CHRONICALLY ELEVATED GLUCOCORTICOIDS IN CONJUNCTION WITH A HIGH FAT DIET: A MODEL OF NON ALCOHOLIC FATTY LIVER DISEASE

ANNA M. D'SOUZA

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Abstract

Non alcoholic fatty liver disease (NALFD) is characterized by lipid excess and is believed to be the hepatic component of the metabolic syndrome (MetS). Previous studies have linked chronically elevated levels of glucocorticoids (GCs) with features of MetS, including abdominal obesity, insulin resistance and elevated plasma FFA (9, 8, 24). Little is known of the interactive effects of chronically elevated GC levels with poor lifestyle choices, including consumption of a high fat diet, and sedentarinism, especially in the context of NAFLD. Thus, we explore the effects of a short-term exposure to chronically elevated CORT levels in the presence of a high fat diet. Male Sprague Dawley rats were randomly divided to receive either corticosterone (CORT), via subcutaneous implantation of 400 mg of CORT pellets, or the equivalent amount of wax pellets to serve as a control for pellet treatment. Animals from each pellet group were then randomly assigned to either a 60% high fat diet (HFD) or normal rodent chow. In the presence of chronic CORT, HFD exacerbated hepatic steatosis. Hepatic fibrosis, plasma alanine aminotransferase (ALT) levels and visceral fat mass were dramatically elevated with chronically elevated plasma CORT. These effects were further exacerbated in the presence of HFD. Chronic CORT in conjunction with HFD also resulted in elevated fasting insulin and glucose values, severe whole body insulin resistance, and impairments to hepatic insulin signalling. These results suggest that HFD in the presence of chronic CORT can exacerbate development of NAFLD in an interim period.

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List of Abbreviations

11βHSD1	11 Beta-Hydroxysteroid Dehyodrgenase 1
ACTH	Adrenocorticotropic Hormone
ALT	Alanine Aminotransferase
CD36	Cluster of differentiation 36
CPT-1	Carnintine Palmityl Transferase 1
CRH	Corticotrophic Releasing Hormone
CORT	Cortisol/Corticosterone
CBG	Cortisol/Corticosterone Binding Globulin
EGP	Endogenous glucose production
ELISA	Enzyme linked immunosorbent assay
FASN	Fatty acid synthase
FATP5	Fatty acid transport protein 5
FFA	Free fatty acid
FOXO1	Forkhead box protein O1
G6Pase	Glucose 6 Phosphatase
GIR	Glucose Infusion Rate
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
GC	Glucocorticoid
HFD	High fat diet
HOMA-IR	Homeostasis Model Assessment For Insulin
HPA axis	Hypothalamic Pituitary Adrenal axis
IKKβ	I Kappa B Kinase Beta
IRS-1	Insulin Receptor Substrate 1
L-FABP	Liver Fatty acid binding protein
LPL	Lipoprotein lipase
MetS	Metabolic Syndrome
mRNA	Messenger Ribonucleic Acid
NAFLD	Non alcoholic fatty liver disease
NASH	Non alcoholic steatohepatitis
NEFA	Non esterified fatty acids
NFKβ	Nuclear Factor Kappa Beta
OGTT	Oral glucose tolerance test
PGC1α	Peroxisome proliferator-activated receptor coactivator 1-alpha
PEPCK	Phosphoenolpyruvate Carboxykinase
PI3-K	Phosphoinositide 3 Kinase
R_A	Rate of glucose appearance (hepatic glucose production)
R_D	Rate of glucose disposal (peripheral glucose utilization)
RIA	Radio immunoassay
SCN	Suprachiasmatic nuclei
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SREBP-1	Sterol regulatory binding protein 1
T2DM	Type 2 Diabetes Mellitus
TG/TAG	Triglycerides/Triacylglycerol
TNFα	Tumor Necrosis Factor Alpha
VLDL	Very low density lipoprotein

Introduction 1

Parallel with the growing rate of obesity is the rising occurrence of other related metabolic disorders including type 2 diabetes and the metabolic syndrome (MetS). Approximately one in four individuals suffers from the metabolic syndrome (MetS). MetS was first described more than 80 years ago as a way to classify a collection of factors that increase risk of cardiovascular disease (171). Though MetS is commonly characterized by abdominal obesity, insulin resistance, hypertension, hyperlipidemia and reduced HDL levels, lipid accumulation in the liver (steatosis) is also commonly amongst individuals with MetS. Approximately 70-75% of all individuals with MetS have steatosis (144). This ancillary condition, once believed to be benign, is now being recognized in the pathogenesis of non alcoholic fatty liver disease (NAFLD).

NAFLD often referred to as the hepatic component of MetS due to the high incidence of insulin resistance and abdominal obesity amongst individuals with NAFLD. NAFLD is characterized by lipid accumulation in the liver, primarily in the form of triacylglycerols, in individuals who consume less than 20 g of ethanol per day (223). Lipid accumulation that initially occurs with NAFLD can progress into a spectrum of conditions including development of non alcoholic steatohepatitis (NASH), liver fibrosis, and cirrhosis (2).

Amongst the many factors contributing to development of NAFLD, a positive energy balance due to excess caloric intake is the most common (241).

Positive energy balance can occur as a result of a high calorie diet, likely due to increased fat intake. Such diets have been shown to increase adipose accumulation centrally and induce insulin resistance (55).

In addition to excess caloric intake, chronically elevated glucocorticoid (GC) levels are emerging as an important risk factor contributing to metabolic dysfunction. The high incidence of elevated plasma GC levels amongst individuals with obesity or diabetes (148), and common occurrence of insulin resistance, hyperglycemia and hypertension amongst individuals with elevated endogenous GC levels suggests that GCs are a potent contributor to impaired glucose and lipid metabolism (99). In addition, fatty liver has also been characterized among individuals with chronically elevated GC levels (202). However, few studies have examined the interactive effects of GCs with other lifestyle risk factors, in particular, poor dietary choices and their influence on the development of the MetS and NAFLD.

Thus, the purpose of this study was to assess the influence of chronically elevated GC levels in combination with consumption of a high fat diet on the development of NAFLD in a sedentary rodent model. Previous studies have shown that elevated GCs or consumption of a high fat diet are independently capable of inducing insulin resistance, abdominal obesity, elevated plasma FFA and TG levels, all of which are associated with development of fatty liver disease (55, 62, 79, 109). Therefore, it is possible that when combined, chronic GCs and HFD can rapidly exacerbate development of NAFLD.

Review of the Literature 2

2.1 Metabolic Syndrome

The term "metabolic syndrome" was used in the 1970s by Haller (88), describing a phenotype that had been noticed since the 1920s (123). It was first described as the clustering of disease characteristics that included hypertension, hyperglycemia, steatosis and gout (65). Reaven et al. later went on to define this syndrome in 1988 as "insulin resistance syndrome" or "syndrome X" to recognize a group of conditions with a high risk for cardiovascular disease (7, 171). In 2001, the Third Report of the National Cholesterol Education Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (151) defined MetS based on five categorical predictors including: abdominal obesity, insulin resistance, hypertriglyceridemia, reduced HDL-C levels, and increased blood pressure (7). Hepatic steatosis, *per se*, is probably not one of these predictors since it is not as easily screened for as these other measures listed above. It is estimated that one fourth of the world's population suffers from metabolic syndrome (MetS), defined as having 2 or more of the categorical predictors listed above (115). The global epidemic of obesity and diabetes has resulted in an exponential increase in the number of individuals with MetS (138). The increasing rate of central obesity has been the driving force behind the growing prevalence of MetS. Though the clinical definition of MetS does not distinguish between increases in subcutaneous versus visceral central adipose

depots, the predominance of this syndrome amongst populations with greater intra-abdominal adiposity versus those with greater central subcutaneous adiposity suggests that the higher metabolic rate of larger visceral adipose depots may explain the increased lipid delivery to and accumulation in surrounding organs associated with this condition (87, 215). This increase in adipose storage is especially relevant with the concomitant occurrence of insulin resistance, which is the primary characteristic of MetS. With the development of insulin resistance, insulin is no longer able to inhibit lipolysis of triacylglycerol in stored adipose tissue, resulting in a greater amount of free fatty acid released (106). High levels of circulating fatty acids can promote ectopic lipid accumulation in peripheral insulin sensitive tissues, such as the liver and skeletal muscle and lead to modifications downstream of the insulin signalling pathway (42). Thus, it may be that steatosis can result from and simultaneously contribute to insulin resistance syndrome/MetS.

2.2 Non alcoholic Fatty Liver Disease (NAFLD)

Fatty liver has previously been associated with excessive alcoholic consumption. The presence of a fatty liver in patients with MetS has long been reported, and was initially overlooked as an incidental occurrence with little clinical significance (204). Now however, NALFD is emerging as the most common chronic liver condition in the western world, with the potential to lead to major liver damage and loss of function (3). It encompasses a range of

pathological abnormalities starting with simple steatosis (fat accumulation in the liver), which can then progress to non-alcoholic steatohepatitis (NASH) if inflammation occurs. (184). Most cases of NALFD occur in obese (60-95%), type 2 diabetic (28-55%) or hypertriglyceridemic patients (27-92%) (33,32). Amongst individuals with liver disease, the incidence of MetS ranges from 14% in those with fatty liver, to 38% in non-alcoholic steatohepatitis (139). Diagnosis of NAFLD is primarily based on hepatic ultrasound and liver function tests (143), but only liver biopsy is able to confirm the histological type of NAFLD and provide prognosis. The histological criterion for the diagnosis of NAFLD is the presence of fat in more than 5% of the liver (152). Accumulation of fat usually starts in zone 3 of the liver, and may spread to occupy the whole acinus, or functional unit of the liver (31). Plasma levels of alanine aminotransferase, ranging from 2-5 fold elevations (of which normal values range from 9-50 U/L), are used as a marker of hepatic tissue injury (59, 167).

NALFD is attributed to genetic defects or a sustained positive energy balance, possibly through consumption of a high fat diet (125). Several mechanisms may lead to fatty liver including: increased free fatty acid supply due to increased spill over from visceral adipose tissue, decreased fat oxidation, increased de novo hepatic lipogenesis and decreased synthesis and export of very low density lipoproteins from the liver. However, because NAFLD share many of the criteria for MetS, it is possible that many more factors contribute to development of this disease. Studies examining the correlation between hepatic

steatosis and plasma GC levels have shown that the chronic use of corticosteroids as drug therapy contributes to development of fatty liver (75). Similarly, patients with elevated activation of the neuroendocrine hypothalamic pituitary adrenal (HPA) axis, as in Cushing's syndrome, have a higher prevalence of steatosis in comparison to the general population, and as many as 20% have NAFLD suggesting that elevated stress hormone levels may play a role in disease progression (180,216).

2.3 *Glucocorticoids*

The stress response was first described by Hans Selye, the father of stress research (198). Selye characterized stress as a response to a threat to physiological homeostasis. Upon facing a 'stress stimuli', the hypothalamic pituitary adrenal (HPA) axis is activated, and results in the synthesis and release of glucocorticoids (GCs) from the adrenal cortex. Corticotrophin releasing hormone (CRH) is released from the paraventricular nucleus of the hypothalamus, which then acts on the anterior pituitary to release adrenocorticotrophic releasing hormone (ACTH). ACTH enters into circulation, and binds to its respective receptors on the adrenal cortex, stimulating synthesis and release of GCs (corticosterone in rodents, cortisol in humans). GC synthesis is regulated by a negative feedback loop in which GCs act on receptors in the central nervous system to inhibit further secretion of CRH and its downstream activity (209). GCs have pleiotropic effects on the body, including suppression of

immune response, bone tissue loss, and substrate mobilization (26). Overabundance of GCs is linked to metabolic abnormalities including central obesity and insulin resistance, as demonstrated by Cushing's disease, in which an adrenal or pituitary adenoma results in excess GC production. Alternatively, an insufficient level of GCs, which occurs with Addison's disease, is also associated with adverse effects including weight loss, lethargy and hypotension (165). These findings demonstrate the importance of tightly regulated GC levels to avoid adverse metabolic outcomes.

i. Glucocorticoid Receptor and Regulation

GCs are released from the adrenal cortex, primarily as their active form (cortisol in humans, corticosterone in rodents), with a smaller fraction of inactive GCs also released (cortisone in humans, 11deoxycoricosterone in rodents) Because of their lypophilic nature, the majority of GCs are either bound to proteins (90% bound to cortisol binding globulin, 5% bound to albumin) or remain in a conjugated form (as sulphate or glucouronide derivatives) (28, 169). The unbound form of GCs enters freely into cells, and is considered the biologically active fraction that is able to diffuse freely into cells and exert their effects by binding to glucocorticoid receptors located within cells. The plasma half-life of GCs ranges from ~80 minutes (cortisol) to 270 minutes (dexamethasone, a synthetic GC) (190). The liver and kidney are the major sites of hormone inactivation and elimination. Metabolic degradation occurs primarily in the liver via the A-ring reductase series of enzymes that convert the active form of GCs

(cortisol or corticosterone) into its inactive tetrahydrometabolites, either 5α- and 5β-tetrahydrocortisol and 5β-tetrahydrocortisone (61). The kidney excretes 95% of these metabolites and the gut eliminates the remainder (189).

GCs primarily exert their intracellular genomic effects via the GC receptor (GR). The GR is associated with accessory proteins in the cytosol, for example heat shock proteins (HSP90, HSP70). Following binding of GCs, a ligand-receptor complex forms and then enters into the nucleus and binds to glucocorticoid response elements (GREs) to regulate transcription. In addition, the GC-GR complex is also known to induce a non-transcriptional effect through interacting with nuclear factor kappa B and activating protein 1 (169). Within peripheral tissue, GRs are found ubiquitously and exist as two alternative splice variants termed GRα and GRβ. GCs are capable of either up regulating or inhibiting the transcription of hundreds of diverse genes when bound to GRα (176). Activation of GRα requires the formation of a homodimer. GRβ acts as a negative inhibitor of GRα, and instead forms a heterodimer with one GRα, thus preventing homodimerization and activation of GRα (206). GR distribution and concentrations are tissue specific.

Overexposure of peripheral tissue to stress hormones is prevented by a negative feedback response in which GCs released into circulation are able to bind to receptors located in higher brain regions, which then inhibits further release of GCs (53).

GC action is further mediated by the activity of 11 beta hydroxysteroid dehydrogenase (11β-HSD) (63). 11βHSDs are intracellular nicotinamide adenine dinucleotide phosphate (NADPH) dependent enzymes responsible for pre-receptor metabolism of GCs. In man, the 11βHSD isoforms are involved in the inter-conversion of cortisone and cortisol (corticosterone and 11-dehydroxycorticosterone in rodents respectively) (63, 206).The enzyme exists as two isoforms: 11βHSD1 and 11βHSD2, which are tissue-specifically expressed, and are responsible for catalyzing the inter-conversion of hormonally active cortisol and inactive cortisone. 11βHSD2 is predominantly found in the kidney, where it converts the active form of GCs to an inert form, protecting the glucocorticoid receptor from cortisol excess (207). In contrast, 11βHSD1 is widely distributed throughout the body, especially in adipose and liver tissue, and is capable of converting inert GCs into their active form, thus increasing GC action (197). In man, this reaction involves the conversion of cortisone to cortisol; while in rodents, 11-dehydroxycorticosterone is converted to corticosterone (227). In obese individuals, 11βHSD1 levels are reportedly elevated in central adipose stores in comparison to normal individuals (227), resulting in higher levels of circulating GCs, suggesting a potential role of GCs in facilitating the development of obesity and metabolic syndrome.

ii. Diurnal Rhythm of Glucocorticoids

GC release follows a pulsatile and circadian rhythmicity that is necessary to fulfill the many roles it plays in maintaining whole body homeostasis (46).

Circadian rhythms in most organisms have evolved as a result of the earth's axial rotation (i.e. light/dark cycle) (89). The master circadian clock lies in the suprachiasmatic nuclei (SCN) of the ventral hypothalamus. The rhythmic production and release of hormones within the endocrine system is necessary to regulate many biological processes including reproduction and metabolism. Signalling from the SCN directly regulates activities of the HPA axis. The SCN activates rhythmic release of CRH from the paraventricular nucleus while also generating neural signals that act on the adrenal cortex to regulate glucocorticoid production (157). However, the central nervous system is not the only factor contributing to regulation of GC secretion from the adrenal cortex. Andrews and colleagues first alluded to this concept when suggesting that adrenal glands in culture exhibited circadian rhythms for metabolic activity and GC release (9). The rhythmicity of these regulatory sites, both in the CNS and at the level of the adrenals, implies that there are fluctuations in plasma GC concentrations, depending on environmental and internal cues. The peak of GC release is dependent on the activity phase of the animal. In humans, the peak occurs in the early morning, while in nocturnal rodents, this peak occurs in early evening (43, 163,130,114). Alterations to the circadian rhythm result in impaired HPA axis negative feedback and the dysregulation of GC-controlled metabolic processes. One method of altering circadian rhythm is via administration of exogenous corticosterone (238). Interestingly, this dysregulated GC functioning, as a result of exogenous corticosteroid therapy, has been associated with larger waist

circumference (29); thereby suggesting that abolishment of the diurnal rhythm facilitates metabolic abnormalities.

iii. Conditions of Chronic Glucocorticoid Excess

Chronically elevated circulating GC levels may occur as a result of Cushing's disease, exogenous administration of synthetic glucocorticoids, or as a result of increased activity of 11βHSD-1 in obese individuals (134). In Cushing's syndrome, GC regulation is impaired due to tumours in the pituitary or adrenal gland resulting in chronic stimulation of GC synthesis. This chronic synthesis is detrimental to peripheral tissue function as elevated levels of circulating GCs lead to excess abdominal fat deposition, reduced muscle mass and bone strength, and impaired insulin sensitivity, with the chance of developing type 2 diabetes (111, 180, 189). Interestingly, Cushing's syndrome patients share the identical spectrum of diagnostic criteria that MetS patients have (e.g. central obesity, hyperglycaemia, hyperlipidemia) and as many as 80% of Cushing's patients develop glucose intolerance (214) .

It is likely that central obesity and excess circulating cortisol levels and/or excess local GC reactivation are interrelated even in non-Cushing's patients. Indeed, circulating levels of cortisol are higher in patients suffering from MetS compared with healthy weight controls (146, 166, 199). These elevated cortisol levels are a result of larger adipose tissue mass, which results in an increased generation of active GCs via 11βHSD1, this in turn promotes other features of MetS including glucose intolerance and hypertriglyceridemia (173).

While obesity and MetS are more common scenarios in which GC levels may be elevated, there is also a subset of the population that use chronic exogenous GC therapy that have the potential to produce detrimental side effects induced by GC. GCs are one of the most prescribed medications worldwide. The use of GCs as therapy first began in 1948, in which they were used to suppress the clinical manifestations of rheumatoid arthritis. Today, GCs are the standard therapy for reducing inflammation, immune activation, as well as other systemic diseases (190). However, with chronic GC therapy, there is potential for adverse effects. The most common include: muscle atrophy, growth inhibition, development of osteoporosis, and altered metabolism. Chronic doses of GCs, as occurs with asthma treatment in children, have been shown to suppress growth; while removal of GC therapy resulted in enhanced growth rates (23). As little as 2.5 mg of prednisolone, a commonly used form of GC therapy, can cause reduced statural growth and altered body composition (greater fat to lean mass ratio) (233). Additionally, patients exposed to long term GC treatment can undergo bone loss of as much as 13% in the first few months (137). Studies have also shown that both acute and chronic administration of GCs resulted in a transient increase in plasma insulin concentrations and skeletal muscle insulin resistance (97, 155, 162). Excess GC exposure has also been shown to predispose individuals to develop overt diabetes, especially in persons with diminished β-cell function (94), and GC therapy late in pregnancy may predispose the offspring to glucose intolerance later in adulthood (154).

Adrenal GCs stimulate phenylethanolamine N-methyltransferase (PNMT) to convert norepinephrine to epinephrine in the adrenal medulla. By suppressing GC synthesis, GC therapy might also suppress catecholamine synthesis. However, a study using dexamethasone with or without stress stimuli found that without stress dexamethasone was able to induce synthesis of PNMT, suggesting that neural stimulation may be involved overriding hormonal regulation of epinephrine synthesis during stress (201).

iv. Regulation of Food Intake by Glucocorticoids

Glucocorticoids were initially defined for their role in mobilization of substrates for gluconeogenesis from peripheral tissue stores (44). However, studies have also shown that high cortisol levels are also related to anti-anabolic effects and linked to increasing intake of palatable, high fat foods. Epel et al. found increases in caloric consumption in energy dense "comfort foods" when daily stressors induced in a laboratory setting in healthy premenopausal women induced elevated cortisol levels (68). The effects of GCs on food intake and preference are best examined in adrenalectomized rodent models in which endogenous CORT is replaced with a specific dosage of exogenous CORT. In these animals, chow intake is increased with increased dosage of CORT (208). In the presence of elevated insulin levels, this effect is further amplified (41). CORT is believed to increase drive for calories, while insulin increases preference for high fat foods. This is due to insulin's effects on increasing the

palatability of lard, through their influence on cells in the brain's reward center, the nucleus accumbens (74).

Over nutrition is cited as the most common cause of NAFLD and is a central feature of the modern lifestyle that predisposes development of obesity (125). Because of the potential for this intervention to induce alterations not only in fat mass, but also in glucose homeostasis and overall metabolic function, it is a preferred treatment used in animal models of obesity and NAFLD (92). Specifically, Sprague Dawely rats administered a high fat diet *ad libitum* for 6 months developed steatosis and increased visceral adiposity in addition to elevated serum glucose and insulin levels (213). High fat feeding, in addition to causing visceral obesity, also results in development of insulin resistance, dyslipidemia, and altered adipokine and cytokine profile, as well as oxidative stress, which may all contribute to liver injury (40). While the use of high fat feeding as a model for MetS represents an accurate reflection of clinical development of metabolic disorders, the prolonged duration required to induce effects (often several months), makes it a problematic model to use in animal models of metabolic dysfunction.

2.4 Hepatic Glucose Metabolism

The liver is part of a network of organs including the brain, pancreas, intestines, peripheral adipose and muscle tissue, that each plays a critical role in glucose homeostasis. Under fed conditions, excess glucose from the diet

undergoes glycogenesis and excess glucose is stored as glycogen in liver and muscle to maintain euglycemia (blood glucose levels ~4-7 mmol). A drop in blood glucose levels signals the release of glucagon from the pancreas. Glucagon stimulates the liver to undergo glycogenolysis to breakdown glycogen stores and export glucose into the blood for transport to other tissues. Under fasting conditions and with the depletion of glycogen stores, the liver activates another set of enzymes involved in the production of glucose, a process known as gluconeogenesis. The main substrates involved in gluconeogenesis are alanine, lactate, glycerol and glycogen. Prior to glucose production, phosphoenolpyruvate carboxykinase (PEPCK) is involved in the rate-limiting step of gluconeogenesis involving decarboxylating and phosphorylating oxaloacetate into phosphoenolypyruvate. The penultimate step involved in glucose production is the cleavage of a phosphate group from glucose-6-phosphate. This can only be done in the liver due to the presence of glucose-6-phosphatase (G6Pase) (66). The process of gluconeogenesis is tightly regulated by the opposing actions of insulin and glucagon. While insulin inhibits excess glucose synthesis in fed conditions by regulating the actions of downstream transcription factors involved in regulation of gluconeogenesis, glucagon promotes hepatic glucose production in conditions of fasting. Many studies have suggested that the disrupted hepatic glucose homeostasis associated with type 2 diabetes is directly a result of increased expression of gluconeogenic enzymes, in particular the rate limiting enzymes PEPCK and G6Pase (122, 136, 226). Though one study found that

mRNA expression of PEPCK and G6Pase in liver biopsies of type 2 diabetics did not differ in expression compared to healthy controls (186), the vast majority of studies show that type 2 diabetes is associated with increases in rates of gluconeogenesis (101, 136,182).

i.Influence of Insulin and Glucagon in the Liver

Hepatic glucose metabolism is largely regulated in the post absorptive state by a balance between insulin and glucagon secretion (230). Under post prandial conditions, insulin concentrations rise, resulting in suppression of endogenous glucose production and stimulation of hepatic glycogen synthesis. In contrast, during periods of low blood sugar, or hypoglycemia, plasma insulin levels drop and glucagon concentrations rise, resulting in breakdown of glycogen stores and stimulation of EGP (135). Glucagon has also been shown to stimulate PEPCK expression (107).

Under normal, fed conditions, insulin binds to its receptor (Figure 1), undergoing auto phosphorylation on its tyrosine substrates, thus catalyzing the phosphorylation of downstream cellular proteins, such as the insulin receptor substrate (IRS) family (192). Signalling of IRS is mediated by the phospho-inositide-3-kinase (PI3K) pathway. PI3K is a central mediator of the effects of insulin on hepatic glucose production (183). Activation of PI3K through insulin signalling leads to AKT association with PDK1. PDK1 is then able to phosphorylate AKT on Threonine 308, one of two stimulatory phosphorylation sites. AKT is a master mediator of insulin signalling and influences glucose

transport, glycogen synthesis, protein synthesis, lipogenesis and hepatic gluconeogenesis. Following phosphorylation, AKT is able to translocate from the cytoplasm into the nucleus, where it may then act on transcription factors within the nucleus (192). Transcription factors such as forkhead box 1 (FOXO1) and PPAR gamma co-activator 1 alpha (PGC1α) regulate the activation of enzymes involved in gluconeogenesis. The FOXO family of transcription factors are capable of binding to the promoter regions of gluconeogenic genes, including phosphoenolpyruvate carboxykinase (PEPCK) and G6Pase (glucose 6 phosphatase), thus increasing their transcription. In the presence of insulin, phosphorylation of FOXO1 by AKT results in FOXO1 export from the nucleus, thus inhibiting gluconeogenic gene transcription. PGC1α appears to interact with FOXO by co-activating it. Studies in fasted or insulin resistant mice have shown increased expression of PGC1α (237), indicating that both FOXO1 and PGC1α work together to promote gluconeogenic gene transcription. A failure of the liver to respond to insulin signalling enables these gluconeogenic transcription factors to be chronically active, and thus increase rates of gluconeogenesis.

ii. Effect of Glucocorticoids on Hepatic Glucose Metabolism

GCs are also capable of stimulating gluconeogenesis via promotion of gluconeogenic gene transcription (12, 77, 126,78). The importance of GCs in regulating endogenous glucose production (EGP) is underscored in a study in which transgenic mice with liver specific inactivation of GR developed severe hypoglycaemia after prolonged fasting due to impaired gluconeogenesis (156).

GCs are capable of antagonizing insulin's suppressive effects on gluconeogenesis by activating a large number of genes involved in augmenting endogenous glucose production. GCs are able to bind to glucose response elements located in the promoter regions of the PEPCK gene (225), thus directly increasing their transcription. GCs have also been shown to interfere with the insulin signalling pathway by decreasing receptor tyrosine phosphorylation of IRS-1 in rodent muscle treated with cortisone, and interfering with AKT phosphorylation through down regulation of PI3K in rat myocardium (234) and adipocytes (34). Additionally, long term GC exposure increases proteolysis, thus increasing supply of substrates for gluconeogenesis (121). GCs have also been shown to increase glucagon secretion in Cushing's patients and obese individuals treated with dexamethasone (232) and can potentiate the effects of other counter regulatory hormones such as glucagon and epinephrine, which can further enhance hepatic glucose production (224).

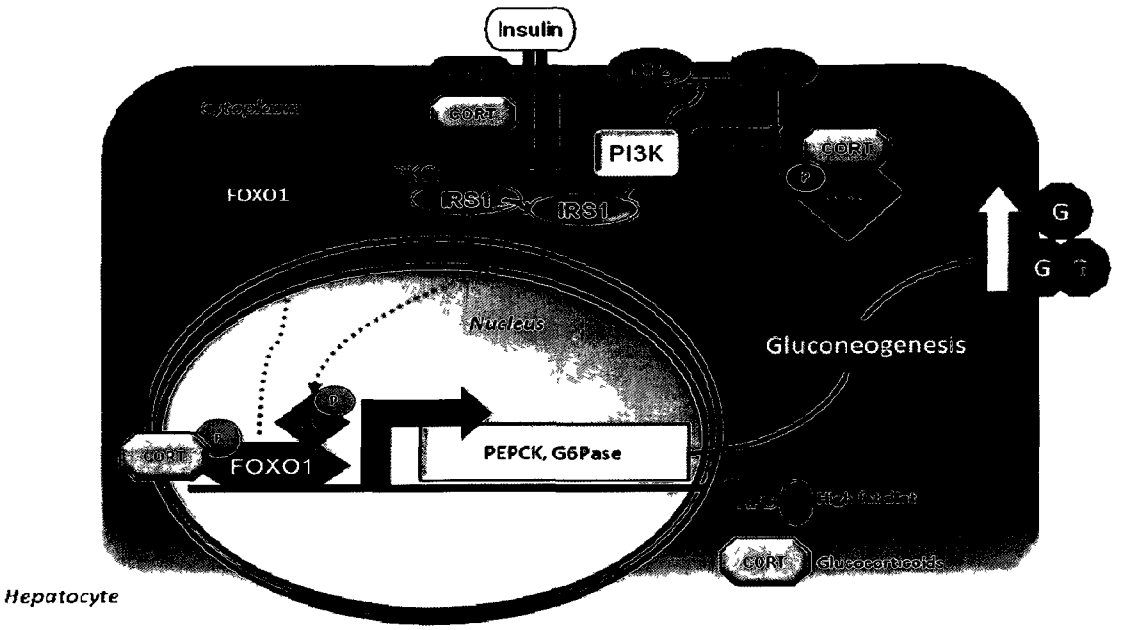

Figure 1: Hepatic insulin resistance. GCs, intracellular FFAs (via consumption of a high fat diet) and activation of PKCs result in reduced insulin signalling. GCs and FFAs interfere with insulin receptor phosphorylation of IRS1. Additionally, PKCε interferes with IRS1 tyrosine phosphorylation thus preventing activation of PI3K. Activation of PI3K is required for AKT phosphorylation. Without phosphorylation by Akt, FOXO1 continues to promote transcription of gluconeogenic enzymes including G6Pase and PEPCK. Additionally, GCs can act directly on gluconeogenic genes to promote transcription via GREs.

iii. PKCε mediates Hepatic Insulin Resistance

Previous studies have implicated protein kinases (85, 239) and specifically protein kinase C epsilon (PKCε) as the link between lipid accumulation and insulin resistance. Schmitz-Peiffer et al (194) and others have found increased PKCε levels in the livers of Zucker diabetic fatty rats and chronic activation of PKCε in skeletal muscle of high fat diet fed rats (48). Furthermore, Samuel et al found that the PKCε isoform mediate hepatic insulin resistance induced by three days of high fat feeding. PKCε is normally activated by diacylgylcerol (DAG),

which causes its translocation to the plasma membrane from the cytosol. Once translocated, it is able to prevent IRS-1 tyrosine phosphorylation, thus prevent downstream insulin signalling (187). Prevention of hepatic steatosis by use of the mitochondrial uncoupler 2, 4-dinitrophenol (DNP), abrogated development of insulin resistance by preventing membrane translocation, and thus activation of PKCε (187). Once activated, PKCε has the potential to induce activation of c-Jun N-terminal kinase 1 (JNK1), which has been shown to play a key role in insulin resistance by phosphorylation of IRS-1 on serine 307 (96, 127). This impairs further downstream signalling of insulin, thus inhibiting insulin's regulation of gluconeogenesis.

GCs have been implicated in PKCε activation, and thus may play an indirect role in promoting insulin resistance. Immature thymocytes treated with glucocorticoids resulted in increased translocation and activation of PKCε from the cytosol, and as a result, increased apoptosis. Alternatively, the addition of the GC inhibitor RU486 resulted in reduced PKC activation and reduced apoptosis (104).

2.5 Hepatic lipid metabolism

In addition to regulating glucose metabolism, the liver serves as a central hub for lipid metabolism. Dietary fat constitutes a large proportion of lipid metabolites received by the liver. Dietary fatty acids are derived from chylomicron remnants that transport dietary fat to other parts of the body for further

processing or storage. On the way to the liver, chylomicrons undergo metabolism by lipoprotein lipase. Under fed conditions, chylomicron synthesis is high, and storage of TGs and FFA in adipose tissue is increased.

As the liver is intimately involved in regulating transport, processing and export of lipids, liver dysfunction directly impacts the development of dyslipidemia. The liver is the primary site of very low density lipoprotein (VLDL) synthesis and cholesterol biosynthesis. To produce both, the liver synthesizes apo-lipoprotein B and requires lipid metabolites in the form of free fatty acids obtained from peripheral sources. The flux of fatty acids into the liver can come from 3 sources: triglycerides in adipose tissue hydrolyzed by hormone sensitive lipase, free fatty acids from dietary triglycerides in the form of chylomicrons (which are hydrolyzed by lipoprotein lipase), and endogenous fatty acid synthesis within the liver (75). Two major metabolic pathways exist in the liver that either facilitate fatty acid storage, or metabolism. Fatty acids may be incorporated into glycerol-3-phosphate and stored through the esterification of acetyl-CoA with glycerol-3-phosphate to form triglycerides. VLDLs are then formed when these triglycerides are packaged with specific apo-proteins. Some of the TGs present in VLDLs are formed from dietary carbohydrates that are converted to lipids via *de novo* lipogenesis in the liver. These VLDLs may then be exported to adipose tissue for storage. Alternatively, fatty acids may proceed down a catabolic pathway to undergo β-oxidation and conversion into energy by the tricarboxylic acid cycle (75). Hepatic lipid accumulation occurs as a result of an imbalance

between lipid availability and lipid disposal via fat oxidation or lipoprotein secretion. Therefore, GCs and high fat feeding have the potential to contribute to fat flux in the liver by altering adipose spill over, availability of dietary sources, endogenous fatty acid synthesis, or mitochondrial beta oxidation, which may all contribute to the pathology of hepatic steatosis.

i. Lipid Import from Surrounding Adipose Tissue and Facilitated Transport

Circulating free fatty acids, either from the diet or through lipolysis of adipose tissue stores, provide a majority of the fatty acids in the liver. Fatty acid transport into the liver is a complex process involving multiple steps including dissociation from albumin, transport across the plasma membrane, binding to intracellular proteins and esterification to form acyl-CoA (67, 211). FFAs enter the cell via passive diffusion, by binding to G protein coupled receptor 40 or fatty acid transporters (39). Fatty acid transporters are proteins that are found across a number of tissues and sit on the basal side of the plasma membrane to facilitate uptake of fatty acids. Over expression of these transporters is associated with increased fatty acid uptake (58). In the liver, the most abundant transport proteins are: fatty acid transporter 5 (FATP5), cluster of differentiation 36 (FAT/CD36) and liver fatty acid binding protein (L-FABP). Deletion of FATP5 in the liver has been shown to be protective against TAG accumulation and insulin resistance (58). FAT/CD36 accelerates fatty acid dissociation from albumin in circulation and results in accumulation of fatty acids outside the phospholipid bilayer of the plasma membrane. This creates a diffusion gradient promoting

increased fatty acid influx into the liver. In addition, CD36 is also present in the mitochondria where it facilitates fat transport for β- oxidation. Studies by Bonen et al. have shown increases in FAT/CD36 expression in models of high fat feeding via activation of PPARα, a transcription factor that regulates CD36 expression (24). In gastrocnemius muscle tissue, the synthetic glucocorticoid dexamethasone induces significant increases in CD36 expression (116), demonstrating the potential for GC therapy to increase CD36 expression and thus facilitate increased transport. Recent studies have also demonstrated that induction of CD36 transporters in the liver may be critical for the development of steatosis under pathological conditions including obesity and diabetes (118, 132).

Once inside hepatic cells, fatty acids are then distributed by liver fatty acid binding protein (L-FABP) to either be oxidized, or esterified into triglycerides for storage and packaged as VLDLs. Fatty acid binding proteins (FABPs) are the most common proteins in the cytosol of most cells involved in long chain fatty acid (LCFA) uptake and metabolism. Of these FABPs, liver fatty acid binding protein (L-FABP, also called FABP1) is the most broadly distributed mammalian FABP and is expressed at very high levels in tissues most active in LCFA metabolism (14). L-FABP is involved in a number of cell functions including enhancing cellular LCFA uptake, binding LCFAs and LCFA-CoAs to minimize toxic effects, enhancing intracellular transport/diffusion through the cytoplasm, delivering LCFAs to mitochondria for oxidation, targeting LCFAs to peroxisomes for beta oxidation and for hepatic secretion in VLDLs. The physiological function

of L-FABP has been assessed using loss of function studies in which L-FABP -/- mice were protected against hepatic steatosis accompanying a prolonged fast (153).The authors conclude that this protection may be a result of altered saturated fatty acid metabolism. This may be a result of an up regulation of enzymatic pathways that generate fatty acid intermediates, or reduced hepatic lipid oxidation and decreased VLDL excretion. Non-esterified free fatty acids (NEFAs) are among the most potent of lipid metabolites due to their potential to induce oxidative stress and activate pro-inflammatory kinases including NFkB. Activation of NFkB also has the potential to interfere with insulin signalling (103). This alteration of insulin signalling in hepatic tissue results in increased rates of gluconeogenesis and VLDL secretion (129).

ii. De novo *Lipogenesis and other Mechanisms Contributing to Lipid Accumulation within the Liver*

Mammals preferentially burn carbohydrates when energy intake is abundant. After replenishing glycogen stores, the surplus glucose is converted into fatty acids and stored as TGs in adipose tissue (175). In normal individuals, fatty acid oxidation is inhibited by insulin and promoted in the presence of glucagon. Oxidation primarily occurs in the mitochondria, but can also occur in the peroxisomes of the endoplasmic reticulum (170,175). After being transported into the mitochondria, long chain fatty acids then undergo β-oxidation. The primary products of oxidation are ketone bodies and CO_2, which can then be used as fuel for other tissues in the body. In individuals with NALFD, there are

increases in hepatic beta oxidation, but they are insufficient to compensate for the increased accumulation of lipids (10, 20). GCs may promote fat accumulation in the liver by impeding certain enzymes in the multistep oxidation process. Treatment of mice with dexamethasone 21-phosphate (a synthetic GC) resulted in inhibition of medium and short chain acyl-CoA dehydrogenation and hepatic lipid secretion in mice (128).

The liver produces lipids in a process known as *de novo* lipogenesis (DNL) from glucose, in the event that all glycogen stores are full and there is an excess of glucose. Lipogenesis is triggered by insulin and glucose signalling, which act on the transcription factors sterol regulatory element binding protein (SREBP1) and carbohydrate responsive element binding protein (ChREBP) respectively. These transcription factors regulate expression of lipogenic enzymes including glucokinase, malic enzyme (ME), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FASN) (195). ACC and FASN are believed to be the rate limiting enzymes involved in DNL, which convert acetyl CoA to malonyl CoA and malonyl CoA to fatty acyl CoA respectively (93). In fasted, healthy conditions, the liver contributes less than 5% of total circulating triglyceride content from de novo lipogenesis (222). However, in insulin resistant states, DNL increases and accounts for 14% of liver triglyceride content in patients identified with NALFD (57) and up to 26% of liver TG content in hyperinsulinemic subjects (59).

Under normal conditions, the liver takes up triglycerides packaged in chylomicrons and packages them into very low density lipoproteins (VLDLs) to be exported into the circulation. VLDLs enable transport of water insoluble triglycerides to other tissues by combining them with protein. The most commonly synthesized protein produced and secreted by the liver is Apo lipoprotein B-100 (apoB-100). Fatty acids not oxidized are esterified into triglycerides and combined with apoB-100 to form lipoproteins. With NAFLD, an increase in lipolysis in surrounding adipose tissue and an increased rate of lipogenesis contribute to a greater supply of FFAs to the liver (217). To maintain a balance of lipid kinetics within the liver, there needs to be an increase in VLDL export, however the VLDL kinetics in individuals with NAFLD are controversial. While most studies report increases in VDL secretion (4, 70), not all have found increases in NAFLD patients (57). Furthermore, VLDL secretion appears to reach a plateau in NAFLD, which differs from the linear relationship seen in individuals with normal hepatic triglyceride content (71).

iii. Effect of Glucocorticoids on Lipid Metabolism

GCs have been shown to target multiple pathways affecting lipid transport and metabolism within the liver. Among the pathways altered by lipid transport, previous reports have shown that GCs negatively affect L-FABP expression. Dexamethasone indirectly limits β-oxidation by down regulating expression of L-FABP (76, 145). GCs can contribute to increased VLDL secretion by augmenting the secretion of Apo lipoprotein (apo-B) and increasing VLDL synthesis. Using

isolated perfused livers, Martin-Sanz and colleagues found that treatment with dexamethasone increased apo-B secretion by four fold, and caused an increased secretion of VLDLs by hepatocytes (140). These results were similar to those found by Cole et al., who also study perfused rodent livers treated with dexamethasone, and found increased esterification of VLDLs (47).

Within the liver, fat accumulation may be attributed to increased lipid synthesis. A study in which dexamethasone, was administered to broiler chickens found elevations in hepatic fat content, and attributed these elevations to increased transcription of FASN (35). More recently, dexamethasone induction of hepatic steatosis has been shown to require liver X receptors, which are nuclear receptors that respond to cholesterol metabolites and regulate the expression of a subset of glucocorticoid target genes (160).

In addition, previous studies have reported that GCs produce normal (84) or enhanced responses to a variety of hormones (72). GCs appear to act synergistically with insulin to up regulate lipogenesis in adipose tissue and liver (5, 6, 35). The synergistic effects of GCs and insulin in lipogenesis were first reported well over fifty years ago in a study using pharmacological amounts of insulin and GCs that found enhanced lipogenesis in perfused liver (5). Insulin is necessary for GCs to exert their lipogenic effects. Amatruda et al found increased lipogenesis and expression of acetyl coA carboxylase and fatty acid synthase in hepatic cells treated with dexamethasone and insulin, while only

minor increases in lipogenesis occurred with GC treatment alone (6). Thus, GCs are necessary for maximum expression of insulin-stimulated lipogenesis in the liver. GCs are capable of inducing hepatic insulin resistance both directly by interfering with insulin signalling, and indirectly by elevating plasma NEFA and TG supply to the liver (158). In vitro, GCs have been shown to suppress β-oxidation and increase TG synthesis, thus leading to increased intrahepatic TG accumulation (82). In addition to increasing hepatic lipid content, GCs may also augment hepatic VLDL secretion, resulting in increased plasma levels of TGs (47).

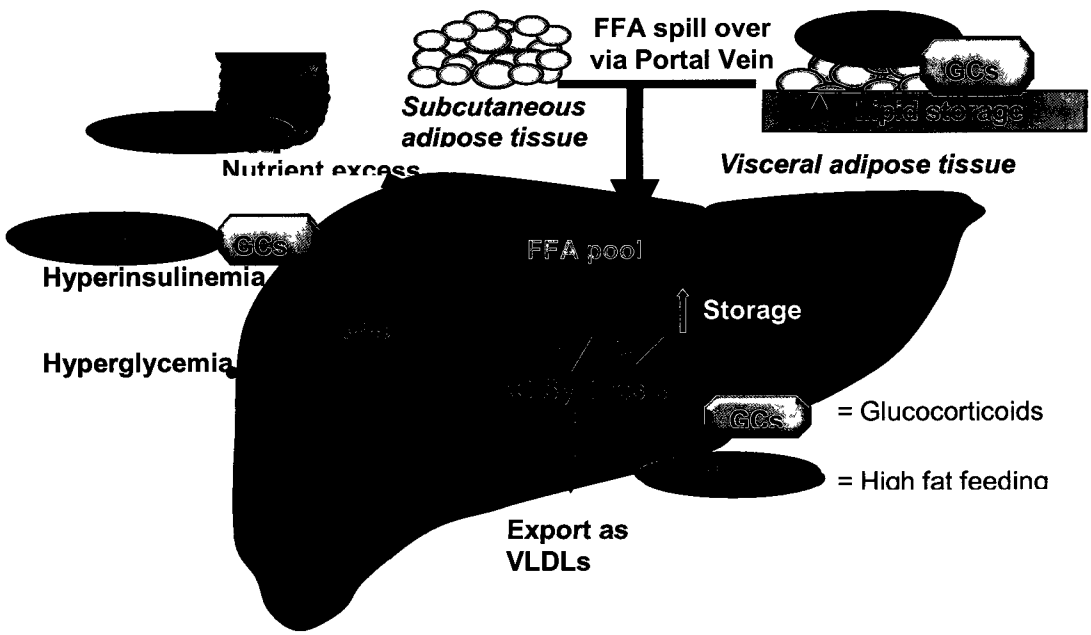

Figure 2: Sources of fat accumulation in the liver resulting in NALFD. The majority of fat pooling in the liver is derived from spill over from adipose tissue, either from subcutaneous or visceral stores. In the presence of insulin and GCs, there is also an increase in lipogenesis and suppression of beta oxidation, thus promoting increase accumulation and less utilization of lipids.

iv. Bifurcation of the Insulin Signalling Pathway

Based on the above literature review, it is clear that insulin's actions in the liver extend well beyond increasing gluconeogenesis. They also include effects on synthesis of fatty acids and triglycerides in a process known as lipogenesis. In many metabolic disorders including diabetes and obesity, elevations in fasting insulin levels are observed when there are also increases in circulating GCs (1). In normal individuals, elevated insulin levels following consumption of a meal are necessary to suppress hepatic gluconeogenesis, while concomitantly promoting lipid storage via lipogenesis. With insulin resistance, insulin fails to suppress gluconeogenesis and continues to promote lipogenesis (30). This paradox is explained by a bifurcation downstream of the insulin receptor. As mentioned previously, insulin resistance results in a reduction in AKT phosphorylation. While this leads to inhibited suppression of FOXO1, the lipogenic transcription factor SREBP-1 remains responsive to insulin via the mammalian target of rapamycin complex 1 (mTORC1) (185). The increase insulin levels, and potential increase in fasting glucose levels provide the perfect climate for lipogenesis to occur when GCs are also elevated. With increases in lipogenesis, there is increased lipid accumulation, potentially further contributing to hepatic steatosis.

2.6 Contribution of Central Adipose Depots to Lipid Accumulation

i. Glucocorticoids and Lipolysis

GCs have been shown to increase rates of lipolysis (with elevated GC concentrations), promoting increased release of lipid metabolites. At the same

time, elevated GCs also promote differentiation of pre-adipocytes to adipocytes, which may lead to increased number of smaller adipocytes and increased size of adipose depots overall (91). In the presence of the GR inhibitor RU486, lipase activity in adipocytes was inhibited, suggesting that GCs are necessary to mediate lipolysis (235). The mechanisms by which GCs increase lipolysis are believed to be due to increases in transcription of lipolytic enzymes (36). It should be noted that these effects on increased lipolysis occur in mature adipocytes and not pre-cursor adipocytes, and that the effects of GCs are more profound on visceral adipose tissue than subcutaneous adipose tissue, likely due to the increased rate of GC turnover in visceral adipose tissue (207). As mentioned above, a majority of patients with NAFLD have concomitant elevations in abdominal adiposity. Recent studies have shown that this characteristic is more than just a correlation with elevated hepatic lipid content. The close proximity of visceral fat to the liver makes it a contributor to lipid influx into the liver via the portal vein (13). A clinical study in obese women found increased visceral lipolytic rates of cultured visceral adipocytes corresponding with increased hepatic lipid content (218). What's more, GCs have been shown to increase lipolytic rates in visceral adipose tissue more so than in subcutaneous adipose tissue, thus providing a possible mechanism by which chronic GC excess may lead to increase influx of lipids to facilitate hepatic lipid accumulation (38). These findings suggest that with chronically elevated GCs, there is an increased rate of

lipolysis in mature adipocytes in subcutaneous and visceral adipose depots, which could potentially contribute to hepatic lipotoxicity.

ii. Link between dysfunctional adipose tissue and NAFLD

Data from metabolic studies in patients with NAFLD and NASH suggest adipose tissue insulin resistance is a common occurrence with these conditions, which creates the conditions for a lipotoxic environment (49). An excess of energy intake leads to a failure of fat cells to adequately adapt with sufficient proliferation and differentiation to meet increased lipid content. As a result, these fat cells undergo a considerable amount of metabolic stress that impairs glucose and lipid homoeostasis. Alterations in lipid kinetics in visceral fat tissue are a key determinant of progression onto NALFD. As these tissues increase their rates of lipolysis, the available room to store fat is decreased and the result is more lipid spill over from subcutaneous and visceral fat stores into circulation and a higher concentrations of lipids in the blood. Interestingly, chronically elevated GCs have been shown to increase visceral lipolytic rates (38), thus potentiating increased lipid spill over. In peripheral fat tissues GCs induce activation of hormone sensitive lipase and reduce intravascular lipoprotein lipase (LPL) activity; but in visceral tissue, GCs appear to increase TG synthesis and LPL activity, thus facilitating increased mobilization of lipids to central regions, where they may be stored or influence surrounding tissue (148). A study by Donnelly et al. has confirmed that serum FFAs are the major contributors to hepatic TAG content,

and free fatty acid delivery to the liver accounts for as much as two thirds of its lipid accumulation (59).

Adipose tissue is also a major producer of inflammatory cytokines, which have the potential to interfere with insulin sensitivity (203). For example, adiponectin released from adipocytes is involved in regulation of hepatic stellate cell fibrogenesis and has systemic anti-inflammatory effects (108). In NAFLD patients, plasma adiponectin levels are severely decreased, while leptin (which has opposing effects to that of adiponectin) levels are increased with increased adiposity.

2.7 Consequences of Liver Disease: Inflammation and Fibrosis

Although fatty liver disease was initially considered a benign condition, current evidence suggests that development of NAFLD increases the risk of mortality due to development of cirrhosis and hepatic carcinoma (168). Among the most common outcomes that result from the development of fatty liver is the development of inflammation and fibrosis, which occurs in approximately 32% to 37% of the population (73, 90). Whether or not consequential cirrhosis or cancer develops, and the severity of their development, depends on the presence of other confounding risks including obesity and diabetes.

i. Inflammation

Inflammation represents the homeostatic response to protect and prevent from further injury by isolating and eliminating the infecting agent. Low grade

inflammation is present in chronic metabolic conditions (54), and is evident by heightened release of acute phase markers from the liver, including haptoglobin and C reactive protein (164). Haptoglobin levels can increase by as much as 10 fold in response to inflammatory stimuli (18), and is a commonly used marker of inflammation in rodents (80). The liver is composed of a variety of heterogeneous cells including hepatocytes, Kupffer cells, stellate cells and sinusoidal endothelial cells. Interaction between the diverse cell types occurs via hormonal signals as in the case of Kupffer cells and stellate cells, which are primarily involved in release of pro-inflammatory cytokines (219). GCs are well established to be anti-inflammatory in action (193), thus it is unclear if GC-induced steatosis would develop into an inflammatory state.

Release of cytokines including interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) are linked with increased hepatic inflammation and insulin resistance. Chronic exposure to TNF-α promotes the accumulation of inflammatory cells in the liver (120). Previous studies have suggested that inflammation may precede development of steatosis. The absence of either IL-6 or TNF- α was shown to decrease high fat diet-induced liver lipid accumulation and inflammation, as assessed by infiltration with macrophages and neutrophils (221). Additionally, patients with severe NASH who are given an anti-TNF antibody report improved steatosis (220).

Enhanced cytokine release may be a result of increased adiposity. Adipose tissue represents a potent endocrine organ with the capacity to produce

adipokines and cytokines, of which some are beneficial or detrimental, depending on their downstream effects. Expression of IL-6 and TNF-α is increased in the fat cells of obese individuals, and is believed to mediate an insulin resistance state (98). These cytokines elicit their detrimental effects by activating Kupffer cells and serine/threonine inflammatory kinases including PKC and inhibitory kinase kappa beta (IKKβ) (203). Normally, the transcription factor NFkβ resides in the cytoplasm bound by an inhibitory protein known as IKB. Phosphorylation of IKB by IKK-β releases NFkB, which then moves into the nucleus to act on regulatory genes involved in the immune response.

These proinflammatory cytokines initiate insulin resistance by increasing phosphorylation of serine 307, the inhibitory phosphorylation site on IRS-1, thereby inhibiting the stimulatory phosphorylation of tyrosine, and further downstream signalling. Rodent models of insulin resistance have shown that PKC is also capable of inducing insulin resistance by preventing tyrosine 612 phosphorylation (188) and activating IKKB and JNK1, either directly or via oxidative stress (240). Inhibition of PKC- ε has been shown to prevent insulin resistance and steatosis in rats fed a high fat diet (188).

ii. Fibrosis

Hepatic fibrosis is a wound-healing response characterized by inflammation, activation of matrix producing cells, extracellular matrix deposition and remodelling (15). It is common to all chronic liver diseases, and precedes cirrhosis, the final common stage of liver injury. Advanced fibrosis is

characterized by an accumulation of fibrillar collagen in the extracellular matrix (102). Injury to hepatocytes results in recruitment and stimulation of Kupffer cells, which are resident inflammatory cells. Factors released by these inflammatory cells lead to activation of hepatic stellate cells and accumulation of scar extra cellular matrix. This induces loss of tissue architecture, namely by loss of microvilli and endothelial fenestrae, and thus loss of normal organ function. As a result of fibrosis accumulation, liver function is impaired and portal hypertension occurs. Additionally, progression of NAFLD into NASH reduces the ability of mature hepatocytes to proliferate and repair from injury (60).

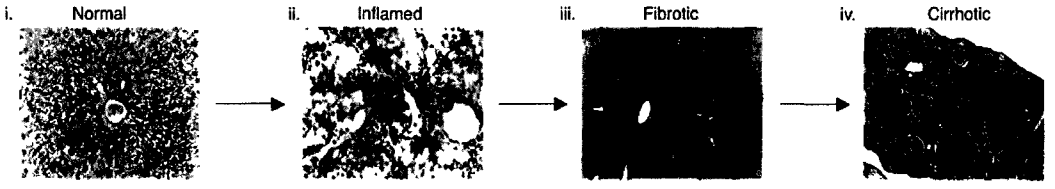

Figure 3: Stages of hepatic injury. NAFLD consists of a range of liver malfunctions beginning with inflammation, as result of activation of Kupffer cells, progression to fibrosis, and finally cirrhosis, a condition of scar formation and impaired function. Image adapted from (102) with permission.

Rationale, Purpose and Hypotheses 3

Rationale

The occurrence of chronically elevated GC levels is steadily increasing due to increased use of GCs to treat anti-inflammatory conditions and increasing rates of obesity. Elevated GC levels induce characteristics of MetS including hepatic steatosis, central obesity and insulin resistance (8). Consumption of a HFD is known to independently induce symptoms of NAFLD and MetS (17, 92). Additionally, GCs have also been shown to increase the consumption of energy dense foods and contribute to increased weight gain (124). Thus, it is very likely that GCs, coupled with a poor diet, can exacerbate the development of NAFLD (17, 141).

Purpose

The purpose of this thesis is to examine the combined effects of chronically elevate glucocorticoid levels and consumption of a 60% high fat diet on NAFLD development.

Hypotheses

Based on the rationale, it is hypothesized that *ad libitum* consumption of a 60% high fat diet in the presence of chronically elevated GC levels (corticosterone, the main active endogenous GC in rodents) will:

1. Rapidly and synergistically induce NALFD marked by hepatic steatosis, hepatic fibrosis, and increased plasma ALT levels.

2. Result in impaired hepatic lipid metabolism, due to increased influx of FFA and TG spill over from peripheral and central adipose stores, in addition to increased *de novo* lipogenesis.

3. Synergistically contribute to whole body insulin resistance, reduced peripheral glucose tolerance and increased hepatic gluconeogenesis.

High Fat Feeding Rapidly Exacerbates the Development of NAFLD that Occurs as a Result of Chronically Elevated Glucocorticoids

Anna D'souza[1], Jacqueline Beaudry[1], Laelie Snook[3], Andrei Szigiato[1], Arend Bonen[3], Stephen J. Trumble[2], and Michael Riddell[1].

[1] School of Kinesiology and Health Science, Faculty of Health, Muscle Health Research Center and Physical Activity and Chronic Disease Unit, York University, 4700 Keele St., Toronto, ON, Canada, M3J 1P3

[2] Department of Biology, Baylor University, One Bear Place 97388, Waco, TX, United States, TX 76798

[3] Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, ON, N1G 2W1

Corresponding Author: Dr. Michael Riddell, School of Kinesiology and Health Science, Faculty of Health, Muscle Health Research Center and Physical Activity and Chronic Disease Unit, York University, 4700 Keele St., Toronto, ON, Canada. Tel: 416-736-2100 Fax: 316-736-5774. Email: mriddell@yorku.ca

Key terms: high fat diet, insulin resistance, steatosis, visceral obesity, glucose intolerance

Contribution by the Authors

For this thesis project, I was involved in developing the research design with my supervisor. I collected all anthropometric measurements, ran all of the hyperinsulinemic euglycemic clamps, plasma hormone assays, q-PCR and protein expression analyses, in addition to collecting all of the tissue. I conducted all data and statistical analysis, including composition of all graphs, tables and write up of this thesis and edits. Jacqueline Beaudry and Carly Gordon, graduate students in Dr. Riddell's lab, assisted with the cannulation surgeries. Jacqueline Beaudry also assisted with the glucose tolerance test, plasma collection and animal euthanization. Carly Gordon assisted with sectioning of liver and staining with Oil Red O. Andrei Szigiato, a summer NSERC student in Dr. Riddell's laboratory, also helped with sectioning frozen liver and staining for fibrosis. Protein expression of CD36 and L-FABP were assessed by Laelie Snook, a technician in Dr. Arend Bonen's laboratory at the University of Guelph in Guelph, Ontario. Plasma measurements of alanine aminotransferase levels, total bilirubin and cholesterol were conducted by Clearstone Laboratories in Toronto, Ontario. Total hepatic lipid content was analyzed by Dr. Stephen J. Trumble's laboratory at Baylor University in Waco, Texas. Dr. Michael Riddell is the primary investigator and supervisor of this project.

INTRODUCTION

The occurrence of non alcoholic fatty liver disease (NAFLD) is common amongst individuals with the metabolic syndrome (MetS), a condition marked by abdominal obesity, insulin resistance and hypertriglyceridemia (7). NAFLD is defined as a chronic condition that ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), and can progress to fibrosis, cirrhosis and hepatocellular carcinoma (131, 196). Larger central adipose depots are closely related to development of hepatic steatosis due to the 'portal hypothesis' in which there is an increased delivery of FFAs due to increased lipolytic activity of adipose tissue (22). Positive energy balance through excess consumption of calories and sedentarism (174, 177) are believed to potentiate MetS symptoms in addition to hepatic lipid accumulation. Animal models of NAFLD have previously used prolonged periods of high fat feeding (92), lasting weeks or months to induce increased lipid accumulation that is typically evident in NAFLD. However, liver damage, marked by elevated plasma alanine aminotransferase (ALT) levels and fibrosis, typically does not develop with short-term feeding studies (< 4 weeks in duration) (11,40, 212). Specialized methionine and choline deficient diets are commonly used to model the development of NAFLD in rodents (92), but do not appear to induce insulin resistance (113,179) Studies that include both hepatic injury and insulin resistance, in addition to reflecting lifestyle factors, such as sedentarism and poor dietary choices, or side effects of chronic pharmacological therapy, are necessary to better understand the natural development of NAFLD.

Glucocorticoids (GCs) are stress hormones that, when chronically elevated, can promote both steatosis (160, 216) and several features of the MetS (181, 202) and type 2 diabetes (216,147). Interestingly, GCs have also been shown to promote increased desire for high fat foods (50), a secondary effect that may accelerate steatosis.

The present study sought to determine if a HFD, when combined with chronically elevated GCs, would rapidly result in NAFLD in otherwise healthy, male, six week old rats. To our knowledge, this represents the first investigation of the synergistic effects of elevated GCs and a high fat diet (HFD) on the development of NAFLD. Our results demonstrate that GCs and HFD additively and rapidly promote hepatic steatosis, insulin resistance, abdominal obesity, hyperinsulinemia and hyperglycaemia, thus providing a novel disease model of NAFLD. These findings illustrate the important interactive effects that chronic GC exposure may have with other lifestyle factors in contributing to metabolic dysfunction.

EXPERIMENTAL DESIGN AND METHODS

Animals, housing and pellet implantation

Adult male Sprague Dawley rats (six weeks of age, initial weight 225-250g, Charles Rivers Laboratories (Quebec, Canada) were individually housed in standard cages on a 12-h light, 12-h dark cycle. After a 7 day habituation period, animals were randomly assigned to receive either corticosterone (CORT; via four, 100 mg pellets of corticosterone, Sigma, St. Louis, MO) or wax (Placebo)

pellets of the same amount, as previously described (124). Animals were anesthetised under 2% isoflourane and aseptic conditions, and pellets were implanted below the subcutaneous layer between their scapulae (38). Immediately following pellet implantation surgery, animals within each group were randomly allocated to receive either standard rodent chow (consisting of 60% calories from carbohydrates, 13% from fat and 27% from protein, Purina Lab Diet, 5012), or a high fat diet (HFD, comprised of 60% calories from fat, 21% from carbohydrates and 18% from protein, Harlan Laboratories, TD.06414). During this study, individual rats were placed in one of four treatment groups (Figure 1): exogenous CORT treatment and a high fat diet (CORT HFD), CORT treatment alone (CORT chow), high fat diet alone (placebo HFD), and a group serving as a control for both diet and pellet treatment (placebo chow). Body mass (g) and total caloric intake (kcal/g/day) were measured every other day. All animals underwent an oral glucose tolerance test (OGTT) on day 8. After 16d, animals were euthanized and liver, epididymal adipose tissue, and adrenal glands were rapidly removed, weighed to the nearest second decimal place and flash frozen either for histological analysis or for protein and/or mRNA expression. All experiments were approved by the York University Animal Care Committee in accordance with regulations set forth by the Canadian Council for Animal Care.

Glucose Tolerance Test

Prior to GTT on day 7, animals underwent an overnight fast for a 12-hour period before receiving an oral gavage of 1.5 g/kg body weight glucose. At 0-, 30-, 60-, 90- and 120-minutes after gavage, a drop of blood collected from the tail vein was used to determine blood glucose concentrations using a hand held glucose meter (Contour blood glucose meter, Bayer, Toronto). An additional forty microlitres of blood was collected into EDTA-coated microvette tubes (Sarstedt, Montreal, Canada) for analysis of insulin. Plasma was separated and stored at -20°C. The homeostatic model assessment of insulin resistance (HOMA-IR) method was used to evaluate the degree of insulin resistance induced by treatment (110). A higher HOMA-IR value is indicative of a greater degree of insulin resistance (25).

Plasma analyses

Plasma CORT levels were measured one day prior to, and 7 days after, pellet implantation surgery at peak (one hour after onset of dark phase; 2000h) and nadir (one hour after onset of light phase; 0800h) time points. Plasma collected on day 7 via the tail vein was used for the determination of fasting insulin and glucose values. Immediately following sacrifice, blood from the hepatic and portal vein was also collected in EDTA microvette tubes on ice and plasma was stored at -20°C. Trunk blood (blood collected at decapitation) was also collected for measures of ALT, total bilirubin, haptoglobin and cholesterol.

Commercially available kits were used to assess plasma CORT (Corticosterone double antibody RIA, MP Biomedical, OH), non esterified FFAs (HR Series NEFA-HR, Wako Chemicals, Richmond, VA), and triglycerides (TR0100, Sigma Aldrich, ON). An ELISA was used to measure insulin (INSKR020, Crystal Chem, IL) and haptoglobin (Life Diagnostics, West Chester PA) in plasma. A Roche/Hitachi automated clinical chemistry analyzer was used to assess total cholesterol, ALT, and total bilirubin (all automated analyzer reagents from Cobas Inc., ON Canada).

Histology

Liver tissue from euthanized animals was snap frozen, sectioned 10μm thick, and stained for lipid content using Oil Red O as previously described (119). Briefly, liver sections were fixed with 3.7% formaldehyde for 1 hour at room temperature followed by immersion in an Oil Red O solution composed of 0.5g Oil Red O powder and 100 ml of 60% triethyl phosphate for 30 minutes at room temperature. Collagen content was determined in a similar manner. Liver sections were stained for one hour in Sirius Red dissolved in picric acid and subsequently washed in acified water, dehydrated in 70% ethanol and cleared with xylene. All slides were allowed to air dry for 10 minutes and sealed with Permount. All solutions were purchased from Sigma Aldrich (Oakville, ON Canada). Images for Oil Red O staining were acquired at a magnification of 10x, and for collagen using a 20x magnification using a Nikon Eclipse 90i microscope

(Nikon Canada) and Q-Imaging MicroPublisher 3.3 RTV camera with Q-Capture software. Analysis was based on conversion of coloured pictures to a grey scale, followed by quantification of optical density performed with Adobe Photoshop CS version 8.0. Staining intensity was converted into a percentage relative to the average intensity of the control group.

Quantification of hepatic lipid content

Frozen whole liver tissue (n=5 per group) was homogenized with sodium sulphate (approximately 10 g, J.T. Baker, Phillipsburg, NJ) before running through an Accelerated Solvent Extractor (Dionex ASE 350, Sunnyvale, CA), using a 2:1 chloroform-methanol solvent (VWR, West Chester, PA). The homogenization was conducted using a Wheaton tissue homogenizer with a Teflon pestle that was powered by a household drill (2500 r/min (1 r = 2prad)) in an ice bath to prevent evaporation. Excess solvent was evaporated under a steady stream of nitrogen (1.5 L/min at 37°C). Each tissue sample was weighed (to the nearest 3rd decimal place) on an XP56 microbalance (Mettler Toledo) before and after extraction to obtain total lipid content for each liver sample. Liver lipid content was expressed as a percentage of total liver sample weight.

Immunoblotting

Liver protein was extracted and analyzed for various mediators of insulin signalling, gluconeogenesis, and fat transport as previously described (112, 159).

Briefly, 50 mg of frozen liver tissue was homogenized in 1 ml of lysis buffer consisting of 20 mM Tris HCl, 150 mM NaCl, 2 mM EDTA, 20 mM NaF, 1% triton x-100, 10% glycerol, protease inhibitor cocktail and phosphatase inhibitor cocktail (both cocktails purchased from Sigma Aldrich, Oakville, ON) and centrifuged at 12,000g for 30 minutes. Protein concentration from collected tissues was determined using the Bradford method on the collected supernatant. Protein extracts were then subject to an SDS-PAGE (10% polyacrylamide SDS gel) and transferred to PVDF membranes. Membranes were blocked with 5% BSA for 1 hour at room temperature followed by overnight incubation at 4°C in primary antibody: Akt (1:1000 Cell Signaling Technology, Boston, MA), p-Akt^{thr308} (1:1000 Cell Signaling Technology), FOXO1 (1:1000; Cell Signaling Technology), p-FOXO1^{ser256} (1:1000; Cell Signaling Technology), PEPCK (1:1000; Santa Cruz Antibody, Santa Cruz, CA), G6Pase (1:1000; Santa Cruz, Santa Cruz, CA), CD36 (Mo25, gift from N. Tandon), L-FABP (1:1000; Abcam, Cambridge, MA), followed by a 1 hour incubation at room temperature with the appropriate secondary antibody. Blots were visualized by chemiluminesence (GE Healthcare, Baie d'Urfe, QC) with use of the Caresteam in vivo pro imaging system (Rochester, NY). β-actin (1:20,000, Abcam) was used as a loading control for all protein analyses. Quantification of relative band intensity was done by dividing the intensity of the target protein by the loading control intensity, and then making intensity of each sample relative to the average intensity of the placebo chow

controls. Band intensity was assessed using Carestream molecular imaging software (Rochester, NY).

Cytosolic and membrane protein fractions for determination of PKCε expression

To assess protein kinase C epsilon activity, protein from cytosolic and membrane fractions of liver tissue was separated as previously described with minor adaptations (112). Briefly, frozen liver tissue was homogenized in 1 mL of lysis buffer "A" (20 mM Tris HCl, 150 mM NaCl, 2 mM EDTA, 20 mM NaF, protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma Aldrich)) and centrifuged at 13,000 g for 1 hour at 4°C. Following centrifugation, the supernatant was collected as the cytosolic fraction. The pellet was immersed into buffer B (buffer A + 1% triton X-100) and then centrifuged for 1 hour. The supernatant was then collected to represent the membrane fraction. Protein concentrations and immunoblotting were performed as described above. PKCε (1:1000, Cell Signaling Technology) translocation was determined by the relative expression of membrane fractions to total (membrane and cytosol combined) expression from each sample.

RNA isolation, cDNA synthesis, and real-time qPCR analysis

Total RNA was extracted from 50 mg of powdered liver tissue using Trizol reagent (Invitrogen, Burlington, Canada). Following isolation, integrity of the RNA

was checked using a nano drop spectrophotometer (Thermo Scientific, Rockford, IL). RNA from all samples were then treated with DNAse I (Sigma Aldrich) and reverse transcribed into cDNA with random hexamers using Superscript II Reverse Transcriptase (Invitrogen). Primer sequences for fatty acid synthase and β-actin were analyzed (primer sequences shown in Table 1), and were validated using a Taq Polymerase Reaction and run onto a 3% agrose gel. Real-time quantitative PCR reactions were performed using the My IQ Single Color Real Time PCR Reaction System (Bio Rad) in 96 well plates containing 2.5 μg of cDNA, 5 μM each of the forward and reverse primer, and 12.5 μL of SYBR Green PCR Master Mix (Bio Rad) in a total volume of 25 μL. Relative mRNA levels were corrected with β-actin as the reference gene and calculated using the standard curve and delta CT method.

Hyperinsulinemic-Eugylcemic clamp

The hyperinsulinemic eugylcemic clamp procedure was performed as previously described (45). Ten to twelve days after pellet implantation surgery, animals were anesthetized and cannulated by inserting a catheter into the right external jugular vein and left carotid artery. Following a 3 day recovery period and an overnight fast, catheters from the jugular vein and carotid artery were extended to connect to infusion pumps (Harvard Apparatus). Radioactive tracer ([3-H^3]-glucose, 20 μCi/ml; Perkin Elmer Life Science, Boston MA) was infused for 90 minutes prior to insulin infusion to achieve equilibrium, and maintained at a

constant rate of infusion throughout the clamp (0.4 mL initial bolus plus 7.5 μL/min infusion). After 90 minutes, insulin was infused at a constant rate of 10 μL/min. Plasma glucose levels were measured every five minutes throughout this period using a glucose analyser (YSI, Yellow Springs, OH) to adjust the rate of infusion of exogenous labeled glucose (48 μCi/g). Aliquots of plasma were collected at 60, 70, 80 90, 150, 160, 170 and 180 min for determination of plasma 3-H^3 glucose concentrations. Animals were euthanized following the clamp. Under steady state conditions, the rate of glucose appearance (the ratio between 3-H^3 infusion rate and plasma 3-H^3 specific activity) is equivalent to the rate of glucose disappearance. The difference between glucose appearance and glucose infusion rate (GIR) yields the rate of endogenous glucose production.

Statistics

All data are represented as mean ± SE, with a criterion of $p<0.05$ to determine statistical significance. A two-way factorial analysis of variance (ANOVA) was used to determine statistical significance for anthropometric measurements, relative protein and mRNA expression, all plasma hormone and lipid analyses, HOMA-IR scores, total hepatic lipid and hepatic triglyceride content. To determine significance of glucose and insulin values over time during the OGTT, a one-way repeated measures ANOVA was used. Food intake and body mass over a six day period was assessed with a three way ANOVA (diet type, pellet type and day all served as variables). A one way ANOVA was used to

compare glucose infusion rate, percent suppression of EGP and glucose disposal and fasting glucose and insulin values amongst groups. An unpaired t-test was used to determine pellet mass differences between CORT groups. All statistical tests were conducted using Statistica 6.0 software. Post-hoc analyses such as Fisher-LSD or Bonferroni tests were used when ANOVA detected differences among treatments.

RESULTS

Chronic CORT treatment increases plasma corticosterone levels and induces atrophy of the adrenal glands.

At baseline (day 0), all treatment groups had lower basal plasma corticosterone values relative to peak values and displayed a normal diurnal rhythm (Figure 2A, $p<0.05$ between peak and trough for all groups except placebo chow, where $p=0.07$). Eight days following pellet implantation, CORT-treated animals demonstrated an abolished diurnal rhythm, with elevated CORT levels at both peak and basal time points (Figure 2B, $p<0.05$ based on a three way ANOVA). Short term exogenous CORT treatment also resulted in significant reductions in relative adrenal mass (Table 2; three way factorial ANOVA, $p<0.05$) indicating an expected suppression of endogenous hypothalamo-pituitary adrenal activity. Wax pellet implantation and consumption of a high fat diet did not alter the normal diurnal rhythm (Figure 2B), nor did it affect adrenal mass (Table 2; $p>0.05$). The amount of CORT dissolved over the treatment period did not differ

significantly between the CORT-treated groups (76.41±3.1% vs. 67.51±3.2% of pellet remaining in CORT chow vs. CORT HFD; $p>0.05$ using an unpaired t-test).

Hypercortisolemic animals experience increased food consumption and increased visceral adipose mass, but decreased body mass.

Chronic CORT administration over a 12-16 day period resulted in significant increases in caloric intake (Figure 3B; $p<0.05$ Day 2 versus Day 6 for both CORT groups). Chronic CORT or HFD alone resulted in a 1.5-fold increase in epididymal fat mass, which was exacerbated by 2 fold when CORT was combined with a HFD (Table 3; $p<0.05$ relative to placebo chow). In contrast to increased VAT stores, subcutaneous fat tissue was virtually non-existent in CORT-treated animals (based on our observations). Despite increases in visceral adipose mass, CORT-treated animals experienced a decrease in overall body mass (Figure 3A and Table 3; $p<0.05$) and gastrocnemius muscle mass (Table 3; $p<0.05$ relative to the placebo chow group).

HFD and CORT rapidly induce development of NAFLD without hepatic inflammation

After 16 days of treatment, CORT and HFD alone both resulted in ~2 fold increases in relative hepatic lipid content (g fat/g liver mass). In combination however, the CORT HFD group experienced an additive effect of both treatments, and resulted in a ~4 fold increase in hepatic lipid content relative to

controls (Figures 4A and 4B; $p<0.05$). Both hepatic lipid extraction and Oil Red O staining (Figures 4C and 4D) confirmed that the combination of CORT and HFD resulted in the greatest increases in lipid content. Excess lipid accumulation often is associated with development of fibrosis and abnormally elevated plasma ALT and total bilirubin levels, which indicate liver injury (168). While HFD alone did not produce any increases in ALT and total bilirubin relative to controls, the combination of elevated CORT levels with HFD resulted in severe liver injury, as evidenced by 2.8- and 2.3-fold elevations in plasma ALT and total bilirubin levels in CORT HFD relative to the control group ($p<0.05$; Table 4). Fibrosis, assessed by collagen staining of liver sections, was significantly elevated in the CORT HFD group (Figures 4E and 4F; $p<0.05$ relative to placebo chow), but did not differ from controls in the CORT-Chow and placebo-HFD groups ($p>0.05$).

Haptoglobin, a common marker of hepatic inflammation, was measured to determine whether increased lipid accumulation was associated with increases in hepatic inflammation. Both CORT groups experienced reductions in plasma haptoglobin levels relative to the placebo chow group, but the CORT chow group was the only group to experience significant reductions in content ($p<0.05$; Table 4). HFD alone did not significantly alter haptoglobin concentrations as the placebo HFD and placebo chow groups were comparable.

Cholesterol, FFA and triglyceride content were measured from trunk blood in a fed state. CORT alone resulted in 1.5 and 1.3 fold increases to plasma cholesterol and TG levels. While HFD alone did not lead to significant alterations

to FFA, Cholesterol or TG concentrations, the addition of a HFD to elevated COT further amplified cholesterol production and induced hypertriglyceridemia, evident by a 2.0 fold increase in plasma cholesterol and 3.7 fold increase in TG concentrations in the CORT HFD group relative to the placebo chow group (Table 4; $p<0.05$).

Chronic CORT results in hepatic lipid accumulation by increasing spill over of free fatty acids from the portal vein

Prior to sacrifice, blood samples were taken from the portal and hepatic vein, representing delivery into and export out of the liver, respectively. Chronically elevated CORT levels, either alone or in combination with a HFD, resulted in elevated portal vein concentrations of plasma FFAs relative to placebo chow controls (Figure 5A; $p<0.05$). HFD alone did not significantly increase portal vein FFA concentrations. Portal vein TG concentrations were comparable between all groups (Figure 5C; $p>0.05$)

FFA export, as measured by hepatic vein concentrations, was greatest in the CORT HFD group (Figure 5B; $p<0.05$), however the net uptake of FFA, determined by the net differences in FFA concentrations in the hepatic versus portal veins (Figure 5E), were comparable between both CORT groups. In contrast, the CORT Chow group was the only group to experience a significant increase in hepatic vein TG levels (Figure 5D; $p<0.05$). Net up take of TGs (measured as the difference between portal vein and hepatic vein TG

concentrations) were negative for all groups except the CORT HFD group (Figure 5F). This positive net uptake suggests a greater amount of TGs taken up and stored in the liver, than secreted from the liver.

Chronically elevated CORT results in increased protein expression of CD36, and acts synergistically with a HFD to increase FASN mRNA

Within the liver, chronically elevated CORT levels resulted in elevated expression of the lipid transporter CD36 (2.5-fold increase relative to placebo chow, Figure 6A; $p<0.05$). HFD did not independently or additively alter CD36 expression. No differences in L-FABP protein expression relative to controls were evident (Figure 6B; $p>0.05$ relative to placebo chow). Expression of hepatic fatty acid synthase (FASN) mRNA, was assessed by real time quantitative polymerase chain reaction (qPCR) of frozen liver tissue. CORT treatment resulted in a significant increase in mRNA expression of FASN (Figure 6C; $p<0.05$). Together, HFD and elevated CORT resulted in a synergistic increase in FASN mRNA expression that was 2 fold higher than CORT treatment alone, and 14-fold higher relative to the placebo chow group (Figure 6C; $p<0.05$).

Chronic CORT induces hyperinsulinemia and glucose intolerance, which is further exacerbated by consumption of a high fat diet.

CORT HFD animals endured hyperglycaemia after an overnight fast (blood glucose 10.92 ±1.23 mmol, $p<0.05$; Figure 7A), while all other groups

maintained euglycemia. CORT treatment alone resulted in fasting hyperinsulinemia, evident by a 7.7 fold increase in plasma insulin levels relative to the placebo chow group. The addition of a HFD to elevated CORT resulted in a 13 fold increase in fasting plasma insulin levels relative to the placebo chow group (Figure 7B; CORT-HFD = 5.78±1.92 ng/ml; CORT-Chow=3.12±0.58 ng/ml, $p<0.05$ relative to placebo chow). Increases in plasma CORT concentrations (at the basal time point on day 8) correlated with higher fasting insulin levels (Figure 7C, $r^2=0.69$, $p<0.05$). HOMA-IR scores, which are an indicator of insulin resistance, and are based on fasting insulin and glucose scores, were elevated 12 fold as a result of elevated CORT alone (Figure 7D; $p<0.05$). HFD worsened insulin resistance in the presence of elevated CORT levels as the CORT-HFD group endured a 26 fold increase in HOMA-IR scores relative to placebo chow controls (Figure 7D; $p<0.05$). The OGTT, performed after an overnight fast, revealed that while the CORT-HFD had elevated glucose levels throughout the 2 hour test, glucose intolerance, as assessed by area under the curve, was similar between both CORT groups (Figure 7E, 7F; $p<0.05$ relative to placebo chow).

Hepatic insulin signalling is impaired as a result of chronically elevated CORT levels, which is impaired with the addition of a HFD

The hyperinsulinemic euglycemic clamp was used to evaluate hepatic insulin sensitivity and endogenous glucose production. The glucose infusion rate (GIR) during the clamp is a direct measure of insulin sensitivity, with higher

infusion rates suggesting more insulin sensitivity. While the placebo group experienced an average GIR of 25.6 mg/kg/min, this rate was significantly reduced with CORT treatment and CORT and HFD combined (Table 5; $p<0.05$). Similarly, the CORT-HFD group experienced the least suppression of endogenous glucose production during the clamp period (in the presence of exogenous insulin), as compared to the basal period (no exogenous insulin infusion; Table 5). The signalling pathway involved in insulin regulation of gluconeogenesis was assessed via immunoblotting. Significant reductions in phosphorylation of AKT at the threonine 308 site occurred in both groups with elevated CORT levels (Figure 8A; $p<0.05$ relative to placebo chow). FOXO1 phosphorylation was dramatically reduced in all treatment groups (Figure 8B), but had the greatest degree of attenuation in the CORT-HFD group (75% decrease relative to placebo chow, $p<0.05$). This corresponded with a 44% increase in G6Pase protein expression by the CORT HFD group compared to a 21% increase with CORT and HFD alone (Figure 8C; $p<0.05$). Both CORT-treated groups experienced similar increases in PEPCK protein expression relative to the placebo groups (Figure 8D; $p<0.05$), while HFD alone did not significantly increase PEPCK protein expression.

Measurement of hepatic PKCε translocation revealed no significant differences in PKCε activity as a result of CORT or HFD (Figure 9; $p>0.05$ relative to the placebo chow group).

DISCUSSION

In this study, we use a rodent model to demonstrate the influence of chronic CORT treatment in combination with a HFD on the development of NAFLD. The chronically elevated plasma CORT concentrations, when combined with a 60% high fat diet rapidly accelerated development of NAFLD, resulting in a 4 fold increase in hepatic lipid content, 2.8 fold increase in plasma ALT levels , and 1.5 fold increase in fibrosis after only 16 days of treatment, and in comparison to the placebo chow group. Along with steatosis, combined treatment facilitated development of other MetS characteristics including synergistically exacerbating development of insulin resistance and additively increasing central adiposity.

CORT treatment resulted in an abolished diurnal rhythm, increased caloric consumption and Cushing's phenotype.

Through CORT pellet implantation, we were able to achieve an abolished diurnal rhythm with basal CORT values 20-60% above placebo-treated peak values in CORT-Chow and CORT-HFD groups respectively (Figure 2B). These increased CORT concentrations mimic the increases endured by patients given 25 mg of prednisone, a commonly prescribed synthetic GC. After 5 days of GC therapy, they experienced an increase in baseline values which were similar to peak values that occurred prior to GC exposure (210).

As a result of chronic CORT levels, caloric intake was significantly higher in CORT groups relative to placebo controls (Figure 3B; $p<0.05$). Exposure to HFD alone facilitated the opposite response and resulted in a compensatory reduction in caloric intake over time. This hyperphagic effect of chronic CORT has been observed by Dallman and colleagues, who show that elevated GCs increase caloric intake, and in combination with insulin, increases preference for high fat foods (19, 51, 161, 229). Despite increases in food intake, chronically elevated CORT levels resulted in a reduction in overall body mass (Figure 3A; $p<0.05$). This reduction in body mass is attributed to decreases in lean muscle mass (Table 3; $p<0.05$ relative to place chow controls), of which chronic CORT treatment has previously been shown to induce muscle atrophy (16, 133). Additionally, the decline in muscle mass may also be due to CORT's suppressive effects on growth hormone secretion from the anterior pituitary (64, 150). In contrast, visceral adipose mass (measured from the epididymal adipose depot) was significantly increased with CORT treatment and further exacerbated by the addition of a high fat diet (Table 3). Taken together, the decline in muscle mass and increased central adiposity resembles the common Cushing's phenotype of central obesity and atrophied periphery (Table 3; relative to placebo controls) that often occurs as a result of chronically elevated CORT levels (21, 56). These results therefore suggest that the exogenous CORT treatment given was sufficient to induce Cushing's syndrome in a relatively rapid period of time.

CORT and HFD additively induce development of NALFD, marked by steatosis and fibrosis, which occurs independent of hepatic inflammation

Our findings clearly show that that a HFD can exacerbate the development of NAFLD that occurs as a result of chronically elevated CORT levels. HFD alone increased lipid content by 2 fold but did not cause significant alterations to ALT and bilirubin (Table 4, $p<0.05$). In contrast, our findings indicate that when HFD is given in the presence of chronically elevated CORT, the outcomes are exacerbated, and result in 4 fold increases in hepatic lipid content and 2.8 fold elevations in ALT after only 16 days (Figure 4A, Table 4). A similar effect on fibrosis development occurred when CORT and HFD were combined. While CORT treatment alone and HFD alone did not elicit fibrosis, the combination of both resulted in a 1.5 fold increase in collagen content relative to placebo chow controls (Figure 4E; $p<0.05$). These surprising findings may be a result of the 'toxic' environment produced by CORT and HFD. Though we did not measure reactive oxygen intermediates (ROIs), previous studies have suggested that increased ROI as a result of lipid accumulation in the liver may facilitate increased activation of hepatic stellate cells (231), thus facilitating increases in collagen content. Further studies are necessary to determine if ROI content is increased in the present model of NAFLD.

GCs are known for their capacity to suppress inflammation and are often used to treat inflammatory conditions including asthma and arthritis (178). Our findings demonstrate an immunosuppressive effect of GCs by showing

decreased expression of plasma haptoglobin, a marker of hepatic inflammation, in CORT-treated groups (Table 4). CORT alone resulted in a significant decline in haptoglobin content. In contrast, HFD combined with chronically elevated CORT levels resulted in less of a reduction in plasma haptoglobin levels (Table 3; p=0.07). These results suggest that fibrosis is able to develop in this model of NAFLD independent of hepatic inflammation. Whether CORT results in a similar immunosuppressive effect on cytokines released from surrounding adipose tissue warrants further investigation.

CORT drives FFA spill over from peripheral adipose stores, increases facilitated lipid transport expression and acts synergistically with a HFD to increase *de novo* lipogenesis

Our results show that 16 days of CORT treatment increased delivery of FFA into the liver via the portal vein, while HFD, either alone or combined with CORT, did not significantly increase FFA spill over (Figure 5A). Jensen et al (105) reported that portal vein FFAs predicted the proportion of hepatic FFAs that originated from adipose tissue lipolysis, including subcutaneous and visceral depots. Measurement of glycerol concentrations in the portal vein (Table 4) support the notion that these FFAs being 'spilled' into the liver are a result of increased lipolysis of surrounding visceral adipose tissue (VAT). VAT can contribute to increases in lipid spill over into the liver due to its close proximity to the liver and high metabolic activity (172, 142). CORT alone resulted in a 1.5 fold

increase in visceral adipose tissue (VAT), which was further increased to 2 fold when combined with a HFD (Table 3; $p<0.05$). The apparent paradox of increased lipolytic activity despite increases in adipose mass has been previously shown in 3T3 cells cultured with CORT and is believed to occur due to CORT's ability to promote pre-adipocyte differentiation (increasing hyperplasia of adipose tissue), while also increasing activity of enzymes involved in lipolysis (38). CORT treatment also resulted in depletion of subcutaneous adiposity, suggesting a re-distribution of adipose mass to the central adipose depots (38). Though the CORT-HFD group had access to a greater amount of dietary lipids relative to the CORT chow group, this did result in further increases in portal vein concentrations of FFAs and TGs, suggesting that primary source of FFA spill over into the liver is from adipose tissue lipolysis and not dietary fat.

Hepatic lipid metabolism is a balance between lipid import into the liver, and export from the liver, primarily as VLDLs. The elevated hepatic vein TG concentration in the CORT chow group could suggest increases in VLDL export, a phenomenon that is known to occur with CORT treatment, through increases in synthesis and release of apo-B100, a key protein component of VLDLs (228). However, it is difficult to attribute the hepatic vein TGs as a direct contribution from VLDLs as the hepatic vein can include both TGs from VLDLs synthesized and exported from the liver, and TGs that are not re-esterified by the liver. Future studies are warranted to determine how VLDL export is altered with this model.

The induction of facilitated lipid transporters in the liver may be a critical factor involved in steatosis development (117, 132). CD36 has been shown to facilitate fatty acid influx into the liver (25), and thus increased expression may contribute to steatosis. While FFA are able to enter into the liver via passive diffusion, it is differences in concentrations of facilitated transporters that contribute to significant alterations in FFA uptake by the liver (27). In our study, chronic CORT treatment resulted in a 2.5 increase in CD36 expression (Figure 6A). The increases in CD36 expression paralleled increases in hepatic lipid accumulation suggesting that CORT may facilitate steatosis through increasing facilitated transporter expression. These findings are similar to those of Komamura et al., who found that dexamethasone (a synthetic GC) increased expression of CD36, a facilitated lipid transporter, in gastrocnemius muscle (116). In contrast, differences in L-FABP expression amongst treatment groups were not observed (Figure 6B). L-FABP is predominantly found in the cytosol where it facilitates intercellular transport of long chain fatty acids for beta-oxidation (14). Interestingly, Foucaud et al. found that daily dexamethasone treatment for 5 days down-regulated L-FABP expression in rat liver *in vivo* likely via increases in lipid provision (76). This suggests that despite increases in lipid content in the liver, facilitated transport within the liver does not undergo compensatory increases.

Also within the liver, and as a result of insulin stimulation, lipid synthesis via *de novo* lipogenesis can occur. CORT acts synergistically with elevated insulin levels to increase *de novo* lipogenesis (95). With our treatment model, we see a synergistic increase in mRNA expression of FAS, a rate limiting enzyme involved in hepatic lipogenesis, in the CORT-HFD group (Figure 6C; $p<0.05$, relative to placebo chow). This corresponds with previous studies that have found increases in DNL with NAFLD (195) and hyperinsulinemia (59). Thus, HFD in the presence of CORT may directly exacerbate increased lipid accumulation in the liver directly by increasing FAS mRNA expression, and indirectly by promoting a greater degree of hyperinsulinemia.

If not utilized in oxidation, the hepatic FFA pool contributes to cholesterol synthesis. Increased plasma cholesterol concentrations were evident in the CORT treated groups, and were even greater when CORT and HFD were combined. Cholesterol content was highest in the CORT-HFD group (Table 4; $p<0.05$), suggesting that high fat feeding and chronic CORT treatment are not only capable of rapidly inducing liver damage, but may also perpetuate development of atherosclerosis and heart disease due to increased cholesterol levels.

Consumption of a HFD in the presence of chronic CORT rapidly promotes a diabetic phenotype and reduces hepatic insulin signalling

CORT alone did not induce fasting hyperglycemia, but it did increase insulin levels 7.6 fold (3.31 ng/ml) above placebo chow controls (0.43 ng/ml). These results are consistent with a previous report (109) that found elevated insulin levels as a result of exogenous CORT administration. The combination of CORT and HFD rapidly induced a 13-fold increase in fasting hyperinsulinemia (average = 5.73 ng/ml), 2.5 fold increase in fasting hyperglycemia (average = 10.9 mM), and 26-fold increase in HOMA-IR score relative to placebo chow (Figure 7A-C). Our results extend these findings by showing that high fat feeding is capable of exacerbating insulin resistance, hyperinsulinemia and hyperglycemia with CORT treatment to produce a diabetic phenotype. To determine the cause of fasting hyperglycemia, we assessed gluconeogenesis using the hyperinsulinemic euglycemic clamp and protein expression of enzymes involved in the insulin signalling cascade. The glucose infusion rate during the hyperinsulinemic euglycemic clamp was reduced with CORT and HFD alone, and was further reduced when CORT and HFD were combined. In the presence of exogenous insulin infusion during the clamp, hepatic glucose production was significantly reduced in both the placebo chow and CORT chow group, but not the CORT-HFD group (Table 5; $p<0.05$). This suggests that insulin regulation of gluconeogenesis is impaired when elevated CORT is combined with a HFD. To elucidate whether altered hepatic insulin signalling occurs in this model of

NAFLD, we measured protein expression of downstream targets of the insulin receptor. In the presence of insulin, Akt induces phosphorylation and removal of FOXO1 from the nucleus, preventing transcription of gluconeogenic enzymes (149). However, with insulin resistance, this inhibition is lost and FOXO1 remains active in the nucleus (138, 200). Previous studies report that CORT is a strong agonist of gluconeogenic gene transcription because it is able to bind to glucocorticoid response elements on the PEPCK and G6Pase gene (69, 205). As well, CORT may also interfere with insulin receptor autophosphorlyation (81) and abrogate phosphorylation of Akt in the presence of insulin (234), thus preventing insulin regulation of gluconeogenesis. Consistent with these studies, we found decreased phosphorylation of Akt and FOXO1 with CORT treatment (Figure 8A-B; $p<0.05$). While CORT and HFD alone resulted in reduced FOXO1 phosphorylation, HFD in the presence of CORT resulted in the greatest reductions in FOXO1 phosphorylation (Figure 8B; $p<0.05$). This corresponded with elevations in G6Pase protein expression that occurred with CORT and HFD alone, but where greatest when CORT and HFD were combined (Figure 8C; $p<0.05$). While CORT treatment resulted in increases in protein expression of PEPCK, the addition of a HFD did not further amplify PEPCK protein expression in CORT treated animals (Figure 8D; $p<0.05$).

PKCε is emerging as a significant player in the link between steatosis and insulin resistance. PKCε activation is believed to be initiated by fatty acids (52), consumption of a high fat diet (188), and also CORT (104). To determine

whether PKCε was involved in promoting hepatic insulin resistance, we measured PKCε activation via its translocation to the plasma membrane in the liver. PKCε translocation did not significantly differ between groups, suggesting that CORT and HFD induce insulin resistance independent of PKCε activation. (Figure 9, $p>0.05$).

The increases in PEPCK and G6Pase protein expression experienced as a result of chronic CORT and HFD combined are in contrast to the high HOMA-IR scores experienced by this group. This suggests that insulin resistance in this model is not solely attributed to impaired hepatic insulin sensitivity, but rather a collective dysfunction of other tissues involved in glucose homeostasis, including impaired peripheral glucose uptake or altered pancreatic insulin secretion.

Conclusion

In summary this study demonstrates for the first time the interactive effects of high fat feeding and chronic CORT in rapidly facilitating hepatic lipid accumulation, fibrosis and liver injury. We highlight the influence of CORT and HFD in provoking increased lipid spill over from more metabolically active adipose depots as a major contributor to steatosis. Within the liver, we show that CORT and HFD impair lipid metabolism by increasing expression of enzymes involved in facilitated lipid transport and *de novo* lipogenesis. In addition to hepatic steatosis and injury, the combination of CORT and HFD rapidly induces a diabetic phenotype marked by hyperinsulinemia, whole body insulin resistance,

and reduced glucose infusion rate within 16 days of exposure. Thus, evidence that dietary modifications significantly exacerbate development and progression of NAFLD and MetS in the presence of chronically elevated CORT represents a key finding that permits further evaluation of the role of poor diet and sedentarinism in accelerating progression of liver disease, and other ancillary conditions including development of diabetes and heart disease.

Figures and Tables

Figure 1: Experimental Design and Timeline

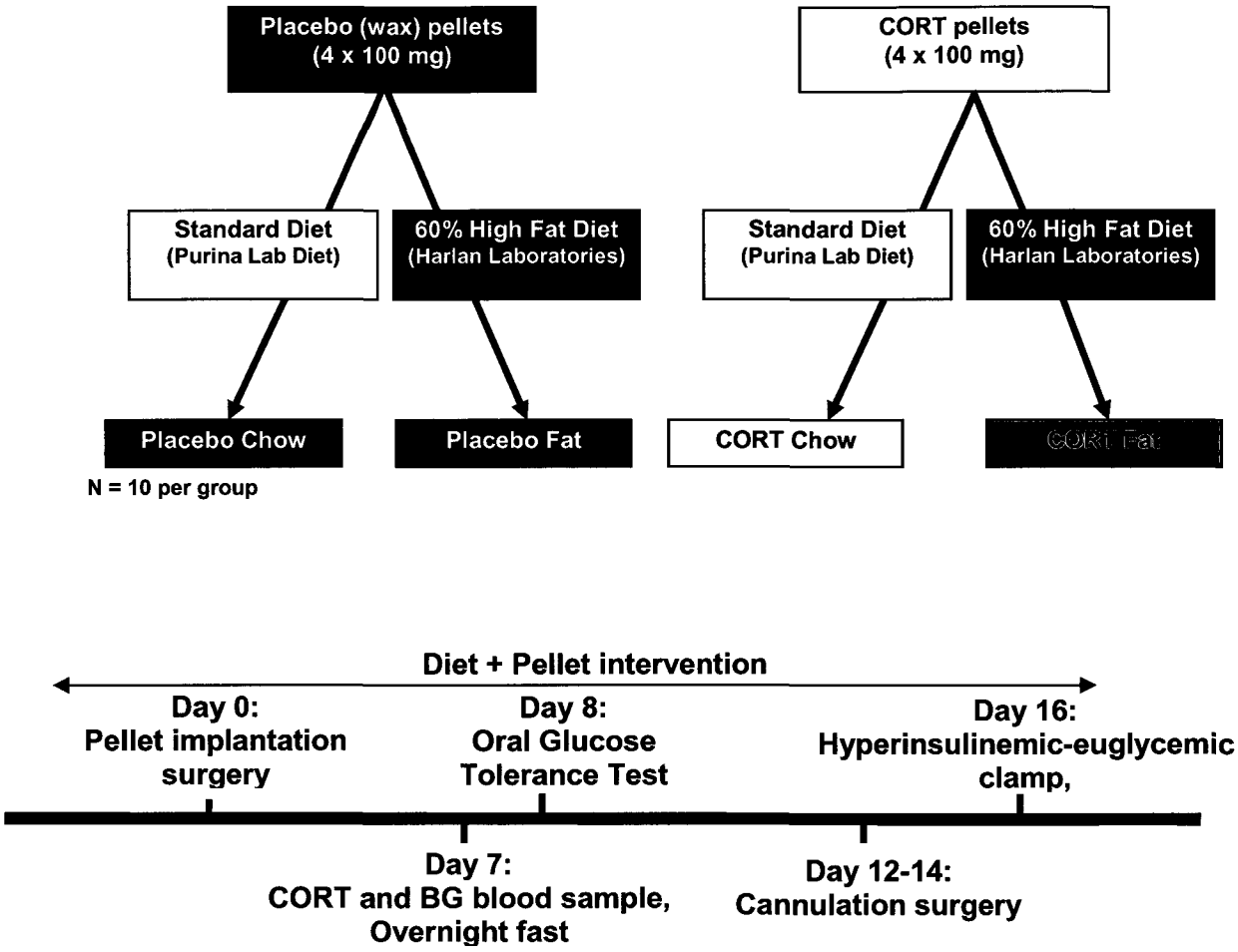

Figure 1: Experimental design. Healthy male Sprague Dawley rats (6 weeks of age) were subject to a pellet implantation surgery in which they either received four, 100 mg CORT pellets or four 100 mg pellets made of wax. Animals from each pellet group were then randomly assigned to receive a standard chow diet or a 60% high fat diet for 2 weeks.

Figure 2: Day 0 and Day 8 basal and peak plasma CORT concentrations

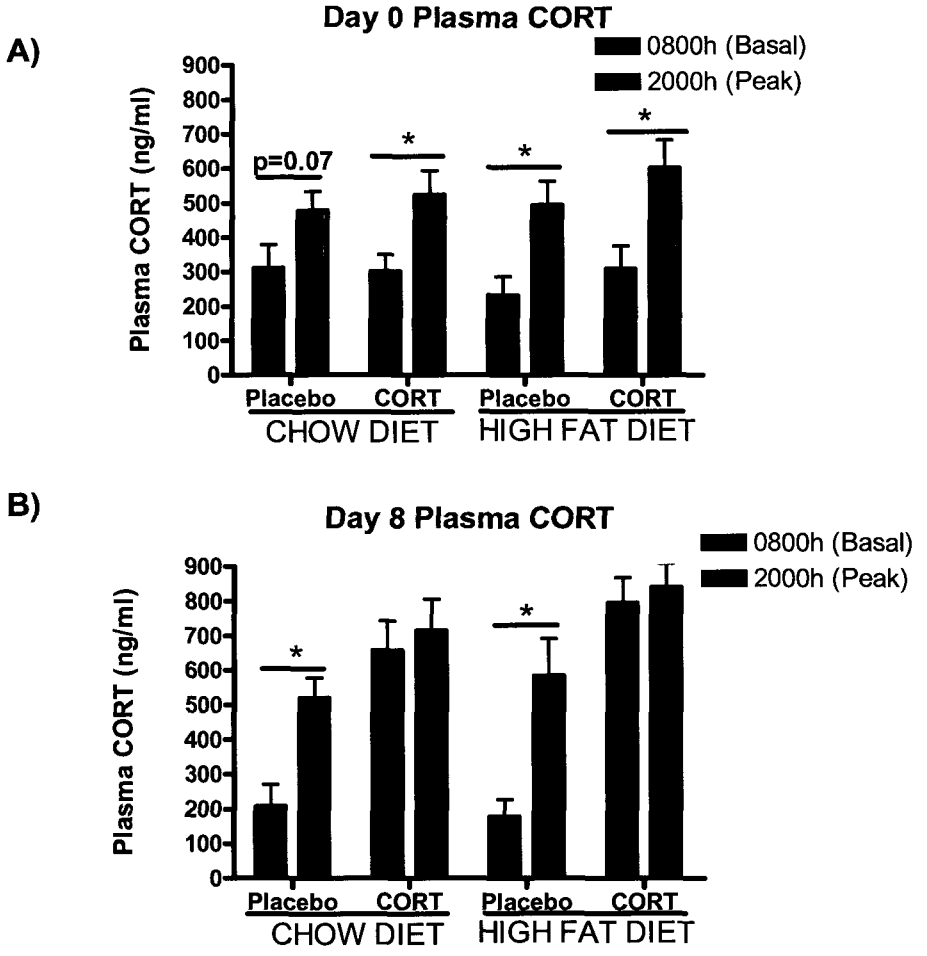

Figure 2: Corticosterone diurnal rhythm. Prior to pellet implantation (day 0; A), all groups demonstrated a similar diurnal rhythm; with significant differences between basal and peak plasma CORT levels. One week following CORT pellet implantation (day 8; B), CORT-treated animals experienced an abolished diurnal rhythm; while placebo (wax) treated animals maintained a normal diurnal rhythm. *N ≥8 per group; a three way ANOVA was performed where * indicates p<0.05 at 2000h relative to 0800h; all values are ± SEM).*

Figure 3: Body Growth and Food Intake

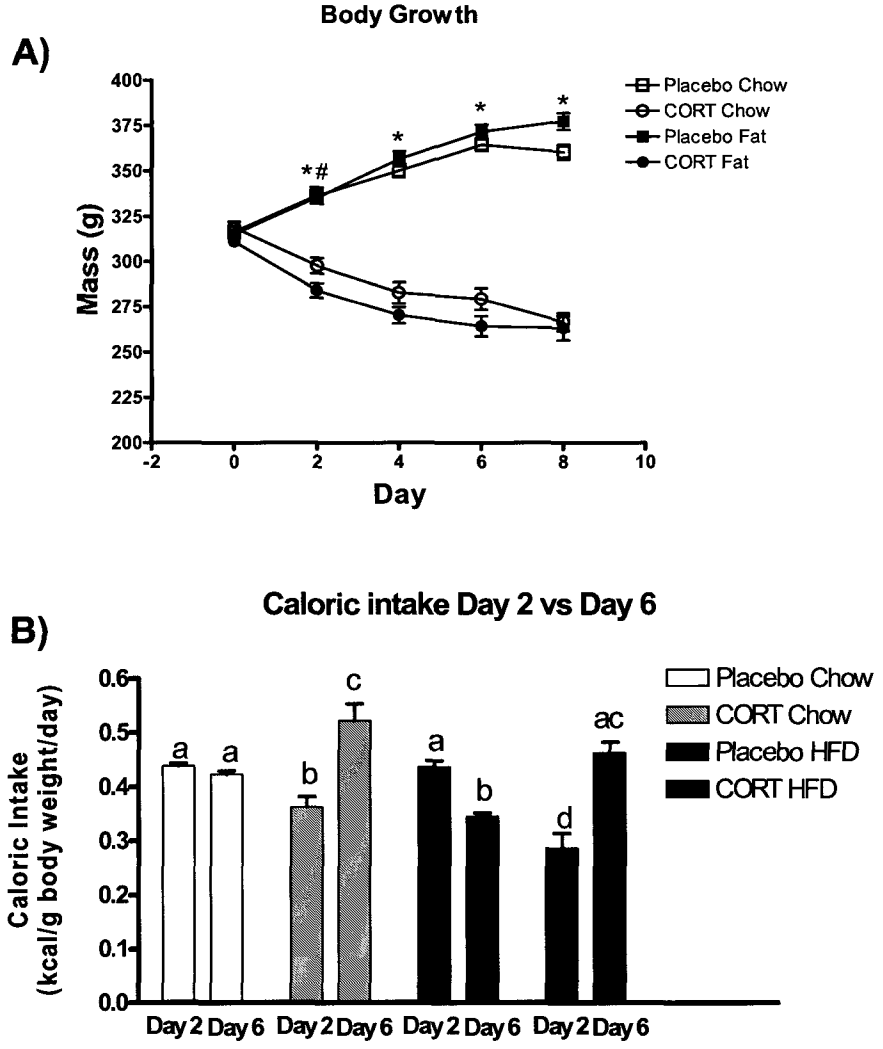

Figure 3: Body Growth and Food Intake.
CORT treatment resulted in a significant decrease in body mass over time (**A**) in comparison to the placebo-treated animals. Caloric intake did not differ in the placebo chow group, but was significantly increased in CORT treated animals. This was in contrast to the decrease experienced by the placebo-HFD group relative on day 6 versus day 2. (**B**). *N=5-10 per group; a three way ANOVA was performed at each time point with LSD post hoc tests where * indicates main effect of CORT; # indicates p<0.05 CORT chow vs. CORT HFD. Different letters indicate p<0.05. All values are ± SEM.*

Figure 4: Hepatic Lipid and Collagen Content

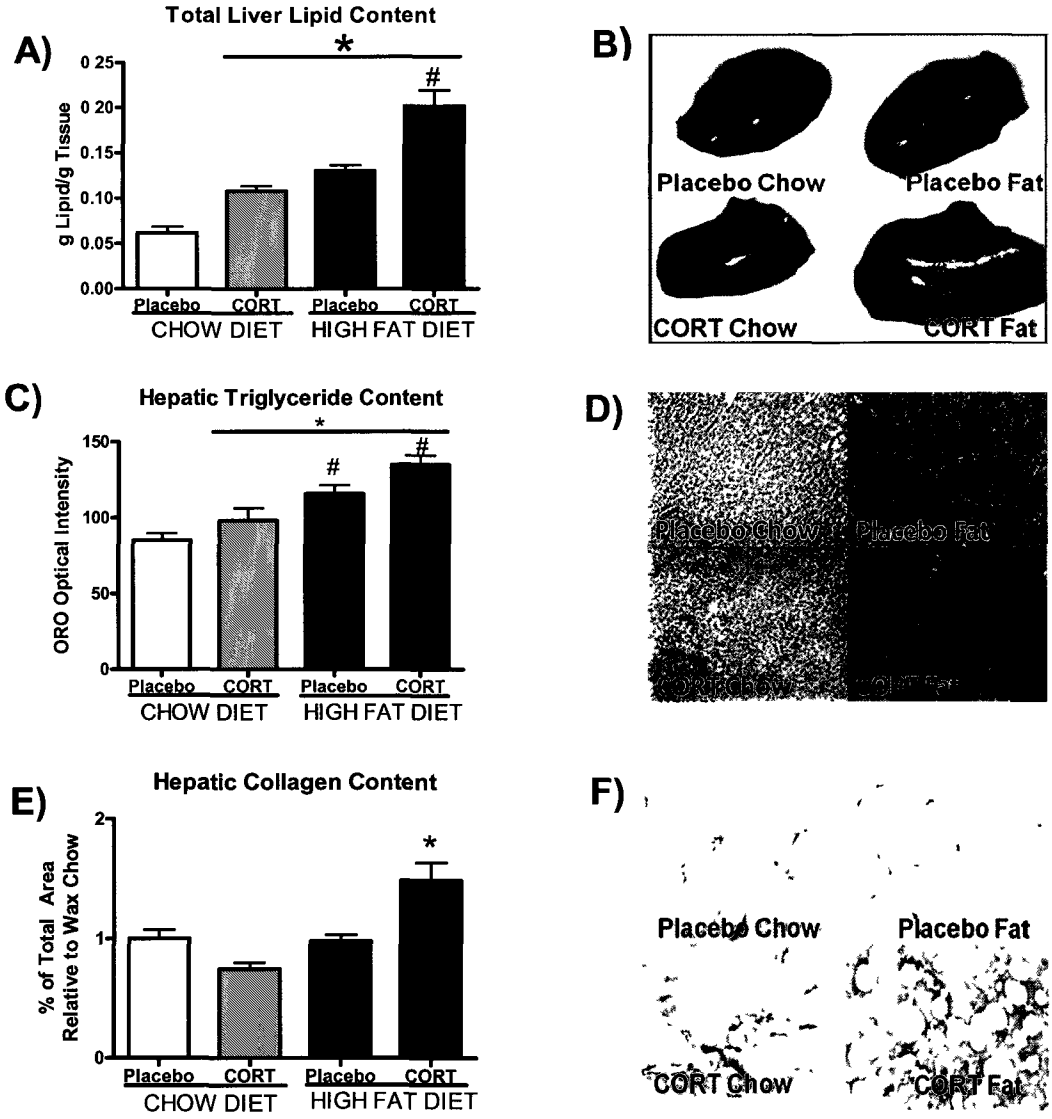

Figure 4: **Hepatic lipid and collagen content.** Chronically elevated CORT levels combined with HFD resulted in severe hepatic steatosis, indicated by total liver lipid extraction **(A, B)** and Oil Red O staining of lipid content **(C, D)**. This occurred after only 16 days of pellet and diet exposure. High fat feeding in the presence of chronically elevated CORT levels resulted in increased hepatic fibrosis, evident by Sirius red staining for collagen content **(E, F)**. Quantification of red stain via color density revealed marked increases in fibrosis in the CORT-HFD group (**E**; $p<0.05$). *$N \geq 5$ per group; a two way ANOVA was performed with Bonferonni post hoc tests where * indicates $p<0.05$ relative to placebo chow; # indicates $p<0.05$ relative to CORT-Chow. All values are ± SEM.*

Figure 5: Transport of FFAs and TGs to and from the Liver

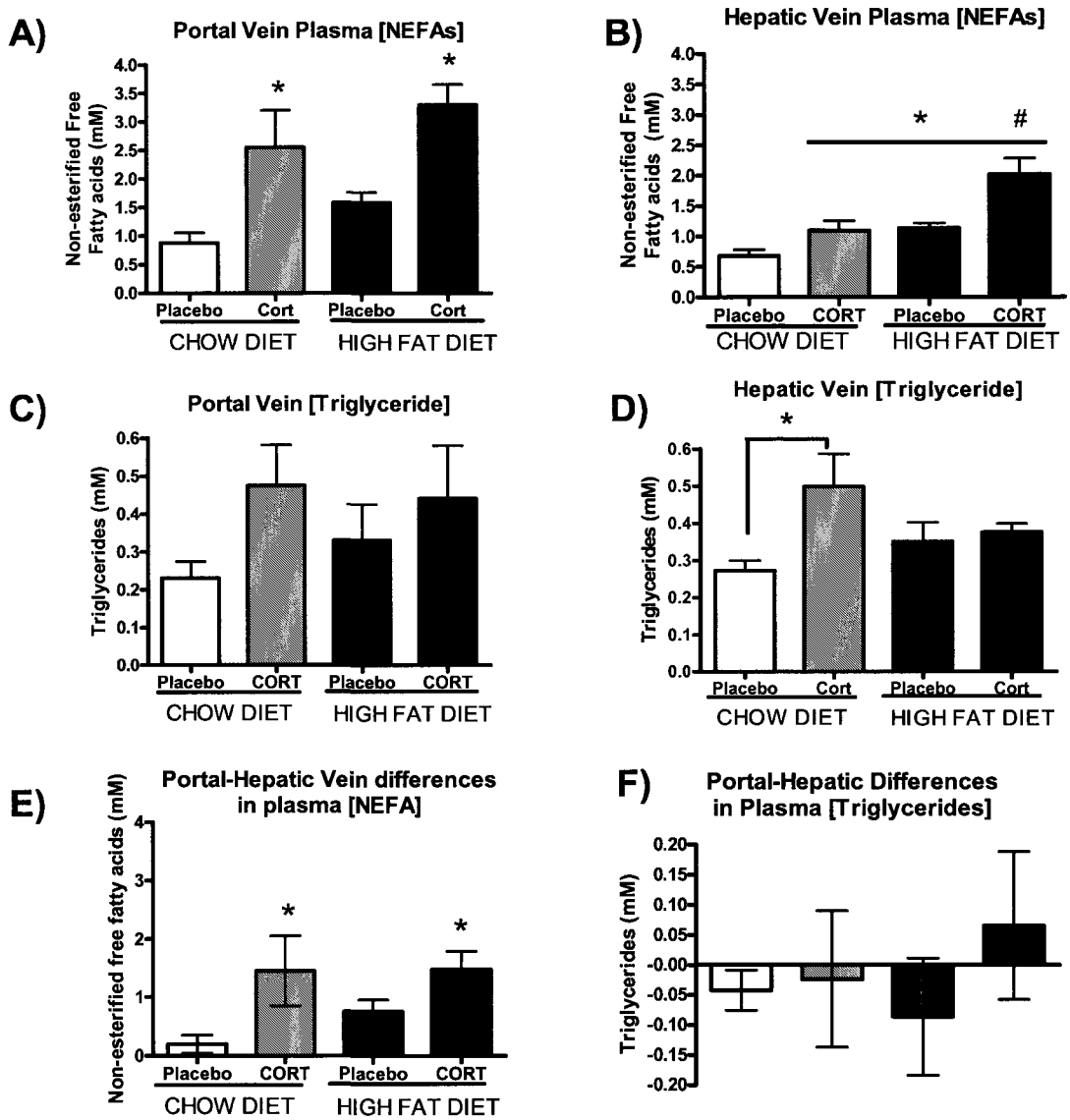

Figure 5: Lipid transport into the liver. The portal vein delivers lipid metabolites including FFA and TG from the intestines and surrounding adipose tissue to the liver. CORT treatment results in elevated NEFAs concentrations in the portal vein (**A**; $p<0.05$), but no significant increases in TG import (**C**). CORT and HFD alone increased FFA export, and effect was additive in combination (**B**). CORT alone resulted in the only significant increase in hepatic TG concentration (**D**). Net differences in FFA and TG content were measured by subtracting hepatic vein values from portal vein values. A positive net value indicates greater uptake/storage in the liver. CORT treatment resulted in a significant increase in net FFA uptake (**E**). In contrast, only the CORT HFD group experienced a positive net uptake of TGs (F). A two way ANOVA and Bonferonni post hoc tests were performed. ** indicates $p<0.05$ versus placebo chow. # indicates $p<0.05$ versus CORT chow. All values are ±SEM.*

Figure 6: Protein expression of Facilitated Lipid Transporters within the liver and FAS mRNA expression

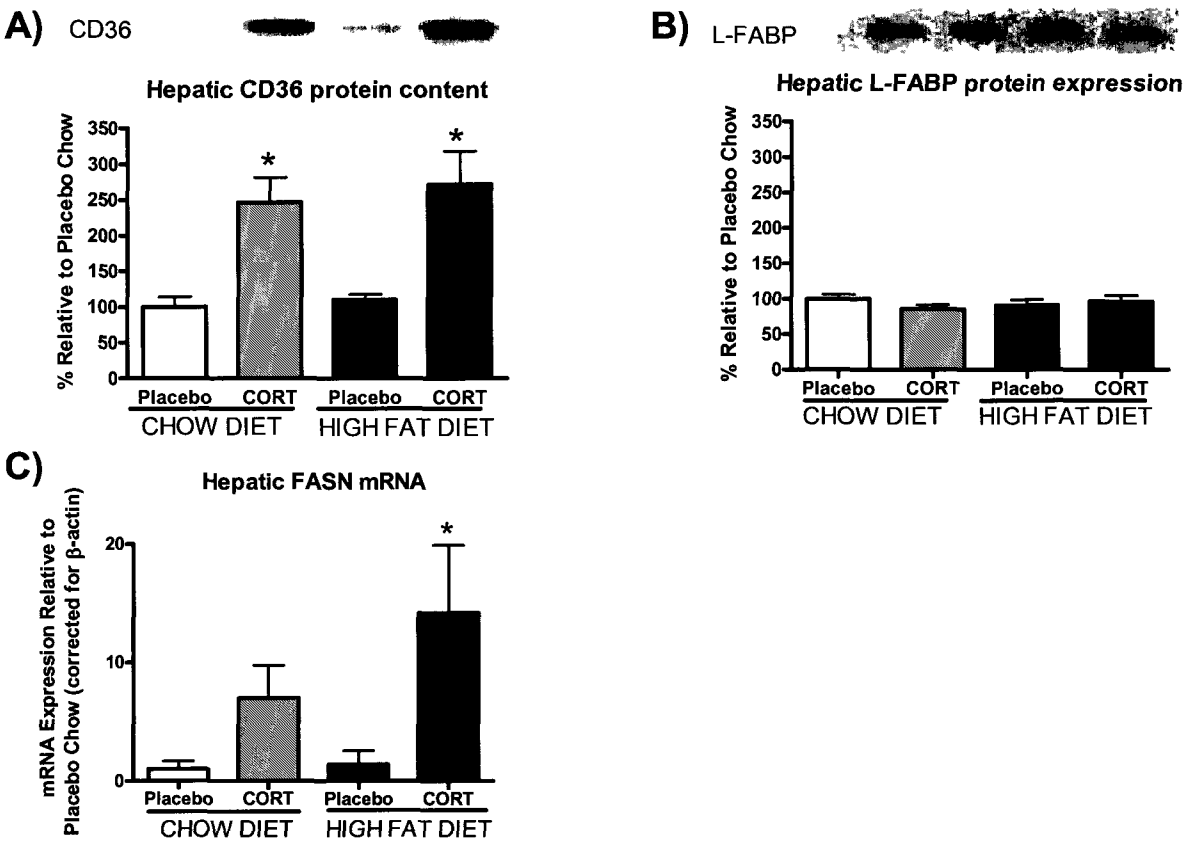

Figure 6: Lipid transport into the liver. Protein expression of facilitated lipid transporters CD36 and L-FABP were measured from frozen liver tissue. Expression of CD36 was increased as a result of CORT treatment (**A**), but L-FABP expression was not altered (**B**).The relative mRNA expression of FAS, a marker of hepatic de novo lipogenesis showed a trend for increase as a result of CORT alone, and was synergistically increased when CORT and HFD were combined (**C**). *N = 4-7 per group; a two way ANOVA was performed with Bonferonni post hoc tests where * indicates $p<0.05$ relative to placebo chow; # indicates $p<0.05$ relative to CORT Chow. All values are ± SEM.*

Figure 7: Whole body insulin sensitivity and glucose tolerance

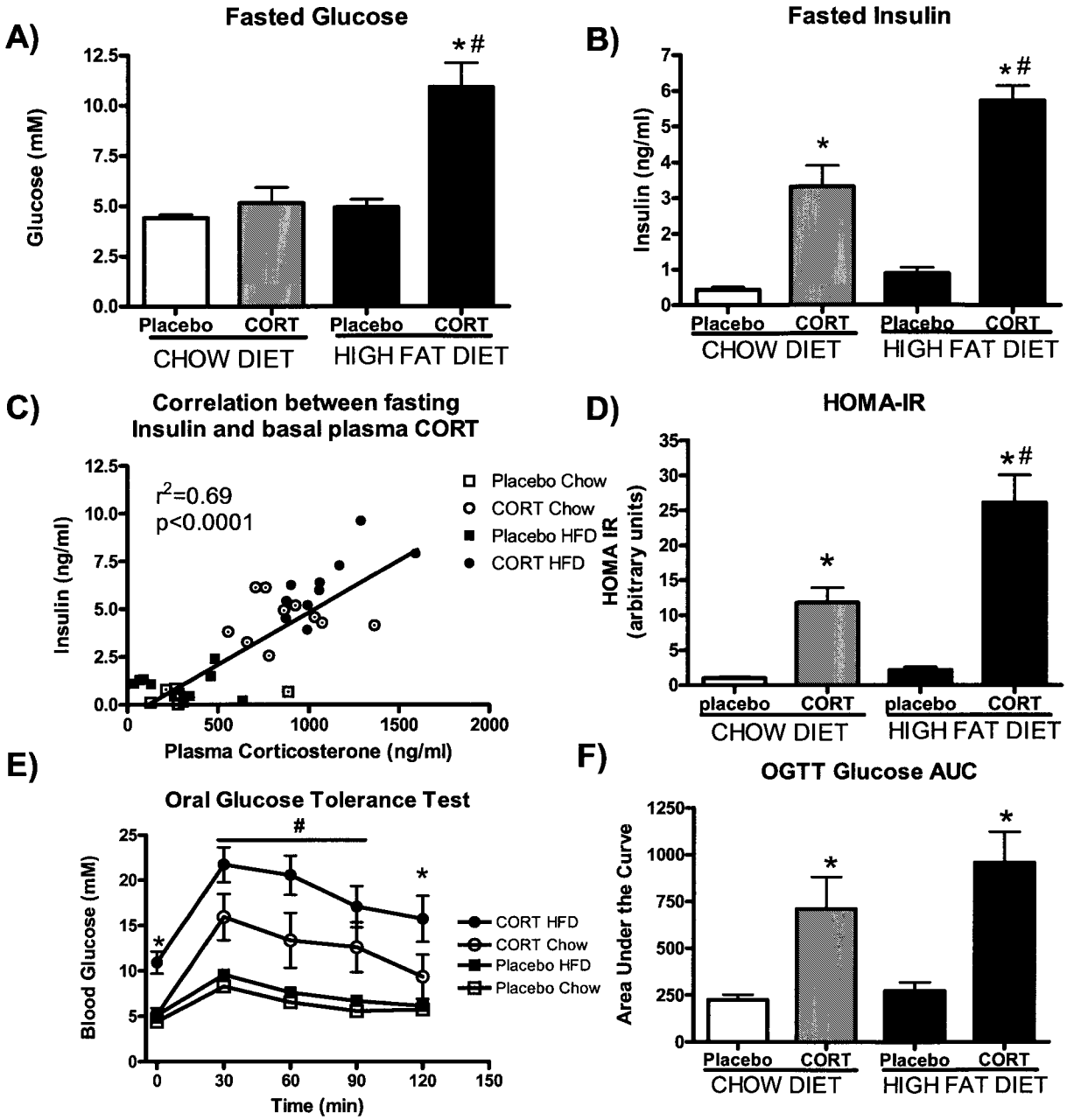

Figure 7: Whole body insulin sensitivity and glucose tolerance. Eight days after diet and pellet intervention, animals were subject to an overnight fast to measure fasting glucose (**A**) and insulin (**B**) values. Chronic CORT treatment induced severe insulin resistance, and was exacerbated with a HFD, as measured by HOMA-IR scores (**C**; $p<0.05$ using a 2 way ANOVA). Increases in plasma CORT levels correlated with elevated fasting insulin levels (**D**; $p<0.05$ using linear regression). An OGTT was performed following the fast, and demonstrated severe glucose intolerance as a result of CORT treatment, which was exacerbated with HFD (**E**, **F** $p<0.05$ using a repeated measures ANOVA and LSD Fisher post hoc analyses). *$n \geq 8$ per group, * indicates $p<0.05$ vs. Placebo Chow, # indicates $p<0.05$ vs. CORT Chow; all values are ± SEM.*

Figure 8: Hepatic Insulin Signalling and Expression of Gluconeogenic Enzymes

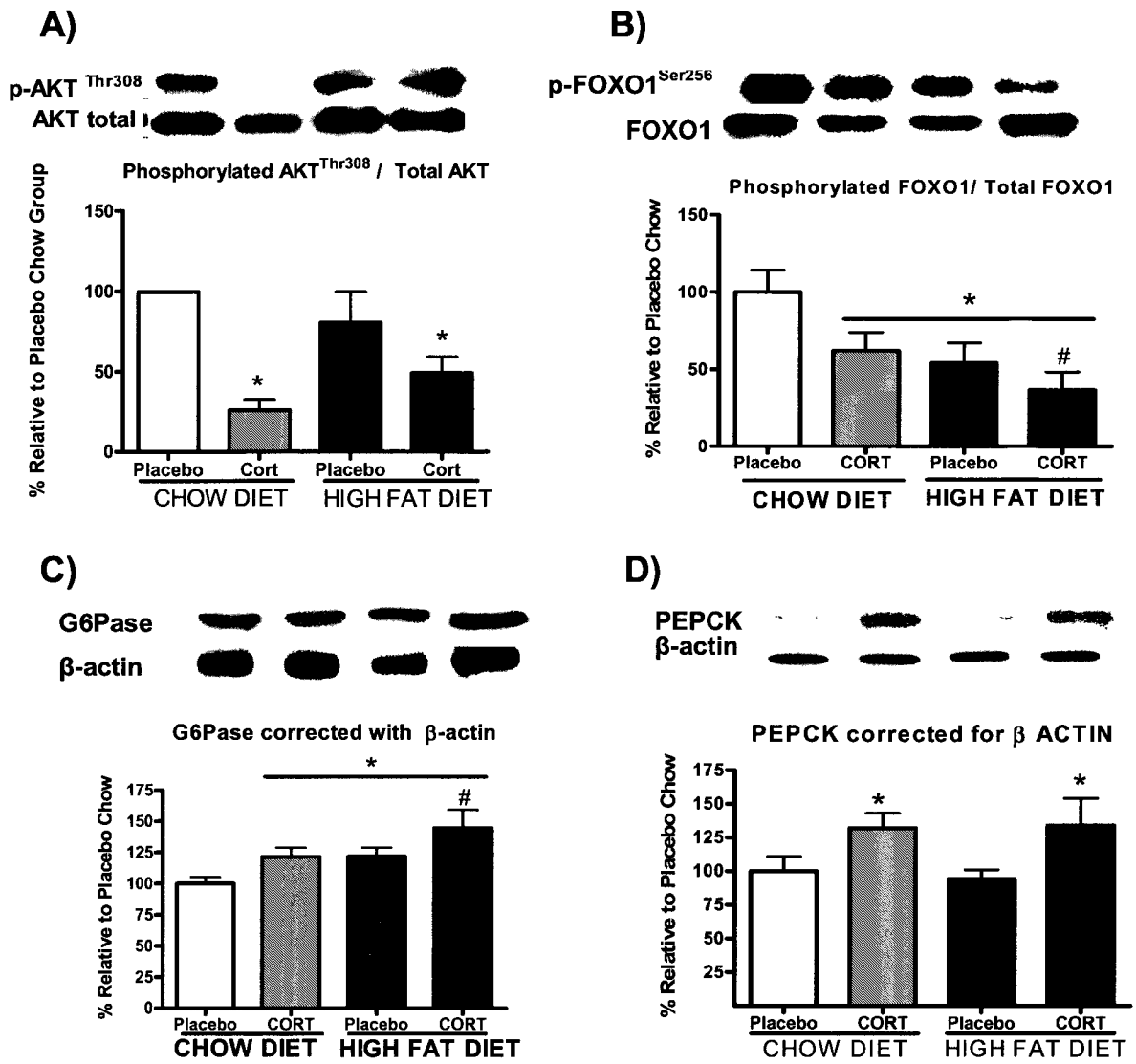

Figure 8: Hepatic Insulin Signalling and Gluconeogenic Enzyme Protein expression. Chronically elevated CORT levels resulted in decreased Akt^{Thr308} phosphorylation (**A**). This coincided with reduced $FOXO1^{ser256}$ phosphorylation (**B**) experienced by all groups, but was most severe with CORT and HFD combined. Reduced phosphorylation of FOXO1 impaired inhibition of FOXO1 transcription activity. Analysis of protein expression of the gluconeogenic enzymes PEPCK and G6Pase demonstrated increases with CORT treatment in PEPCK (**C**) and an additive increase of G6Pase and PEPCK combined (**D**). *N= 4-7 per group; a two way ANOVA was performed with Bonferonni post hoc tests where * indicates $p<0.05$ relative to placebo chow; # indicates $p<0.05$ relative to CORT Chow. All values are ± SEM and relative to the placebo chow group.*

Figure 9: PKC epsilon expression in cytosolic and membrane fractions of liver tissue

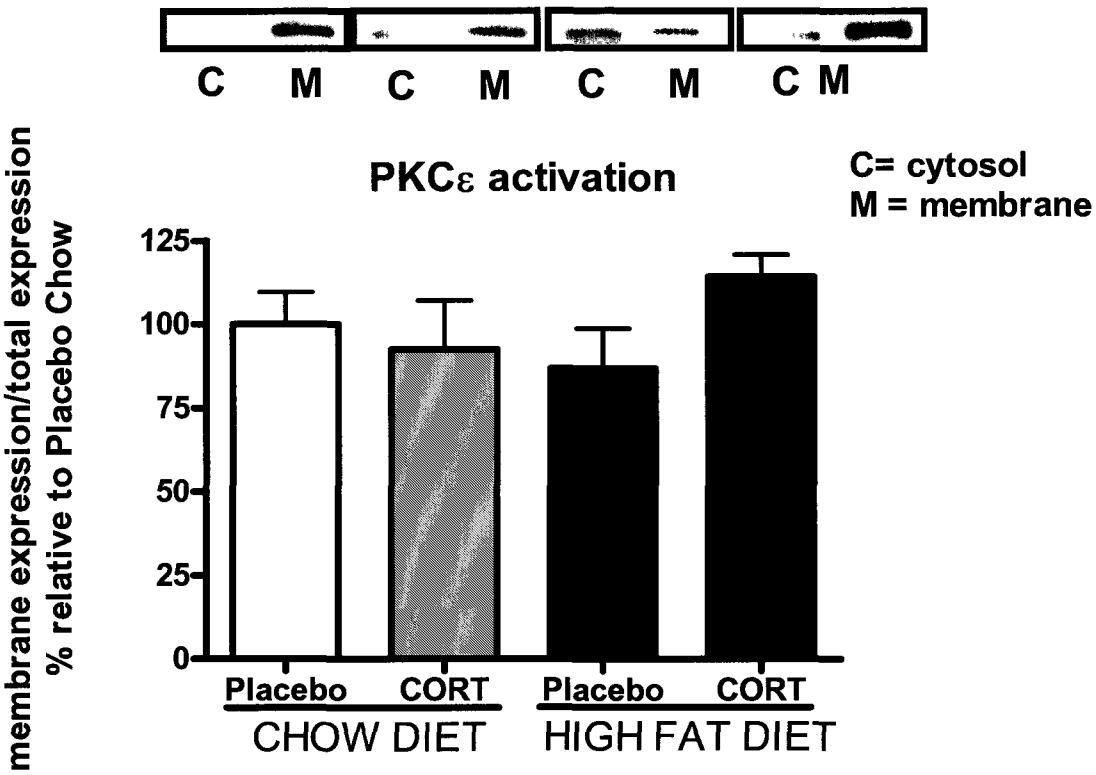

Figure 9: PKCε translocation from the cytosol to the membrane. PKCε activation is measured as its translocation from the cytosol to the membrane. Translocation was determined as the relative ratio of membrane expression to total (cytosolic and membrane expression). No significant increase in PKC translocation occurred. *N = 4-7 per group; A two way ANOVA was performed followed by Bonferonni post hoc analysis. No differences were found between groups. All values are ± SEM relative to the placebo chow group.*

	Forward Sequence	Reverse Sequence
FASN	5'-CAACCTGCATTTCCACAACCCCAA-3'	5'-ACCTCCGAAGCCAAACGAGTTGAT-3'
β-actin	5'-CCAACCGTGAAAAGATGACC-3'	5'-ATCACAATGCCAGTGGTACG-3'

Table 1: Primer sequences for RT q-PCR. Primer sequences were designed using Primer 3 software, and verified for expression in the liver by using a BLAST search.

	Placebo Chow	CORT Chow	Placebo HFD	CORT HFD
Left adrenal mass (g/kg body weight)	0:099±.01	0.039±.006*	0.095±.011	0.041±.004*
Right adrenal mass (g/kg body weight)	0.097±.006	0.033±.004*	0.10±.011	0.042±.004*

Table 2: Relative adrenal mass. Adrenal tissues were excised upon euthanization, weighed and corrected relative to body mass. Values are represented as mean ±SEM. N=10/group; * indicates different from Placebo Chow, $p<0.05$; # indicates different from CORT Chow, $p<0.05$.

	Placebo Chow	CORT Chow	Placebo HFD	CORT HFD
Body Mass (g)	342.57±7.24	245.56±7.37*	315.4±1.89	249.38±11.07*
Epididymal adipose tissue (g/kg B.W.)	8.59±1.11	12.59±1.01*	12.77±0.85*	18.54±1.42#
Liver (g/kg B.W.)	37.991±1.13	51.92±2.61*	40.87±2.07	60.11±2.81#
Gastrocnemius (g/kg B.W.)	5.18±0.11	4.23±.10*	4.93±0.16	4.17±.09*

Table 3: Relative tissue mass. Tissues were excised following euthanization, weighed and immediately flash frozen. Values are represented as mean ±SEM. B.W. = body weight. N=10/group; * indicates different from Placebo Chow, $p<0.05$; # indicates different from CORT Chow, $p<0.05$.

	Placebo Chow	CORT Chow	Placebo HFD	CORT HFD
Triglycerides	0.12±0.01	0.24±0.06*	0.18±0.03	0.47±0.13*#
Free fatty acids (mM)	0.89±0.13	0.67±0.06	0.80±0.12	0.64±0.04
Cholesterol (mM)	1.86±0.16	2.80±0.40*	1.83±0.21	3.86±0.54*#
Total Bilirubin (µmol/L)	0.75±.07	0.96±0.18	0.90±0.18	1.76±0.44*
ALT (U/L)	49.9±5.05	94.64±12.40*	48.3±4.14	138.3±32.0*#
Haptoglobin (mg/ml)	1.19±.36	0.35±0.11*	1.18±0.29	0.48±0.15
Glycerol† (mM)	0.20±.05	0.42±.10*	0.29±.08	0.46±.12*

Table 4: Plasma lipid metabolites and parameters of liver damage. Trunk blood triglycerides and free fatty acids were collected in a fasted stated. Cholesterol, total Bilirubin, ALT, and Haptoglobin were measured from trunk blood collected in a fed state. Bilirubin and ALT are markers of liver injury, while Haptoglobin is a marker of hepatic inflammation. Glycerol, a measure of lipolysis, was measured from the portal vein in a fed state. Values are represented as mean ± SEM; $n \geq 6$ per group. * indicates $p<0.05$ vs. Wax Chow; # $p<0.05$ vs. CORT Chow. † indicates portal vein glycerol concentrations with a main effect of CORT ($p=0.04$); and a trend for significant differences in CORT-treated animals relative to the Placebo-Chow group ($p=0.07$).

Group	Glucose Infusion Rate (mg/kg/min)	Glucose disposal rate (mg/kg/min)		Endogenous Glucose production (mg/kg/min)		% Supression of HGP
	Clamp	Basal	Clamp	Basal	Clamp	Clamp
Placebo Chow	25.66 ±2.7	8.2 + 1.5	34.84 + 5.84#	11.15±4.833	6.44±.054	59.37
CORT Chow	19.43±1.1*	16.39 ± 3.4	33.23 ± 6.62#	13.521±3.13	7.77±1.60	40.33±7.30
Placebo HFD	22.10±4.9	9.64 ± 2.9	23.94 ± 3.00#	7.99±0.15	4.86±1.35#	39.59±15.7
CORT HFD	12.65±2.3*	13.97 ± 4.2	17.51 ± 2.06	12.84±1.88	9.14±3.22	32.73±13.4

Table 5: Glucose infusion rate, Glucose disposal, Endogenous Glucose Production and Percent Suppression of EGP during the Hyperinsulinemic Euglycemic clamp. Values are averages from the last 30 minutes of the hyperinsulinemic euglycemic clamp. Glucose infusion rate, glucose disposal and glucose production are represented as mean ± SEM. Percent suppression is an average of the difference between basal and clamp period endogenous glucose production values. *N=1-3 per group, * indicates p<0.05 relative to placebo chow using a two way ANOVA; # indicates p<0.05 relative to basal period using a student's t-test.* See Appendix for calculations.

Summary, Limitations & Future Directions 5

The results of this study suggest that chronically elevated plasma CORT levels interact with a high fat diet to create a toxic environment in liver tissue, rapidly inducing NALFD as marked by hepatic steatosis, and severe whole body insulin resistance. Though high fat diets and GC administration via daily injections have been shown to induce these outcomes independently (55, 62, 79, 109), the present investigation is the first to show that when combined, these two treatments interact and rapidly exacerbate features of NAFLD, MetS and Type 2 Diabetes Mellitus.

One of the most striking findings of this thesis was the delineation of the role of surrounding visceral adipose tissue, which increases in size as a result of both CORT and HFD, as a significant contributor to the development of NAFLD in this model. We observed that CORT treatment in conjunction with a high fat diet dramatically increased visceral fat stores and resulted in depleted subcutaneous fat. CORT treatment increased lipolysis in adipose tissue, as measured by increased glycerol concentrations in the portal vein, and also increased delivery of FFAs to the liver via the portal vein, which correlated with increased fat in the liver.

Within the liver, we observed increased CD36 protein expression as a result of CORT treatment, demonstrating another mechanism by which steatosis

is rapidly facilitated in this rodent model. We also report that *de novo* lipogenesis, as measured by mRNA expression of FASN, is synergistically increased by CORT and HFD. These increases in FASN may in fact be an indirect effect of elevated insulin levels caused by CORT exposure (195), which were most severe in the CORT-HFD group. The resulting steatosis, induced by combined CORT and HFD treatment also resulted in the greatest increases in plasma ALT levels, and liver fibrosis, which are also common clinical features of NAFLD.

Though we saw clear evidence of peripheral insulin resistance in the CORT-HFD group, (based on 30-fold elevations in HOMA-IR scores) and similar impaired glucose tolerance (based on results from the OGTT), we cannot confirm that this occurred solely as a result of increased hepatic gluconeogenesis. Through the hyperinsulinemic euglycemic clamp, as well as protein and mRNA expression of gluconeogenic enzymes, we were able to quantify some deleterious alterations in hepatic glucose metabolism such as decreased suppression of endogenous glucose production during the clamp, decreased phosphorylation and inhibition of FOXO1 and increased protein expression of PEPCK and G6Pase. Our findings suggest that chronic CORT has the potential to reduce hepatic insulin sensitivity, which is mildly exacerbated with addition of a HFD. These reductions to hepatic insulin sensitivity may account for some, but not all of the mechanisms that produce the hyperglycemia and glucose intolerance experienced with combined treatment. Further studies are warranted

to determine whether CORT and HFD induce hyperglycemia through alterations in peripheral glucose uptake and glucose metabolism.

These findings together indicate that chronic CORT combined with a HFD provides a novel, valuable model to study development of NAFLD. Chronic CORT and HFD combined produced the common characteristics of NAFLD including: steatosis, insulin resistance, and fibrosis, and all within a rapid time period. This is in contrast to the current animal models of NAFLD using HFD alone, or specialized diets, which often fail to induce all of the characteristics associated with NAFLD and require a prolonged period of intervention to see such effects (92).

From a clinical perspective, the findings from this study suggest that individuals on chronic GC therapy or with spontaneously elevated GC levels as an ancillary effect of obesity and diabetes must be vigilant of other lifestyle factors that may interact with elevated GC levels to accelerate development of metabolic impairments. The majority of individuals in western society consume an excessive amount of calories (86), of which saturated fat intake has been found to be increased in NAFLD patients (236). These lifestyle patterns, when paired with altered glucose and lipid metabolism as a result of chronically elevated GC levels, produce detrimental metabolic effects. The core belief of decreased caloric intake and increased energy output is very pertinent to prevention of GC and HFD-induced NAFLD.

Limitations of the Study

This study has a number of important limitations that need to be acknowledged:

1. The present study is a reflection of chronically elevated stress hormone levels. To achieve these elevations, animals were given four pellets containing 100 mg of exogenous CORT. While this approach is common amongst studies exploring the effects of chronic CORT on metabolic outcomes (109), the dosage of corticosterone in our study was such that it may have raised plasma CORT levels above the normal physiological range (50-400 ng/ml). Thus, the effects of CORT in this study may be an exaggeration of the chronically elevated CORT levels occurring in rodents under psychological stress (191) or rodent models of diabetes mellitus (37) .

2. The younger age of the animals used in this study may have be a confounding variable that explains the dramatic decreases in body mass that occurred as a result of chronic CORT treatment. Animals were roughly 7-8 weeks of age during CORT exposure, the equivalent of early adolescents which is a period of rapid growth. CORT has been shown to suppress growth hormone (83), and thus may have resulted in attenuated growth and thus dramatically lower body weights relative to the placebo-treated groups. To eliminate the effects of attenuated growth in this disease model, older animals should be used.

3. The hyperinsulinemic euglycemic clamp represents the gold standard of hepatic insulin sensitivity, and a way to measure the contribution of endogenous

glucose production to whole body glucose homeostasis. Hepatic insulin resistance has been shown to result in uncontrolled endogenous glucose production, which is measured using labelled glucose. The invasive procedure of the pellet implantation surgery and severe effects of GCs resulted in a low survival rate following cannulation surgeries and clamps. Studies in which cannulated animals are introduced to CORT treatment or studies in which the chronic CORT treatment is given at a lower dosage so that it does not have as profound effects on anthropometry are required to successfully run the hyperinsulinemic euglycemic clamp and characterize endogenous glucose production using this model.

Future Directions

This study has shed light on the roles of chronically elevated CORT levels and high fat feeding in the development of NAFLD. While we saw increases in fasting insulin concentrations and severely elevated insulin resistance HOMA-IR scores as a result of combined treatment, we are unable to elucidate whether chronic CORT combines with the effects of a HFD to induce hepatic insulin resistance prior to pancreatic β cell dysfunction, which explains the concomitant hyperinsulinemia. Use of an insulin sensitizer would help to clarify whether peripheral insulin resistance precedes beta cell dysfunction. Additionally, measurement of alternative markers of impaired hepatic lipid metabolism would help to clarify the specific cause of hepatic lipid accumulation. Of primary importance is the measurement of hepatic VLDL export. Understanding whether

or not VLDL export is increased will help to explain where the imbalance in lipid metabolism lies. Glucocorticoids have been shown to impair hepatic beta oxidation (128). Future studies using this model should include RT-PCR analysis of markers such as CPT-1 to measure beta oxidation, or SREBP1, a transcription factor involved in lipogenesis, to further clarify the influence of chronic CORT in combination with HFD on these processes. In addition, measurements of hepatic VLDL export (done by suppressing hepatic TG uptake via injections of triton-x 100) would help to clarify whether or not GCs in the presence of a HFD result in impaired VLDL export, and whether this is a contributing factor to hepatic steatosis.

The *in vivo* nature of this study meant that we were unable to tease apart the direct impact of chronic CORT administration and excess caloric content on the liver from the influence of a toxic environment created by increased visceral adipose mass along with other characteristics of the metabolic syndrome that are present. In this regard, future work should focus on use of an *in vitro* model to characterize the specific effects of GCs and fat metabolites on hepatic cell function. Additional studies could also determine the influence of a toxic environment by incubating hepatic cells in serum from adipose tissue treated with CORT and given FFAs to better define the effects of surrounding adipose tissue and altered adipokine profile on hepatic cell function.

Studies of metabolic disorders and diabetes often suggest an exercise intervention to protect against or attenuate the disease state. Indeed, exercise has been shown to improve insulin sensitivity (100), improve plasma CORT levels (37), and reduce uncontrolled gluconeogenesis (112), in addition to increasing overall energy expenditure. Thus, future studies using this disease model, with the addition of an exercise intervention or exercise agonist (e.g. the AMPK mimetic, AICAR) are warranted to determine whether exercise protects against steatosis.

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Appendix 7

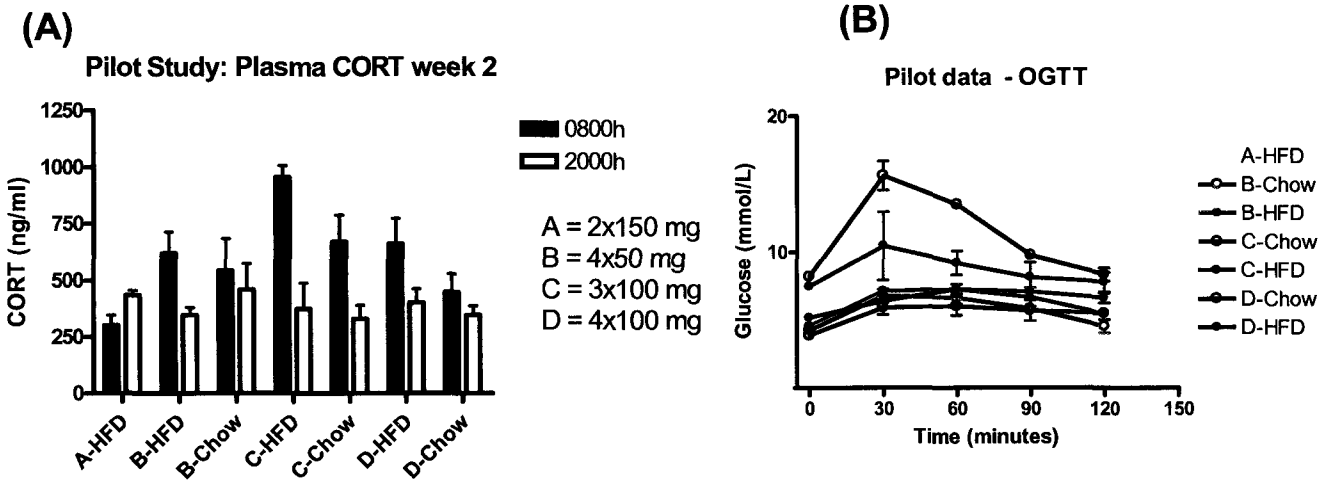

Figure 7.1: Pilot data for pellet dosage. CORT pellets at 4 different doses and pellet numbers (2x150mg, 4x50mg, 3x100 mg and 4x100 mg) were implanted into the subcutaneous layer of rodents who were then either given a normal rodent diet (Chow) or a 60% high fat diet (HFD). Plasma CORT levels were measured 2 weeks following pellet implantation (A), along with performance of an OGTT (B).

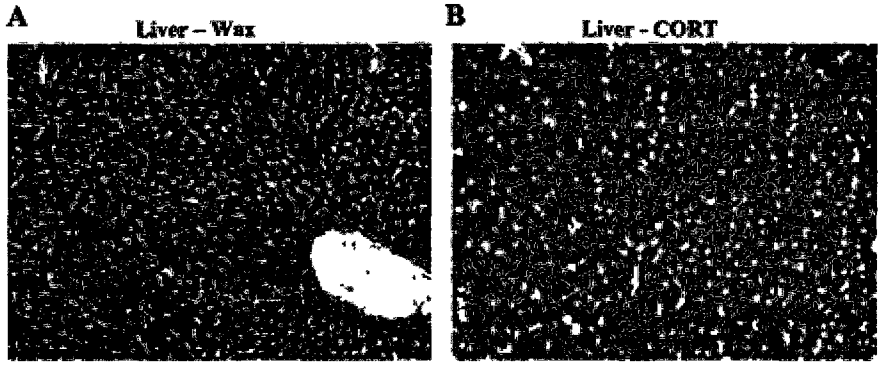

Figure 7.2 Liver sections stained with hematoxyling and Oil Red O from Placebo-Chow and CORT-Chow animals.
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Additional Methodology and Results for the Hyperinsulinemic Euglycemic Clamp

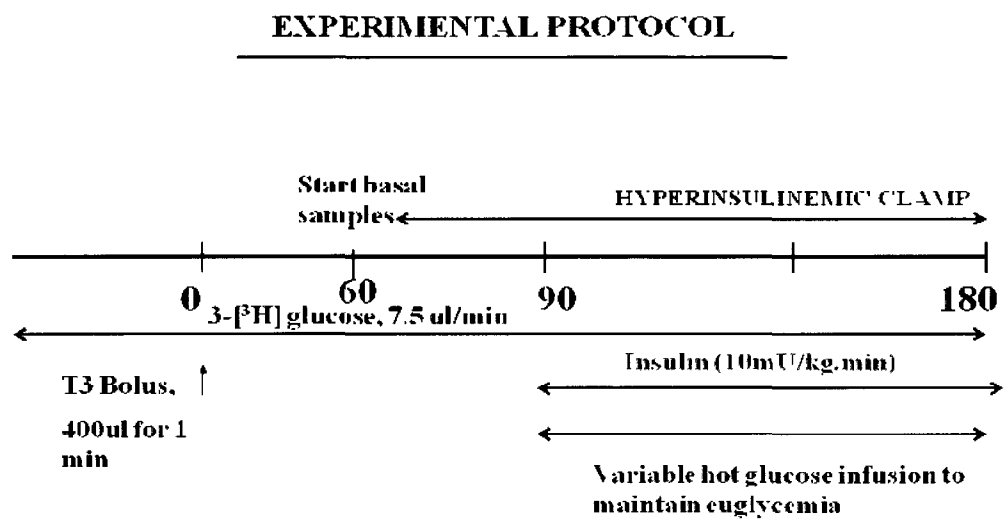

Figure 7.3: Hyperinsulinemic Euglycemic Clamp protocol

7.1 Additional Methods

i. Cannulation surgery

Two weeks post pellet implantation surgery, another subset of identically treated animals underwent a cannulation surgery to implant catheters into the carotid artery for blood sampling, and jugular vein for infusion during a hyperinsulinemic eugylcemic clamp (n= 1-3/group). Animals were anaesthetized under 2% isoflourane diluted in oxygen (similar to previous pellet surgeries). Five millimeters of the jugular vein vessel was immobilized and isolated from a ventral cervical skin incision made at the level of the clavicle. A sterile cannula, 3.7 cm in length, was inserted and

secured in place above and below the point of insertion into the vessel. The same procedure was done to cannulate the left common carotid artery. Both cannulas were then drawn from the ventral side of the neck through the scapular incision, tested for patency and infused with 4% heparinzed saline solution before being sealed with steel pins.

ii. Tracer Assay

Plasma radioactivity from 3-3H glucose was measured after deproteinization with $Ba(OH)_2$ and $ZnSO_4$. Samples were dried overnight to remove tritiated H_2O. Aliquots of the TINF (3-^{3}H glucose), and GINF (tritiated glucose infusate) were assayed together with the dried plasma samples and counted in 1ml of H2O combined with 10 mls of scintillation cocktail using a beta counter (Perkin Elmer, Boston, MA).

iii. Calculations

Calculations for rate of endogenous glucose production (R_A) and glucose disposal (R_D) are previously described by Chintoh et al (44) and based on the average of values taken between 60-90 min and 150-180 min of the hyperinsulinemic euglycemic clamp. Briefly, rate of glucose infusion (GIR) was based on each animal's weight and rate of glucose infusion pump rate. Glucose turnover is based on the rate of glucose appearance (Ra) and was calculated by taking into account the extra tracer infused with the glucose infusate using steady state formulae (44). Endogenous glucose production was calculated as the difference between the total rate of glucose appearance and the exogenous glucose infusion rate. At steady

state, the R_D is equivalent to the R_A. At euglycemia, the glucose disappearance rate corresponds to glucose uptake by tissue because renal clearance is zero.

iv. Composition of TINF, GINF and Insulin used for the Hyperinsulinemic-Euglycemic Clamp

TINF

Stock solution:

- *5 mCi in 5 ml of 3-^{3}H Glucose (#NET331C, Perkin Elmer)*
- *250 ml of tracer diluent solution (5 g D-glucose, 2 g Sodium Benzoate, 1L saline)*

Final concentration = 20 µCi/ml (or 5 mCi/250 ml)
Per clamp = 5 mL of TINF stock solution
Infusion rate = bolus of 400 µL for one minute , followed by 7.5 µL/min for remainder of clamp

GINF (Hot glucose)

Stock solution:

- *5 mCi in 5 ml of 3-^{3}H Glucose (#NET331C, Perkin Elmer)*
- *5 g D-glucose, 2 g Sodium Benzoate, 1L saline*
- *125 ml of tracer diluents solution (5 g D-glucose, 2 g Sodium Benzoate, 1L saline)*

Final concentration = 40 µCi/ml
Per clamp: 5 ml of 50% glucose + 3 ml of GINF stock solution
Infusion rate = variable (range between 5 µL/min – 30 µL/min)

Insulin

0.1% BSA solution (volume depending on body weight) + 10 U of insulin
Dose required: 5 mU/kg/min
Infusion rate = 10 µl/min

Table 7.1: List of hyperinsulinemic-euglycemic clamps

Animal	Clamp Result
WC13	Did not reach steady state
WC18	Did not reach steady state (BG too low)
WC20	Steady state achieved
WC60	Variability in tracer assay
WC64	Steady state achieved
WC66	Clamp aborted early
WC116	Steady state achieved
CC33	Did not reach steady state (BG too low)
CC34	Did not reach steady state
CC36	Stroke 165 min into clamp
CC37	Stroke 93 min into clamp
CC32	Steady state achieved
CC38	Tail sampling used instead of catheter
CC39	Cannulas pulled out prior to clamp
CC81	Stroke 170 min into clamp
CC83	Steady state achieved
CC137	Steady state achieved
CC138	Steady state achieved
WF25	Partial steady state achieved (BG during clamp just under normal)
WF26	Did not reach steady state (BG too low)

WF27	Did not reach steady state
WF28	Stroke 49 min into clamp
WF73	Insulin infusion did not reduce BG
WF75	Stroke 150 min into clamp
WF130	Steady state achieved
WF131	Steady state achieved
CF43	Cannula destroyed at 105 min
CF44	Steady state achieved
CF46	Steady state achieved (BG slightly higher)
CF48	Did not reach steady state
CF93	Clamp aborted at 170 min
CF143	Steady state achieved
CF147	Steady state achieved