Consumption of a High Fat Diet Rapidly Exacerbates the Development of Fatty Liver Disease that Occurs with Chronically Elevated Glucocorticoids

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Running Head: High Fat Feeding Exacerbates Glucocorticoid-Induced NAFLD
ABSTRACT

Chronically elevated glucocorticoids (GCs) and a high fat diet (HFD) independently induce insulin resistance, abdominal obesity and non-alcoholic fatty liver disease (NAFLD). GCs themselves have been linked to increased food intake, particularly energy dense “comfort” foods. Thus, we examined the synergistic actions of GCs and HFD on hepatic disease development in a new rodent model of chronically elevated GCs. Six-week-old male Sprague Dawley rats received exogenous GCs, via subcutaneous implantation of four 100 mg corticosterone (CORT) pellets, to elevate basal GC levels for 16 days (n=8-10 per group). Another subset of animals received wax pellets (placebo) to serve as controls. Animals from each group were then randomly assigned to receive either a 60% HFD or a standard high carbohydrate diet (13% fat; 60% carbohydrate). CORT and a HFD in combination resulted in central obesity, despite a relative weight loss, a 4-fold increase in hepatic lipid content, hepatic fibrosis and a 2.8-fold increase in plasma alanine aminotransferase levels relative to placebo chow controls. Development of hepatic injury occurred independent of inflammation, as plasma haptoglobin levels were reduced with CORT treatment. Insulin resistance and hepatic steatosis occurred with CORT exposure alone; these outcomes were further exacerbated when HFD was given in the presence of elevated CORT. In addition to fatty liver, the CORT HFD group also developed severe insulin resistance, hyperinsulinemia, hyperglycemia, and hypertriglyceridemia, which was not evident with either HFD or CORT treatment alone. Thus, a HFD dramatically exacerbates the development of NAFLD and characteristics of the metabolic syndrome in conditions of chronically elevated CORT.
INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is defined as a chronic condition that ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), which can progress to fibrosis, cirrhosis and hepatocellular carcinoma (37, 59). NAFLD is commonly attributed to a positive energy balance through excess consumption of calories and sedentary lifestyle (52, 53). As is the case of most complex metabolic conditions, energy balance is not the only mediator contributing to disease development.

Glucocorticoids (GCs) are stress hormones that, when chronically elevated, result in hepatic lipid accumulation (48, 66), the metabolic syndrome (1, 42) and eventually type 2 diabetes mellitus in some circumstances (43, 66). GCs exert their actions predominantly through the transcriptional regulation of a number of metabolic genes including glucose 6 phosphatase (G6Pase) and phosphoenolpyruvate carboxy kinase (PEPCK), the rate limiting enzymes involved in gluconeogenesis. GCs have also been shown to have a paradoxical role in lipid metabolism causing increased lipolysis and increased adiposity (49). Lipolysis is facilitated through GC activation of adipose tissue lipases (10), while expanded central adiposity is achieved either by up regulation of adipose cell proliferation along with increased food consumption (49), both of which can accelerate development of NASH. The effects of GCs on hepatic inflammation and fibrosis are unclear, particularly in situations where lipid stress increases, such as with a HFD.
The present study sought to determine if a HFD, in the presence of chronically elevated corticosterone (CORT, the naturally occurring GC in rodents), would rapidly and synergistically exacerbate the development of steatosis and NAFLD in otherwise healthy young Sprague Dawley rats. We hypothesized that a HFD would worsen the detrimental effects of chronically elevated CORT by potentiating hepatic steatosis, fibrosis, and severe insulin resistance. With a confirmation of our hypothesis, these findings illustrate the important interactive effects that chronic GC exposure has on liver metabolism with altered nutritional intake.

EXPERIMENTAL DESIGN AND METHODS

Animals, housing and pellet implantation

Adult male Sprague Dawley rats (age 6 weeks old, initial weight 225-250g, Charles Rivers Laboratories, Quebec, Canada) were individually housed in standard cages on a 12-h light-dark cycle. After a 7 day habituation period, animals were randomly assigned to receive either CORT (via four 100 mg pellets of corticosterone, Sigma, St. Louis, MO) or wax (placebo) pellets, as previously described (41). Animals were anesthetised with 2% isoflourane and under aseptic conditions. CORT or placebo pellets were then implanted below the subcutaneous layer between their scapulae (10). Immediately following pellet implantation surgery, animals within each group were randomly allocated to receive standard rodent chow (Purina Lab Diet 5012), or a 60% HFD diet (Harlan Laboratories, see Table 1). The treatment groups (n=12-15/group with a subset per group used for various analyses) were as follows (see Fig. 1): exogenous
CORT treatment and a high fat diet (CORT HFD), CORT treatment with a normal chow diet (CORT Chow), placebo pellet and a high fat diet (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/d) were measured every other day. Eight days following pellet implantation, all animals underwent an oral glucose tolerance test (OGTT). Sixteen days after pellet implantation, animals were killed via decapitation. Liver, epididymal adipose tissue, and adrenal glands were rapidly excised, weighed and flash frozen in liquid nitrogen for histological, protein and/or mRNA expression analysis. All experiments were approved by York University’s Animal Care Committee in accordance with regulations set forth by the Canadian Council for Animal Care.

**Plasma analyses**

Plasma CORT levels were measured at the peak of the diurnal rhythm (~2000h, one hour after onset of dark phase of light cycle) on days 0 and 7, and at the nadir of the diurnal rhythm (~0800h, one hour after onset of light phase) on days 1 and 8 from a tail nick involving a superficial poke made at the tip of the animal’s tail to collect ~50µL of whole blood. This procedure does not involve any restraint and care is taken to minimize stress on the animal, however, due to the short nature of this procedure, animals experience little handling prior to the first blood collection, and thus exhibited higher than normal GC levels compared with surgically implanted indwelling catheters (16, 46). Plasma collected on day 7 via tail nick was used for the determination of fasting insulin and glucose concentrations. Trunk blood was collected after decapitation
on day 16 for measures of plasma alanine aminotransferase (ALT), total bilirubin, haptoglobin and cholesterol. In a subset of animals under anaesthesia just prior to decapitation, blood from the hepatic vein and portal vein was collected in EDTA microvette tubes on ice and spun down to separate plasma, which was stored at -20°C for plasma free fatty acids (FFA) and triglyceride (TG) analyses. Commercially available kits were used to assess plasma CORT (corticosterone double antibody RIA, MP Biomedical, OH), FFAs (HR Series NEFA-HR, Wako Chemicals, Richmond, VA), and triglyceride levels (TR0100, Sigma Aldrich, ON). An enzyme linked immunosorbent assay (ELISA) were used to measure plasma insulin (INSKR020, Crystal Chem, IL) and haptoglobin (Life Diagnostics). 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).

126 Oral Glucose Tolerance Test

On day seven, animals underwent an overnight fast for 15 h. Baseline blood samples were collected prior to an oral gavage of 50% glucose (Abbott Laboratories Limited, Montreal, Canada) at a dosage of 1.5 g/kg body weight. Blood was collected from the tail vein at 30 min intervals over a two hour period to determine blood glucose concentrations (Contour glucose hand-held meter, Bayer, Toronto). An additional forty microlitres of whole blood was collected via tail vein for analysis of insulin (see above). The homeostasis model assessment of insulin resistance (HOMA-IR) was used to evaluate the degree of insulin resistance induced by treatment (29). A higher HOMA-IR
value is indicative of a greater degree of insulin resistance and in particular, hepatic insulin resistance (6, 68).

Histology

Liver tissue from euthanized animals was snap frozen, sectioned 10 m thick, and stained for lipid content using Oil Red O as previously described (35). Liver sections were fixed with 3.7% formaldehyde for 1 h 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. Briefly, slides were stained for 1 h 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). Images for Oil Red O and collagen staining were acquired at a magnification of 10x, and 20x, respectively, 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 grey scale format, followed by quantification of optical density performed with Adobe Photoshop CS version 8.0. Staining intensity was converted into a percentage and expressed as relative to the average intensity of the placebo chow group.

Hepatic total lipid and ceramide content
Total hepatic lipid content was determined gravimetrically in duplicate after extraction with a chloroform–methanol solution, as previously described (67). Briefly, frozen 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 = 2 prad]) 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 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.

In addition to total lipid content, pure standards of endogenous ceramide subspecies (C14, C16, C18, C18:1, C20, C24 and C24:1) were assessed liquid chromatography electrospray ionization tandem mass spectrometry, as described by Kasumov et al. (28). Non-naturally occurring internal standards (C17 and C25 ceramides) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL; purity > 99%) and served as internal standards. Frozen liver tissue was first homogenized in ice cold NaCl before undergoing extraction with chloroform/methanol (1:2 v/v). Tissue samples were vortexed and collected eluent was dried and reconstituted in HPLC elution buffer and analyzed by mass spectrometry. Linear regression equations were derived from calibration curves and were used to calculate ceramide concentrations in tissue samples.
**Immunoblotting**

Liver protein was extracted and analyzed as previously described (31, 47).

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 (P8340, Sigma Aldrich, Oakville, ON), phosphatase inhibitor (P5726, Sigma Aldrich) and centrifuged at 12,000g for 30 min. Protein concentration from collected tissues was determined using the Bradford method (7) on the collected supernatant. Protein extracts were then subject to an SDS-PAGE (10% polyacrylamide gel) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% BSA for 1 h at room temperature followed by overnight incubation at 4°C in primary antibody: AKT (1:1000 Cell Signaling Technology, Boston, MA), p-AKT<sup>Thr308</sup> (1:1000 Cell Signaling Technology), FOXO1 (1:1000 Cell Signaling Technology), p-FOXO1<sup>ser256</sup> (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 h incubation at room temperature with the appropriate secondary antibody. Blots were visualized by chemiluminescence (GE Healthcare, Baie d’Urfe, QC) with use of the Kodak in vivo pro-imaging system. Beta actin (1:20,000, Abcam) was used as a loading control. Quantification of relative band intensity was done using the Carestream molecular imaging system (Rochester, NY).
Cytosolic and membrane protein fractions for determination of PKCδ and PKCε expression

The delta (PKCδ) and epsilon (PKCε) isoforms of protein kinase C have been implicated as the link between lipid accumulation and insulin resistance in the liver (20, 55). PKCδ is activated and translocated from the cytosol to the membrane in response to elevated cellular DAG levels (36), while PKCε translocation occurs in the presence of elevated hepatic ceramides (55). In the current study, translocation of both PKCδ and PKCε was measured by determining relative protein expression in cytosol and membrane fractions of liver tissue, as previously described, and assuming the same purity of fractions as has been previously obtained (31, 36). 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 (P8340, Sigma Aldrich, Oakville, ON), and phosphatase inhibitor (P5726, Sigma Aldrich). Following centrifugation, the supernatant was collected as the cytosolic fraction while the pellet was immersed into buffer B (buffer A + 1% triton X-100). The pellet and buffer solution were then centrifuged for 1 h, and the supernatant was collected to represent the membrane fraction. PKCε (1:1000, Cell Signalling Technology) activity was determined by the relative expression in membrane and cytosol fractions from each hepatic tissue sample.

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 (Thermo Scientific). 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). Primers for fatty acid synthase (forward sequence: 5’-CAACCTGCATTTCACAACCCAA-3’; reverse sequence: 5’-ACCTCCGAAGCCAAACGAGTTGAT-3’) and β-actin (forward sequence: 5’-CCAACCGTGAAAAGATGACC-3’; reverse sequence: 5’-ATCACAATGCCAGTGGTACG-3’) were used in real time quantitative PCR reactions 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 calculated using the 2 delta CT method, and corrected with β-actin mRNA expression.

Statistics

All data are represented as mean ± SE, with a criterion of p<0.05 to determine statistical significance. All fold differences are expressed as relative to the Placebo Chow group unless otherwise stated. Food intake and body mass gains over time were assessed by a use of a three-way analysis of variance (ANOVA). An unpaired t-test was used to determine pellet mass differences between CORT treatments. A two-way factorial ANOVA was used to determine statistical significance for body weight, food consumption, tissue mass, relative protein and mRNA expression, plasma FFAs, TG levels, HOMA-IR values, total hepatic lipid and hepatic TG content. A one-way repeated measures ANOVA was used to determine significance of glucose and insulin values
during the OGTT. All statistical tests were conducted using Statistica 6.0 software. Bonferroni or LSD Fisher post hoc tests were used where appropriate when ANOVA detected differences among treatments.

RESULTS

Exogenous CORT treatment abolishes endogenous diurnal rhythm and induces adrenal atrophy

At baseline (day 0), all treatment groups had lower basal plasma corticosterone levels relative to peak values (i.e. displayed a diurnal rhythm), but with slight elevations in GC levels, possibly because of some handling stress associated with blood sampling (Fig. 2A, p<0.05 between peak and trough for each group). Eight days following pellet implantation, CORT-treated animals demonstrated an abolished diurnal rhythm, (p>0.05 comparing plasma CORT at basal and peak time points in CORT-treated animals) with significant elevations in basal, but not peak, corticosterone levels compared to day 0. Peak CORT values (2000h) on day 8 were not statistically significant between groups (Fig 2B, p>0.05). Exogenous CORT treatment resulted in significant reductions in relative adrenal mass (Table 2; p<0.05, main effect of CORT pellet), as expected, since endogenous adrenocorticotropic hormone (ACTH) and GC production is suppressed. The total amount of CORT dissolved over the treatment period did not differ between the two CORT-treated groups (93.16±11.23mg vs. 131.3±13.01mg in CORT Chow vs. CORT HFD; p>0.05 based on an unpaired t-test). Wax pellet implantation and consumption of a HFD did not alter the normal diurnal rhythm of GCs (Fig. 2B), nor did it
affect adrenal mass (Table 2; p>0.05). Though previous studies have demonstrated increases in plasma CORT levels in response to a HFD (65), we did not find any significant increases in plasma CORT as a result of HFD. These differences may be attributed to differences in rat strain, method of blood collection or experience in handling the animals prior to hormone stress measurements.

Chronically elevated CORT levels result in reduced body and muscle mass, but increase central adiposity

CORT pellet implantation resulted in a significant reduction in body mass over the course of treatment (Fig. 3A). This occurred despite an increase in caloric intake after 6 days of treatment (Fig. 3B; p<0.05 day 2 compared to day 6). This is in contrast to the 23% reduction in caloric intake experienced by the placebo HFD group (Fig. 3B). CORT alone resulted in a 1.5 fold increase in epididymal adipose mass, which was further augmented to a 2-fold increase with the addition of a HFD (Table 2; p<0.05). In contrast, both CORT-implanted groups experienced a similar reduction in muscle mass compared to the two placebo treated groups (1.2 fold decrease; p<0.05).

CORT and HFD together increases hepatic steatosis, fibrosis and plasma ALT levels, but do not increase hepatic inflammation

Both CORT and the HFD alone resulted in a 2-fold increase in hepatic lipid content, as assessed by lipid extraction from whole liver tissue. The combination of
CORT and HFD resulted in an additive effect on hepatic steatosis and resulted in a 4-fold increase in total lipid content (Fig. 4A-4B; p<0.05). A similar trend for ceramide content was also found. More specifically, the CORT HFD group experienced a 3.7-fold increase in ceramide content relative to the placebo chow group (Fig. 4C; p<0.05). Oil Red O staining of neutral lipids (predominantly triglycerides) demonstrated a visible increase in hepatic lipid content in the CORT HFD group as compared to the placebo chow controls (Fig 4D). In addition to steatosis, the combination of elevated CORT with HFD resulted in a 1.5-fold increase in hepatic fibrosis, as quantified by collagen staining of liver sections (Fig. 4E and 4F; p<0.05), a 2.8-fold elevation in plasma ALT and a 2.3-fold elevation in total bilirubin levels (Table 3; p<0.05) when compared to the placebo chow group. While the CORT Chow group also experienced increases in plasma ALT levels (1.9 fold), fibrosis was not present within this timeframe of study (16 d). In addition, the HFD alone did not produce any significant increases in fibrosis or injury relative to the placebo chow group.

Haptoglobin, thought to be a sensitive acute phase marker of inflammation (19), was measured to determine if increased lipid accumulation was associated with increases in inflammation. Haptoglobin content was significantly reduced in the CORT Chow group (Table 3, p<0.05) relative to Placebo Chow controls, and showed a trend for reductions in the CORT HFD group (p=0.07), thereby suggesting that inflammation was reduced in the face of elevated hepatic lipid exposure and insulin resistance. Moreover, the HFD alone did not significantly alter haptoglobin concentrations within the timeframe of this study (16 d) (p>0.05).
Chronic CORT increases FFA spill over into the liver via the portal vein

Prior to sacrifice and under anesthesia, blood samples were taken from the portal and hepatic vein, representing delivery into and export out of the liver, respectively. Chronically elevated CORT levels resulted in increased portal vein and hepatic vein concentrations of plasma FFAs (Fig. 5A-B; both p<0.05). Portal vein TG concentrations were not significantly increased as a result of CORT pellets, nor were they increased with a HFD (Fig. 5C, p<0.05). HFD alone did not significantly increase portal vein FFA and TG concentrations, either alone or in conjunction with CORT (Fig. 5A and 5C, p>0.05). Only the CORT Chow group experienced a significant increase in hepatic vein TG concentrations (Fig. 5D, p<0.05). Net uptake of FFAs, based on the difference between hepatic and portal vein concentrations, were greatest in the CORT treated groups (Fig. 5E, p<0.05), indicating that a greater FFA clearance occurs with CORT treatment.

Chronic CORT drives increases in CD36 protein expression and acts in combination with a HFD to synergistically increase FASN mRNA

CD36 and L-FABP are proteins that facilitate uptake of fatty acids into a variety of tissues including skeletal muscle and liver. CD36 mediates FFA transport across the plasma membrane (5), while L-FABP distributes fatty acids within the liver, and has been shown to facilitate delivery of lipids for oxidation (3). Immunoblotting was used to determine if CORT or HFD altered relative protein expression of CD36 and L-FABP in the liver. After 16 d, both CORT-treated groups experienced a similar 2.5-fold increase
in CD36 expression (Fig. 6A; p<0.05) while HFD did not independently or additively alter CD36 protein expression. No differences in L-FABP protein expression relative to controls were evident (Fig. 6B; p>0.05 relative to placebo chow).

Hepatic fatty acid synthase (FASN) is the rate-limiting enzyme involved in de novo lipogenesis (23). Expression of FASN mRNA, was assessed by real time quantitative PCR of frozen liver tissue to determine if either pellet or diet treatment altered the rate of hepatic de novo lipogenesis. HFD and CORT synergistically increased FASN mRNA expression 14-fold higher than the placebo chow group (Fig. 6C; p<0.05). Though CORT Chow animals also experienced an increase in hepatic FASN mRNA, this increase was not significant (p>0.05)

**High fat diet exacerbates plasma TG and cholesterol levels that are increased with chronic CORT treatment**

CORT treatment alone resulted in a 1.5-fold increase in plasma cholesterol and TG concentrations, compared with placebo treated animals by the end of the experimental timeline (Table 3; p<0.05). The addition of a HFD resulted in a further increase in circulating cholesterol and TG levels in the CORT HFD group, to ~2-fold above values observed in the placebo chow animals (p<0.05). In contrast, plasma FFA levels did not significantly differ between treatment groups (Table 3 p>0.05).
Whole body insulin resistance, hyperglycemia and hyperinsulinemia occur when CORT and HFD are combined

The CORT HFD group experienced hyperglycaemia in a fasted state (blood glucose 10.9 ±1.2 mmol, p<0.05, Fig. 7A), while all other groups maintained fasting euglycemia. Fasting insulin levels were highest in the CORT-HFD group (insulin=5.78±1.92 ng/ml; 13-fold higher relative to controls; p<0.05, Fig. 7B) and as a result of CORT treatment alone (3.12±0.58 ng/ml 7.7 fold increase; p<0.05). Basal CORT levels correlated strongly with fasting plasma insulin levels ($r^2=.69$, p<0.05, Fig. 7C), indicating that the sustained elevations in CORT promoted increased insulin resistance and elevated beta cell insulin secretion in concert. HOMA-IR scores were used to quantify the degree of insulin resistance, primarily in the liver (68), and were increased by 13-fold with CORT alone, but were synergistically increased by 26-fold when CORT and HFD were combined (Fig. 7D, p<0.05). Glucose tolerance, assessed by an OGTT performed on day 8, revealed a significant elevation of blood glucose levels (Fig. 7E; p<0.05) and greater area under the curve (Fig. 7F) throughout the duration of the 2h test in the CORT-treated animals compared to the placebo groups.

Development of steatosis is complimented by reduced hepatic insulin signalling

CORT treatment resulted in significant reductions in phosphorylation of AKT at threonine 308 compared to the Placebo Chow group, although this did not differ between CORT-treated groups (Fig. 8A, p<0.05). Treatment failed to significantly alter basal phosphorylation of AKT at serine 473, although there was a tendency for the high
fat diet to lower phosphorylation status (Fig. 8B). Downstream of AKT, phosphorylation of the transcription factor FOXO1 was dramatically reduced in all treatment groups (Fig. 8C), but was most severely hindered 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 or HFD alone (Fig. 8D, p<0.05). Both CORT-treated groups experienced similar increases in PEPCK protein expression relative to the placebo groups (Fig. 8E, p<0.05).

To investigate the mechanisms linking elevated hepatic lipid content and insulin resistance, we measured translocation of the delta (δ) and epsilon (ε) isoforms of PKC. Both these isoforms of PKC are activated by translocation from the cytosol to the membrane in response to DAGs or ceramides, respectively. No differences in PKCε expression were found between treatment groups (Fig. 9A, p>0.05). In contrast, CORT treated animals experienced a significant increase in PKCδ translocation to the membrane, as determined by increased membrane expression of PKCδ relative to its cytosolic expression (Fig. 9B).

**DISCUSSION**

In the present study, we show that two weeks of high fat feeding, in the presence of elevated basal CORT levels, rapidly induces development of fatty liver disease without any evidence of acute hepatic inflammation in young male Sprague Dawley rats. Development of fatty liver disease involved dramatic hepatic steatosis (Fig. 4A-D), fibrosis (Fig. 4E,F), and increased plasma ALT levels (Table 3). While the elevation in
circulating CORT levels by itself, or the consumption of a HFD alone, also produce some degree of steatosis, they do not cause significant fibrosis or liver damage during this time frame of exposure (Fig. 4E-F). Along with liver injury, we also show that the combination of sustained elevation in CORT and a HFD rapidly induces a type 2 diabetic phenotype characterized by hyperinsulinemia and hyperglycemia, which does not occur when CORT or a HFD are administered on their own. Moreover, while the CORT Chow and Placebo HFD group did experience some degree of whole body insulin resistance (Fig. 7D) and increased visceral adiposity (Table 2), these effects were greatest when CORT and HFD were combined.

Animal models of NAFLD have previously used prolonged periods of high fat feeding (21), lasting weeks or months, to induce increased lipid accumulation. However liver damage, indicated by fibrosis and elevated plasma alanine aminotransferase (ALT) levels, typically does not develop with short-term high fat feeding (< 4 weeks in duration) (2, 11, 64). Similarly, the use of specialized methionine and choline deficient diets are commonly used to model the development of NAFLD in rodents (21). Though these diets have been shown to cause steatosis and fibrosis, they rarely induce insulin resistance, an important clinical characteristic of NAFLD patients (32, 54). Recently, Karatsoreos et al., have proposed a new GC-induced mouse model for the study of metabolic syndrome using CORT dissolved in drinking water, which was characterized by accelerated ectopic lipid deposition and whole body insulin resistance (27). We have previously reported similar results (10) using Sprague-Dawley rats treated with exogenous GC pellets, but we have not characterized the development of NAFLD in this new model. The use of exogenous GC pellets, rather than providing CORT in the
drinking water, allows for greater control of CORT dosage and involves a minimally invasive procedure to produce an abolished diurnal CORT rhythm. Though care was taken to avoid imposing stress on these animals during the blood collection, the novelty of being handled and enduring a tail nick to collect blood for plasma CORT analysis may have contributed to higher basal and peak plasma corticosterone than what is typically observed when using indwelling carotid catheters in the same species (16, 46). These acutely elevated stress hormone levels at the time of blood sampling may have masked subtle changes in plasma CORT levels that could have occurred as a result of the HFD alone. However, it is important to note that only the CORT treated animals developed the characteristic steatosis and insulin resistance evident in fatty liver disease.

Our present findings are an extension of previous work utilizing exogenous CORT treatment to induce a metabolic syndrome phenotype in young adult rodents (27). In this paper, we show that consumption of a HFD, in the presence of chronically elevated CORT levels, in young growing rats exacerbates a number of characteristics presented in NALFD including steatosis, fibrosis and liver injury. It also results in severe whole body insulin resistance, impairments to hepatic insulin signalling and fasting hyperglycemia. These features are strikingly similar to the metabolic syndrome phenotype observed in a large percentage of patients with pre diabetes or type 2 diabetes mellitus (12). Unlike patients with metabolic syndrome, the CORT HFD treated animals experienced attenuated body mass gains over time, relative to the placebo chow group. This likely reflects the catabolic nature of CORT on lean mass and the
overall reduction in circulating growth hormone levels (25), in addition to the potential for glycosuria associated with the severe hyperglycemia.

NALFD occurs without hepatic inflammation.

Our findings show that a HFD, in the presence of chronically elevated CORT, results in a 4-fold increase in hepatic lipid content (Fig. 4A), a 2.8 fold increase of plasma ALT levels and a 2.3-fold increase of total bilirubin content (Table 3) after only 16 days of exposure. In contrast, the HFD alone increased hepatic lipid content by ~2 fold but did not cause significant alterations to plasma ALT or bilirubin levels. Importantly, this increase in lipid content and liver injury in the CORT HFD combined group was accompanied by significant increases in fibrosis, an effect that was not evident with CORT or HFD treatment alone (Fig. 4E). Thus, it would appear that a HFD rapidly induces severe liver damage in the face of elevated GC levels, a finding that has important implications in the dietary management of patients with hyperglucocorticoidemia.

NAFLD is commonly associated with the development of hepatic inflammation (18) and type 2 diabetes (62, 69). Interestingly, GCs are well established to be anti-inflammatory in action (57) despite promoting steatosis (48). As such, it was unclear if GC-induced NAFLD would be associated with hepatic inflammation in this animal model. To test this, we measured plasma haptoglobin levels, a well-known acute phase marker of acute hepatic inflammation (19), and found that CORT treatment alone resulted in significant reductions in plasma haptoglobin concentrations (Table 3;
p<0.05), while CORT and HFD combined had haptoglobin levels similar to the placebo chow group. Plasma TNFα was also measured but levels did not differ between groups (data not shown). These results suggest that development of fibrosis and liver injury in this model of NAFLD occurs independent of hepatic inflammation.

**CORT drives lipid spill over from central adipose tissue, which is greatly increased with consumption of a HFD**

Hepatic lipid metabolism is a balance between lipid import via very low density lipoproteins (VLDL) into the liver and export out of the liver. Donnelly et al. (13) suggest that up to 60% of lipids in the liver are from FFA spill over from central adipose depots. Since CORT has been shown to increase both central adiposity and the lipolytic activity of adipose tissue (10, 40, 51), we speculated that one possible mechanism for severe steatosis in the CORT HFD group would be increased lipid uptake from more metabolically active adipose tissue. In support of this, we show that CORT treatment alone resulted in a 1.5 fold increase in visceral adipose tissue mass, which was further exaggerated (>2.5-fold) in the presence of HFD (Table 2; p<0.05). Jensen et al. (26) reported that portal vein FFA concentrations predicted the proportion of hepatic FFAs that originated from surrounding central adipose tissue. Based on their findings, we investigated delivery of lipids into the liver using FFA and TG concentrations in the portal vein as markers of lipid spill over. Our results suggest that elevated CORT exposure drives lipolysis (10) and thus “spill over” of FFA into the liver via the portal vein (Fig. 5A). We also show that chronic CORT treatment induces increases in central
adipose tissue lipolysis, based on increased glycerol concentrations in the portal vein (Table 3), which occurs despite increases in overall adipose tissue mass. We speculate, therefore, that CORT treatment results in a greater, and more metabolically active, central adipose tissue depot that promote lipid accumulation in the liver.

Although hepatic vein TG concentrations did not differ between the two CORT groups (Fig. 5D), the CORT HFD group had significantly elevated trunk blood TG and cholesterol levels (Table 3). These increased levels may increase risk of metabolic syndrome. Though previous studies have suggested that the synthetic GC dexamethasone (dex; a synthetic GC) has no significant effect on LPL expression (8), it remains to be determined if prolonged exposure to exogenous CORT has a similar effect, which contributes to the increased plasma TG experienced by the CORT HFD group.

CORT increases facilitated transporter protein expression and combines with HFD to synergistically increase de novo lipogenesis.

The induction of facilitated lipid transporters in the liver may be a critical factor involved in steatosis development (34, 38). Komamura et al. (33) found that dex increased expression of CD36, a facilitated lipid transporter, in gastrocnemius muscle. In our study, a 2.5 fold increase in hepatic CD36 expression occurred as a result of CORT treatment (Fig. 6A). In contrast, differences in L-FABP expression amongst treatment groups were not observed (Fig. 6B). A previous study by Foucaud et al. (17) that used repeated dex injections to study the effects of CORT on L-FABP expression
found that 5 days of GC treatment down-regulated L-FABP expression in rat liver. This
down regulation of L-FABP was abolished when oleic acid was added in culture. Thus, it
was speculated by these authors that the \textit{in vivo} changes in L-FABP expression by dex
were likely due an indirect effect of GCs on lipid provision (17). In contrast, we did not
find any alterations to L-FABP expression, despite increases in hepatic lipid content as
a result of CORT alone or CORT and HFD combined. This indicates that facilitated
transport in the liver, potentially towards oxidation, is not increased, thus likely further
contributing to the observed steatosis in this model.

In the presence of elevated insulin levels, hepatic lipid synthesis may occur via
de novo lipogenesis (4). Previous reports have found that de novo lipogenesis is
increased with NAFLD (58) and as a result of elevated CORT concentrations (24). Our
results confirm these findings as we demonstrate a significant increase in mRNA
expression of fatty acid synthase, a marker of hepatic lipogenesis, in the CORT HFD
group relative to placebo chow controls (Fig. 6C; p<0.05). This finding clearly
demonstrates the synergistic effect of CORT and HFD in exacerbating de novo
lipogenesis, which contributes to increased lipid accumulation in the liver, possibly as a
direct effect of CORT or as an indirect effect by promoting hyperinsulinemia (Fig. 7I).
This increase in de novo lipogenesis may help to promote increased synthesis of
ceramides, the potentially toxic by-product of excessive lipids accumulation in insulin
sensitive tissues. Ceramides are indeed deemed to be toxic to hepatic function and
contribute to liver lipoxicity and insulin resistance (50). We observed that CORT
treatment results in increased hepatic ceramide content, particularly in the environment
of a HFD, a finding that is in line with the observed deterioration in insulin sensitivity.
Consumption of a HFD in the presence of chronic CORT exposure rapidly promotes a diabetic phenotype and hepatic insulin resistance

The combination of CORT and HFD rapidly induces fasting hyperglycemia (Fig. 7A), hyperinsulinemia (Fig. 7B) and severe insulin resistance (Fig. 7D), all features of type 2 diabetes. Interestingly, several rodent models of type 2 diabetes have elevations in circulating GCs, including the Zucker Diabetic Fatty rat (9) and the diabetic Goto-Kakizaki rat (71), suggesting that CORT may play a role in promoting the diabetes phenotype. As expected, CORT alone did not induce fasting hyperglycemia, but did result in hyperinsulinemia and insulin resistance (Fig. 7B). Prior studies have demonstrated that two weeks of HFD is sufficient to induce altered hepatic insulin sensitivity with no changes to peripheral insulin sensitivity (22). Our findings are in line with these results as we did not observe any significant differences in peripheral insulin sensitivity despite decreased hepatic insulin sensitivity in response to 2 weeks of high fat feeding without elevated CORT exposure. Our results are also consistent with a previous report (27) that found elevated basal and post glucose load insulin levels as a result of exogenous CORT administration. Our data extend these findings by revealing that high fat feeding is capable of producing hyperglycemia and hyperinsulinemia in a young “diabetes resistant” rodent model when combined with elevated CORT levels. To determine whether the cause of fasting hyperglycemia was due to impairments in insulin regulation of gluconeogenesis in the liver, we assessed expression of proteins in the insulin-signalling pathway in the liver. In the presence of insulin, AKT induces phosphorylation and removal of FOXO1 from the nucleus, impairing it from inducing
transcription of gluconeogenic enzymes (44). However, with insulin resistance, this inhibition is lost (39, 60). Previous studies report that CORT is a strong agonist of gluconeogenic gene transcription (14, 61), and is able to abrogate phosphorylation of AKT in the presence of insulin (70). Similarly, an increase in hepatic lipid metabolites, particularly ceramides, has been shown to impair AKT phosphorylation, and thus perpetuate insulin resistance (15). Our results show that CORT-treated animals developed impaired insulin signalling and increased protein expression of gluconeogenic enzymes along with increased ceramide levels and reduced AKT phosphorylation on threonine 308, but not serine 473. We also show that HFD exacerbates CORT’s effects on hepatic insulin signalling by resulting in greater reductions in FOXO1 phosphorylation (Fig. 8C) and increased G6Pase protein expression (Fig. 8D), despite no further down regulation in AKT serine 308 phosphorylation beyond what was observed with CORT treatment alone (Fig. 8A). These findings suggest that a HFD, combined with CORT, may be acting downstream of AKT to impair insulin’s regulation of gluconeogenesis, but the exact mechanisms warrant further clarification.

To further elucidate the mechanisms linking excess lipid accumulation and hepatic insulin resistance, we measured activation of two PKC isoforms –δ and –ε, believed to mediate lipid-induced insulin resistance. Previous studies have reported that hypertriglyceridemic diabetic rats have greater hepatic PKC activity than lean rats (55). Furthermore, steatosis is associated with activation of PKCε (45). It is believed that PKCδ mediates insulin resistance by having an inhibitory effect on tyrosine kinase activity of the insulin receptor in human embryonic kidney cells (30). Similarly, PKCε
has been shown to interfere with insulin receptor activation, thus preventing downstream signalling (56). In the present study, we found no differences in activity of PKCε as a result of CORT or HFD (Fig. 9A). In contrast, elevated FFA and triglyceride levels in the CORT-treated animals were associated with increased PKCδ translocation to the membrane (Fig. 9B). These results suggest that PKCε does not mediate the attenuated insulin signalling that is observed in CORT treated animals but activation of PKCδ as a result of hypertriglyceridemia may contribute to the alterations in insulin signalling as a result of CORT treatment.

Conclusion

In conclusion, the present study has shown that short-term (2 wk) exposure to elevated CORT, along with a HFD, results in a phenotype that mimics severe steatosis, NAFLD and type 2 diabetes mellitus in young rodents. The young age of these rodents (6-8 weeks of age) suggests that caution should be taken when interpreting these results to metabolic conditions experienced by adults, however. Further studies are warranted to examine whether this model of chronically elevated CORT and HFD have similar effects on aged rats. Nonetheless, the increased CORT levels experienced by the CORT treated group mimic the abolished diurnal rhythm that occurs in patients prescribed exogenous GCs (e.g. prednisone) after five days of treatment (63) and parallels the hyperglucocorticoidemia that is observed in humans and animal models of type 2 diabetes. Though further studies are required to determine whether the altered adipose lipid metabolism and whole body insulin sensitivity that result from CORT and
HFD combined precede development of hepatic insulin resistance and steatosis, our current findings point out the important interactive effects of poor diet and elevated stress hormones in exacerbating metabolic disease.

**GRANTS**
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**DISCLOSURES**
No potential conflicts of interest relevant to this article are reported.

**AUTHOR CONTRIBUTIONS**
AD and MCR designed the study. AD and JB collected and analyzed the data, made the graphs and tables, contributed to discussion and wrote the first manuscript draft. ST performed the analysis of the liver lipid and ceramide content, while LS performed the analysis of the lipid transporters. AS performed the histological analysis. MCR reviewed and edited the final manuscript. All authors approved the final manuscript submission.
Figure 1: Experimental design. Healthy adult male Sprague Dawley rats (6 weeks of age) were subjected to a pellet implantation surgery in which they either received four 100 mg CORT pellets or four 100 mg pellets made of wax (i.e. placebo). Animals from each pellet group were then randomly assigned to receive a standard chow diet or a 60% high fat diet for 16 d.

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

Figure 3: Body mass gain and caloric intake. Fed body mass was taken on days 0, 2, 4, 6 and 8 and fasted mass (terminal mass) was taken on day 16. CORT treatment resulted in a significant decrease in body mass over time, while Placebo treated animals gained mass with time (group by time interaction, p<0.05) (A). Food intake was measured on day 2 and day 6 and caloric intake was estimated based on diet energy density (B). Estimated caloric intake did not differ in the placebo chow group, but significantly increased with CORT. N=5-10 per group; * indicates Placebo greater than CORT treated animals. Different letters indicate significantly different mean values at p<0.05. All values are ± SEM.

Figure 4: Hepatic lipid and collagen content. Chronically elevated CORT levels combined with HFD resulted in severe hepatic steatosis (A, B) and increased ceramide content (C) as indicated by liver lipid extraction and images of hepatic tissue, and by oil red O staining of frozen liver sections (D). Quantification of Sirus red stain for hepatic collagen content via color density revealed marked increases in fibrosis in the CORT-HFD group (E, F; p<0.05). N=5-7 per group; * indicates p<0.05 relative to placebo chow; # indicates p<0.05 relative to CORT-Chow. All values are ± SEM.

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 resulted in elevations in NEFAs concentrations in the portal vein (A; p<0.05). CORT and HFD alone increased FFA levels in the hepatic vein (i.e. export). These effects were additive when CORT and HFD were combined (B). CORT significantly increased portal vein TG (C) but only CORT alone significantly increase hepatic vein TG concentrations (D). Net differences in plasma NEFA 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 into liver tissue (E). N = 5-7 per group. * Indicates p<0.05 versus placebo chow. # indicates p<0.05 versus CORT chow. All values are ±SEM.

Figure 6: Hepatic lipid transporter protein content. Protein expression of the facilitated lipid transporters, CD36 and L-FABP, were measured in frozen liver samples.
Expression of CD36 was increased as a result of CORT treatment (A), but L-FABP expression was unchanged (B). The relative mRNA expression of FASN (C), a marker of hepatic de novo lipogenesis, showed a trend for increase as a result of CORT alone (p=0.08) and was synergistically increased when CORT and HFD were combined (p<0.05). N = 4-7 per group; * 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. Eight days after pellet implantation, animals were subject to an overnight fast to measure fasting glucose (A) and insulin (B) values. Increases in plasma CORT levels correlated with elevated fasting insulin levels (C; p<0.05 using linear regression). Chronic CORT treatment induced severe insulin resistance as measured by HOMA-IR scores, which were exacerbated when combined with a HFD (D; p<0.05 using a 2 way ANOVA). An OGTT demonstrated severe glucose intolerance as a result of CORT treatment, which was worsened with a HFD (E, F p<0.05 using a repeated measures ANOVA and LSD Fisher post hoc analyses). N=8-10 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 gluconeogenic enzyme protein expression. Chronically elevated CORT levels resulted in decreased Akt\textsuperscript{Thr308} phosphorylation (A). In contrast, AKT\textsuperscript{Serine 473} was unchanged significantly with treatment, although there was a tendency for reduced phosphorylation with a HFD alone (B, p>0.05). The reduction in AKT\textsuperscript{Thr308} coincided with reduced FOXO1\textsuperscript{ser256} phosphorylation (C) experienced by all treatment groups relative to placebo chow, but was most severe when CORT and HFD were combined. Reduced phosphorylation of FOXO1 indicates impaired inhibition of FOXO1 transcriptional activity on G6Pase and PEPCK. Analysis of protein expression of these gluconeogenic enzymes confirmed increases in G6Pase (D) and PEPCK (E) with CORT treatment. N= 4-7 per group; * 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\textgreek{d} and PKC\textgreek{e} membrane protein expression. PKC\textgreek{d} and PKC\textgreek{e} activation is determined by its translocation from the cytosol to the membrane. Translocation was measured as the relative ratio of membrane expression to total (cytosolic and membrane expression). No significant increase in PKC\textgreek{e} translocation occurred as a result of CORT, HFD or both combined (A; p>0.05). Blot images for PKC\textgreek{e} are rearranged from the original blot, and grouped based on treatment group. PKC\textgreek{d} had a trend for increased translocation in the CORT treated group, but was not significant (B). N = 4-7 per group; All values are ± SEM relative to the placebo chow group.
Table 1: Diet composition

<table>
<thead>
<tr>
<th></th>
<th>Standard Chow (%)</th>
<th>High Fat Diet (%)</th>
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</thead>
<tbody>
<tr>
<td><strong>Protein</strong></td>
<td>27.1</td>
<td>27.1</td>
</tr>
<tr>
<td><strong>Carbohydrate</strong></td>
<td>59.7</td>
<td>13.2</td>
</tr>
<tr>
<td><strong>Fat</strong></td>
<td>13.2</td>
<td>59.7</td>
</tr>
<tr>
<td><strong>Kcal/g</strong></td>
<td>4.9</td>
<td>3.4</td>
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Table 2: Relative tissue mass.

<table>
<thead>
<tr>
<th></th>
<th>Placebo Chow</th>
<th>CORT Chow</th>
<th>Placebo HFD</th>
<th>CORT HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Mass (g)</strong></td>
<td>342.57±7.24</td>
<td>245.56±7.37*</td>
<td>315.4±1.89</td>
<td>249.38±11.07*</td>
</tr>
<tr>
<td><strong>Epididymal adipose tissue (g/kg B.W.)</strong></td>
<td>8.59±1.11</td>
<td>12.59±1.01*</td>
<td>12.77±0.85*</td>
<td>18.54±1.42#</td>
</tr>
<tr>
<td><strong>Liver (g/kg B.W.)</strong></td>
<td>37.99±1.13</td>
<td>51.92±2.61*</td>
<td>40.87±2.07</td>
<td>60.11±2.81#</td>
</tr>
<tr>
<td><strong>Gastrocnemius (g/kg B.W.)</strong></td>
<td>5.18±0.11</td>
<td>4.23±1.01*</td>
<td>4.93±0.16</td>
<td>4.17±0.09*</td>
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<tr>
<td><strong>Average adrenal mass (g/kg body weight)</strong></td>
<td>0.19±0.02</td>
<td>.07±0.01*</td>
<td>0.19±0.011</td>
<td>0.08±0.004*</td>
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Table 3: Plasma lipid metabolites and parameters of liver damage

<table>
<thead>
<tr>
<th></th>
<th>Placebo Chow</th>
<th>CORT Chow</th>
<th>Placebo HFD</th>
<th>CORT HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triglycerides</strong></td>
<td>0.12±0.01</td>
<td>0.24±0.06*</td>
<td>0.18±0.03</td>
<td>0.47±0.13*#</td>
</tr>
<tr>
<td><strong>Free fatty acids (mM)</strong></td>
<td>0.89±0.13</td>
<td>0.67±0.06</td>
<td>0.80±0.12</td>
<td>0.64±0.04</td>
</tr>
<tr>
<td><strong>Cholesterol (mM)</strong></td>
<td>1.86±0.16</td>
<td>2.80±0.40*</td>
<td>1.83±0.21</td>
<td>3.86±0.54*#</td>
</tr>
<tr>
<td><strong>Total Bilirubin (µmol/L)</strong></td>
<td>0.75±0.07</td>
<td>0.96±0.18</td>
<td>0.90±0.18</td>
<td>1.76±0.44*</td>
</tr>
<tr>
<td><strong>ALT (U/L)</strong></td>
<td>49.9±5.05</td>
<td>94.64±12.40*</td>
<td>48.3±4.14</td>
<td>138.3±32.0#</td>
</tr>
<tr>
<td><strong>Haptoglobin (mg/ml)</strong></td>
<td>1.19±0.36</td>
<td>0.35±0.11*</td>
<td>1.18±0.29</td>
<td>0.48±0.15</td>
</tr>
<tr>
<td><strong>Glycerol† (mM)</strong></td>
<td>0.20±0.05</td>
<td>0.42±0.10*</td>
<td>0.29±0.08</td>
<td>0.46±0.12*</td>
</tr>
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TABLE LEGENDS

Table 1: Macronutrient composition of standard rodent chow and high fat diet.

Table 2: 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.

Table 3: Trunk blood triglycerides and free fatty acids were collected in a fasted state. 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>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).


30. Kellerer M, Mushack J, Seffer E, Mischak H, Ullrich A and Haring HU. Protein kinase C isoforms alpha, delta and theta require insulin receptor substrate-1 to inhibit


56. **Samuel VT, Liu ZX, Wang A, Beddow SA, Geisler JG, Kahn M, Zhang XM, Monia BP, Bhanot S and Shulman GI.** Inhibition of protein kinase Cepsilon prevents


Figure 1
Figure 2
Figure 3
Figure 4
Hepatic CD36 protein content

![CD36 Image]

Hepatic L-FABP protein expression

![L-FABP Image]

Hepatic FASN mRNA

![FASN Image]

Figure 6
Figure 7

A) Fasted Glucose

B) Fasted Insulin

C) Correlation between fasting insulin and basal plasma CORT

D) HOMA-IR

E) Oral Glucose Tolerance Test

F) OGTT Glucose AUC
Figure 8
Figure 9