# Introduction

Obesity is chronic disease that affects approximately 40% of the US population (7). Obesity increases one’s risk of type 2 diabetes, hypertension and insulin resistance. Insulin resistance is also a common side effect of glucocorticoid action which is important because insulin promotes adipocyte differentiation and lipogenesis (10, 11). Obesity is defined by excess adipose tissue and glucocorticoids can also play a role in both catabolic and analobic effects on adipose tissue. Though it is well-known that glucocorticoids induce muscle weakness, it is unclear how obesity modifies muscle atrophy in response to glucocorticoids.

Skeletal muscle is vital for most everyday basic functions and maintenance of health. However, many factors including poor nutrition, lack of exercise, and a myriad of diseases can lead to loss of skeletal muscle (15). One causal factor in muscle loss is elevated glucocorticoids. Glucocorticoids are steroid hormones that function through the Glucocorticoid Receptor (GR, encoded by *Nr3c1*) to alter tissue-specific gene expression (21). Exogenous glucocorticoids induce muscle atrophy through increased muscle proteolysis and inhibition of protein synthesis in lean mice (29). The estimated prevalence of oral glucocorticoids usage in the United States is 1.2% for a variety of health concerns including asthma, chronic stress, chronic obstructive pulmonary disease, and a range of autoimmune disorders (20). Elevated levels of glucocorticoids within the human body have shown to cause skeletal muscle atrophy. This muscle atrophy stems from an upregulation of atrogenes and other factors which promote muscle protein breakdown (22, 27, 29).

Muscle proteolysis is in part caused by glucocorticoid induction of atrogenes, a set of E3 Ubiquitin ligases including MuRF1 and Atrogin-1, downstream of the FOXO pathway (13, 28). These E3 ligases target muscle proteins for degradation, under normal circumstances to provide substrates for gluconeogenesis. The inhibition of protein synthesis is believed to be directed by inhibition of the mTORC1 pathway associated with muscle growth by glucocorticoids (29).

Preliminary work from our group and others have demonstrated that glucocorticoids promote adipocyte gene transcription, lipolysis, glucose production and insulin resistance in obese, relative to lean animals (8, 11). In this thesis, I demonstrate that both lean and obese mice develop reductions in lean mass, muscle mass, and grip strength when treated with dexamethasone and these effects are higher in obese mice. We also show that obese, dexamethasone treated mice had transient induction of muscle degradation transcripts including *Fbxo32* and *Trim63*, (Atrogin-1 and MuRF1 respectively) and their upstream regulator *Foxo3*. Lastly we will show the obese dexamethasone-treated mice are profoundly insulin resistant, even after accounting for reductions in muscle mass.

# Methods

## Animal Husbandry

Male C57BL/6J mice were purchased from The Jackson Laboratory at 9 weeks of age and randomized into groups of 4 animals/cage. All animals were on a light/dark cycle of 12 hours and housed at 22°C. At 10 weeks of age, mice were placed on a high-fat diet (HFD; 45% fat from lard, 35% carbohydrate mix of starch, maltodextrin, and sucrose, and 20% protein from casein, Research Diets cat # D12451) or kept on a normal chow diet (NCD; 13% fat, 57% carbohydrate, and 30% protein,Teklad 5LOD) for 12 weeks. At 22 weeks, mice were either treated with vehicle (water) or 1 mg/kg/d of dexamethasone (Sigma-Aldrich; catalog #2915) dissolved in their drinking water. All mice were provided with *ad libitum* access to food and their respective waters throughout the study. Food and liquid consumption were measured weekly to determine the concentration of dexamethasone consumed per cage and volumes were averaged per mouse per cage. All procedures were approved by the University of Michigan or University of Tennessee Health Sciences Center Institutional Animal Use and Care Committees.

## Grip Strength

Mice were tested using a grip strength meter with a Chatillon digital force gauge (AMETEK). These mice were treated for six weeks with their respective waters. A grip strength baseline was established per mouse and all measurements were reported in force (N). Mice were placed on a grid attached to the meter and once all four paws had contact with the grid, the mice were slowly pulled backwards by the tail until they left the grid. Each mouse was tested five times and given approximately 10 seconds rest in between each test. Final measurements for grip strength were assessed by taking the average of the five trials and reported as average peak force (N).

## Contractile Measurements

All contractile properties were measured for gastrocnemius muscles *in situ*. After the mouse was anesthetized using isoflurance, the right gastrocnemius muscle was carefully isolated and a 4–0 silk suture was tied around the distal tendon. After the tendon was secured, the tendon was cut so the hindlimb could be secured at the knee to a fixed post. Animals were placed on a temperature-controlled platform with continual drip of saline over the gastrocnemius at 37°C to keep with muscle warm and moist. The distal tendon of the gastrocnemius muscle was tied to the lever arm of a servomotor (6650LR, Cambridge Technology). In order to measure force generated at the nerve, a bipolar platinum wire electrode was used to stimulate the muscle at the tibial nerve.

The voltage of the electrode pulses was incrementally adjusted to find maximum isometric twitch and the muscle length was altered to find the optimal length (Lo). Optimal length is the length of the muscle in which the maximal twitch force was obtained. Once Lo was found, gastrocnemius muscles were kept at that length (Lo) and the frequency of pulses was increased in increments of 300-ms to obtain maximum isometric tetanic force (Po). In order to measure force generated at the muscle, an electrode cuff was placed around the mid-belly of gastrocnemius for muscle stimulation. The process was then repeated as done for the nerve.

After all force measurements, both gastrocnemius and quadricep muscles were dissected, weighed, and snap frozen in liquid nitrogen. Mice were sacrificed under anesthesia via removal of vital organs and muscles were stored at -80℃.

## Histology and Fiber Type Quantifications

Quadriceps were collected and frozen in 2-methyl-butane cooled under liquid nitrogen. Quadricep samples were sectioned using a CryoStar NX350 HOVP Cryostat (Thermo Scientific) at -20°C with a thickness of 10um through the mid-belly and mounted on SuperFrost glass slides (Electron Microscopy Sciences, 71882-01). For analysis of fiber cross-sectional area (CSA), fibers were assessed by hematoxylin and eosin (H&E staining) and for fiber-type, muscles were stained using NADH-NBT staining (9, 31). Each section of mouse quadricep was imaged in four times; topleft, topright, bottom-left and bottom right photos were taken. The images were taken using a 20x objective of an EVOS XL digital inverted microscope. Muscle fibers were indivdiually counted in each image and the cross-sectional area was measured by outlining 150 randomly chosen fibers per image and using ImageJ (2).

## Cell Culture

C2C12 cells were cultured in 10% Fetal Bovine Serum (FBS), Dulbecco's Modification of Eagle's Medium (DMEM; 4.5 g/L D- glucose; Fisher Scientific; catalog #11965118) with penicillin, streptomycin and glutamine (PSG). Cells were split at approximately 75% confluency and differentiated using DMEM, 1x PSG with 2% Horse serum until myotubes were obtained. Media was replenished as needed until myotube differentiation was complete around one week. Myotubes were treated with 250 nM dexamethasone for either 2, 4, 8, 12, or 24 hours or left untreated. All cells were kept in a 5% CO2 regulated incubator at 37 °C. After treatment, cells were homogenized in TRIZol using a TissueLyser II (Qiagen) and prepared for RNA extraction using a PureLink RNA kit (Life Technologies, cat # 12183025).

## mRNA Quantification

Cells and tissues were lysed in TRIzol using a TissueLyser II (Qiagen) and RNA was extracted using a PureLink RNA kit (catalog no. 12183025; Life Technologies) following manufacterer’s instructions. Complementary DNA (cDNA) was synthesized using the High Capacity cDNA Reverse Transcription Kit without RNAse inhibitor (catalog no. 4368813; Life Technologies). Quantitative Real-Time Polymerase Chain reaction (qPCR) was performed using a QuantStudio 5 (Thermo Fisher Scientific) with primers, complementary DNA, and Power SYBR Green PCR Master Mix (catalog no. 4368708; Life Technologies) per manufacturer’s instructions. Messenger RNA (mRNA) expression levels were normalized to control gene, *Rplp13*, and analyzed.

LIST OF PRIMERS

|  |  |  |
| --- | --- | --- |
| **Gene** | **Forward 5’-3’ Sequence** | **Reverse 5’-3’ Sequence** |
| *Fbxo32* | CTTCTCGACTGCCATCCTGG | GTTCTTTTGGGCGATGCCAC |
| *Trim63* | GAGGGCCATTGACTTTGGGA | TTTACCCTCTGTGGTCACGC |
| *Foxo1* | AGTGGATGGTGAAGAGCGTG | GAAGGGACAGATTGTGGCGA |
| *Foxo3* | AAACGGCTCACTTTGTCCCA | ATTCTGAACGCGCATGAAGC |
| *Rplp13* | GCGGATGAATACCAACCCCT | CCTGGCCTCTCTTGGTCTTG |

Assessment of Insulin Tolerance

Insulin tolerance testing took place between ZT8 and ZT10 following a 6-hour day-time fast. Mice were assessed for basal glucose levels using a handheld glucometer (Accu-chek) from the tail vein. Insulin was then quickly administered 0.75IU per kg of lean mass for lean mice as determined by MRI and 1.5IU per kg of lean mass for obese mice via intraperitoneal injection (8). Glucose was measured in 15 minutes intervals for a total of two hours following insulin administration.

## Lean Mass Determination

The animal’s lean mass was determined weekly using a EchoMRI 2100. Mice were placed in plastic holding tube without sedation or anesthesia. The holder is then inserted into the EchoMRI machine.

## Statistics

All results are represented as mean ± SEM. Two-Way ANOVA analyses, mixed linear models and Chi-squared tests were performed to test for significance and determine interactions between diet and dexamethasone treatment. Pairwise testing was performed after assessing normality and equal of variances. If Shapiro-Wilk test was insignificant, a Levene’s tests was performed and followed by either a Welch’s or Student’s *t*-test as noted in the figure legends. Any p-value under 0.05 was considered significant. All statistical tests were conducted using R version 3.5.0 (24).

# Results

## Greater Losses in Grip Strength in Obese-Dexamethasone Mice

As a test to assess the effect of glucocorticoids on muscle strength, we treated lean and obese male mice with dexamethasone for five weeks and measured grip strength. Dexamethasone treatment resulted in reductions in grip strength in both lean and obese mice when compared to their counterparts (Figure 1A-B). Obese dexamethasone-treated mice had greater overall losses in grip strength when compared to the lean animals. For mean grip strength, we saw a 4.8% reduction in lean animals (p=0.007) but a 26.2% reduction in grip strength for obese animals (p=3.6x10-5).

## Reductions in Strength are Related to Smaller CSA

In order to expand upon these results, we measured the force generated by gastrocnemius muscle *in situ* both by stimulation of the nerve and by direct electrical stimulation of the muscle. In NCD animals, the force generated by nerve stimulation was reduced 10.2% when treated with dexamethasone. However in HFD animals, force generated by nerve stimulation was reduced 32.2% when treated with dexamethasone, with a significant interaction between pre-existing obesity and dexamethasone treatment (p=.009 Figure 1C). These results are concordant with results from direct muscle stimulation. In NCD animals, force generated by direct muscle stimulation was reduced 10.6% when treated with dexamethasone, while in HFD animals, the force generated by direct muscle stimulation was reduced 30.2% when treated with dexamethasone (pinteraction=.024, Figure 1D).

In order to examine whether changes in muscle strength were proportional to declines in muscle size, we plotted a regression of force (mN) versus whole-muscle cross-sectional area (CSA). The cross-sectional area explained 59% and 58% of the variance in force at the nerve and muscle respectively. As cross-sectional area declined muscle force by both stimulations decreased in proportion. Pre-existing obesity did not modify this force-area relationship (p=0.42). These data indicate that pre-existing obesity causes more dramatic dexamethasone-induced muscle weakness, but this is largely explained by reductions in muscle size.

## Enhanced Muscle Loss in Obese Mice

To determine whether obesity and glucocorticoid treatment promoted losses in muscle mass, we treated lean and obese male mice with dexamethasone for five weeks. Dexamethasone caused a reduction in lean mass in both lean and obese mice. Consistent with losses in strength, obese-dexamethasone treated mice had greater losses in lean mass (pinteraction = 6.32e-14; Figure 2A). This loss in lean mass is consistent with previously reported effects of glucocorticoids on muscle atrophy (23) (28). At sacrifice, the NCD animals quadricep and triceps surae weights were smaller by 17.6% and 11.5% in the dexamethasone treated. While in HFD animals, quadricep and tricep surae weights were smaller by 42.3% and 33.1% in the dexamethasone treated mice (for quadriceps pinteraction = 1.50×10-5; for tricep surae pinteraction = 0.003 Figure 2B).

We next evaluated short-term dexamethasone treated animals by placing male mice on vehicle or dexamethasone for two weeks to match our isometric force testing. The obese, dexamethasone-treated animals had enhanced reductions gastrocnemius weights and whole-muscle cross-sectional area (Figure 2C-D). At sacrifice, the NCD animals’ gastrocnemius weights were smaller by 12.7% in the dexamethasone treated group but 27.2% in the HFD group (pinteraction=0.021). Similarly, cross-sectional area of the muscle was reduced 13.1% in the NCD group and 22.9% in the HFD group though the modifying effect of obesity did not statistical significance (pinteraction=.110).

## Obesity with Dexamethasone Treatment Resulted in Smaller Muscle Fibers

In order to assess changes at the individual muscle fiber-level, we cryosectioned the 5-week dexamethasone-treated mice quadriceps at the mid-belly and H&E stained these samples (Figure 2E). The NCD animal’s muscle fibers were smaller by 17.4% in the dexamethasone treated and in HFD animals muscle fibers were smaller by 54.7% in the dexamethasone treated mice (pinteraction=.001; Figure 2F).

## Dexamethasone did not Induce Changes Fiber-Type Composition

In order to assess any changes in the ratio of oxidative versus non-oxidative fiber-types, we stained muscle sections and quantified the muscle fibers based upon their oxidative capacity. Mouse skeletal muscle is made up Type I, Type IIa, Type IIb, and Type IIx fibers (30, 31). Oxidative fibers or Type I fibers stain the darkest (Figure 2G). We found no significant change in the ratio of oxidative to total fibers in the mice quadriceps in lean or obese mice treated with dexamethasone (Figure 2H).

## Dexamethasone Reduced Type II Fiber Cross-Sectional Area

Though we did not see changes in composition of fiber types, we did observe fiber-type specific reductions in fiber size. Dexamethasone-treatment reduced type IIa or light-stained fibers CSA in lean and obese mice by 28.0% and 39.8% respectively though the moderating effect of obesity did not reach statistical reference(pinteraction=0.494). Dexamethasone treatment also reduced type IIb or medium-stained fibers CSA in lean and obese by 35.1% and 32.3% respectively (pinteraction=0.584). As for type I or dark-stained fibers, dexamethasone treatment only reduced fiber CSA in NCD animals. Though dexamethasone treatment reduced type I fiber CSA by 20.7% in lean, the treatment increased fiber CSA in obese mice by 14.2% (pinteraction=p=0.003; Figure 2I). This outcome is consistent with previous data shown in which plantares muscles from mice treated with dexamethasone for 13 days showed significant atrophy in Type IIb and Type IIa and not in Type I fibers (26).

## Short-term Dexamethasone-Treatment Induced Muscle Degradation Transcripts as seen *in vitro*

It is well established that dexamethasone treatment induces expression of muscle atrophy-related genes (13, 27, 33). To better understand these cell-autonomous effects we treated C2C12 myotubes with dexamethasone over time in order to assess the expression of *Foxo1*, *Foxo3*, and the atrogenes, MuRF1 and Atrogin-1 (encoded by *Trim63* and *Fbxo32* respectively)*.* Relative expression of all genes were elevated after 2 hours of treatment with dexamethasone (Figure 3A).

To evaluate the molecular effects of dexamethasone *in vivo* and how this was moderated by obesity, we treated lean and obese mice with dexamethasone and evaluated atrogene expression. After one week of dexamethasone treatment, we observed a greater induction of both *Foxo3* and the atrogenes, *Trim63* and *Fbxo32*, in obese mice as compared to their lean counterparts though the interaction between obesity status and dexamethasone treatment did not reach statistical significance for these transcripts (Figure 3B). The expression of *Trim63, Fbxo32,* and *Foxo3* was elevated in obese mice than their lean counterparts*.* However we did not observe an increase in *Foxo1* or *Ncr31*, glucocorticoid receptor. These data suggest that the obesity-sensitizing effects on muscle atrophy could be concordant with elevations of FOXO3 and these two atrogenes.

### Obese Dexamethasone-Treated Mice are Insulin Resistant

Since we have highlighted that obesity can enhance steroid-induced skeletal muscle atrophy, we next evaluated insulin resistance as the majority of all postprandial glucose uptake occurs within the muscle (4). In lean animals, there was no significant change in fasting blood glucose with a reduction of 6.20% between treatment groups however there was a 43.5% increase in fasting blood glucose in obese animals given dexamethasone (pinteraction=0.033; Figure 4A), consistent with our previous report (8).

In order to evaluate whether the dexamethasone-treated animals were insulin resistant, we treated the same lean and obese mice with insulin at doses relative to their lean mass composition to account for their difference in muscle mass between dexamethasone treated and control mice. In both NCD and HFD animals, dexamethasone induced near complete insulin resistance (p= 8.8 x 10-12 for NCD and 7.7 x 10-7 for HFD; Figure 4B). Notably HFD mice and NCD mice were given different doses of insulin, so that near-equivalent insulin responses could be observed. These data suggest that even after accounting for change in muscle mass, glucocorticoids still cause insulin resistance.

# Discussion

Here I show that dexamethasone treatment in concert with pre-existing obesity caused elevated reductions in muscle strength, size and insulin sensitivity. Muscle weakness is a common side effect of exogenous glucocorticoid consumption as well as continually elevated levels of endogenous glucocorticoids (6, 29). For example, adult who had elevated salivary cortisol had a significantly higher risk of loss of grip strength than their peers (21). My research could be particularly important because those with obesity are more likely to have reduced muscle function (1, 12, 17, 35) Notably people with obesity are also more likely to have elevations in endogenous glucocorticoid levels (25, 34).

We show, consistent with previous reports that glucocorticoid-dependent reductions are more dramatic in type II muscle fibers(6, 26). In addition to steroid-induced atrophy, there are a variety of conditions and lifestyle factors such a bed-rest that also lead to other significant myofiber changes. For instance, disuse atrophy as a result of denervation or immobilization of a limb, reduces type I fiber size and muscle mass (19, 26, 32). Even though we saw no change in fiber composition when treating animals with dexamethasone, an other study has shown that dexamethasone reduces both that proportion and size of type II fibers in muscles in rats (3). This discrepancy could be due to the proportion of dexamethasone provided to the animals; our dose is consistent with that of a human taking a high prescription dose and their dose was approximately 60% of our dose and administered via intraperitoneal injection (8). The discrepancy could also be due fiber composition of the muscle they chose to evaluate, gastrocnemius and flexor digitorum superficialis combined, compared to our examination of the quadriceps.

It is also important to note that glucocorticoids induce muscle atrophy in a muscle specific manner. Researchers often test on mouse hindlimb muscle because they are fairly large and accessible load bearing muscles. Specifically, type II fibers are more prone to the effect of glucocorticoids (6, 14, 29) so it is possible that muscles with higher concentrations of type II fibers may be more vulnerable to obesity and steroid-induced atrophy. For example, rats treated with dexamethasone for two weeks had no significant reduction in mean fiber CSA in their solei but had significant reduction in their plantares muscles, which have higher type II fiber composition (26). A study has shown increased losses in contraction force of muscle wither higher percentages of type IIa fibers with and without a DHPR blocker on muscles with different fiber type composition (18).

This work demonstrates that obesity enhances the ability of glucocorticoids to reduce muscle function and size. How this increased responsiveness occurs is not currently clear. One possibility is that obesity remodels the chromatin landscape, allowing for easier GR access. Another is that the effects of GR-dependent signaling are promoted by insulin resistance. A third possible theory is that first excess adipose tissue contributes pro-inflammatory cytokines that act upon skeletal muscle and then as an additive effect, glucocorticoids function to increase skeletal muscle degradation. Pro-inflammatory cytokines have catabolic effects on protein metabolism and anabolic effects such as reduced *de-novo* protein synthesis. Tumor necrosis factor alpha has been shown to directly act on muscle cells to induce protein degradation in C2C12 myotubes (16). It is possible that excess adiposity could sensitize muscles to degradation and glucocorticoids function as a second-hit of catabolism, which would lead to exacerbated muscle weakness. For example leptin-receptor deficient, obese mouse mice were found to have increase in interleukin 1beta and TNFa in the brain, a lowered threshold for release of pro-inflammatory cytokines and significant microgliosis (5).

Glucocorticoids and obesity have deleterious health effects. These effects include loss of skeletal muscle which may result in reduced motor function, coordination, and energy production (12, 17, 35). Insulin resistance is an additional negative effect associated with both elevated glucocorticoids and excess adiposity in the body (8, 10, 22). The process by which these factors induce insulin resistance is not yet fully understood. In this study, I have highlighted that dexamethasone-induced muscle atrophy is exacerbated in an obese mouse model, as evidenced by synergistic reductions in muscle function, muscle mass, and fiber-specific cross-sectional area.

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# Figure Legends

**Figure 1. Obese-Dexamethasone Treated Mice Lost Significant Muscle Strength**

Grip strength (N) in lean (A) and obese (B) male mice over the course of six weeks of dexamethasone treatment. N=4-8 per group. Data collected by Innocence Harvey. Force generated by nerve stimulation (C) and by direct muscle gastrocnemius stimulation (D) in lean and obese mice treated with dexamethasone for 15-21 days. Force plotted relative to whole gastrocnemius cross-sectional area (E-F). Asterisks indicate significant interaction between diet and dexamethasone treatment by two-way ANOVA (n=5-8 per group).

**Figure 2. Obese-Dexamethasone Treated Mice had Reduced Lean Mass, Muscle Weights, and Type II Fiber CSA.**

Lean mass determined via EchoMRI (A) and muscle weights (B) in lean and obese mice following 6 weeks of dexamethasone treatment (n=8-22 per group). Data collected by Innocence Harvey. Gastrocnemius weights (C) and cross-sectional area (D) in lean and obese mice treated with dexamethasone for 15-21 days (n=5-8 per group). H&E stained section of quadriceps (E) from mice treated with vehicle (water) or dexamethasone for six weeks. Average fiber cross-sectional area (F) averaged from 200 fibers per quadricep section (n=4 mice per group). NADH-NBT stained section of quadriceps (G) from mice treated with vehicle (water) or dexamethasone for six weeks. Percent of oxidative or type I fibers to total fibers (H; n=4). Average fiber cross-sectional area separated by NADH-NBT staining density with dark fibers indicating oxidative or type I muscle fibers (I). Asterisks indicate significant interaction between diet and dexamethasone treatment by two-way ANOVA.

**Figure 3. Short-term Dexamethasone Treatment Induced Muscle Degradation Transcripts unlike Chronic or Long-Term Treatment**

Relative atrogene (*Fbxo32, Trim63, Foxo1* and *Foxo3*) expression in C2C12 myotubes treated with 250 nm dexamethasone for 2, 4, 8,12, or 24 hours or left untreated(a). After treatment, cells were homogenized and prepared for RNA extraction.

Atrogene expression in mice treated for either 72 hours, one week, or two weeks with vehicle(water) or 1mg/kg/d dexamethasone (b). RNA was extracted from the quadriceps. \*=Significance identified by Student’s T-Test and in mice treated for six weeks with vehicle (water) or dexamethasone. \*=Diet-Treatment interaction identified by Two-Way ANOVA. N=8 per group.

**Figure 4. Dexamethasone Treatment Induced Insulin Resistance** Blood glucose values taken from the tail vein in lean and obese male mice after a 6-hour fast and two weeks of dexamethasone or vehicle (water) treatment (a). \*=Diet-Treatment interaction identified by Two-Way ANOVA. N=4 mice per group. Glucose values after insulin administration at time 0, following a 6-hour fast (b). Insulin was given via intraperitoneal injection at .75g/kg lean mass for lean mice and 1.5g/kg for obese mice.N=4 mice per group.