. **RESULTS AND DISCUSSION:** Both GLP-1R mRNA and protein were detected on primary human hepatocytes, and receptor was internalized in the presence of GLP-1. Exendin-4 increased the phosphorylation of PDK-1, AKT, and PKC-ζ, in HepG2 and HuH-7 cells. siRNA against GLP-1R abolished the effects on PDK-1 and PKC-ζ. Treatment with Exendin-4 quantitatively reduced triglyceride stores compared to control-treated cells. **CONCLUSION:** This is the first report that the G-protein coupled receptor (GPCR) GLP-1R is present on human hepatocytes. Furthermore, Exendin-4 appears to have *in vitro*, the same beneficial effects we have seen in our previously published *in vivo* study in *ob/ob* mice, directly reducing hepatocyte steatosis. Future use for human non-alcoholic fatty liver disease, either in combination with dietary manipulation or other pharmacotherapy, may be a significant advance in treatment of this common form of liver disease.

Background

GLP-1 is a peptide product of the ***L***-cells of the small intestine and proximal colon and has been the subject of considerable laboratory research over the past two decades. Although the primary function of GLP-1 is to serve as an incretin in β cells of the mammalian pancreas, the functioning peptide is quickly cleaved by the dipeptidyl peptidase IV (DPPIV) rendering the peptide functionally inactive (*98-100*). The principle pleotropic effects of GLP-1 include enhanced satiety, delayed gastric emptying (*101, 102*) and increased lower gastrointestinal motility (*98, 103*). GLP-1 binds to its cognate receptor, glucagon-like peptide-1 receptor 1 (GLP-1R), a G-protein coupled receptor (GPCR) that has been found in many tissues including the brain and pancreas (*101, 104*). However, great consternation persists about whether GLP-1 has a functioning receptor on hepatocytes. Mice which lack GLP-1R (DIRKO) do not seem to have marked hepatic metabolic changes (*105-109*). Exendin-4 is a 39 amino acid agonist of GLP-1R, and is derived from the saliva of the Gila monster, *Heloderma suspectum*. At present Exendin-4 is being used to augment insulin production in type 2 diabetics (*110*). While we recently published that Exendin-4 significantly reduced hepatic steatosis found in *ob/ob* mice we did not elucidate a cellular mechanism, nor determine if the effects were the result of direct action on hepatocytes, or is such beneficial effects were related to non-hepatic effectors (*36*).

Non-alcoholic fatty liver disease (NAFLD) is strongly associated with other clinical features of the metabolic syndrome including obesity, type 2 diabetes mellitus, hypertension, and dyslipidemia. Insulin resistance is a central feature of "metabolic syndrome." Hepatocyte insulin resistance in particular—in part related to impaired

insulin signal transduction—may be a key problem in the development of hepatocyte steatosis. Here we positively identified the GLP-1 receptor in not only transformed hepatocytes HuH7 and Hep-G2 cells, but also in primary human hepatocytes. We have also demonstrated, as with other GPCRs, on binding to its ligand, GLP-1R internalizes (*100*). GLP-1 or Exendin-4 can activate key signaling molecules downstream of insulin receptor substrate-2 (IRS-2). Furthermore, in the absence of insulin, we demonstrated a significant loss of triglycerides from steatotic hepatocytes following Exendin-4 treatment. To our knowledge this is the first report that convincingly demonstrates GLP-1R on hepatocytes, and provides a signaling mechanism whereby GLP-1 proteins can independently reduce hepatocyte triglyceride accumulation.

Materials and Methods

Hepatocyte cultures: Hep-G2 and HuH7 cells were purchased from ATCC (Manassas, VA) and cultured using DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Hyclone, Logan, Utah). Cells were treated with 10nM of GLP-1 or Exendin-4 10nM (Sigma, St. Louis, MO) for varying time intervals from 5 minutes to 12 hours in accordance with previously published reports (*111, 112*).

Primary Hepatocyte Culture: Primary hepatocytes, purchased from Lonza (Allendale, NJ), were grown to confluence in media (HMM CC-3197 with HMM single quots CC-4192) on collagen coated plates (BD-Biosciences, Bedford, MA), at a density of 0.15 mL cells/0.5 mL media. RNA and protein were subsequently extracted. This was done in the absence of insulin.

RT-PCR: Total RNA was extracted from HuH7 and human hepatocytes by TRIzol ® reagent (Invitrogen). PCR was performed using primers for GLP-1R: 5'- TTG GGG TGA ACT TCC TCA TC-3' for forward and 5'-CTT GGC AAG TCT GCA TTT GA-3' for reverse, and real time PCR was performed.

Immunoblot assay to detect GLP-1R and Exendin-4 signaling pathway: Lysate from HuH7 and HepG2 cells were prepared after treatment of the cells with Exendin-4 or GLP-1 for 5, 15, 30, 60, 90, 180, and 360 minutes. Equal amounts of protein were resolved on SDS-PAGE (*113*), transblotted, and subjected to immuno-detection using primary antibody for GLP-1R which was purchased from abcam (ab39072; 1:500), the

phosphorylated and total species of PDK-1, AKT, and PKC-ζ. β-actin served as a loading control (*113*).

Sub-cellular fraction analysis: HuH7 cells were treated with Exendin-4 for 30 min and one h. Cells treated with pre-immune serum served as controls. Cytosolic, membrane and nuclear fractions were separated using manufacturer's instructions (Biovision # K270). These fractions (20 μg) were resolved on SDS- PAGE and subjected to immuno-detection against the GLP-1R antibody.

Bioluminescence Assay: Cell surface expression assays were performed as previously described by Xu *et al* (*114*). Briefly, 35 mm^3 collagen coated dishes (BD#354459) were used to plate an equal number of HuH7 cells. The cells were treated with GLP-1 or Exendin-4 for 4 min, 10 min and 30 min. After cells were formalin fixed, cells were treated with 3% non-fat dry milk in PBS and subsequently incubated with primary antibody, Anti-GLP1-R [1:500] for 1 h, followed by secondary antibody [1:1000]. Antibody-receptor binding was detected by supersignal Elisa pico enhanced chemiluminescence (ECL) reagent (Pierce, Rockford, IL). The luminescence, which corresponds to the amount of receptor on the cell surface, was determined by using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). Control cells were treated with pre-immune serum.

Immunoflorescence and Confocal Microscopy: To visualize GLP1-R , HuH7 cells were grown on chamber slides and treated with GLP-1 or Exendin-4 for 4 min, 15min, 30 min and 1h and routine immunostaining was performed. Briefly, the cells were fixed with paraformaldehyde, permeabilized with 0.5% triton X-100 + 0.08% saponin in H^+ at 25^o

for 40 min and then incubated with 50 µl of rhodamine phalloidin diluted 1:60 at 25^{o} for 45 min. Cells were blocked with 2% BSA for 1 h at 25^{o}, followed by incubation with primary antibody, GLP1-R [1:200] overnight at 4^{o}C. After washing, cells were incubated with secondary antibody (Anti-rabbit FITC). Fluorescence and confocal microscopy were performed.

Oil Red O staining and triglyceride assays: HuH7 and Hep G2 cells were exposed to medium containing 1% free fatty acids (FFA)-free BSA, and fat loaded with 400µM of palmitic and oleic acid (Sigma). After 12 h, cells were treated with 20 nM of Exendin-4 for 6 h and stained with Oil Red O staining (Polyscience, Niles, IL) to visualize triglyceride (TG) accumulation. TG quantification assay was performed following manufacturer's instructions (Biovision # K622-100). These experiments were conducted in the absence of insulin.

Methionine-choline Deficient (MCD) Media Treatment and Flow Cytometric Analysis: After serum starvation for 24 h HepG2 cells were exposed to either control media or MCD medium (**Gibco)** supplemented with 10% FBS as previously described (*115*). These experiments were also conducted under insulin-free conditions. Cells were treated with Exendin-4 for up to 24 h and stained with Nile Red (MP Biomedical, Solon, Ohio) at a concentration of 0.5µg/ml and incubated for 15 min at 37^{o}C as previously described (*116*). Flow cytometry was performed. Briefly, cells were resuspended in PBS plus 0.5% BSA and analyzed on a FACS Calibur (Becton Dickinson, San Jose, CA) on the FL3 channel (670LP filter with 488nm excitation) and FL4 channel (660/20 filter with 633nm excitation).

siRNA against GLP-1R. Three primer pair sequences for siRNA-GLP-1R and negative control (Stealth Negative control) were purchased from Invitrogen as shown in Table 1.

Table 2.1. Primer Pairs for siRNA Experiments

siRNA–Glp-1R	Forward	Reverse
Primer 1	5′-UCCACCAAGAGCCAGUAGUAAUUGG-3′	5′-CCAAUUACUACUGGCUCUUGGUGGA-3′
Primer 2	5′-AUAAUGAGCCAGUAGUUCAUGUUGG-3′	5′-CCAACAUGAACUACUGGCUCAUUAU-3′
Primer 3	5′-AUGACCCGAACAAAGAUGAGGAAGU-3′	5′-ACUUCCUCAUCUUUGUUCGGGUCAU-3′

HuH7 cells were transfected using Lipofectamine® RNAiMAX reagent (Invitrogen) following the reverse transfection protocol by the manufacturer. Cells were plated at 50% confluency and transfected with the siRNA sequences at 30 nM and maintained for 48 h. GLP-1R knock down was confirmed by immunoblot. Cell lysates were prepared and subjected to immunoblot analysis for GLP-1R, PDK1, AKT and PKC-ζ.

***Statistical Analyses*:** The data are presented as the mean ± SE. Statistical analysis was performed using Graphpad Instat 3 software (www.graphpad.com). Groups were compared using parametric tests (paired Student *t* test or one-way ANOVA with posttest following statistical standards). *P* values of less than .05 were considered statistically significant.

Results

Confirmation of GLP-1R on HuH7 cells and human hepatocytes. Western blot analysis revealed the presence of GLP-1R in HuH7 cells and primary human hepatocytes. **(Figure1A).**

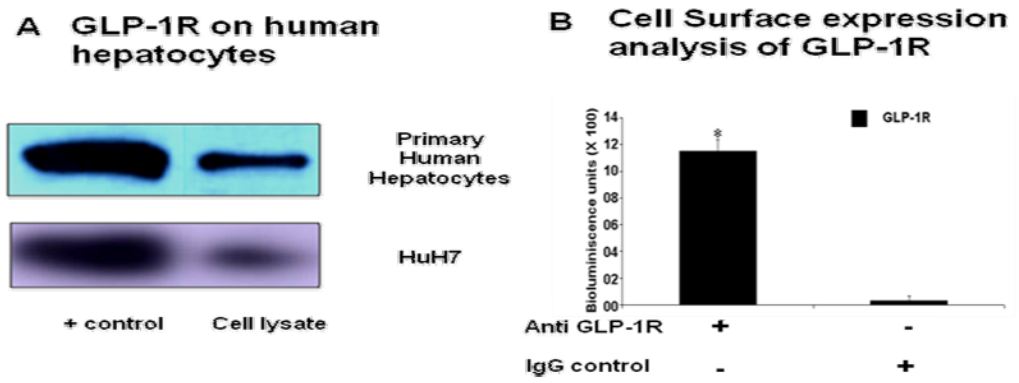


Figure 2.1: Identification of GLP-1R on hepatocytes. A: Primary human hepatocyte and HuH7 cells show the presence of GLP-1R by western blot analysis. Brain lysate was used as a positive control. **B:** Bioluminescence analysis for GPCRs demonstrates the presence of GLP-1R on HuH7 cells. Bars represent percent increase in bioluminescence in GLP-1R as compared with no- primary antibody treatment. Data are presented as the mean ± SE; *$p < 0.05$ versus control no primary antibody monolayer. The experiment was repeated three times in triplicate.

Bioluminescence confirms specific GLP-1R localization. As shown in **Figure 1B** there was a multifold increase of the GLP-1R for HuH7 cells as compared to the pre- immune serum treated controls ($p<0.05$).

GLP-1R is internalized in HuH7 cells. GLP-1R is internalized on stimulation by GLP-1 or Exendin-4 (**Figure 2**). This was first demonstrated by cell surface expression analysis (bioluminescence assay) as described previously in Figure 1C. Upon stimulation with either GLP-1 or Exendin-4, there was a decrease in the amount of receptor seen on the cell membrane as seen by confocal microcopy. We then confirmed the microscopic findings by sub-cellular fractionation as seen in **Figure 2B.** This demonstrated that following GLP-1R exposure to its agonist, the membrane-bound fraction was reduced. These data suggest that there is loss of the receptor from the cell membrane.

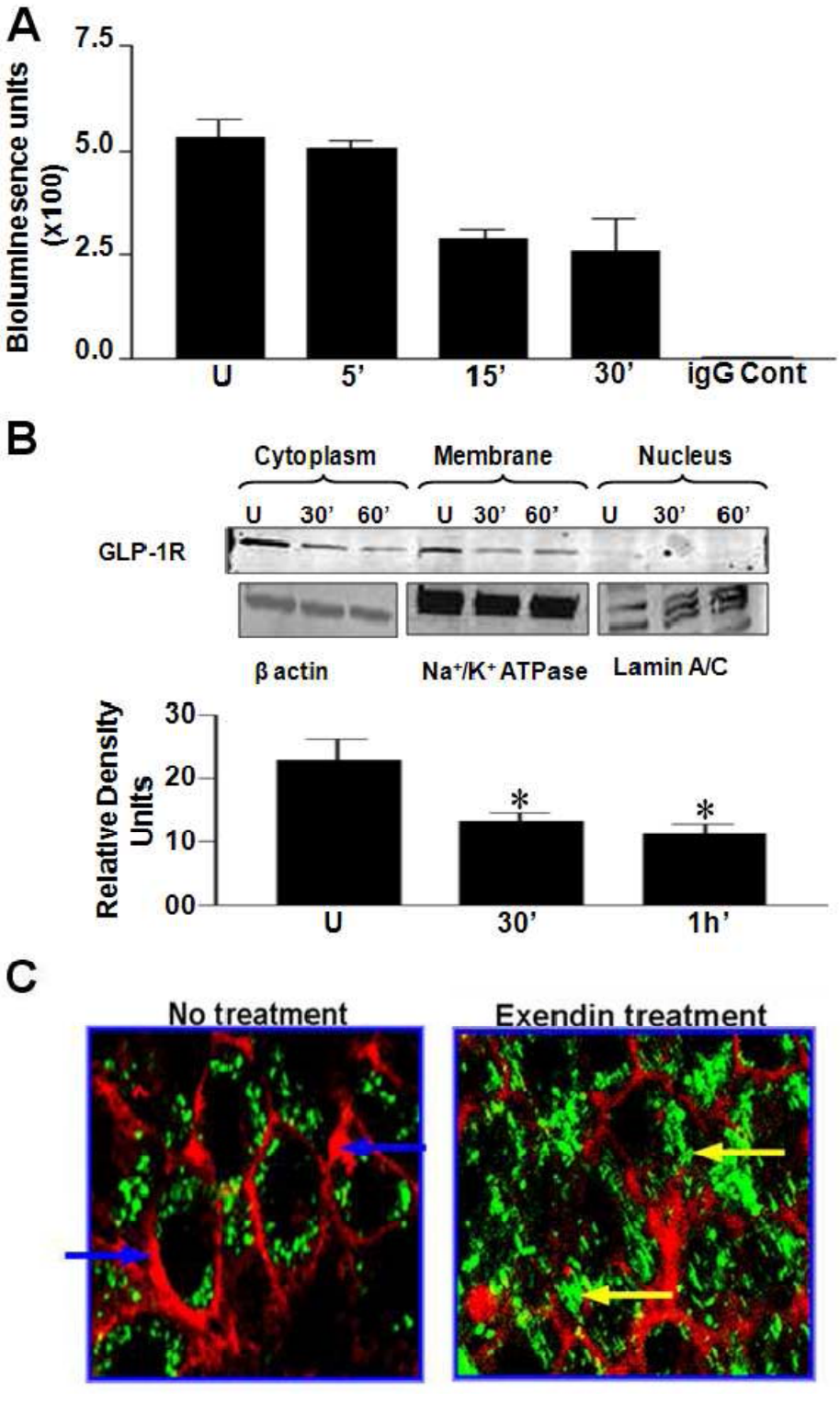

A
Bioluminescence units (x100)
7.5
5.0
2.5
0.0
U
5'
15'
30'
igG Cont
B
Cytoplasm
Membrane
Nucleus
U 30' 60' U 30' 60' U 30' 60'
GLP-1R
β actin
Na+/K+ ATPase
Lamin A/C
Relative Density Units
30
20
10
00
*
*
U
30'
1h'
C
No treatment
Exendin treatment

Figure 2.2: GLP-1R is present on the plasma membrane and was internalized upon agonist stimulation. A: Monolayers were stimulated with GLP or Exendin-4 for 5 min, 15 min, 30 min. (10 nM) as described in MATERIALS AND METHODS. Bars show % decrease in bioluminescence compared with unstimulated monolayer (means ± SE; $*p < 0.05$ compared with unstimulated monolayer). The experiment was repeated three times and compared with untreated controls and pre-immune serum treated controls. **B**: Cell fractionation of HuH7 monolayers was performed as described in MATERIALS AND METHODS. Samples from membrane (M), cytoplasm(C) and nuclear (N) fractions were subjected to Western blot analysis using anti-GLP-1R antibody (1:500). Blots were also probed for Na^+-K^+-ATPase, Lamin A/C and β-actin to confirm equal protein loading. The results are representative of 2 independent experiments. **C**: Confocal imaging of GLP-1R was performed on filter grown monolayers of HuH-7 cells. Cells were stained with rabbit polyclonal antibody against GLP-1R (1:200) followed by Alexa Fluor secondary antibody. Rhodamine (blue arrow) was used to stain the cytoskeleton. In **1)** GLP-1R (yellow arrow) is seen localized to the membrane and in **2)** upon agonist stimulation GLP-1R is decreased from the membrane.

Fluorescence and Confocal Microscopy confirm bioluminescence and sub-cellular fractionation results. Both confocal and fluorescent imaging confirmed that GLP-1R is internalized. In **Figure 2C** (left panel) displays untreated cells where GLP-1R (detected in green) is seen lining the cell membrane. On treatment with GLP-1 or Exendin-4 the receptor (Figure 2C, right panel) was detected primarily in the cytoplasm than on the plasma membrane (yellow arrows). These data support the detection of internalization of the receptor by bioluminescence assay which was also confirmed by sub-cellular fractionation analysis.

Exendin-4 reduces triglyceride stores in HuH7 and HepG2 cells. To determine whether a physiologic endpoint of putative GLP-1 receptor signaling could be achieved, we explored whether, following Exendin-4 treatment there was a significant reduction in the cellular triglyceride content by several approaches. As seen by images following Oil Red O staining (**Figure 3A),** following engorgement of HuH7 cells with palmitate and oleate, Exenidin-4 greatly reduced TG stores; and, this was further corroborated by TG quantitation (**Figure 3B).** The reduction in cellular lipid content (both neutral and polar lipids) by Exendin-4 was also confirmed using flow cytometry, with Nile Red staining in cells rendered steatotic by methionine-choline deficient media (**Figure 3C).**

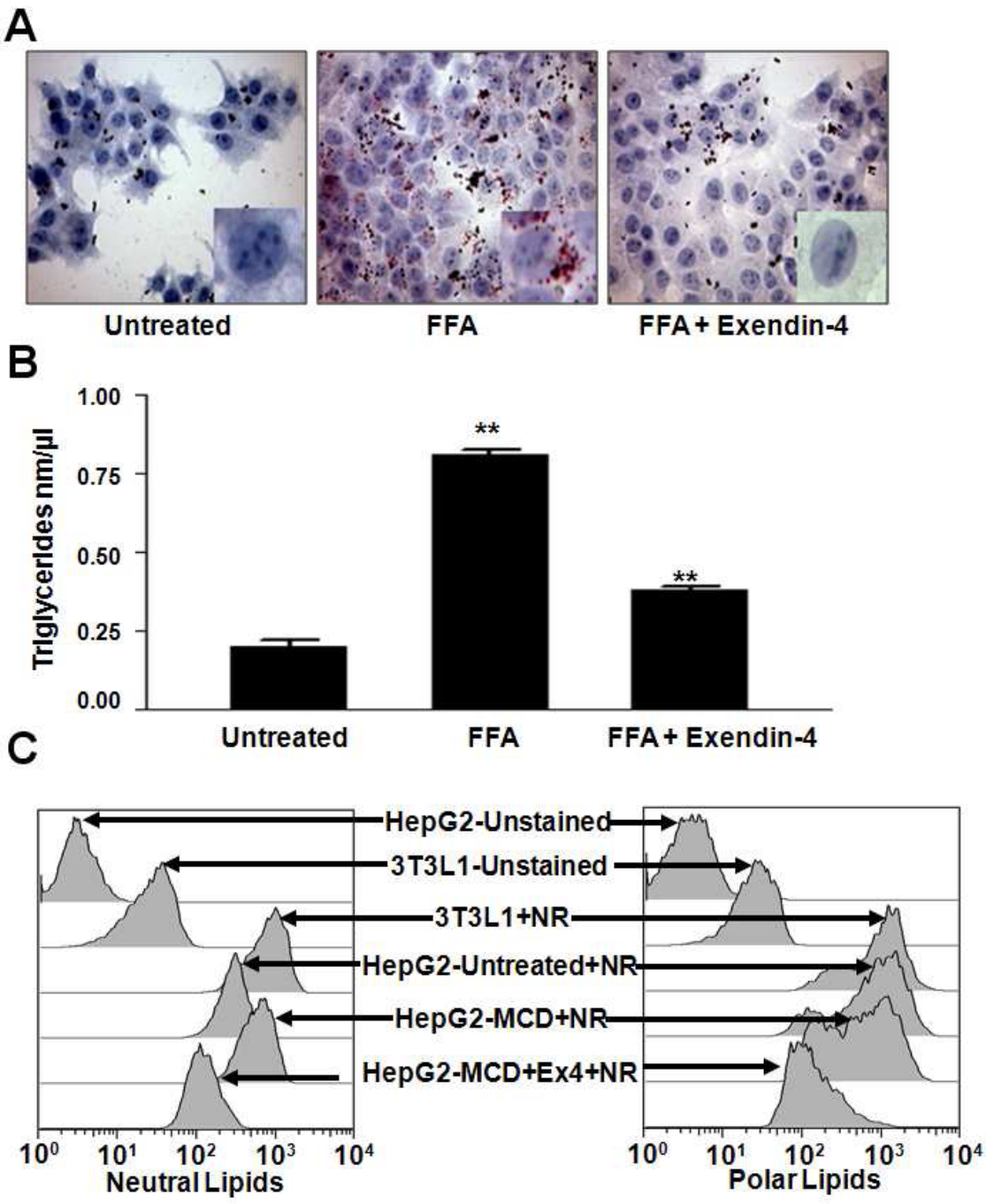

A
Untreated
FFA
FFA + Exendin-4
B
Triglycerides nm/µl
1.00
0.75
0.50
0.25
0.00
**
**
Untreated
FFA
FFA + Exendin-4
C
HepG2-Unstained
3T3L1-Unstained
3T3L1+NR
HepG2-Untreated+NR
HepG2-MCD+NR
HepG2-MCD+Ex4+NR
10⁰ 10¹ 10² 10³ 10⁴
Neutral Lipids
10⁰ 10¹ 10² 10³ 10⁴
Polar Lipids

Figure 2.3: Reduction of steatosis on exposure to exendin-4. (A) Huh7 cells were treated with palmitic acid (400 μM/L) and oleic acid (400 μM/L) for 12 hours under insulin-free conditions and subsequently exposed to exendin-4 (20 nM) for 6 hours. A marked increase in Oil Red O–stained droplets (red) are visible in Huh7 cells treated with free fatty acids (FFA) compared with untreated cells. Exposure to exendin-4 resulted in a significant loss of fat droplets (original magnification ×40). (B) TG assay was performed on Huh7 cell lysate after treatment with palmitic and oleic acid followed by exposure to exendin-4 as described in Materials and Methods. Bars represent the percent increase in TG content and the percent decrease on treatment with exendin-4. Data are presented as the mean ± SE. $^{**}P < 0.05$ versus untreated steatotic cells. The experiment was repeated three times in triplicate and compared with free fatty acids exposed and untreated controls. (C) HepG2 cells were grown in either control media or methionine-choline–deficient media. Cells were then treated with exendin-4 for 24 hours. Following treatment, intracellular lipids (polar and neutral) were stained with Nile Red (0.5 μg/mL). Flow cytometry was performed as described in Materials and Methods. 3T3L1 cells served as a positive control. The data are representative of three independent experiments.

Exendin-4 increases phosphorylation of PDK-1, AKT, and PKC-ζ proteins. Exendin-4 resulted in a significant increase in phosphorylation at 60 min of PDK-1, and AKT **(Figure 4)** ($p<.05$,). The phosphorylation of PKC-ζ was significantly increased at 30, 60, and 90 min ($p<.05$) (**Figure 4**). siRNA against GlP-1R (Supplemental figure) was used to abolish effects seen in Huh7 cells treated with Exendin-4. The knockdown of GLP-1R abolished the effects for PDK-1 and PKC-ζ (**Figure 5),** $p<.05$, n=3**)**, but not AKT (data not shown).

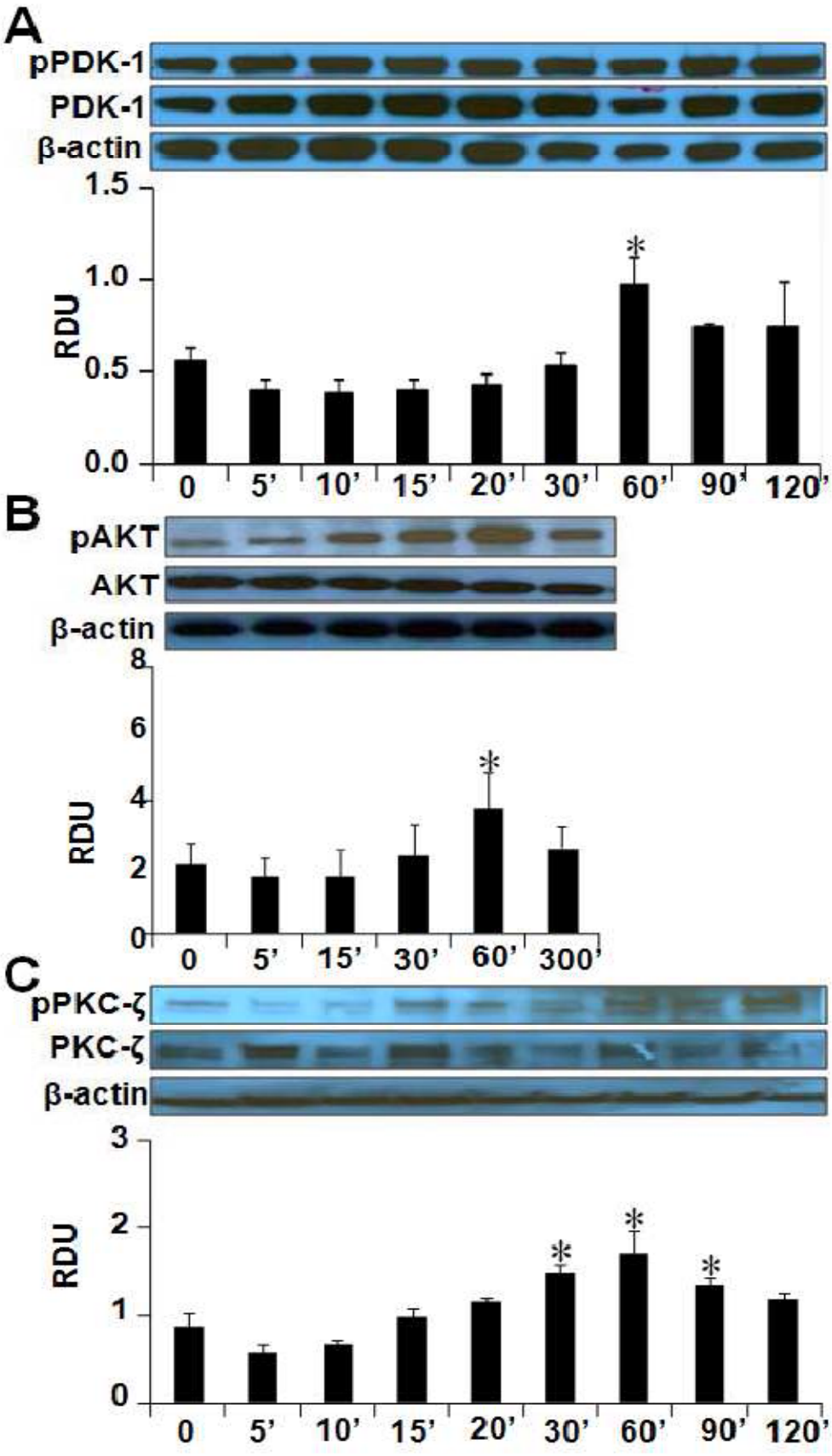

A
pPDK-1
PDK-1
β-actin
RDU
1.5
1.0
0.5
0.0
0
5'
10'
15'
20'
30'
60'
90'
120'
B
pAKT
AKT
β-actin
RDU
8
6
4
2
0
0
5'
15'
30'
60'
300'
C
pPKC-ζ
PKC-ζ
β-actin
RDU
3
2
1
0
0
5'
10'
15'
20'
30'
60'
90'
120'

Figure 2.4: GLP-1R signals through key insulin signal transduction elements. Huh7 cells were treated with GLP/exendin-4 (10 nM) following the time course indicated, and western blot analysis was performed. β-Actin was used as a loading control. (A) Phosphorylation of PDK was induced. (B-C) AKT phosphorylation was also increased in a time-dependent manner, as was the phosphorylation of PKC-ζ. All data are presented as the mean ± SE of at least three experiments. $*P < 0.05$ versus basal or untreated cells.

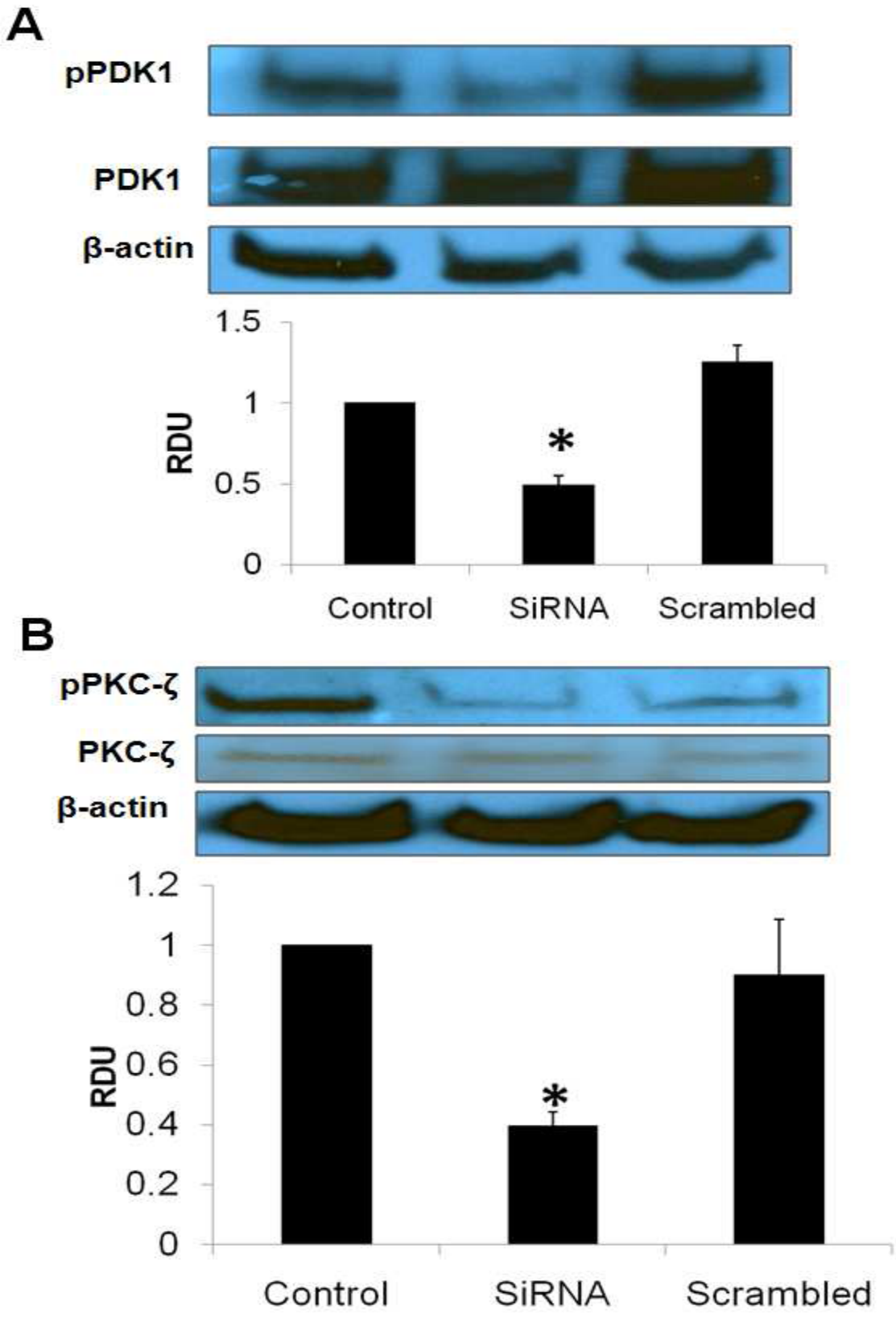

A
pPDK1
PDK1
β-actin
1.5
1
0.5
0
RDU
*
Control
SiRNA
Scrambled
B
pPKC-ζ
PKC-ζ
β-actin
1.2
1
0.8
0.6
0.4
0.2
0
RDU
*
Control
SiRNA
Scrambled

Figure 2.5: Knockdown of GLP-1R by siRNA. Huh7 cells were transfected with siRNA (30 nM) against GLP-1R, and western blot analysis with β-actin serving as a loading control was performed. (A) Knockdown was achieved as compared with control with 30 nM siGLP-1R. (B) Transfected Huh7 cells were treated with exendin-4 (10 nM) for 60 minutes. siRNA GLP-1R abolished the exendin-4–mediated effects on PDK-1 and PKC-ζ. The data are representative of multiple independent experiments. $*P < 0.05$ versus control.

Discussion

A key problem facing biologists and clinicians includes a plausible molecular basis for metabolic syndrome and its hepatic complications. It is widely believed that NAFLD is a component of this epidemic and is the commonest reason a patient sees a gastroenterologist in developed countries. While we previously published intriguing findings in which the long-acting GLP-1 agonist, Exendin-4, significantly reduced hepatic triglycerides stores in the livers of *ob/ob* mice, we did not provide a molecular mechanism for how GLP-1 proteins mediated this beneficial effect (*36*). Furthermore there was a lack of evidence—particularly with regard to human liver—as to whether or not GLP-1 receptors are present, specifically on hepatocytes, and whether or not they are biologically active, though recent data since our publication demonstrates presence of GLP-1R on cholangiocytes (*117*).

In this report we provide a direct molecular explanation for the effects of GLP-1 or a long-acting homologue, Exendin-4, in steatotic liver cells. Our data strongly suggest that as in other mammalian tissues, the GLP-1 receptor is present in human hepatocytes. These data are corroborated not only by conventional analysis (RT-PCR, Immunoblot) but also by bioluminescence, which also demonstrates internalization of GLP-1R. These data are supported by confocal microscopy and sub-cellular fractionation which suggest that the receptor is internalized. Future work is ongoing to directly measure ligand-receptor interactions, which we recognize gauge specific properties than the antibody-receptor analyses presently conducted. On the other hand, the physiologic data indicating a direct reduction of cellular TG is a strong corollary to the receptor work presented herein.

GLP-1R is a member of the seven transmembrane family of G protein coupled receptors (*81*) and their signaling and functioning capabilities have been well defined. Several elegant studies by Widmann *et al* have demonstrated that GLP-1R is internalized on stimulation with its agonist and recycles back to the plasma membrane after several hours following endocytosis (*100*). They have also reported that the receptor after endocytosis is partly internalized into an endosomal compartment such as endoplasmic reticulum, desensitized (*118*) or recycled back to the plasma membrane. However other target organelles for internalization cannot be excluded. Several mechanisms of internalization have been proposed and β-arrestin-1 may be an important adapter protein for several GPCRs. (*119, 120*). Sonoda et al (*121*) postulated a role of β-arrestin-1 in receptor signaling but not in trafficking, a hypothesis consistent with a report by Syme et al (*122*). Internalization was measured by the loss of surface expression of the receptor (as in our study) and has also been seen in response to partial GLP-1R agonists (*123*). While the confocal data is convincing and is corroborated with our blots from the membrane and nuclear fraction, the immunoblot data regarding transfer of GLP-1R to the cytoplasmic fraction is not as robust as visualized in the confocal data. Future work to clarify the internalization results will need to be performed. For example, self-labeling protein tags that will covalently be linked with fluorophores would selectively label the specific pool of GPCRs present at the plasma membrane without labeling any of the internal pools. Thus a non-permeable SNAP-tag substrate will label only the plasma membrane bound SNAP-GPCR proteins. This selective labeling approach may significantly reduce the signal intensity obtained by the confocal microscopic examination of cells performed with Exendin-4 as we have demonstrated here. While we have demonstrated the hepatic

GLP-1 receptor can be internalized, the data here cannot quantify the degree to which Exendin-4 induces this process.

While we recognize that much of the work performed here was in transformed malignant hepatocyte cell-lines—primarily Huh7 cells, the identification of GLP-1R was also performed in primary human hepatocytes. We suspect that one of the reasons that some, but not all, previous authors have not been able to identify the GLP-1 receptor is the availability of better quality antibodies against the receptor and both the purity and availability of viable human hepatocytes for *in vitro* experimentation.

From a clinical and translation perspective these data are exciting as they offer a plausible explanation as to why GLP-1 or GLP-like proteins may be beneficial in the treatment of metabolic syndrome and NAFLD in particular. Importantly, these data indicate a direct effect of GLP-1 protein, as opposed to an indirect or pleotropic effect. As has been recently reported, patients undergoing bariatric surgery are found to have higher circulating levels of GLP-1 with significant histological improvement in their livers (*124-127*) especially those who undergo ileal transposition (*126*) . In the present work we provide evidence for direct cellular effects of GLP-1 proteins. First by potentiating hepatocyte steatosis *in vitro* by supplementing HuH7 cells with palmitic and oleic acids, and gauging the reduction of steatosis by Oil Red O staining and supportive TG quantification . Flow cytometric analysis demonstrated that MCD increased cellular neutral lipid content, which was significantly decreased by Exendin-4 treatment. Interestingly, polar lipid content was only slightly altered by MCD, but was dramatically decreased by Exendin-4 treatment, which also warrants further investigation.

The fact that treatment with Exendin-4 at concentrations seen in either treated diabetics (*128*), or at levels of GLP-1 seen in post-bariatric surgery patients (*129, 130*), results in decreased hepatic TG content. These data clearly underscore that GLP-1 has a direct, independent, and novel action on steatotic hepatocytes.

Our work also provides a molecular mechanism to explain the signal effectors of GLP-1 in its potential role in hepatocyte TG reduction. A key signaling effector for insulin signaling downstream from insulin receptor signaling protein 1(IRS-1) is AKT. Based on our data we have outlined a proposed molecular pathway whereby GLP-1 or homologues intersect the insulin signaling pathway in hepatocytes (**Figure 6**), since this and inter-related pathways in hepatocytes has emerged as critical for the molecular basis of the emergence of hepatocyte insulin resistance.

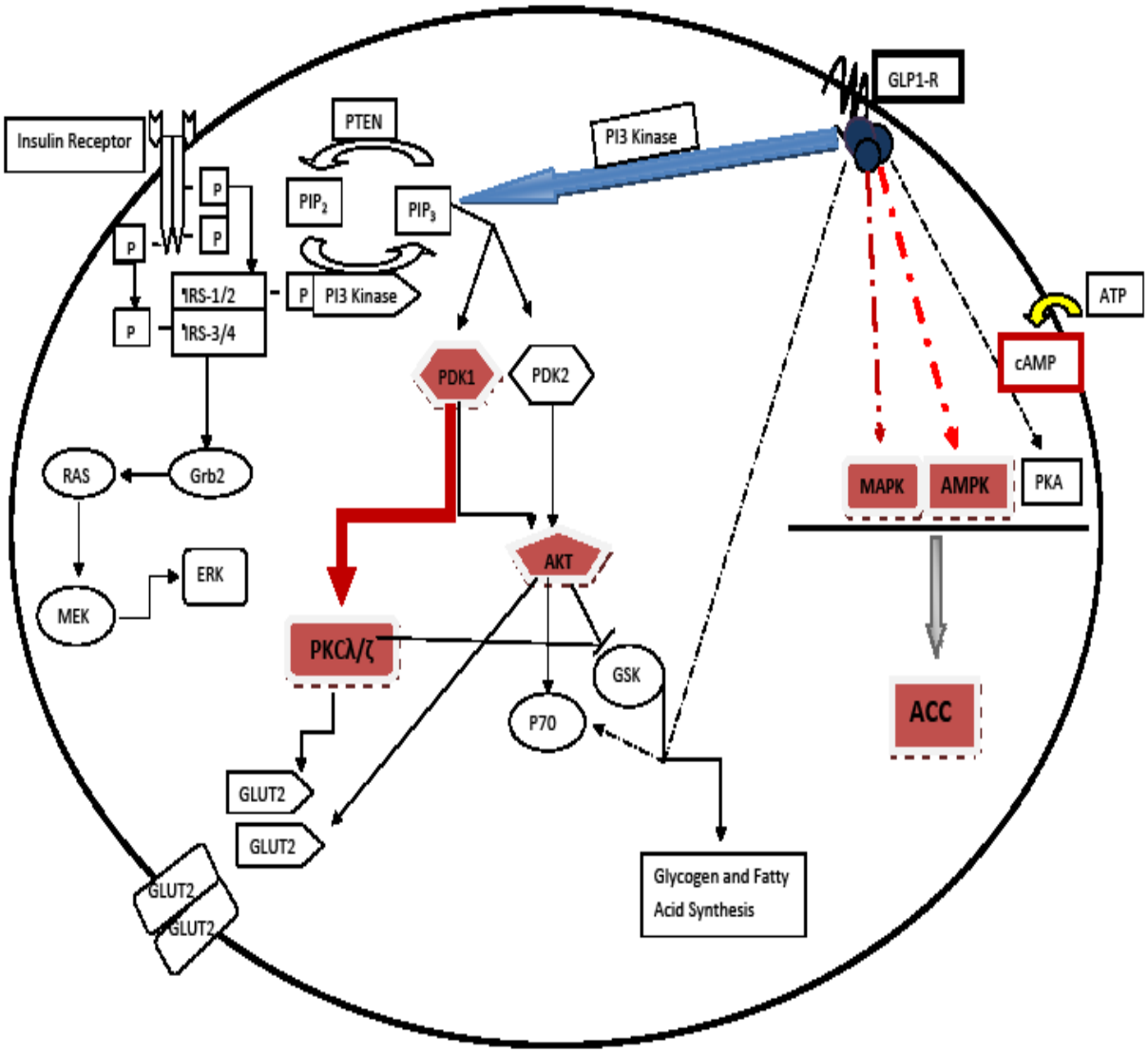

GLP1-R
Insulin Receptor
PTEN
PI3 Kinase
PIP2
PIP3
P
P
P
P
IRS-1/2
IRS-3/4
P
PI3 Kinase
ATP
cAMP
PDK1
PDK2
RAS
Grb2
MAPK
AMPK
PKA
AKT
ERK
MEK
PKCλ/ζ
GSK
ACC
P70
GLUT2
GLUT2
GLUT2
GLUT2
Glycogen and Fatty
Acid Synthesis

Figure 2.6: Proposed GLP-1R signal transduction scheme. In our previous work, we demonstrated that GLP-1 or exendin-4 increased cyclic adenosine monophosphate (cAMP) production. Here we propose that the GLP-1 action shares key downstream components of the insulin signaling pathway, including PKC-ζ, which has been shown to be a key factor in NAFLD.

It has been widely reported that AKT phosphorylation is markedly diminished in steatotic hepatocytes (*131*). Here we show that GLP-1 ligands increase not only the phosphorylation status of AKT but other key molecules downstream. Our signaling studies are noteworthy, because they confirm that Exendin-4 not only activated AKT, but also resulted in robust phosphorylation of both PDK-1 and PKC-ζ. By contrast we failed to knock down AKT phosphorylation by siGLP-1R but successful did so against PKD-1 and PKC- ζ. These data provide a plausible mechanism by which Exendin-4 may be bypassing AKT activation in patients with hepatic insulin resistance.

PDK-1 activates PKC-ζ; moreover PKC-ζ appears to have a significant role in Exendin-4 mediated lipolysis in rat adipocytes. Studies by Arnes *et al* in the rat liver showed that GLP-1 significantly increased Glut 2 mRNA levels increasing lipolysis (*132*). In addition, knockout studies of IRS-1 and IRS-2 in rat hepatocytes by Sajan *et al,* demonstrated that both appear to activate the AKT pathway, however only IRS-2 appears to activate the PKC-ζ (*133*). Our data suggest that GLP-1R activates the same pathway as IRS-2.and may account for why we failed to knock down AKT phosphorylation but were able to significantly knock down PDK-1 phosphorylation and PKC- ζ. What is apparent from our data is that more than one pathway related to insulin signal transduction can act to execute an action of insulin, but in this case such an action (reduction in TG store in liver cells) was executed by GLP-1 proteins. The siRNA studies knocking out the GLP-1R demonstrate a novel insulin action of GLP-1 proteins by up regulating key elements of the hepatocyte insulin signaling pathway. (**Figure 6**)

Future cellular analysis should focus on GLP-1 proteins serve as insulin sensitizing agents in hepatocytes as opposed to an incretin effect seen in pancreatic β cells. These

cellular data may reveal a definitive role for a higher dose distribution of GLP -1 analogues to reduce hepatic steatosis particularly in patients with type 2 diabetes mellitus, and raises the possibility that such agents may, in combination, be safely administered to reduce hepatic TG stores in NAFLD.

Chapter 3: GLP-1 analogue, liraglutide ameliorates hepatic steatosis and cardiac hypertrophy in C57BL/6J mice fed a western diet.

Abstract

Objective to determine if liraglutide (LG), a glucagon-like peptide 1 (GLP-1) long-acting analogue could reverse the effects of a diet containing trans-fat and high fructose corn syrup (HFCS) in mice including reduction in hepatic IR, steatosis and improved cardiac function.

Research Design and Methods Male C57BL/6J mice were fed *ad libitum* either standard chow or a high fat diet containing trans-fat and high-fructose corn syrup (HFCS) for eight weeks, followed by four weeks of concomitant daily LG injections. Hyperinsulinemic-euglycemic clamp studies were performed at 6 weeks. Glucose tolerance and insulin resistance tests were performed at 8 and 12-weeks before and following liraglutide treatment. Cardiac measurements, blood chemistry, RNA and protein analysis were performed.

Results: Clamp studies revealed hepatic insulin resistance after 6 weeks of diet administration. After LG injections, within the ALIOS group, LG treatment significantly improved fasting serum glucose levels as compared with saline-treated mice (122.2 mg/dL ± 6.17 vs. 166 mg/dL ± 50.73, $p<0.0001$), reduced body weight (29.30 g ±0.692 vs. 33.08 g ±4.54, $p<0.005$), reduced liver weight (1.067 g ±0.083 vs. 1.217 g ±0.108, $p<0.05$), and heart weight (0.006845 g/cm ± 0.000302 vs. 0.00755 g/cm ±0.000612). Liver from LG treated mice had significantly higher levels of fatty acid binding protein (LFABP), acyl-CoA oxidase II (ACOX2), very long-chain acyl-CoA dehydrogenase (LCAD), and microsomal triglyceride transfer protein (MTTP).

Conclusions: LG reduces the harmful effects of an ALIOS diet by improving insulin sensitivity and by reducing lipid accumulation in liver and heart through fatty acid uptake, transport, and increase β-oxidation.

Background

Obesity and type 2 diabetes (T2DM), and related co-morbidities are major public health epidemics. According to data collected from the National Health and Nutrition Examination Survey (NHANES), the number of Americans classified as obese (body mass index [BMI] ≥30) continues to increase, with the greatest increases occurring in adult men (30.4%) and among children aged 6 through 19 years (16.0%) (*2*). The prevalence of diabetes has dramatically increased, affecting approximately 20.8 million people in the U.S, and it estimated that by 2050 persons diagnosed with diabetes will increase by 165% (*8, 9*). Accompanying obesity and insulin resistance is a cluster of metabolic derangements, including non-alcoholic fatty liver disease (NAFLD), hypertension, hypertriglyceridemia, low high density lipoproteins (HDL) and hypertension— which are commonly referred to as metabolic syndrome.

Epidemiologic studies in the United States have suggested that the consumption of a Western diet (high in saturated fat, trans-fat and sugar), is strongly associated with the increasing prevalence of NAFLD and cardiovascular disease (CVD) (*61*). In addition, diets containing trans-fat and high-fructose corn-syrup (HFCS) have been shown independently to increase insulin resistance, fat mass and liver lipid accumulation in rodents and humans (*59, 61, 134, 135*). Although dietary changes and moderate exercise are effective in treating obesity and diabetes, pharmacologic treatments, which target multiple aspects of insulin resistance/obesity induced metabolic abnormalities including NAFLD are warranted.

Until recently there was not a suitable mouse model that mimicked the Western diet-induced pathophysiology observed in human populations. Tetri *et al*. reported that

the American-Lifestyle Induced Obesity Syndrome (ALIOS) model resulted in C57BL/6J mice at 16 weeks having severe hepatic necroinflammatory damage (*71*). No data however, was provided on the effect on the cardiovascular system. Tetri's diet was composed of macronutrients similar to what is contained in an American fast-food diet; with forty-five percent of calories derived from fat. Additional calories are derived from (HFCS).

Recently our lab published studies that demonstrated glucagon-like peptide 1 (GLP-1) and its cognate receptor GLP-1R are present and functional on human hepatocytes (*83*). Moreover, we demonstrated that exendin-4, a long-acting GLP-1 analogue increased phosphorylation of PDK-1, AKT, and PKC-ζ; suggesting that in addition to its incretin properties previously described; GLP-1 analogues have direct insulin sensitizing effects in hepatocytes (*83*). The aim of the present study was to determine if liraglutide (LG), a GLP-1 long-acting analogue could reverse the effects of the ALIOS diet as it relates to the development of metabolic syndrome in mice. This report provides new information related to the role of GLP-1 proteins which promote improved cardiac function, as well as new information as to how GLP-1 affects genes associated with lipid metabolism, and the potential role it has in improving lipid turnover.

Research Design and Methods

Animal studies

Male C57BL/J6 4-5 wk old mice were obtained from Jackson Laboratories (Bar Harbor, ME). The animals were cared for in accordance with protocols approved by the Animal Care and Use Committee of Emory University. The hyperinsulinemic-euglycemic clamp studies were performed at Vanderbilt. Mice were cared for in

accordance with the Vanderbilt Animal Care and Use Committee. Animals were housed in laboratory cages at 23°C under a 12-hour reverse light/dark cycle.

Mice were fed either standard chow or the American Lifestyle-Induced Obesity Syndrome (ALIOS) diet as described by Tetri *et al* (*71*). The ALIOS diet derives 45% of its calories from fat, with 30% of the fat in the form of partially hydrogenated vegetable oil [28% saturated, 57% monounsaturated fatty acids (MUFA), 13% polyunsaturated fatty acids (PUFA); trans fat custom diet TD06303, Harlan Teklad) (*71*). ALIOS mice were also given HFCS equivalents in their drinking water at 42 g/L. (55% fructose, 45% glucose by weight). Control mice were fed standard rodent chow. Food and water consumption was measured by weighing new and remaining food and water three times weekly. Mice were grouped into two groups: control diet and ALIOS diet. After 8 weeks on the diet, mice were injected intraperitoneally (IP) with saline or liraglutide (200 μg/kg) dissolved in saline (Novo Nordisk, Princeton, NJ) of body weight, daily, 1 hour before the onset of the dark cycle, for 4 weeks. In a separate experiment mice were pair fed, food and water were checked daily, ensuring that the ALIOS mice received the same calories as the control mice. At the onset and termination of the study fasting blood samples were obtained, and individual liver and heart samples were snap-frozen in liquid nitrogen and stored at -80°C.

Pathology

Tissue was prepared as describe by Ding *et al* (*36*). Briefly, liver and heart were removed, weighed and divided into three sections: cryosections, formalin fixed, and frozen samples. H&E staining and immunohistochemistry (IHC) were performed on the

cryosections. Frozen samples were used for RNA analysis and Oil Red O staining. Visceral fat tissue was removed, weighed and stored at -80°C (*36, 83*) Heart tissue was excised with great vessels and atria trimmed away before cardiac weights were obtained. The tibia was removed and length measured in mm.

Glucose and insulin tolerance test and hyperinsulinemic-euglycemic clamp

Mice were fasted for 6-8 h. Glucose (2 g/kg) was administered through intraperitoneal administration with a 27-gauge insulin syringe. Glucose levels were measured by tail vein sampling with portable glucometer at 0, 15, 30, 60, 90 and 120 min. Insulin tolerance was measured as described by Tetri *et al.* (*71*). Briefly, mice were fasted for 6-h and injected intraperitoneally 0.6 units/kg human regular insulin at a concentration of 0.2 U/ml with a 27-gauge insulin syringe. Glucose levels were measured by tail vein sampling with portable glucometer at 0, 15, 30, 45, 60 min. Glucose and insulin tolerance tests were administered at 8 weeks (before the treatment period) and at the termination of the study. The hyperinsulinemic-euglycemic clamp procedures were performed by a National Institutes of Health Mouse Metabolic Phenotyping Center (MMPC) at Vanderbilt University as described by Ayala *et al* and others (*136, 137*).

Blood Chemistry

Blood taken from the submandibular vein, at the termination of the study, was used to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, and triglycerides.

Echocardiography and Blood Pressure

Echocardiography and blood pressure measurements were taken 1 week prior to sacrifice. Mice were anesthetized with 1.5% isoflourane, and echocardiography was performed with ultrasonography (Sequoia 512, Acuson 15L8, Oceanside, CA). A 15-MHz linear ultrasound transducer was used. The 2D guided M –mode measurements of the LV internal diameter were taken from three beats and averaged. Blood pressures were measured by tail cuff using Visitech Systems BP-2000 (Apex, NC)

RNA analysis

Total RNA was extracted from liver tissue using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). An equal amount of total RNA was used to synthesize the first DNA strand by iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Real-time PCR was performed with the Eppendorf Realplex4 (Hauppauge, NY), using SYBR green detection. Primer sequences for liver fatty acid binding protein (LFABP), fatty acyl synthase (FASN), acyl-CoA carboxylase-α (ACCα), carnitine palmitoyl transferase (CPT-1A), acyl-CoA oxidase 2 (ACOX2), stearoyl-CoA desaturase-1 (SCD1), microsomal triglyceride transfer protein (MTTP), apolipoprotein B100 (ApoB) peroxisome proliferator-activated receptor-α (PPARα), and peroxisome proliferator-activated receptor-γ (PPARγ) are shown in Table 2. All samples were run in triplicate, PCR products were normalized to 18s rRNA expression levels.

Table 3.1: RNA Primers

Pathway	*Abbreviation*	***GenBank Accession***	***Sequence***
Uptake and transport	*FABPL*	***NM_017399***	*TCTCCGGCAAGTACCAATTG* *TTGATGTCCTTCCCTTTCTGG*
Lipogenesis	*FASN*	***NM_007988***	*CCCCTCTGTTAATTGGCTCC* *TTGTGGAAGTGCAGGTTAGG*
	ACACA	***NM_133360***	*AAGGCTATGTGAAGGATGTGG* *CTGTCTGAAGAGGTTAGGGAAG*
Mitochondrial β-oxidation	*CPT-1A*	***NM_009948***	*CCTCCGAAAAGCACCAAAAC* *GCTCCAGGGTTCAGAAAGTAC*
Peroxisomal β-oxidation	*ACOX1*	***NM_015729***	*CATATGACCCCAAGACCCAAG* *CATGTAACCCGTAGCACTCC*
	ACOX2	***NM_053115***	*AACACAGCATACACAGACCC* *GTCTAGTTTCTCCACAGCTTCC*
Esterification	*SCD-1*	***NM_009127***	*CTGACCTGAAAGCCGAGAAG* *AGAAGGTGCTAACGAACAGG*
VLDL Secretion	*MTTP*	***NM_008642***	*TCCACATACAGCCTTGACATC* *TTAAGCCTTCCAGCCCTTG*
	ApoB	***NM_009693***	*ATTCGAGCACAGATGACCAG* *GTACCTTTCACCATCAGACTCC*
Transcriptional Regulators	*PPARA*	***NM_011144***	*CATTTCCCTGTTTGTGGCTG* *ATCTGGATGGTTGCTCTGC*
	PPARG	***NM_001146***	*TGTTATGGGTGAAACTCTGGG* *AGAGCTGATTCCGAAGTTGG*
	PPARD	***NM_011145***	*GGAAAAGTTTTGGCAGGAGC* *TGTCTTCATCTGTCAGTGAGC*

Immunoblot

Equal amounts of protein from tissue lysates were resolved on SDS-PAGE (*113*), transblotted, and subjected to immuno-detection using primary antibody for MTTP (Santa Cruz), LFABP (Santa Cruz), and medium-chain acyl-CoA dehydrogenase (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD) (Abcam, Cambridge MA). All proteins were normalized to beta-actin.

Statistical Analysis

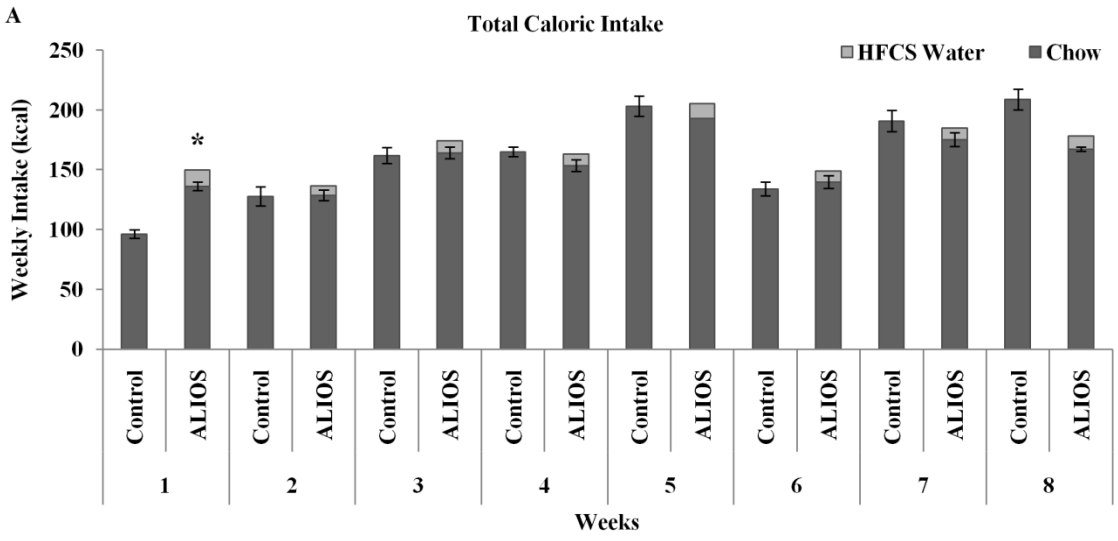
The data are presented as means ± standard error. Statistical analysis was performed using JMP v.8.01 (SAS Institute, Cary N.C.). Means were compared using the Student *t*-test. The Tukey-Kramer test was also used to determine if any group differed significantly each other.

Results

ALIOS diet increases weight and adiposity in mice independent of caloric intake. The caloric intake of the ALIOS mice was significantly higher than the control group during week 1 of the study (Fig. 3.1A) (136.07 kcal ± 3.62 vs. 96.09 ± 3.54 *p<.005)*. However for the remainder of the study there was no significant difference in caloric intake for each of the four mouse cohorts. Despite similar caloric intake patterns between the groups, the ALIOS mice gained significantly more weight (Fig. 3.1B). In a separate experiment mice were pair-fed, and the ALIOS mice still gained significantly more weight than the control fed animals (data not shown). The ALIOS mice group

gained an average of 8.2% of their previous week's weight as compared to the control animals which gained an average of 5.4% of their previous week's weight.

During the four week treatment period with LG or saline all mouse cohorts except the ALIOS + saline group lost a small amount weight (Fig. 3.2A). The ALIOS + saline group continued to gain weight, during the first 21 days of saline injections, however lost weight during the last week of injections (Fig. 3.2A) (increased handling may the reason for the decline). The ALIOS + LG group had no significant change in weight during the 4 weeks of injections. Consequently the significant different in weights observed, Fig. 3.2B between ALIOS groups (saline vs. LG) is a result of the ALIOS + saline group continuing to gain weight throughout the treatment period.


A
Total Caloric Intake
HFCS Water
Chow
Weekly Intake (kcal)
250
200
150
100
50
0
*
Control
ALIOS
1
2
3
4
5
6
7
8
Weeks

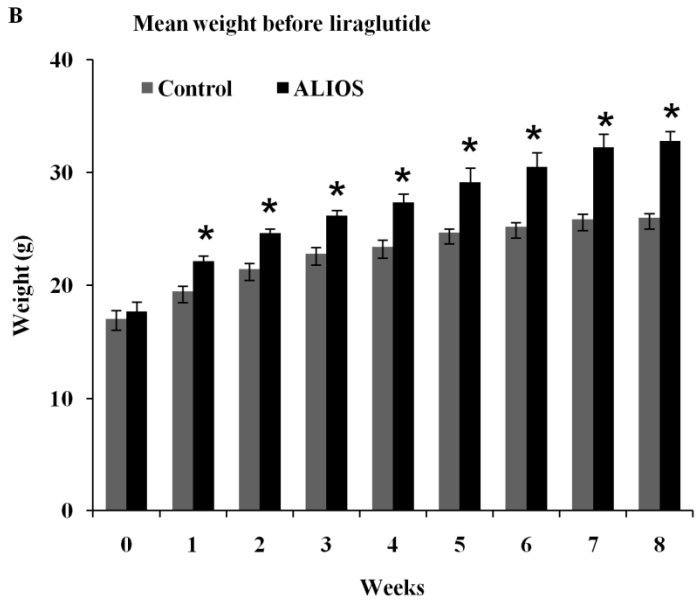

B
Mean weight before liraglutide
Control
ALIOS
Weight (g)
40
30
20
10
0
*
0
1
2
3
4
5
6
7
8
Weeks

Figure 3.1: ALIOS diet increases weight independent of caloric intake. A: Caloric intake of C57BL/6J male mice placed on either control chow or ALIOS diet. There was a significant difference in caloric intake during wk 1 of study. ALIOS mice consumed significantly more calories than control (136.07 kcal ± 3.62 vs. 96.09 ± 3.54, $p<0.005$), however, there was no significant difference in caloric intake for the remainder of the study. **B:** ALIOS mice gained significantly more weight per week than control animals (and 8.2% and 5.47% change in weights respectively, $p<0.005$).

A similar pattern was observed in the liver weights of the animals (Fig. 3.2C). The ALIOS+ saline mouse livers' were significantly heavier than the ALIOS+ LG mouse livers' (1.216 g ± 0.044 vs. 1.066 g ± 0.022, $p<0.05$) and both (LG or saline) livers from the ALIOS mice were significantly heavier than control saline or LG groups (0.8133 g ± 0.0712 and 0.857g ± 0.048, $p<0.005$). The ALIOS diet resulted in a significant increase in visceral adipose tissue (VAT) (Fig. 3.2D). The ALIOS+ LG group significantly less VAT compared to the ALIOS+ saline treated mice (3.596g ± 0.06 vs. 4.78g ± 0.07 $p<0.0001$). However both ALIOS groups had significantly larger VAT than the control chow cohorts, regardless of treatment or placebo.

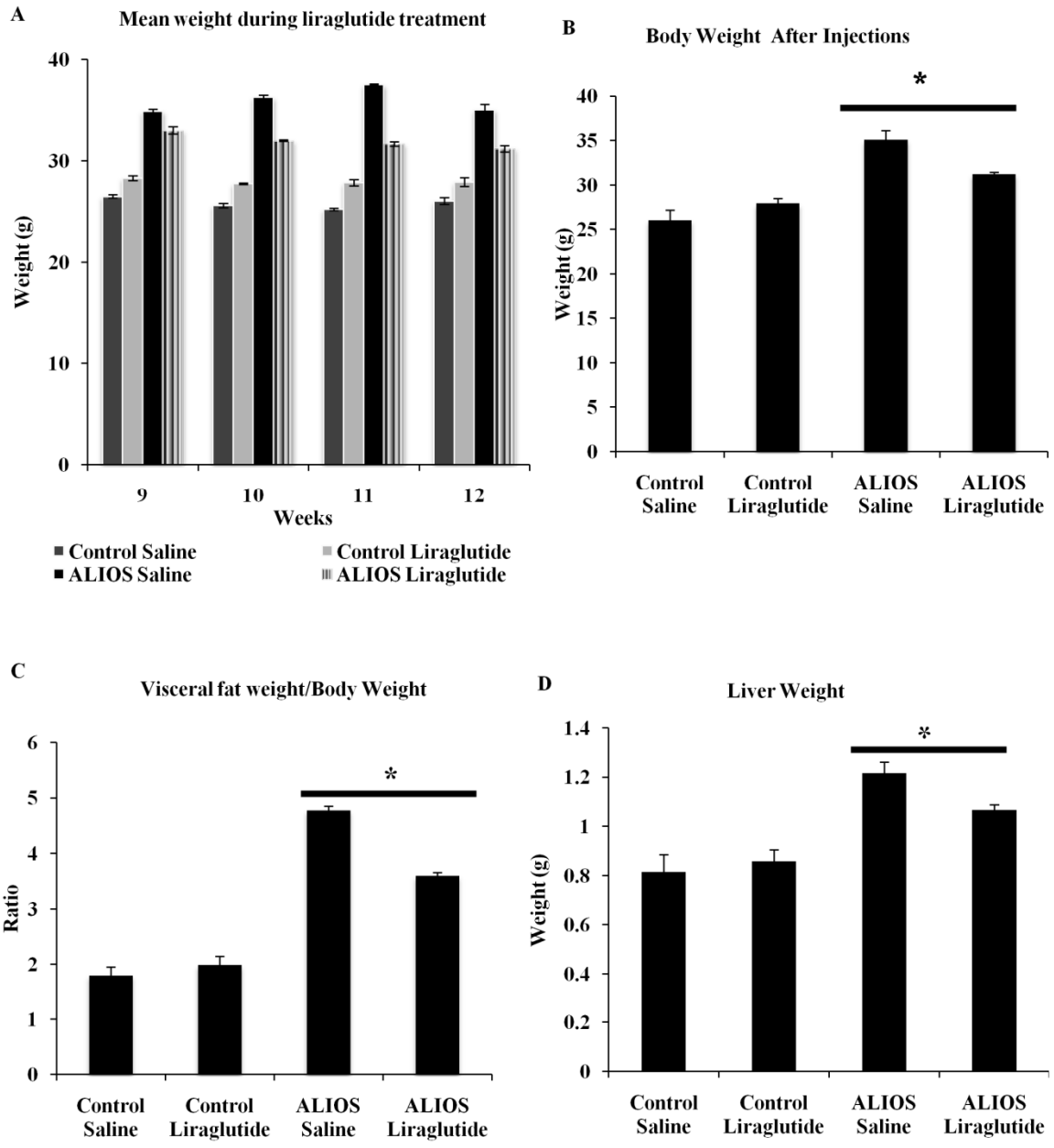

A
Mean weight during liraglutide treatment
Weight (g)
Weeks
9
10
11
12
Control Saline
Control Liraglutide
ALIOS Saline
ALIOS Liraglutide
B
Body Weight After Injections
Weight (g)
*
Control Saline
Control Liraglutide
ALIOS Saline
ALIOS Liraglutide
C
Visceral fat weight/Body Weight
Ratio
*
Control Saline
Control Liraglutide
ALIOS Saline
ALIOS Liraglutide
D
Liver Weight
Weight (g)
*
Control Saline
Control Liraglutide
ALIOS Saline
ALIOS Liraglutide

Figure 3.2: Liraglutide reduces liver weight and VAT in ALIOS mice. A. During injections all groups lost a small amount of weight except the ALIOS + saline injection group, which showed weight gain for first three weeks. The rate of weight gain, however, was not significant for any of the four cohorts. (dark gray= control + saline, light gray= control+ LG, black = ALIOS+ saline, striped = ALIOS+ LG B**:** ALIOS mice had significant reduction in body weight after liraglutide treatment ($p<0.05$). Liraglutide did not reduce body weight in control group. **C:** Liver weight following liraglutide injections lost weight significantly in comparison to livers from ALIOS-fed mice ($p<0.05$). Livers from ALIOS-fed saline-treated mice were significantly heavier than either control groups ($p<0.001$). **D:** While both ALIOS fed groups had significantly more adipose tissue than their control counterparts ($p<0.001$). There was no significant difference in visceral adipose tissue (VAT) weight between control groups. However, there was a significant reduction of VAT weight in ALIOS-fed liraglutide-treated mice compared to ALIOS-fed saline-treated mice ($p<0.0001$).

ALIOS mice developed severe hepatic insulin resistance before systemic insulin resistance. Using hyperinsulinemic-euglycemic clamp studies, the gold standard for detecting hepatic insulin resistance, we evaluated the effect of the ALIOS diet on the development of hepatic insulin resistance in mice. Insulin sensitivity/action was assessed in conscious mice using the insulin clamp (Fig 3.3).

At six weeks, ALIOS mice had significantly higher fasting glucose levels than controls (Fig. 3.3A) (121 ± 3 mg/dL vs. 98 mg/dL ± 8.0 $p<0.01$), however there was no difference in their basal levels of insulin (control: 1.8 ± 0.3 ng/ml; ALIOS 2.5 ± 0.3 ng/ml). During the clamp (Fig. 3.3B), the ALIOS mice required significantly less glucose infusion (approximately 30% less) as compared to the control group ($p<0.01$).

The glucose infusion rate is used to measure the ability of insulin to suppress hepatic glucose production and to increase glucose uptake by tissues. In examining the total glucose turnover rate (Fig. 3.3C) there was no significant difference between any treatment group during the basal and clamp portion of the study suggesting that insulin sensitivity in peripheral tissues could not explain the lower glucose requirements during the clamp.

Assessing hepatic insulin resistance: Basal endogenous glucose turnover was 24% higher in the ALIOS group, but not significant (Fig. 3.3C). During the clamp ALIOS mice had significantly higher levels of insulin as compared to control mice (5.3 ± 0.5 ng/ml vs. 3.3 ± 0.6 ng/ml, $p<0.05$), and the ability of insulin to suppress endogenous glucose production was impaired (Fig. 3.3D) indicating that the liver was resistant to the

action of insulin. Thus failure of insulin to adequately suppress hepatic glucose production during the clamp explains the lower glucose requirements during the clamp.

Since insulin concentration during the clamp was higher in the ALIOS group yet whole body glucose utilization was not increased, the ALIOS diet may also modestly attenuate peripheral insulin action.

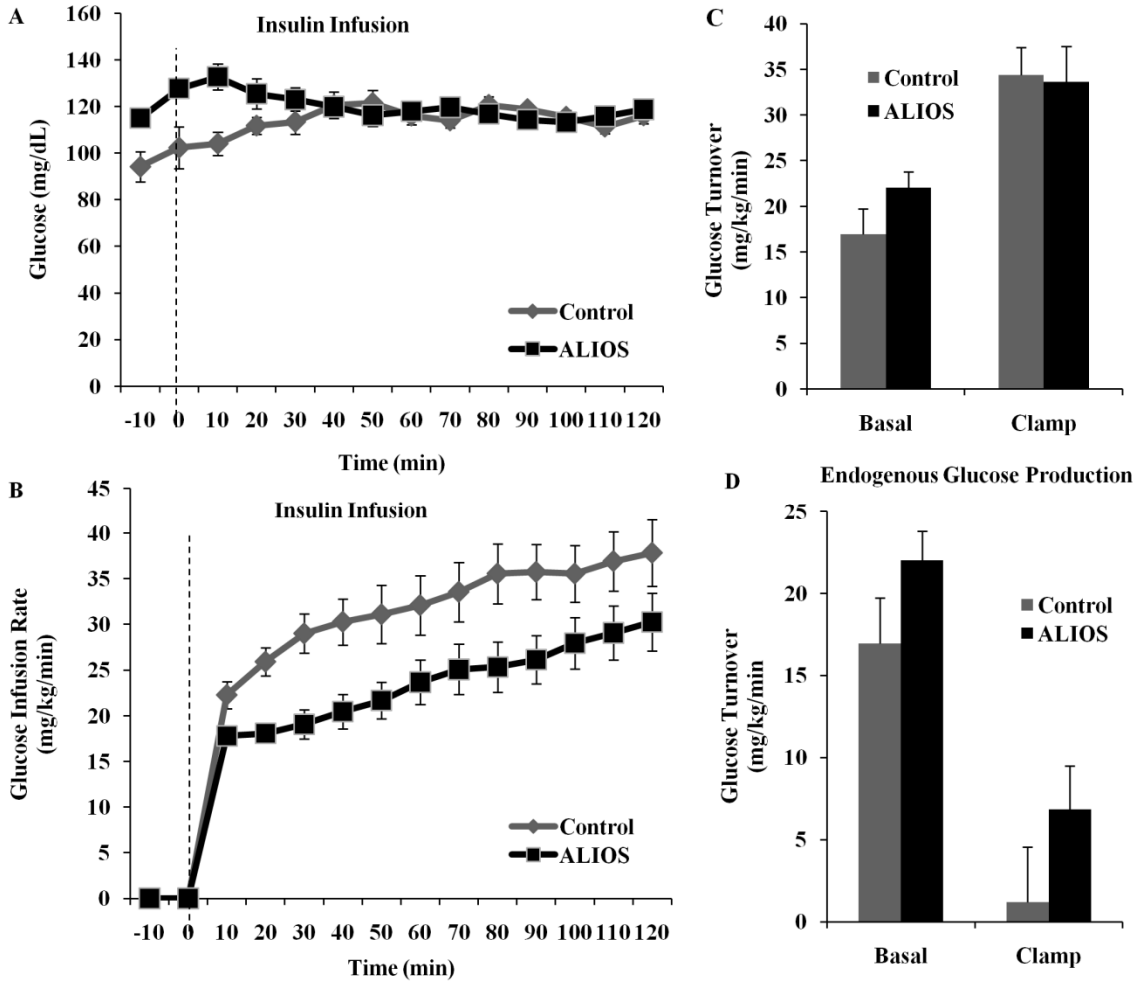


Figure 3.3: Mice fed the ALIOS diet develop hepatic insulin resistance within 6 weeks. **A:** Arterial blood glucose during clamp (black squares= ALIOS group, gray diamonds=control group). **B:** Total body glucose turnover. **C:** Glucose infusion rate during clamp study (black squares= ALIOS group, gray diamonds=control group). **C:** Endogenous glucose turnover.

Liraglutide significantly improved glucose handling and insulin sensitivity in ALIOS treated mice. At the end of 8 weeks of feeding the ALIOS mice had significantly higher fasting glucose levels when compared to control mice (Fig. 3.4A) (167 mg/dL ± 10.15 vs. 107.5 mg/dL ± 4.32 $p<0.0001$). In pair-fed groups this trend persisted (147 mg/dL ± 3.069 vs. 108.37 mg/dL ± 4.17 $p<0.0005$). The ALIOS fed mice had significantly higher fasting glucose than the pair-fed counterparts (167 mg/dL vs. 147 mg/dL $p<.05$).

Pair feeding did not result in a significant change in the insulin tolerance of animals (Fig. 3.4B). However there was a significant difference between the ALIOS and control chow fed groups $p<.05$. Liraglutide treatments significantly improved fasting serum glucose levels in ALIOS fed mice as compared to ALIOS saline injected mice (Fig. 3.4C) (122.2 mg/dL ± 6.17 vs. 166 mg/dL ± 50.73, p<.0001), and significantly improved glucose tolerance $(p<.05)$. As expected mice receiving LG had significantly higher insulin levels than their saline counterparts: control + saline vs. control + LG (585.16 pg/ml ± 80.7 vs. 3106.64 pg/ml ± 1094.3 $p<0.05$); ALIOS + saline vs. ALIOS + LG (1459.82 pg/ml ± 618.9 vs. 3823.66 pg/ml ± 564.1 $p<0.05$). However, the was no significant difference in insulin levels between the control + LG and ALIOS + LG.

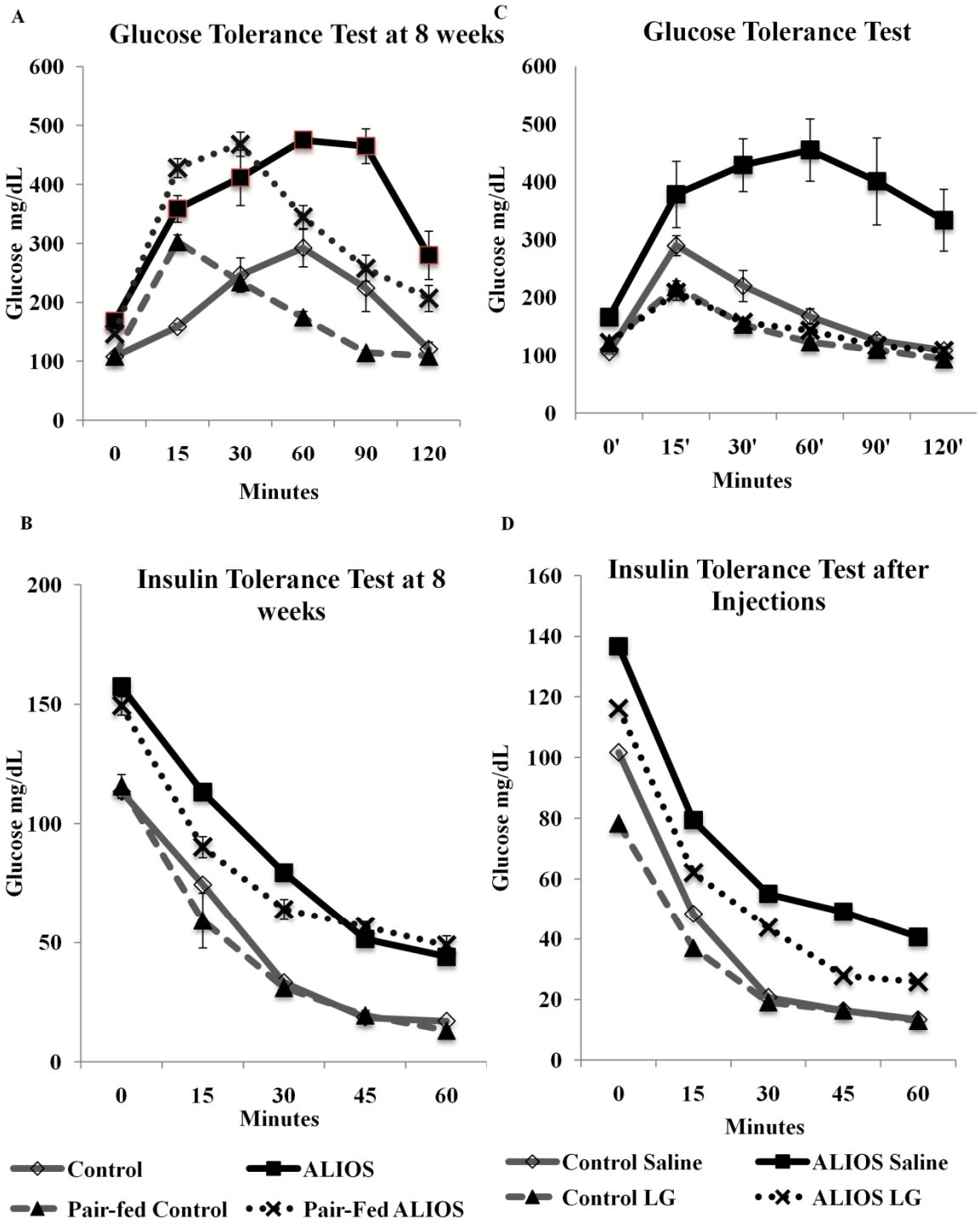
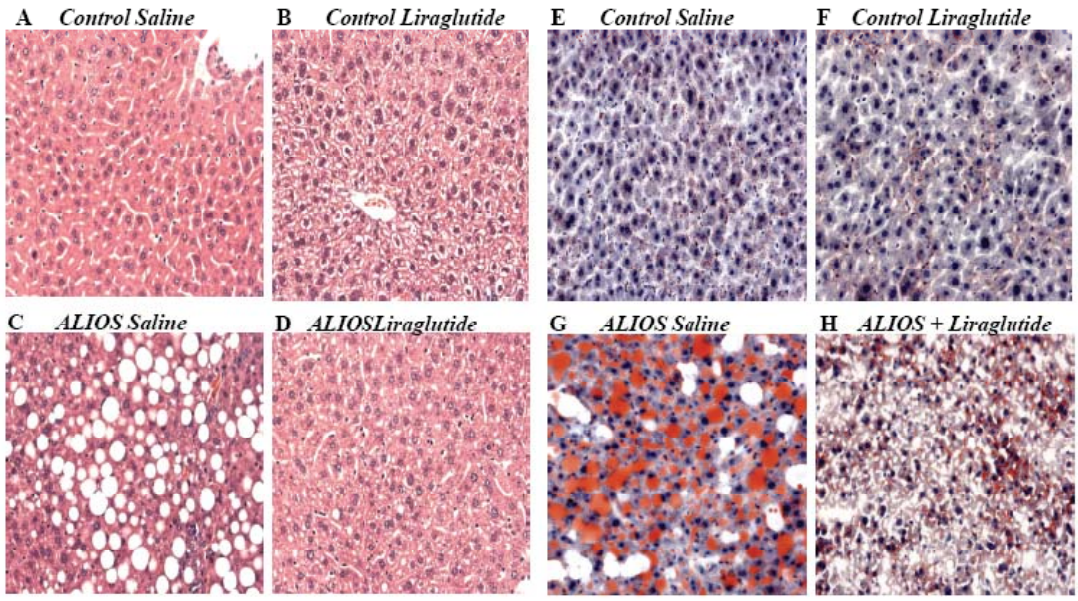
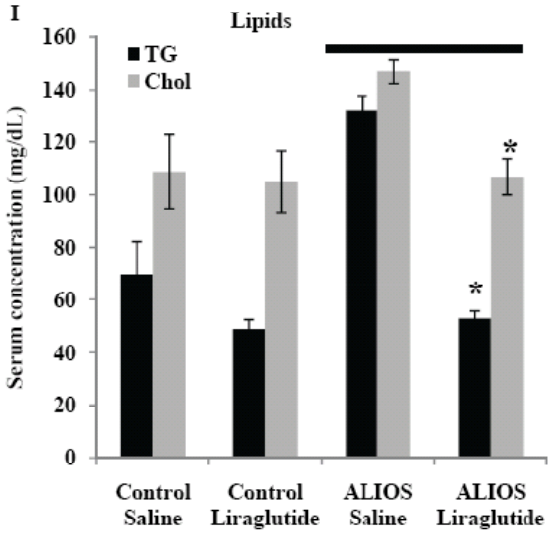

A
Glucose Tolerance Test at 8 weeks
Glucose mg/dL
600
500
400
300
200
100
0
0
15
30
60
90
120
Minutes
C
Glucose Tolerance Test
Glucose mg/dL
600
500
400
300
200
100
0
0'
15'
30'
60'
90'
120'
Minutes
B
Insulin Tolerance Test at 8 weeks
Glucose mg/dL
200
150
100
50
0
0
15
30
45
60
Minutes
D
Insulin Tolerance Test after Injections
Glucose mg/dL
160
140
120
100
80
60
40
20
0
0
15
30
45
60
Minutes
Control
ALIOS
Pair-fed Control
Pair-Fed ALIOS
Control Saline
ALIOS Saline
Control LG
ALIOS LG

Figure 3.4: Liraglutide treatment dramatically improves glucose uptake and insulin sensitivity. A-B: Glucose and insulin tolerance tests performed at 8 week. Mice were fed *ad libitum* (control or ALIOS diet) or pair-fed (ALIOS mice received the same amount of kcal as the control group consumed) for 8 weeks. Both the *ad libitum* and pair-fed ALIOS groups had significantly higher fasting glucose levels as compared to their control counterparts ($p<0.001$). AUC analysis revealed no significant difference between *ad libitum* and pair-fed groups. The ALIOS fed mice had significantly larger AUC than their control counterparts for both the glucose and insulin tolerance tests. (Black open-diamond = Control, Black triangle = Pair-fed control, black square = ALIOS, black cross = pair-fed ALIOS) **C-D:** Glucose and insulin tolerance tests at the end of the study period (12 wk). The ALIOS-fed liraglutide-treated mice had significantly reduced fasting blood glucose compared to the ALIOS-fed saline-treated mice ($p<0.0001$). In the glucose tolerance test there was significant reduction in AUC for the ALIOS-liraglutide group as compared to the ALIOS-saline group ($p<0.001$). (Black open-diamond = control + saline, Black triangle = Control + LG, Black square = ALIOS + saline, and black cross = ALIOS + LG).

Liraglutide treatment prevented ALIOS-induced pathology and lipid accumulation in the liver. Mice fed controls fed standard chow with saline injections for 12 wk (Fig. 3.5A) revealed no evidence of steatosis or steatohepatitis (NASH). As anticipated there was no significant difference between either the LG-treated (Fig. 3.5B), and the saline-treated mice. In contrast ALIOS mice injected with saline showed significant pathology (Fig. 3.5C) including marked hepatic steatosis and ballooning degeneration of hepatocytes; and, Oil Red O staining revealed significant lipid accumulation (Fig. 3.5G). LG treatment significantly reduced hepatic lipid accumulation in the ALIOS fed mice (Fig. 3.5H). The ALIOS saline group had significantly higher serum triglyceride and cholesterol levels (Fig. 3.5I) (132.5 mg/dL ± 5.29 and 147 mg/dL ± 4.58 respectively). LG treatment reduced serum triglyceride and cholesterol levels in the ALIOS fed mice (52.88 mg/dL ± 2.78, $p<.001$ and 106.8 mg/dL ± 6.82, $p<.05$ respectively). The ALIOS saline group had higher ALT values than control saline; LG treatment reduced ALT values in both control and ALIOS-fed mice (p=0.09).


A Control Saline
B Control Liraglutide
E Control Saline
F Control Liraglutide
C ALIOS Saline
D ALIOSLiraglutide
G ALIOS Saline
H ALIOS + Liraglutide


I
Lipids
TG
Chol
Serum concentration (mg/dL)
160
140
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80
60
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Control Saline
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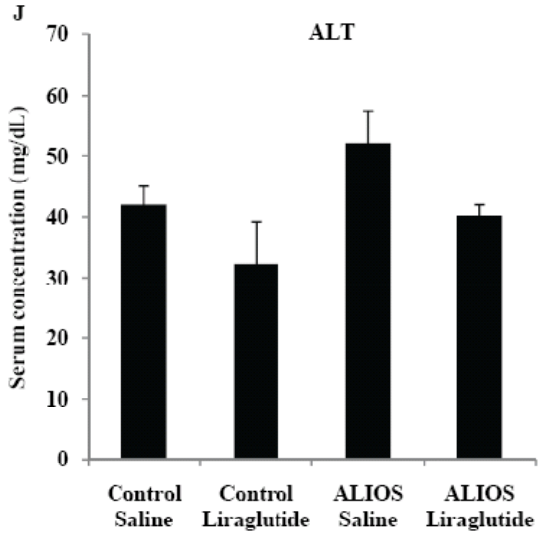
ALIOS Saline
ALIOS Liraglutide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Serum concentration (mg/dL)
70
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Control Saline
Control Liraglutide
ALIOS Saline
ALIOS Liraglutide

Figure 3.5: Liraglutide reduces hepatic steatosis and improves liver function. Hepatic steatosis was observed using Oil red O stain and liver function detected by lipid and ALT concentrations in serum. **A-D:** Liver histology following necropsy (12 wk total including 4 weeks of liraglutide treatment) H&E stain (200X). Mice fed the ALIOS diet for 12 weeks showed significant steatosis **(C)**. ALIOS mice receiving liraglutide show dramatically reduced steatosis **(D)**. **E-F:** There is little lipid accumulation in livers of mice fed control diet. **G:** ALIOS mice injected with saline had significant amount of lipid accumulation, while liraglutide treatment **(H)** significantly reduced the amount of lipid found in ALIOS fed mice. **I:** Liraglutide treatment significantly reduced serum levels of triglyceride and total cholesterol. ALIOS-fed liraglutide group had significantly lower serum triglycerides ($p<0.001$) and total cholesterol ($p<0.05$) when compared with ALIOS-fed saline-treated mice. **J:** There was no difference in ALT values between control fed groups, whereas, ALIOS liraglutide group trended downward, but this reduction was not significant ($p= 0.09$).

Liraglutide treatment reduced blood pressure and cardiac hypertrophy At the end of the study, the ALIOS saline treated mice were found to have significantly higher systolic and diastolic blood pressure (Figure 3.6A and 3.6B respectively). LG treatment significantly reduced systolic blood pressure (117.7 ± 3.40 mmHg vs. 92.46 ± 3.11 mmHg, $p<0.0001$) and diastolic blood pressure (98.32 ± 4.27 mmHg vs. 73.30 ± 1.60 mmHg, $p<0.0001$). ALIOS saline treated mice also had cardiac hypertrophy (Fig. 3.6D). The mean cardiac weight of the ALIOS saline mice was significantly heavier than the control saline group (0.00759 g/cm ± 0.00027 vs. 0.00651 g/cm ± 0.00016 $p< 0.001$). LG treatment significantly reduced cardiac hypertrophy in the ALIOS mice (0.00651 ± 0.00014 $p<0.05$).

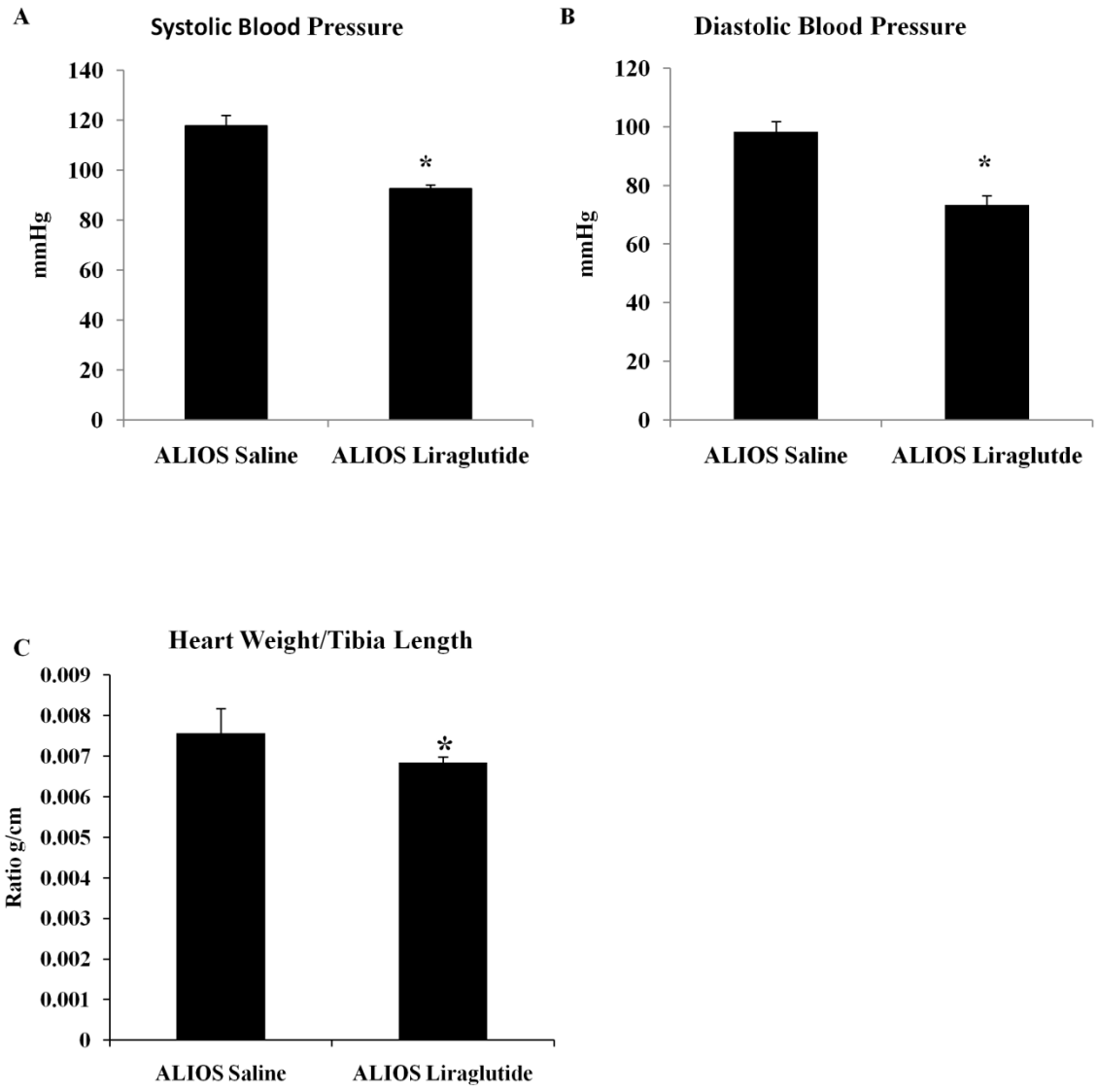
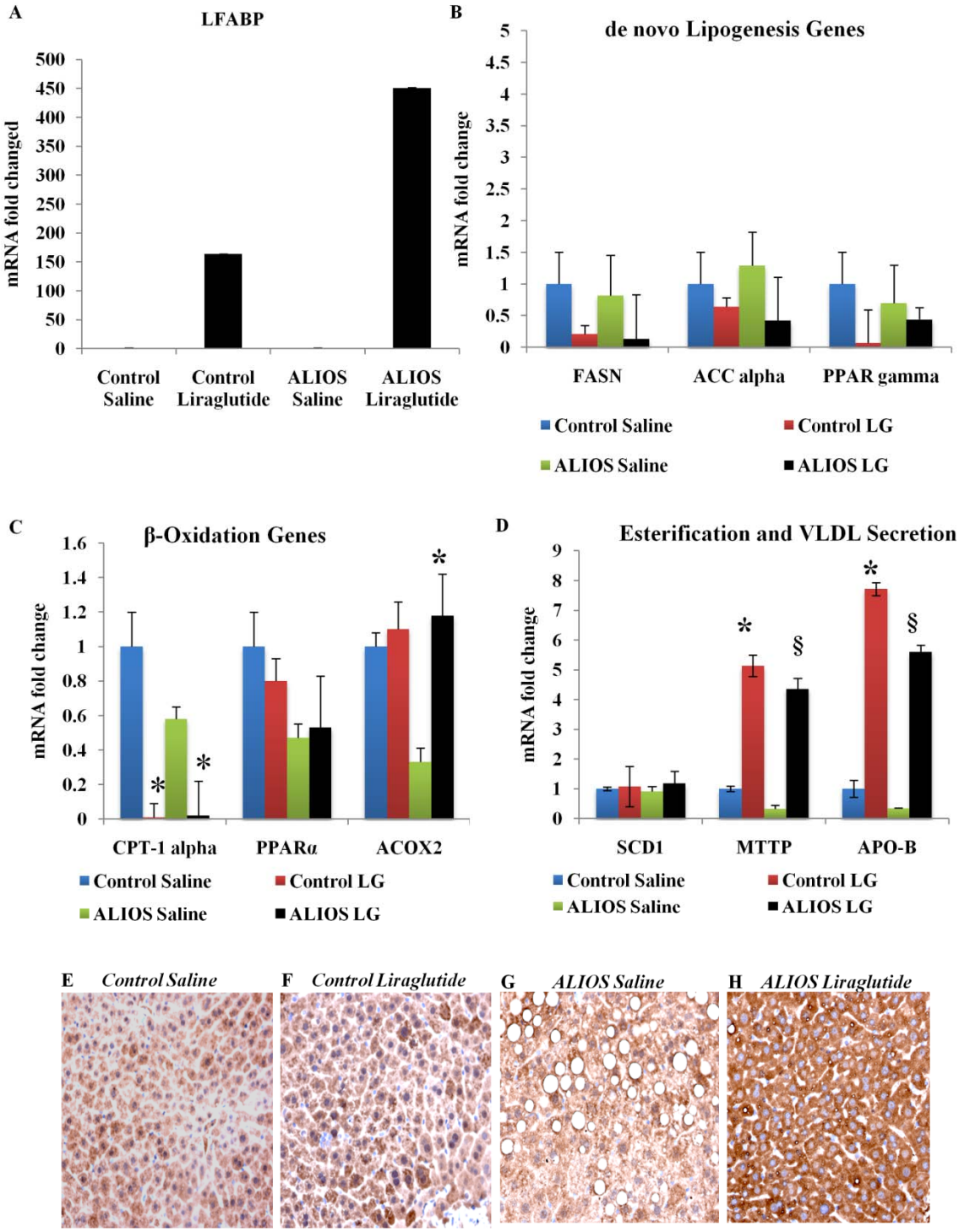


Figure 3.6: Liraglutide treatment reduces blood pressure and reduces cardiac hypertrophy. A: Systolic blood pressure **B.** Diastolic blood pressure. **C**. Cardiac hypertrophy: mouse heart weight was normalized to tibia length to account for growth. ALIOS fed mice treated with saline had significantly increased heart weight than all other groups ($p<0.05$). Liraglutide treatment of ALIOS fed mice significantly reduce their heart weights ($p<0.05$).

LG treatment significantly increased mRNA and proteins related to fatty acid uptake, peroxisomal β –oxidation, and VLDL transport. qRT-PCR was performed on RNA harvested from mouse livers. IHC and western blots were also performed. LG treatment resulted in a significant increase mRNA in LFABP, a protein critical for fatty acid uptake (Fig. 3.7A), in both control and ALIOS fed mice *($p<0.0001$)*. IHC of liver samples (Fig. 3.7E-F), revealed that LG strongly up-regulated LFABP in comparison to saline treated ALIOS-fed mice. LG reduced mRNA of genes associated with *de novo* lipogenesis including FASN, ACC alpha, and PPAR-gamma however these reductions were not significant (Fig. 3.7B). LG increased genes and proteins responsible for peroxisomal fatty acid β–oxidation (ACOX2) (Fig. 3.7C) in ALIOS fed mice ($p<0.05$), while decreasing genes related to mitochondrial β-oxidation (CPT-1alpha) in both control and ALIOS fed mice ($p<.05$).


A
LFABP
mRNA fold changed
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Control Saline
Control Liraglutide
ALIOS Saline
ALIOS Liraglutide
B
de novo Lipogenesis Genes
mRNA fold change
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3
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0
FASN
ACC alpha
PPAR gamma
Control Saline
Control LG
ALIOS Saline
ALIOS LG
C
β-Oxidation Genes
mRNA fold change
1.6
1.4
1.2
1
0.8
0.6
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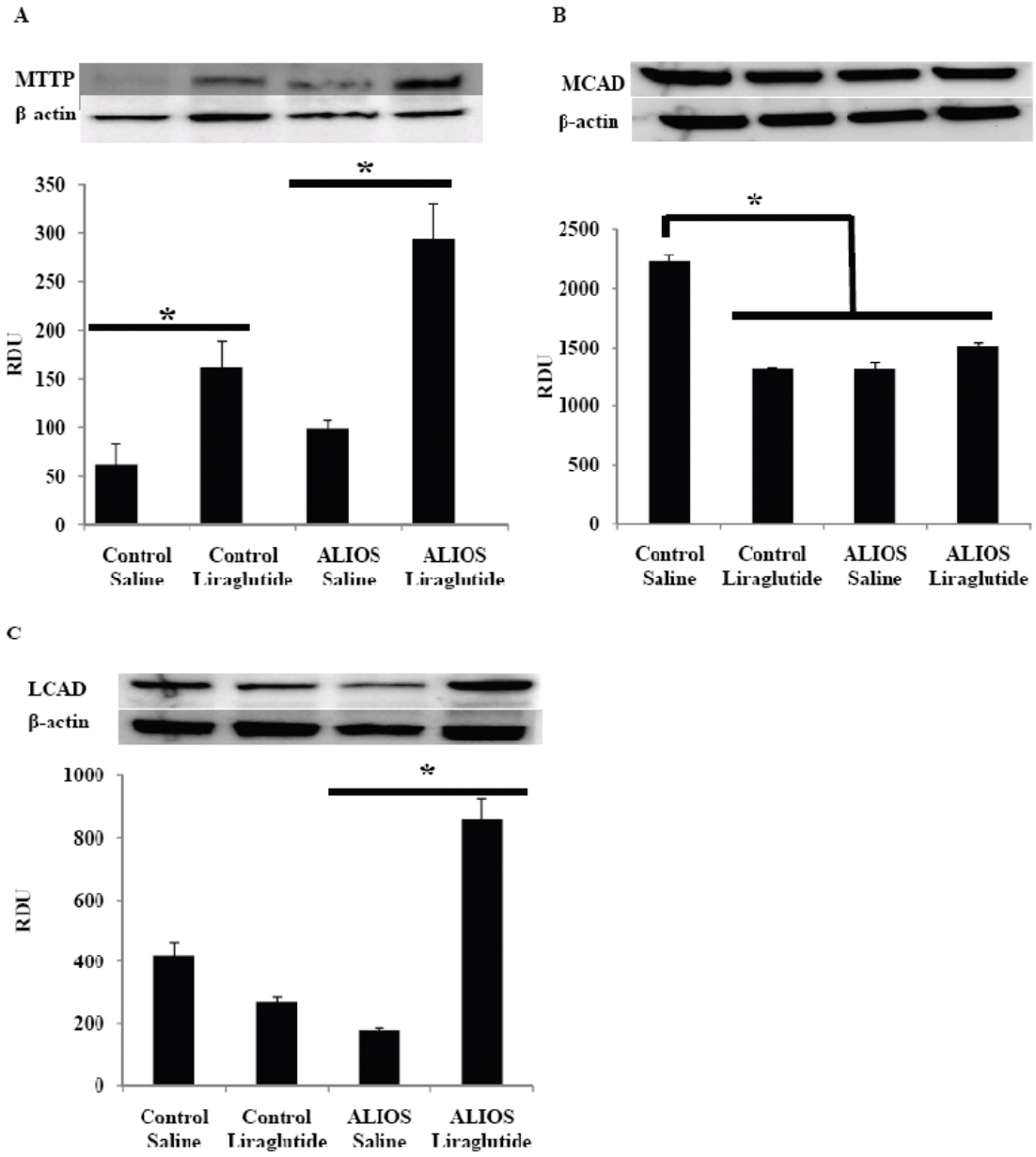
CPT-1 alpha
PPARα
ACOX2
Control Saline
Control LG
ALIOS Saline
ALIOS LG
D
Esterification and VLDL Secretion
mRNA fold change
9
8
7
6
5
4
3
2
1
0
SCD1
MTTP
APO-B
Control Saline
Control LG
ALIOS Saline
ALIOS LG
E Control Saline
F Control Liraglutide
G ALIOS Saline
H ALIOS Liraglutide

Figure 3.7: Liraglutide increases genes related to fatty acid uptake, β-oxidation, and VLDL transport. Liraglutide treatments were compared to their respective saline injected groups. **A:** Liraglutide treatment resulted in a significant increase of fatty acid binding protein (LFABP) in both control and ALIOS fed mice ($p<0.0001$). **B:** Fatty acid synthase [FASN], Acyl CoA Carboxylase Alpha (ACC-α), PPARγ, were all down regulated following liraglutide treatment in ALIOS-fed mice though these did not reach statistical significance compared to the ALIOS-fed saline treated cohort. **C:** CPT-1alpha was down-regulated after liraglutide treatment in both the control and ALIOS fed groups. In the ALIOS-fed liraglutide group ACOX2 was strongly up-regulated ($p<0.05$). **D:** There was no significant change in the expression of SCD1 between control and liraglutide treated groups; however, ALIOS-fed liraglutide mice have significant increase in both MTTP and Apo-B mRNA expression when treated with liraglutide. **E-H:** LFABP was evaluated using IHC. Liraglutide treatment resulted in a significant increase in LFABP expression in liver tissue.

Because the ALIOS diet included trans-fat we examined MCAD and LCAD, (both critical for desaturation of fatty acids) protein expression (Fig. 3.8B and 3.8C). Control fed animals had significantly higher levels of MCAD than all other groups ($p<0.05$) (Fig. 3.8B). Liraglutide treatment significantly increased LCAD protein expression in ALIOS-fed mice as compared to ALIOS-saline mice ($p<0.0001$) (Fig. 3.8C),

LG treatment also increased mRNA expression and protein for genes involved in lipid transport specifically MTTP (Fig. 3.7D) ($p<0.0001$). Protein analysis of MTTP (Fig. 3.8A) showed the same relationship. Liraglutide treatment increased protein levels of MTTP above the saline-matched controls ($p<0.001$ for both groups).


A
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Control Liraglutide
ALIOS Saline
ALIOS Liraglutide
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MCAD
β-actin
RDU
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Control Saline
Control Liraglutide
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ALIOS Saline
ALIOS Liraglutide

Figure 3.8: Liraglutide increases protein related to fatty acid uptake, β-oxidation, and VLDL transport. A: Liraglutide treatment significantly increased MTTP expression in both the control and ALIOS fed groups and also significantly increased MTTP mRNA protein expression in the ALIOS-fed liraglutide treated mice when compared to saline-treated ALIOS-fed mice. **B:** Liraglutide has no effect on MCAD expression; control had a significantly higher level of expression than all other groups ($p<0.05$). **C:** Liraglutide treatment significantly increased LCAD expression in the ALIOS fed group ($p<0.0001$).

Discussion

The increase in incidence of NAFLD and CVD patients closely parallels the increased consumption of HFCS and trans-fat in the U.S. (*61*). The present study provides new insights into the dysregulation of lipid handling as a result of a Western diet; and potential mechanistic insights whereby the long-acting GLP-1 analogue LG can reverse several critical failures associated with metabolic syndrome.

As anticipated the ingestion of the ALIOS diet resulted in ALIOS-induced insulin resistance; and cardinal features of metabolic syndrome including dyslipidemia, obesity, glucose intolerance, hepatic steatosis, and cardiac damage- including high blood pressure and cardiac hypertrophy. LG reversed or significantly ameliorated all major parameters associated with metabolic syndrome and insulin resistance. Importantly we have demonstrated several additional findings worthy of discussion including that hepatocyte insulin resistance predates the development of significant systemic insulin resistance (Fig. 3.3); the development of metabolic syndrome in mice leads to high systolic and diastolic blood pressure, and cardiac hypertrophy, (Fig. 3.6), and we have begun to elucidate potential pathways to account for recently published data that GLP-1 analogues are capable of reducing hepatocyte FFA stores *in vitro* and *in vivo* as we and others have recently described (Fig.3.7-3.8) (*36, 83*).

Our present work demonstrates that hepatic insulin resistance appears to precede the clinico-pathologic features of systemic insulin resistance including steatosis and peripheral glucose intolerance. Hepatic insulin resistance is defined as the impairment of insulin-mediated suppression of glucose production (*24*); and accumulating data indicate hepatic insulin resistance is a pivotal pathophysiologic mediator in the development of

systemic insulin resistance (*25, 26*). The findings of this study are the first to demonstrate hepatic insulin resistance within six weeks of ALIOS administration in normal wild type mice. While the exact mechanism by which this may occur is beyond the scope of this manuscript, there is relatively little existing data regarding the temporal relationship between systemic insulin resistance and hepatic insulin resistance in the context of diet induced obesity (*18*).

An important pathophysiologic change attributed to hepatic insulin resistance is perturbations in VLDL production (*138, 139*). Increased production of VLDL particles in response to increased adipocyte-derived FFA is commonly seen in T2DM. However, the role of VLDL secretion in NAFLD and NASH is not entirely clear. Studies by Fujita *et al*. in Japanese men and women, with either non-alcoholic fatty liver (NAFL) or non-alcoholic steatohepatitis (NASH) (*34*) demonstrated NASH patients had significantly lower expression of apoB100, and lower serum VLDL-TG. In the same study MTTP mRNA levels were significantly lower (*34*). Similar findings were reported in de Piano *et al*. in an intervention study in obese adolescents (*37*). NAFL patients had higher levels of TG and VLDL cholesterol even following a twelve-month intervention, indicating that like the model used in our work there are clearly metabolic perturbations in VLDL synthesis and release. For those patients at a higher risk for developing NASH, our data indicate that GLP-1 synthetic analogues may be a viable treatment option. Future work will provide additional information concerning the relationship of GLP-1, insulin and VLDL synthesis and transport.

The ALIOS diet resulted in higher systolic and diastolic blood pressure and cardiac hypertrophy within 12 weeks of feeding. The finding that LG improves cardiac

function is in agreement with early studies from Yellon's lab and emerging studies from the Drucker laboratory, which have shown that GLP-1 and its analogues protect against myocardial ischemia perfusion injury, and increase left ventricular function, through GLP-1 receptors found in the myocardial cells (*140-142*). While we found other features of metabolic syndrome including elevated (ALT) levels, recapitulating all of the various clinical findings of metabolic syndrome in mice has proven difficult for investigators, and usually requires genetic manipulation or nonphysiologic diets (e.g. methionine-choline deficient diets) (*143, 144*). We show that combining HFCS and trans-fat accelerates the effects seen in longer studies examining the nutrients separately. Data related to serum ALT values and statistically insignificant data, e.g. mRNA expression of FASN, ACC-alpha and PPAR-gamma, may have resulted from the abbreviated feeding and or treatment periods as conceived herein.

On the other hand we also confirmed in the model how primary genes involved in hepatocyte lipid handling can be manipulated by GLP-1. These results have significant implications for work we performed recently *in vitro* in primary human hepatocyte culture demonstrating the GLP-1 analogue exendin-4 significantly reduced triglyceride and FFA stores (*36, 83*). LG administration decreased ACC-alpha mRNA expression levels after four wk of treatment; this is in accordance with our previous findings in *ob/ob* mice treated with exendin-4 for sixty days. (*36*). Based upon our previous studies, we hypothesized that GLP-1 through its cognate receptor may impair *de novo* lipogenesis and enhance peroxisomal β-oxidation (*36, 83*). Data from our qRT-PCR analysis support this hypothesis: mRNA for both PPAR-gamma, a transcriptional regulator of fatty acid

storage and FASN a multi-enzyme complex essential for fatty acid synthesis were down-regulated in ALIOS-fed mice treated with LG. This is in agreement with a recently study by Shlomo *et al*, showing that GLP-1 reduces hepatic lipogenesis via activation of AMPK (*145*).

Another important result was the dramatic increase in LFABP and MTTP (Fig. 7-8). This is the first published study linking GLP-1 analogues with increased LFABP and MTTP expression. The critical role of these proteins in the progression of NAFLD may be explained by the emerging concept of FFA lipotoxicity and hepatic flux. Lipotoxicity is defined as cellular toxicity that occurs in non-adipose tissue in the presence of FFA (*42-44*) that leads to hepatocyte death by apoptosis. There is growing support for the concept that triglyceride accumulation is actually a protective mechanism used to abate FFAs toxic effects (*44*). Recent evidence suggests that FFA exposure and overload may be potentially more hazardous to hepatocytes, and can lead to a dysfunctional unfolded protein response and diminished capacity for the endoplasmic reticular (ER) pathway to prevent apoptosis (*42-44*). Hepatic flux or lipid equilibrium is maintained by four inter-related mechanisms: uptake of FFA, *de novo* lipogenesis, secretion of VLDL, and β-oxidation (*27, 39, 40*). A defect in any one of these mechanism leads to aberrant storage of lipid and potential exposure to FFA (*27, 41*).

Dietary fat and glucose have both been shown to up-regulate LFABP expression (*146-148*). LFABP enhances uptake of long chain fatty acids (LCFA), binding both LCFA and respective metabolites targeting them for peroxisomal β-oxidation, or the ER for increased VLDL packaging and secretion (*146, 149, 150*). Increasing LFABP may be a compensatory mechanism used to dispose of excessive FFA, whereby the liver

serves as a "sink" for toxic FFA (*43*). We have shown the expression of LCAD and ACOX2, proteins directly involved in β-oxidation were also up-regulated in the LG-treated ALIOS-fed mice, which should increase the cell's capacity to oxidize fatty acids more rapidly. We recognize that without functional enzyme assays we can only speculate that increased expression of proteins and enzymes associated with increased FFA transport and β-oxidation leads to increased hepatocyte turnover and disposal. However, these data confirm *in vitro* data we published several years ago; and provide a plausible mechanism for the *in vitro* data demonstrating rapid reduction in FFA in primary human hepatocyte culture (*83*). In sum we demonstrate increased FABP, diminished expression of DNL genes and increased expression of genes associated with oxidation in LG-treated ALIOS-fed mice.

MTTP is an essential heterodimer protein located in the lumen of the ER (*18, 151, 152*). MTTP facilitates the transfer of neutral lipid to ApoB (*152, 153*). If FFA induces lipotoxicity- MTTP expression and levels are critical to preventing FFA overload of the ER, since MTTP levels in NASH are suppressed; and, patients with *abetalipoproteinemia* have defects in their MTTP activity, which results in steatotic hepatocytes and almost no circulating ApoB (*34, 151, 152*). Experiments by Shindo *et al.* in Fatty Liver Shionogi (FLS) mice, which exhibit NASH lesions, also demonstrated that hepatic induction of MTTP resulted in a reduction in NASH lesions, improvement in VLDL export, and improved glucose tolerance (*154*). The ALIOS diet appears to down-regulate MTTP mRNA expression; thereby lowering the ability of the hepatocyte to package FFA with ApoB; which leads to increased exposure to FFA within the cell. LG treatment

significantly increases both mRNA and protein expression of MTTP, which in theory should increase the hepatocyte's ability to package FFA.

From our previous work it is clear that GLP-1 is able to reduce lipid accumulation in hepatocytes in the absence of insulin (*83*). However, the up-regulation of LFABP and MTTP and LCAD, ACOX2, and ApoB when taken together imply increased hepatic capacity to handle FFA. Since insulin is known to inhibit MTTP expression, as well as the rate of ApoB synthesis, it is very unlikely that the effects observed in the present studies are derived from the incretin function of GLP-1 or a GLP-1 increase in circulating insulin (*18, 152, 155*).

In conclusion the present investigations provide a robust model to further study not only NAFLD but non-hepatic parameters of metabolic syndrome in a non-genetic, non artificial diet. These data, and recent data by laboratories throughout the world substantiate the multiple and direct effects of GLP-1 analogues that go beyond function as incretins (*36, 97, 141, 145*). Finally this work along with other recent work provide a plausible working hypothesis which suggests that GLP-1 analogues markedly improve hepatocyte handling of free fatty acids by improving hepatocyte uptake, transport and peroxisomal β-oxidation as well as secretion. Such beneficial actions may improve the ability of hepatocytes to more effectively handle FFA, thereby preventing or reversing potential lipotoxic effects including a dysfunctional unfolded protein response and hepatocyte apoptosis—events associated with the progression of non-alcoholic liver disease to steatohepatitis and cirrhosis (*103, 156, 157*).

Chapter 4: Conclusions and Future Directions

Conclusion Summary

As the incidence of obesity and insulin resistance increase, so does the need to develop safe and effective treatments. GLP-1 is an ideal candidate for the treatment of NAFLD because of its pleotropic effects on multiple tissues. My hypothesis for my dissertation is:

Hypothesis: GLP-1 and its synthetic analogues act directly on the hepatocyte to reduce net hepatic triglyceride stores via activation of its cognate receptor.

Specific aim 1: To characterize the cellular interactions of GLP-1 in hepatocytes.

Specific aim 2: To characterize the normal and abnormal pathways of hepatic lipid metabolism.

Novel findings

Our *in vitro* studies were significant because they demonstrated for the first time that GLP-1R is present on human hepatocytes; and functional on hepatocytes and other human hepatocyte-derived cell lines. Data from Taniguchi *et al*. using liver-specific double knockout phosphoinositide 3-kinase (PI3K) mice seem to indicate that activation of this pathway is involved in lipid homeostasis (*158*). Furthermore, we have shown that in the **absence of exogenous insulin** GLP-1 and its analogues activate key elements of the insulin signaling pathway including phosphorylation of Akt, PDK-1 and PKC-ζ (*83*). Since our publication in **Hepatology**, others have shown that GLP-1 has direct action on hepatocytes. Shlomo *et al.* demonstrated in rodent hepatocytes, that GLP-1 decreased genes associated with *de novo* lipogenesis via AMPK activation (*145*). These data

corroborates our *in vivo* data, showing a decrease in the genes associated with *de novo* lipogenesis. There still exists some debate in the field as to the existence of an additional receptor present in the liver for GLP-1; however there has been no publication that has identified this orphan receptor.

The insulin-like action of GLP-1 could explain the clinical data from patients undergoing Roux-en-Y gastric bypass (RYGB) in comparison to those patients who underwent laparoscopic adjustable gastric band (AB). The RYGB procedure is unique because it moves the ileum closer to the stomach and portal circulation; hence the biological effect of increasing the concentration of portal venous GLP-1 could account for consistently shown clinical data that patients undergoing RYGB have significantly higher levels of GLP-1 in their blood; and more importantly, most RYGB patients resolve their T2DM within weeks of surgery, without significant weight loss (*23, 159*).

While the insulin-like mimetic effects of GLP-1 could potentially explain improved glucose tolerance and is itself a novel finding, the latter half of the work sought to determine how GLP-1 affected key proteins that modulate fatty acid. The aim of the ALIOS-liraglutide study was to expand the scope of our research and examine the development of NAFLD *in vivo,* in a model that did not require genetic manipulation or a non-physiologic diet. The ALIOS liraglutide study was an important study, because we successfully recapitulated many of the key features of NAFLD.

There is still considerable debate about the role of hepatic insulin resistance in the overall pathology of NAFLD. A key finding from the study was the discovery that hepatic insulin resistance preceded systemic, or peripheral, insulin resistance. We established a temporal relationship between hepatic insulin resistance and systemic insulin resistance that has been relatively unexplored in the literature. Hepatic insulin resistance occurred fairly rapidly before the onset of systemic insulin resistance-indicating that hepatic insulin resistance may be the pivotal player in the etiology of systemic insulin resistance and NAFLD. These data stand in direct contrast with prevailing theory that attributes the initiation of insulin resistance to a change solely in adipokine secretion (*14*) from white adipose tissue (WAT). Our data support the molecular studies by Michael *et al.* and Taniguichi *et al.* that underscore the concept that the liver is the pivotal player in establishing insulin resistance and diabetes (*25, 158*).

It is clear that GLP-1 and its analogs change how lipid is handled in the hepatocyte. Our data seem to suggest that liraglutide improves the hepatocytes' ability to handle FFA flux. Initially we framed our entire discussion of GLP-1 in terms of its relation to insulin, either as an insulin-sensitizing agent or having insulin-like properties in the hepatocyte. Many GLP-1 investigators are of the opinion that the GLP-1/GLP-1R system is redundant secondary to insulin, as further evidenced by its rapid cleavage by DPPIV. However there also exists the possibility that the GLP-1/GLP-1R system may function as a bridge between insulin and glucagon. The significant increases in LFABP, MTTP, and APO-B, coupled with the significant reductions in lipid accumulation in our cell culture studies suggest that GLP-1 has lipid related effects similar to glucagon.

Liraglutide administration appears to promote three mechanisms associated with total hepatocyte free fatty acids stores: (1) increases β- oxidation of fat, (2) increases VLDL secretion, and (3) reduces *de novo* lipogenesis. As previously mentioned Shlomo et al. demonstrated in rodent hepatocytes that GLP-1 decreased genes associated with *de novo* lipogenesis via AMPK activation (*145*). Additional work in our laboratory, has demonstrated that exendin-4 administration increased the production of ketone bodies in hepatocytes: indicating an increase in β- oxidation. Additional work examining in the role of GLP-1 proteins in fatty oxidation is clearly warranted.

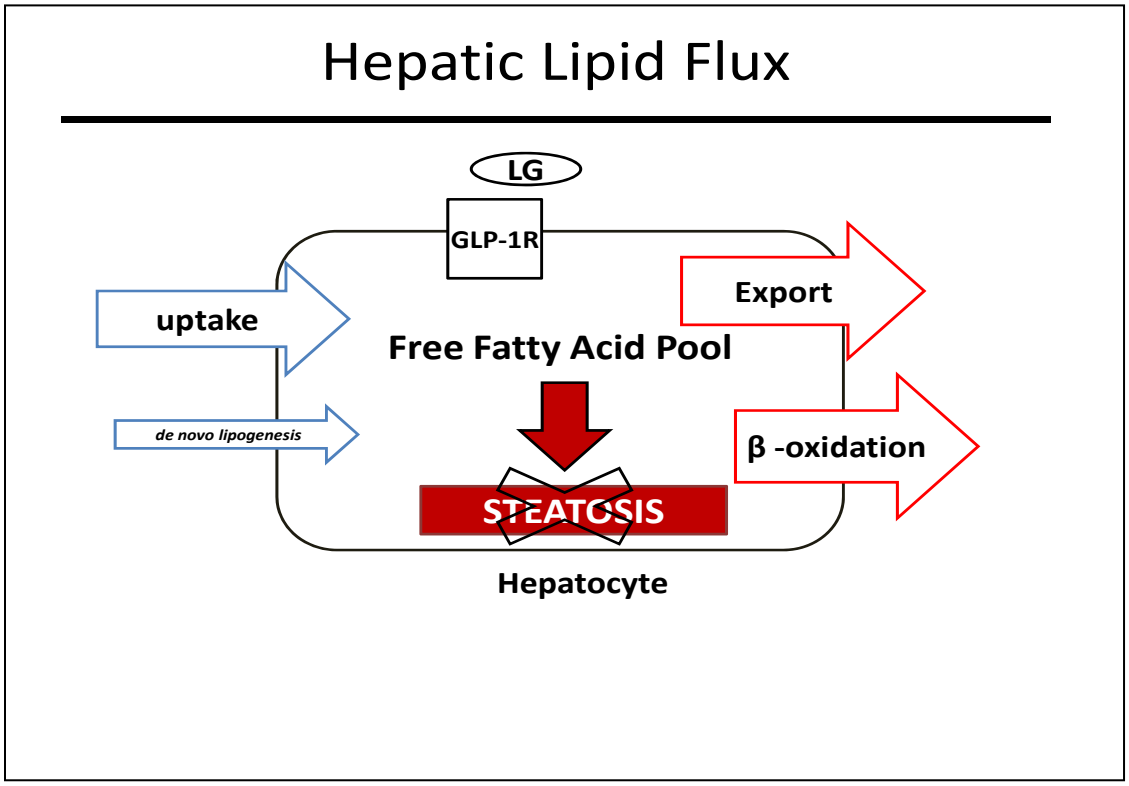

Figure 4.1: Schematic showing the effects of liraglutide on hepatic lipid flux. Liraglutide treatment increase uptake, export and β- oxidation, while decreasing *de novo* lipogenesis.

Future Aims

My dissertation has largely focused on demonstrating that GLP-1 has a functional receptor on the hepatocyte and has direct action on the liver-independent of its incretin effects. Now that we and others have shown that the GLP-1R receptor is present and functional in human hepatocytes, there are several avenues that can be explored. I hypothesize that GLP-1 serves as a bridge between the insulin and glucagon systems; providing insulin-like action in the hepatocyte during feeding or the acute phase, and lipid mobilization and utilization during fasting. To examine this hypothesis studies should first be conducted to determine if activation of these downstream insulin targets serves to mimic insulin's effects or are they acting as an insulin sensitizer.

Specific Aim 1: To determine if GLP-1 acts an insulin mimetic in the liver. In this aim I would test if GLP-1 is able to suppress hepatic glucose production.

Rationale:

From the literature, the term "insulin sensitizer" appears to relate more to tissues with high concentrations of GLUT4 receptors, i.e. brain, muscle and adipose tissue. Pharmacologic agents that result in increased expression of GLUT4 consequently make the tissue more "sensitive" to insulin; thereby facilitating an increase in insulin-mediated glucose uptake. In my work I saw no change in expression of GLUT2, the predominant receptor in the liver. In addition studies Buettner *et al* showed that acute down-regulation of insulin receptors, approximately 95%, did not affect insulin's ability to suppress hepatic glucose production (*160*) – so the idea that GLP-1 sensitizes the liver may be inaccurate; because even with only 5% of receptors available insulin is able to exert its

effects. Our data seems to suggest that GLP-1 may be functioning as an insulin mimetic. Using an *in vitro* system, we can test if GLP-1 and its analogs are able to suppress hepatic glucose production. Potential studies should take advantage of the availability of insulin receptor knockout cell lines. The goal of this approach is to establish if GLP-1 is able to restore insulin-like function to the cell. This can also be tested *in vivo* using our recently developed liver specific GLP-1R knockout mice, and comparing their response to the wild type and LIRKO mice.

Our integrative approach to studying lipid handling via liraglutide administration has opened up several additional avenues for future research. However we still have not answered the question: "where has the lipid gone?"

Specific Aim 2: To determine how GLP-1 administration reduces lipid accumulation in the liver: how are secretion, transport, and β- oxidation affected.

Rationale: We now have several published several papers demonstrating that GLP-1 and its analogs reduce lipid accumulation in hepatocytes in the absence of insulin. However we have not examined the lipid turnover and metabolism in a quantitative manner. Potential studies should first be performed *in vitro*, and take advantage of radio-labeling techniques. Using an *in vitro* model we can use [^{14}C]-acetate incorporation to examine GLP-1's effect on *de novo* lipogenesis. To determine the rate of β- oxidation we can use [^{14}C]-palmitate to quantify the rate of β- oxidation in hepatocytes. Media can be collected, and concentrated to examine APO-B concentrations as a measure of secretion.

Additional studies should explore the two surprising findings from our ALIOS-liraglutide study: the up-regulation of LFABP and MTTP.

Sub aim 1: To characterize the relationship of GLP-1 to LFABP

Sub aim 2: To characterize the relationship of GLP-1 to MTTP

Rationale: There are no published reports linking GLP-1 to LFABP, potential studies should focus on determining how GLP-1 results in increased expression of LFABP. PPARα has been shown to physically interact and up-regulate LFABP (*147, 148*). GLP-1 and its analogs have all been shown to increase PPARα. Studies by Binas *et al.* using liver-specific knockout mice demonstrated that LFABP expression is critical for uptake, oxidation, and esterification of long-chain fatty acids (*149*).

The most promising avenue of research is the regulation of MTTP by GLP-1. MTTP appears to be a nexus between uptake and the processing and packaging of lipids into VLDL particles. It also ties in with on-going work in the lab focusing on autophagy, and ER stress. MTTP is located in the lumen of the ER. Future studies should include *in vitro* studies to elucidate how GLP-1R agonists up-regulate MTTP. An interesting side note, carbon tetra-chloride (CCl_4) is used by our lab and others to induced hepatic fibrosis in mice (*161*). Studies by Pan *et al.* have shown that inhibiting the proteosomal degradation of MTTP prevented CCl_4- induced fibrosis (*162*). The link between MTTP expression and liraglutide administration could explain how GLP-1 and its analogs

increase the hepatocyte lipid handling efficiency. Coupled with the studies in FLS mice- it is reasonable to examine if decreases or defects in MTTP activity determine which patients go on to develop NASH. An additional *in vivo* study that gave CCl4 and liraglutide to determine if liraglutide can prevent NASH is definitely an exciting avenue worth pursuing.

Additional Questions

There remain several questions about GLP-1R regulation and turnover. Our cell fraction and bioluminescence studies provided initial clues into the receptor cycling- however, we still do not know if GLP-1 initiates additional signals upon binding and translocation into the cell. Future studies should include Scatchard binding analysis and, as previously mentioned, self-labeling protein tags linked to fluorophores to clarify how GLP-1 upon binding to its receptor is processed and recycled. Future studies should also include an ALIOS feeding time course to establish, how early hepatic insulin resistance can be established in this mouse model.

Finally, a clinical trial similar to the PIVENS trial needs to be conducted to determine the efficacy GLP-1 analogues in reducing parameters associated with NAFLD (*77*). Currently, both GLP-1 analogues (exendin-4 and liraglutide) are prescribed for diabetic patients, usually in conjunction with Metformin. A recent publication by Maida *et al.* reported that Metformin significantly increased plasma levels of GLP-1 and gene expression of GLP-1R in mice (*163, 164*). Taken together with our findings in primary

human hepatocytes- future studies should focus on establishing GLP-1R agonists as a viable treatment option for NAFLD.

In summary we found that GLP-1 does act directly on hepatocytes via the GLP-1R receptor, and reduces lipid accumulation in hepatocytes by increasing secretion and β-oxidation within the cell.

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