**Introduction**

* Mention glucocorticoid and obesity prevalence
* Mention background on glucocorticoids and lipolysis

Obesity can lead to a multitude of metabolic complications, such as insulin resistance and non-alcoholic fatty liver disease (NAFLD). Similarly, chronically elevated glucocorticoids, such as is seen in Cushing’s disease or glucocorticoid treatment, is often paired with reduced insulin sensitivity and is associated with fatty liver, as well as changes in fat mass and distribution. Though these diseases and their co-morbidities are well characterized, underlying mechanisms caused by the disease subsequently leading to these metabolic complications have not been identified. Moreover, to our knowledge, the combination of obesity and elevated glucocorticoids on the severity of the aforementioned outcomes has yet to be investigated.

It is well known that obesity has become an epidemic in the US and other developing countries, and though Cushing’s disease is rare, it is estimated that 1-2% of Americans are prescribed glucocorticoids at any given time, as they are potent anti-inflammatory drugs used to treat a variety of medical conditions. Comparing this staggering statistic with the fact that many Americans are overweight or obese, it is likely that these two conditions are combined in many instances. Since glucocorticoids were developed in the 1950’s, many of the safety studies were completed at a time when the overall population was in a leaner state. Given the similar co-morbidities associated with obesity and chronically elevated glucocorticoids, we hypothesized that the combinations of these two conditions would lead to even worse metabolic outcomes than either of them alone and indeed, this is what we found. In addition to assessing the results of the combination of these conditions, we also wanted to investigate the underlying mechanisms leading to the enhanced severity of insulin resistance and hepatic lipid accumulation.

Though there has been much speculation as to what exactly is leading to these co-morbidities, there has yet to be a clear answer. Increased adiposity leading to the obese state has been proposed as the likely culprit for insulin resistance and often correlates well with this outcome; however, recent evidence showing that some obese individuals are protected from insulin resistance suggests that this is not the whole story. It is well documented that high circulating glucocorticoids lead to insulin resistance; however, the underlying mechanism has yet to be elucidated. There is an array of physiological changes that occur as a result of chronically elevated glucocorticoids including decreased lean mass, increased fat mass and increased lipolysis, all of which have been associated with decreased insulin sensitivity.

Basal lipolysis has also been shown to be enhanced in obesity and is also a known cause of NAFLD. Lipolysis is the breakdown of tri-, di-, and mono-acylglycerols into fatty acids and glycerol. Once fatty acids are released from the adipose tissue, they can be re-esterified or travel through the circulation to be taken up by other tissues, such as the liver. It has been previously demonstrated that insulin signaling prevents the release of fatty acids from adipose tissue and it is likely that insulin resistance removes this break on lipolysis resulting in higher circulating glycerol and free fatty acids. It is unclear whether lipolysis in turn affects insulin sensitivity.

Here we show that chronically elevated glucocorticoids in the presence of obesity have a synergistic effect on insulin resistance and fatty liver disease. Interestingly, there is a synergistic effect on lipolysis as well. Obese glucocorticoid-treated mice have reduced fat mass compared to all other groups, yet have hyperglycemia and severe insulin resistance with no apparent change in muscle insulin signaling; therefore, we speculate that it is the lipolysis, not the level of adiposity or the loss of lean mass, that is primarily driving the insulin resistance.

**Methods**

* Patient Recruitment

The study was approved by the institutional review board of the University of Michigan Medical System. Written informed consent was obtained from all of the patients. Patients were recruited consecutively from those undergoing a transsphenoidal adenomectomy at the University of Michigan for Cushing's disease or nonfunctioning pituitary adenoma over a 12-month period. Exclusion criteria were age <18, current hormone treatment including glucocorticoids, malignancy, inflammatory disease, diabetes type 1 and established pituitary hormone deficiencies. For each patient, a data sheet was completed including, age, sex, anthropometric measurements, diagnosis of hypertension, diabetes, results of blood tests and medications. Fasting blood samples were assayed for glucose (Siemens Advia 1800, Deerfield, IL, USA) and insulin (Life Technologies) as instructed by the manufacturers.

* Treatment of Animals with Dexamethasone

C57BL/6J adult male mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) at nine weeks of age. Following a one-week acclimation period, mice were treated as described previously (1) or were either kept on normal chow (NCD) or given high fat diet (45% fat; x carbs; x protein) for 12 weeks. Mice stayed on their respective diets and were treated with 1 mg/kg per day of dexamethasone (Sigma–Aldrich) in their drinking water (*n*=x) or used as controls (*n*=x) for six weeks. All animal procedures were approved by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee. Animal body weight and composition was determined weekly using an echoMRI 2100. Food was weighed weekly, with food intake determined as the decrease in food weight per mouse per week per cage. All mice were provided with access to food and water *ad libitum* throughout the study. At the end of treatment, mice were fasted for 16 h and were sacrificed by cervical dislocation at ZT3 after isoflurane anesthesia. Following cervical dislocation, a sagittal incision was made along the medioventral surface of each mouse and the skin was carefully pulled back to expose the subcutaneous fat depots. The incision was extended along the anterior surface of each hind limb to allow careful dissection of the inguinal fat pads. A small incision was then made into the rectus abdominus muscle to expose the abdominal cavity. The epididymal fat pads were identified and carefully dissected out. The right fat pads from each mouse were weighed and snap frozen in liquid nitrogen for later analysis, along with a section of the large lobe of the liver. Small pieces of these tissues, as well as the pancreas were placed in 10% formalin for histology.

* ITT

Insulin tolerance was assessed following five weeks of treatment (27 weeks of age). Following a 6-h fast, mice were given i.p. injections of insulin (Humulin R, Lilly, Indianapolis, IN, USA) at a concentration of 2.5 mU/g. Blood glucose was determined at 15-min intervals post-injection using a One Touch Ultra Glucometer (Lifescan).

* Clamp (get from metabolic phenotyping core?)
* Serum ALT was measured as described previously (1).
* Cell culture

3T3-L1 fibroblasts (pre-adipocytes) were cultured in 10% newborn calf serum, high glucose Dulbecco's Modification of Eagle's Medium (DMEM) with 1% pencilin, streptomycin and glutamine until confluence. A differentiation cocktail including 250nM dexamethasone, 3-isobutyl-1-methylxanthine and insulin in 10% fetal bovine serum, high glucose DMEM with 1% PSG at two days post confluence for four days (2). Media was then replaced including only insulin in the cocktail for an additional three days. The following three days cells remained in FBS media with no additional treatment. To assess lipolysis, cells either remained in FBS media or were treated with an additional dose of 250nM dexamethasone for five days before lysing.

* Assessment of Triglycerides from Cells and Tissue

3T3-L1 ells were grown and treated as described above and a triglyceride assay was performed at the end of the treatment period. Cells were lysed in homogenization buffer (50 mM Tris pH 8, 5 mM EDTA, 30 mM Mannitol, PI inhibitor) undergoing three freeze thaw cycles, liver tissue was homogenized with 5mm stainless steel pellets in this buffer using machine at xHz for 3-5min. Lipids were extracted using KOH and Chloroform:methanol mixture and left to evaporate overnight. A mixture of butanol, methanol and triton were then used to get the lipids into a solution. Triglyceride content was assessed using the Triglyceride Assay Kit (sigma) and absorbance was detected on machine.

* Histology

The liver, IWAT, EWAT, BAT and pancreas were kept in 10% formalin for 24 hours and then stored in 70% ethanol until further processing. Following a series of wash steps, tissues were embedded in paraffin wax and sent to either the University of Michigan University of Michigan Comprehensive Cancer Center Tissue Core or the Unit for Laboratory Animal Medicine In-vivo Animal Core (University of Michigan, Ann Arbor) where they were processed and stained with H&E or trichrome to assess cell morphology/inflammation and collagen formation, respectively.

* Analysis of mRNA

Cells and tissues were lysed in TRIzol and RNA was extracted using the PureLink RNA mini kit (Life Technologies). cDNA was synthesized from 0.5-1g of RNA using the High Capacity Reverse Transcription Kit (Life Technologies). Primers, cDNA and Power SYBR Green PCR Master Mix (Life Technologies) were combined in accordance with the manufacturer’s guidelines and quantitative real-time PCR was performed as previously described (3). mRNA expression level was normalized to *Actb* (Table 1).

* Protein Analysis

Cells and tissues were lysed in RIPA buffer (50 mM Tris, pH 7.4, 0.25% sodium deoxycholate, 1% NP40, 150 mM sodium chloride, 1 mM EDTA, 100 uM sodium orthovanadate, 5 mM sodium fluoride and 10 mM sodium pyrophosphate) on ice then centrifuged for 15 minutes at 13 000 RPM at 4°C. Clarified lysates were loaded on SDS-PAGE gels, transferred and blotted using antibodies raised against ATGL, HSL, pHSL, CGI-58, GAPDH, and ACTIN. Antibody complexes were detected by anti-mouse and anti-rabbit fluorescent conjugated antibodies and visualized using an Odyssey image scanner and blots were quantified using the Odyssey software version 2.1 (LiCOR).

* *In vivo* Lipolysis

Mice were briefly anesthetized with isoflurane and blood was taken via retro orbital bleed from X-week old fed mice at baseline and 15 minutes following an i.p. injection of 10mg/kg isoproterenol (Sigma-Aldrich) in PBS. Glycerol and free fatty acids were assessed via Serum Triglyceride Determination Kit (Sigma-Aldrich) and HR Series NEFA-HR(2) (Wako Diagnostics), respectively, in accordance with manufacturer’s guidelines.

* Stats

For animal studies, two-way ANOVA analyses were performed to test for significance of diet and treatment, as well as their interaction. For cell culture experiments a student’s t-test was used. An alpha level below p value of 0.05 was considered to be significant.

**Results**

# Dexamethasone-Induced Insulin Resistance is Worsened in the Presence of Obesity

Our group has previously published data (1) that illustrates different physiological and gene expression outcomes between those with Cushing’s disease (ACTH-secreting pituitary adenoma) and controls (non-secreting pituitary adenoma). More recently, we speculated that the conditions within the groups may vary according to obesity status. Here we have re-analyzed the data stratifying the Cushingoid and control groups by BMI (Figure 1A), classifying these individuals as “Not obese” (BMI < 30) and “Obese” (BMI ≥ 30). The presence of Cushing’s in individuals with a high BMI leads to increased insulin resistance (measured by HOMA-IR score), above that of Cushing’s or obesity alone (Figure 1B).

To further investigate if obesity status influences insulin sensitivity in the presence of high glucocorticoids we performed an insulin tolerance test (ITT) on lean (NCD) and diet-induced obese (HFD) mice that were untreated (Control) or treated with glucocorticoids (Dexamethasone; Figure 1C-schematic). All groups were given a relatively large dose of insulin (2.5 U/kg) to account for the known insulin resistance typically seen in diet-induced obesity (cite). HFD-fed, dexamethasone-treated mice were significantly more resistant to insulin-stimulated glucose uptake when compared to all other groups (Figure 1D). Though, it is important to note that the NCD-fed, dexamethasone treated animals still experienced some insulin resistance at this high dose. Additionally, HFD/dexamethasone exhibited fasting hyperglycemia, with a significant interaction between diet and drug (p=0.00009).

To evaluate glucose homeostasis in more detail we performed a hyperinsulinemic euglycemic clamp in obese mice (8 weeks of HFD) treated with dexamethasone for 3. This shorter HFD/dexamethasone exposure caused dramatic insulin resistance, hyperglycemia and reductions in lean mass, but no differences in fat mass between the groups (Supplementary Figures 1A-F. As expected, blood glucose levels were level throughout the entire clamp experiment and insulin clearance was not different between the groups (p=XXX Supplementary FIgure 1G-H). During the insulin phase, the infusion rate was 1.5 times lower in obese dexamethasone-treated mice when compared to obese controls (Figure 1E). Basal hepatic glucose production (HGP) was 10% higher in the dexamethasone treated group (p=0.026); moreover, there was a 30% reduction in the ability of insulin to suppress HGP (p=0.0091) resulting in HGP being 5-fold higher during the insulin phase in dexamethasone treated mice (p=0.014) when compared to controls (Figure 1F). Notably, while overall glucose turnover was unchanged in the presence of insulin (Figure 1G), there were significant reductions in 2-deoxyglucose uptake in subcutaneous white adipose (p=0.019), heart (p=0.0003) and gastrocnemius tissues (p=0.00001; Figure 1H). These data suggest that increased HGP, and reduced suppression of HPG by insulin and decreased insulin-stimulated glucose uptake into multiple tissue are the primary causes of the observed insulin resistance and hyperglycemia in these animals.

# HFD-Induced Liver Steatosis is Worsened in Dexamethasone Treated mice

Obesity and chronic elevations in glucocorticoids have been associated with increased liver fat and even non-alcoholic fatty liver disease (NAFLD). We observed slight increases in plasma AST and ALT, which are liver enzymes associated with liver disease (cite?), in Cushing’s patients and obese controls; interestingly, levels were further elevated in obese Cushing’s patients, synergistically so in the case of ALT (Figure 2A).

Since elevated liver enzymes are just one indicator of liver disease, they are not sufficient to lend a diagnosis, we studied this in our mouse model. HFD-fed, Dexamethasone treated mice had significantly elevated liver triglycerides when compared to all other groups (Figure 2B). In support of this, H&E staining of hepatic tissue clearly depicts higher lipid levels in this group (Figure 2C). Collagen/trichrome data…

Expression of genes involved hepatic *de novo* lipogenesis (*Srebf1* and *Fasn*) was assessed via qPCR (Figure 2D). Both transcripts were highly elevated in response to HFD alone; however, levels of both these enzymes were reduced in HFD/dexamethasone livers. This finding indicates that lipid accumulation resulting from dexamethasone treatment is likely occurring via a different mechanism than transcriptional activation of *de novo* lipogenesis.

# Dexamethasone Causes Decreased Fat Mass in HFD-Fed Mice

Dexamethasone treatment lead to decreased body mass in both NCD and HFD groups (FIG 3A). The reduced body mass was primarily due to lean mass loss (figure 3B). Surprisingly, there was also a loss in fat mass in the HFD-fed, dexamethasone treated group (Figs-- MRI and fat pad weights) that was not depot-specific (Figure 3C-D). There were no significant differences in food consumption (Figure 3E).

Fat cell size/inflammation…

Dexamethasone Treatment Results in Increased Lipolysis

Lipolysis has previously been associated with insulin resistance, is a known cause of NAFLD, and has been shown to increase with glucocorticoid treatment. We first assessed whether there was a direct effect of dexamethasone on adipocyte lipolysis in culture (figure 4A-). 3T3-L1 fibroblasts were either kept in media alone (pre-adipocytes), differentiated (mature adipocytes) or treated with dexamethasone following differentiation (mature adipocytes +dexamethasone) over a 15-day period. Dexamethasone treatment following differentiation lead to decreased lipid content and increased glycerol release into the media, indicating increased lipolysis (Figure 4 A,B). To assess this further, we measured lipolytic enzyme mRNA and protein expression levels in these cells (figure 4 C,D). Expression of ATGL (encoded by *Pnpla2*) and HSL (encoded by *Lipe*) were enhanced following dexamethasone treatment in 3T3-L1 cells.

To assess the effects of glucocorticoid-induced lipolysis *in vivo,* we measured the by-products of triglyceride breakdown, glycerol and free fatty acids in basal and stimulated conditions (figure 4 E). Stimulation of lipolysis was achieved via isoproterenol, a -adrenergic receptor agonist, or by a 16-hour fast. For isoproterenol stimulation of lipolysis fed mice were i.p. injected with 10 mg/kg isoproterenol and basal levels were determined prior to injections. Serum free fatty acids and glycerol were measured for each of these conditions. Dexamethasone treatment led to significant increases in basal (p<0.01), fasted (p=0.01) and stimulated (p<0.01) glycerol as well as basal (p<0.01) and stimulated (p<0.01) free fatty acids, indicating enhanced lipolysis.

qPCR lipolytic genes in these mice (if so this will be 4F)

These data support the hypothesis that that glucocorticoids directly stimulate lipolysis in adipose tissue.

To determine whether the effect of dexamethasone-induced *in vivo* lipolysis was exacerbated in the context of obesity we measured serum glycerol following a 16-hour fast (Figure 5A). Similarly, lipolysis was elevated in dexamethasone treated animals, moreso in the obese animals. There was a significant interaction between drug and diet (p=0.017).

We quantified mRNA and protein expression of lipolytic enzymes, ATGL and HSL, in the iWAT of these mice (5B,C). Consistent with the above findings, expression of ATGL was elevated in the dexamethasone-treated groups and there was a significant interaction of drug and diet at both the transcript (p=0.02) and protein (p=0.003) level. These data show that glucocorticoid-stimulated lipolysis and ATGL levels are augmented in the context of obesity.

Why is ATGL increased? Is it via promoter occupancy? Effects on promoter activity in cells.

**Discussion**

Chronic glucocorticoid elevations are associated with, many co-morbidities such as increased fat mass, decreased muscle mass, insulin resistance and non-alcoholic fatty liver disease (NAFLD) in the lean setting, all of which can have a negative effect on metabolism. These side effects are similar those seen in obesity; however, the combination of chronically elevated glucocorticoids in the context of obesity has not assessed. Here we show that glucocorticoid-induced symptoms are exacerbated when paired with obesity, more than the sum of either effect alone.

Obese patients with Cushing’s disease were found to have a higher BMI than obese control patients and this was paired with increases in HOMA-IR score, indicating increased insulin resistance as well as increases in the liver enzyme ALT, a marker of liver disease. However, it is impossible to determine the physiological status of the patients before they got a tumor; therefore, we cannot discern whether obesity was present prior to tumor development or after tumor development, possibly as a result of the disease and contributing to the worsening of comorbidities. For this reason, we designed a mouse study to investigate whether being obese prior to glucocorticoid treatment leads to worsened outcomes.

HFD, dexamethasone-treated mice had hyperglycemia, which was not present in any of the other groups, as well as severe insulin resistance. Clamp data..

Significant elevations in liver fat accumulation was also seen in HFD-fed mice with even further increases in the HFD-fed, dexamethasone treated group, consistent with the elevated ALT levels seen in the obese Cushing’s patients.

To our surprise, the glucocorticoid treatment in obese mice led to an overall reduction in adiposity, which was not depot-specific. Previous work from our lab shows increased fat mass following chronic dexamethasone treatment (1), which has also been reported by others using various glucocorticoids. Therefore, increased fat mass is not the culprit contributing to worse insulin resistance and increased liver fat when comparing HFD control mice to HFD dexamethasone-treated mice.

Lipolysis has been associated with decrease fat mass, fatty liver disease, insulin resistance and is known to be induced with glucocorticoids in both humans (1) and mice. We assessed *in vivo* lipolysis in lean mice given glucocorticoids for six weeks measuring serum glycerol and fatty acid levels in the basal (fed) and stimulated conditions. Stimulation of lipolysis was achieved by a 16 hour fast or by i.p. injections of isoproterenol, a known -adrenergic agonist, to fed mice. Dexamethasone treatment led to significant increases in both glycerol and fatty acid release in all conditions when compared to controls. Elevations in markers of lipolysis were even greater in obese, dexamethasone-treated mice in the fasted state. Lipolysis has been linked to increased gluconeogenesis by several studies. One potential mechanism is that the increased flux of fatty acids, oxidized in the liver to acetyl-CoA, activate pyruvate carboxylase and redirecting TCA cycle intermediates towards gluconeogenesis. This indirect activation of gluconeogenesis is consistent with our observations of synergistic effects of glucocorticoids in adipose, but not liver tissues.

There is some debate as to which genes glucocorticoids are acting on to promote lipolysis.

Downregulation of *Pde3b* (4) and upregulation of -adrenergic receptors and/or lipase transcripts have all been proposed as possible mechanisms.

End: Glucocorticoids are a commonly prescribed drug used to treat a multitude of health issues and are known to induce a variety of metabolic side effects; however, their actions in persons with obesity has not been studied to date. The data presented here shows that obesity does in fact pose a greater risk for the harmful co-morbidities associated with chronically elevated glucocorticoids and this should be considered when determining treatment options. More work is required in the area to assess whether blocking glucocorticoid/lipolytic action in the fat tissue would be beneficial to prevent or enhance recovery of the afore-mentioned glucocorticoid-induced comorbidities.

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