**Introduction**

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

Obesity is often accompanied by a multitude of metabolic complications, such as insulin resistance (1–3) and non-alcoholic fatty liver disease (NAFLD; (4,5)). Similarly, chronically elevated glucocorticoids, such as is seen in Cushing’s disease or corticosteroid treatment, is often matched with impaired glucose tolerance or type 2 diabetes (6,7) and is associated with fatty liver (8,9), as well as changes in fat mass and distribution (10). 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 1-3% of US, UK and Denmark populations are prescribed corticosteroids at any given time and many are taking these medications long-term as treatment for chronic disease (11–14), 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 medicinal corticosteroids 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 seen with obesity and hypercortisolemia, 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 (15). On the other hand, there is evidence that some obese individuals are protected from insulin resistance (16,17). The area of fat storage (ie. subcutaneous vs. visceral) does seem to play a role (18,19), which may provide the link to these inconsistencies. Interestingly, insulin resistance is also common in lipodystrophy (20,21), a phenotype of very little adipose tissue, leading to ectopic fat deposition. These findings suggest that adiposity plays an important role, but the location matters more than the overall quantity… We and others have shown that high circulating glucocorticoids lead to insulin resistance (22–24); however, the underlying mechanism has yet to be elucidated. There is an array of physiological changes that occur as a result of elevated glucocorticoids including decreased lean mass (22,25,26), increased fat mass (22,27,28) and increased lipolysis (29–31), all of which have been associated with decreased insulin sensitivity (15,32,33). Likewise, increased fat mass, insulin resistance and increased fatty acid flux are associated with NAFLD.

Interestingly, it is suggested that higher lipid turnover (i.e. lipolysis) in visceral fat stores and this is what drives poor metabolic health observed in this phenotype, with subcutaneous fat thought to be protective of this (see reviews (18,19)). Indeed, basal lipolysis is enhanced in obesity (34) and is 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 (32) and it is likely that insulin resistance removes this break on lipolysis resulting in higher circulating glycerol and free fatty acids. It has also been shown that when inhibition of lipolysis is prevented, insulin is less able to suppress hepatic glucose production, suggesting lipolysis in turn affects insulin sensitivity (32,35).

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 and data collection

Patients were recruited and data was collected as described in Hochberg et al. (22).

* 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 (22) or were either kept on normal chow (NCD) or given high fat diet (45% fat; 35% carbohydrate; 20% protein; Research Diets, New Brunswick, NJ; ID D12451) for 12 weeks, depending on the experiment. Mice stayed on their respective diets and were treated with 1 mg/kg per day of dexamethasone (Sigma–Aldrich) in their drinking water (*n*=22) or used as controls (*n*=32) 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, unless otherwise noted. 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). Mice were given i.p. injections of insulin (Humulin R, Lilly, Indianapolis, IN, USA) at a concentration of 2.5 mU/g following a six hour fast. Blood glucose was determined using a One Touch Ultra Glucometer (Lifescan) prior to and every 15 minutes post injection.

* Clamp (get from metabolic phenotyping core?) For the Clamp experiment C57BL/6J adult (70d) male mice were fed the same HFD as above for 8 weeks and treated with dexamethasone for 3 weeks prior to the experiment.
* 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 (36). Media was then replaced including only insulin in the cocktail for an additional three days. For 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 using machine 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 (37). 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 (Cell Signaling Technologies), 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

Twenty-one-week-old *ad libitum* chow fed C57BL/6J male mice were briefly anesthetized with isoflurane at 11 weeks post dexamethasone treatment and blood was taken via retro orbital bleed at baseline and 15 minutes following an i.p. injection of 10mg/kg isoproterenol (Sigma-Aldrich) in PBS. Additionally, serum was taken from these mice as well as from another cohort of twenty-eight-week old mice on HFD or chow six weeks post dexamethasone treatment following an overnight fast, just prior to sacrifice. 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 0.05 was considered significant.

**Results**

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

Our group has previously published data (22) 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). There were no significant differences in BMI in the controls compared to the Cushing’s at either level (p=0.19628); however, there were significant differences in BMI status within the groups (p=0.00016), as was expected. Furthermore, obese Cushing’s patients had greater abdominal circumference when compared to obese controls, though this did not reach statistical significance. 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-treated mice exhibited fasting hyperglycemia, with a significant interaction between diet and drug (p=0.00009; Figure 1E). While HFD animals had a 24% increase in fasting glucose when compared to NCD animals, in the presence of Dexamethasone, HFD-fed animals had a 122% increase in fasting glucose relative to NCD controls not treated with dexamethasone. In the chow condition, dexamethasone caused a 18% decrease in fasting glucose.

To evaluate glucose homeostasis in more detail we performed a hyperinsulinemic euglycemic clamp in obese mice (11 weeks of HFD) treated with dexamethasone for three weeks. 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 was kept level throughout the entire clamp experiment and insulin clearance was not different between the groups (p=0.915; Supplementary FIgure 1G-H). During the insulin phase, the infusion rate was xx times lower in obese dexamethasone-treated mice when compared to obese controls (Figure 1E). Basal endogenous glucose production (EGP) was xx% higher in the dexamethasone treated group (p=0.026); moreover, In the dexamethasone group, EGP was reduced XX% but only XX% in the control group (p=0.0091) resulting in EGP 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 (p=0.141; 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 EGP and reduced suppression of EPG by insulin 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 NAFLD. We observed slight increases in plasma ALT, a liver enzyme associated with liver disease, in Cushing’s patients and obese controls; interestingly, levels were synergystically elevated in obese Cushing’s patients (Figure 2A).

Since elevated liver enzymes are just one indicator of liver disease and are not sufficient to lend a diagnosis, we studied this outcome in our mouse model. HFD-fed, Dexamethasone treated mice had drastically elevated liver triglycerides when compared to all other groups and there was a significant interaction of drug and diet (p=0.0000677; Figure 2B). In support of this, H&E staining of hepatic tissue clearly depicts exacerbated lipid levels in this group when compared to HFD-fed controls (Figure 2C).

Genes involved in hepatic *de novo* lipogenesis, *Srebf1* and *Fasn*, were assessed via qPCR (Figure 2D). There was a significant effect of diet and drug on *Fasn* expression (p=0.014). Though both transcripts were somewhat elevated in response to HFD alone, there was no difference between the dexamethasone-treated groups, regardless of diet. 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

We evaluated body mass in these mice via EchoMRI. Contrary to expectations, we observed reductions in fat mass in the HFD-fed dexamethasone treated group (Figure 3A,B). It is important to note that this loss was not depot specific (Figure 3C). There was no change in fat mass in response to dexamethasone treatment in the chow-fed group (Figure 3B). There were no significant differences in food consumption (Figure 3D). As expected, the HFD-fed mice had larger adipocytes compared to chow-fed mice, but there was no effect of dexamethasone on adipocyte size.

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, though the underlying mechanisms are unclear. We first assessed whether there was a direct effect of dexamethasone on adipocyte lipolysis in culture (figure 4A-D). 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 (p=) and increased glycerol release into the media (p=), 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*) was enhanced following dexamethasone treatment in 3T3-L1 cells at the transcript (p=) and protein (p=) level. These data show that glucocorticoids directly stimulate lipolysis in adipocytes.

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 dexamethasone enhances basal and stimulated lipolysis *in vivo* in chow-fed mice. Consistent with these findings, qPCR analysis in the inguinal white adipose tissue (iWAT) shows an upregulation of *Pnpla2* transcripts in the dexamethasone-treated mice compared to controls (p=; Figure 4F).

Since the HFD-fed, dexamethasone-treated mice are subject to more severe insulin resistance and hepatic lipid accumulation, we wanted to determine whether lipolysis was also elevated to a further extent that is seen in the chow-fed mice. To assess lipolysis, we measured serum glycerol following a 16-hour fast (Figure 5A). As expected, serum glycerol was elevated in dexamethasone treated animals, but this effect was even more robust in the obese animals and 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 support the hypothesis that glucocorticoid-stimulated lipolysis is augmented in the context of obesity.

It is important to note that we evaluated other lipolytic transcripts that have been proposed to be involved in glucocorticoid-induced lipolysis. These data can be found in the supplementary information. We were not convinced that these were primarily responsible as we did not find them to be consistently elevated following dexamethasone treatment; therefore, we did not include these data in the main findings.

**Subtitle goes here**

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 (22,27,28), decreased muscle mass (22,25,26), insulin resistance (6,7) and non-alcoholic fatty liver disease (NAFLD; (8,9)), all of which can have a negative effect on health (38–41). These side effects are similar those seen in obesity; however, the combination of chronically elevated glucocorticoids in the context of obesity has not been 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 and abdominal circumference than obese control patients. Moreover, 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. In line with these findings, increased in central adiposity, such as is seen in these patients, has been previously associated with enhanced fatty acid flux (i.e. lipolysis) when compared to lower body fat stores (42), which is thought to contribute to insulin resistance and fatty liver (32,43). However, it is impossible to determine the physiological status of the patients before they developed a tumor; therefore, we cannot discern whether obesity was present prior to or after their Cushing’s status, 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.

We found that HFD-fed, dexamethasone-treated mice had hyperglycemia, which was not present in any of the other groups, as well as severe insulin resistance when compared to all other groups. This was primarily due to increased endogenous glucose production. Normally, elevations in insulin will suppress glucose production (35), but this was not the case in the HFD-fed dexamethasone treated mice. These findings indicate that chronic glucocorticoid treatment in obese mice leads to aberrant or even absent insulin signaling, at least in the liver.

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. Cushing’s disease is often paired with increased fat mass, which has been previously proposed to contribute to fatty liver (44,45). Indeed, obesity is a known risk factor of NAFLD (4,5). Previous work from our lab shows increased fat mass, specifically in the iWAT, following 12 weeks of dexamethasone treatment (22) in chow-fed mice, and increased fat mass has also been reported by others using various glucocorticoids. However, to our surprise, the glucocorticoid treatment in obese mice led to an overall reduction in adiposity, which was not depot-specific. Therefore, when comparing HFD control mice to HFD dexamethasone-treated mice, increased fat mass is not cause of the observed exacerbations in insulin resistance and increased liver fat.

Lipolysis has been implicated in insulin resistance (32,35) and NAFLD and there is evidence to support it is enhanced in the obese state; moreover, glucocorticoids are known to stimulate lipolysis. 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. Dexamethasone treatment led to significant increases in glycerol and fatty acid release in basal and stimulated conditions when compared to controls. Elevations in markers of lipolysis were even greater in obese, dexamethasone-treated mice in the fasted state. Furthermore, we showed that dexamethasone acts directly on adipocytes to stimulate lipolysis. In support of these findings, inhibition of 11b-hsd1, an enzyme responsible for local activation of cortisol, in adipose tissue prevents glucocorticoid-induced lipolysis and hepatic; however, there is no reduction in lipolysis or hepatic steatosis in response to glucocorticoids when this enzyme is knocked out in the liver (46). Though the authors did not assess glycemic responses to exogenous insulin or glucose in the tissue specific knockout mice, total body knockouts had impaired glucose tolerance and were less insulin sensitive, as determined by HOMA-IR. Similarly, mice treated with 11b-Hsd1 shRNA were protected from glucocorticoid-induced reductions in insulin signaling, both in the adipose tissue as well as systemically, and this was paired with reduced epididymal lipolytic signaling in response to glucocorticoids (47). Taken together, adipose tissue lipolysis appears to play an important role in glucocorticoid-induced fatty liver and insulin resistance.

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* (48) 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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