# **Title:** Obesity promotes glucocorticoid-dependent muscle atrophy in male mice.

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# Abstract

# Introduction

Skeletal muscle is vital to normal function and to the maintenance of health. Muscle is central to the regulation of lipid, glucose and amino acid metabolism , processes which are commonly dysregulated during times of illness or disease (44). However, many factors including age, poor nutrition, lack of exercise, medication, stress and diseases can lead to loss of skeletal muscle and function, with attendant reductions in lifespan and health-span (21). One causal factor in muscle loss is elevated glucocorticoids, either pharmacologically or as the result of chronic stress. It is estimated that 1-2% of individuals in the USA and UK are on a prescription glucocorticoid (26, 39). Obesity affects approximately 40% of the American population (10). The disease is characterized by excess adipose tissue that can lead to glucose and lipid dysregulation (17). Obesity increases the risk of type 2 diabetes, hypertension and insulin resistance among other chronic diseases (14). Previous work by our group and others has demonstrated that glucocorticoids and obesity may have synergistically detrimental effects (2, 3, 11, 15, 38).

Glucocorticoids induce muscle atrophy through increased muscle proteolysis and inhibition of protein synthesis in lean mice (15, 35). Glucocorticoids are also elevated during chronic stress or prolonged fasting (7, 40). Elevated levels of glucocorticoids within the human body have shown to cause skeletal muscle atrophy (6, 20, 27, 35). This muscle atrophy stems from an upregulation of atrogenes and other factors which promote muscle protein breakdown (28, 33, 35).

In this manuscript, we provide data that both lean and obese mice develop reductions in lean mass, muscle mass, and grip strength when treated with dexamethasone and these effects are increased in obese mice. I also show that obese, dexamethasone treated mice had elevated induction of key atrophy-inducing 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 reduced muscle mass.

# Methods

## Animal Husbandry

Male C57BL/6J mice were purchased from The Jackson Laboratory at nine weeks of age and randomized into groups of 3-4 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 no. D12451) or kept on a normal chow diet (NCD; 13% fat, 57% carbohydrate, and 30% protein; Teklad catalog no. 5LOD) for 12 weeks. At 22 weeks, mice were either treated with vehicle (water) or 1 mg/kg/d of dexamethasone (Sigma-Aldrich; catalog no. 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 animal 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). 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°C.

## 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, catalog no. 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 as described in (12, 37). Light-stained fibers were labeled as Type IIB fibers, medium-stained fibers as Type IIA and dark-stained as Type I fibers. 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 individually counted in each image by a blinded investigator and the cross-sectional area was measured by outlining 150 randomly chosen fibers per image and using ImageJ (5).

## 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 manufacturer’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 a control gene, *Rplp13* after evaluating eight control gene candidates (Primer sequences in Table 1).

Assessment of Insulin Tolerance

Insulin tolerance testing took place between ZT8 and ZT10 following a 6-hour fast. Mice were assessed for glucose levels using a handheld glucometer (Accuchek) with blood drawn from the tail vein. Insulin (Humulin R, Lilly) was then administered at 0.75IU per kg of lean mass for lean mice determined by MRI and 1.5IU per kg of lean mass for obese mice via intraperitoneal injection. Glucose was measured in 15 minutes intervals for a total of two hours following insulin administration.

## Body, Fat, and Lean Mass Determination

Body weight was measured using a digital scale. Fat and and lean mass were determined using a EchoMRI 2100 (EchoMRI), without sedation or anesthesia.

## 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, followed by Welch’s or Student’s *t*-test as noted in the figure legends. For non-normally distributed data, a Mann Whitney U-test was used. A p-value under 0.05 was considered significant. All statistical tests were conducted using R version 3.5.0 (30). All raw data and analysis scripts are available at <http://bridgeslab.github.io/CushingAcromegalyStudy/>.

# Results

In order to assess diet-induced obesity in mice, we randomized mice into diets of chow or high fat diet. Prior to randomization into dexamethasone treatments animals had approximately the same percent body fat mass at 30% (Table 1). Upon randomization, we also evaluated food intake during the course of treatment to determine the possible origin of changes in adiposity. HFD-dexamethasone animals consumed approximately 70% more calories per day than water controls. Even HFD dexamethasone mice ate the most calories, they lost fat mass when compared to their HFD counterparts (Table 1) consistent with our prior data (11). Our prior work demonstrated substantial elevations of glucocorticoid ingestion over a five-week period, an effect we propose was secondary to their diabetic phenotype. This shorter exposure had elevated dexamethasone exposure in HFD relative to NCD animals, though this did not reach statistical significance.

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

As a test to assess the effect of glucocorticoids on muscle strength, we 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 Cross-Sectional Area

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). 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=0.024, Figure 1D). These data suggest a muscle-dependent phenotype as both nervous and direct muscle weakness was detected.

In order to examine whether changes in muscle strength were proportional to declines in muscle size, we plotted a regression of force versus whole-muscle cross-sectional area (CSA). The cross-sectional area explained 64% and 59% of the variance in force stimulated at the nerve and muscle respectively. As cross-sectional area declined muscle force by both stimulations decreased in proportion. Regression modeling showed that pre-existing obesity did not significantly modify this force-area relationship (Nerve Stimulation: p=0.47, Muscle Stimulation: p=0.42). These data indicate that pre-existing obesity causes elevated dexamethasone-induced muscle weakness, but that this is largely explained by reductions in muscle size.

## Enhanced Muscle Atrophy in Obese Mice

The obese, dexamethasone-treated animals had enhanced reductions in fat free mass (Figure 2A), gastrocnemius weight and whole-muscle cross-sectional area (Figure 2B-C). At sacrifice, the NCD animals’ gastrocnemius weights were smaller by 13% in the dexamethasone treated group but 27% in the HFD group (pinteraction=0.021). Similarly, cross-sectional area of the muscle was reduced 13% in the NCD group and 23% in the HFD group though the modifying effect of obesity did not statistical significance (pinteraction=.11).

## Obesity with Dexamethasone Treatment Resulted in Smaller Type II Muscle Fibers

In order to assess changes at the individual muscle fiber-level, we cryosectioned dexamethasone-treated mice quadriceps at the mid-belly and H&E stained these samples (Figure 2D). The NCD animal’s muscle fibers were smaller by 17% in the dexamethasone treated and in HFD animals muscle fibers were smaller by 55% in the dexamethasone treated mice (pinteraction=.0010; Figure 2E).

In order to assess any changes in the ratio of oxidative versus non-oxidative fiber-types, I 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 (36, 37). Oxidative fibers or Type I fibers stain the darkest (Figure 2F). 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 2G). 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% and 40% respectively though the moderating effect of obesity did not reach statistical reference (pinteraction=0.49). Dexamethasone treatment also reduced Type IIb or medium-stained fibers CSA in lean and obese by 35% and 32% respectively (pinteraction=0.58). 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 21% in lean, the treatment increased fiber CSA in obese mice by 14% (pinteraction=p=0.0031; Figure 2I).

## Obesity and dexamethasone cause elevated atrogene expression

To evaluate the molecular effects of dexamethasone *in vivo* and how this was moderated by obesity, we evaluated atrogene expression in quadriceps. 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, I did not observe a dexamethasone-induced increase in *Foxo1* or *Ncr31*, glucocorticoid receptor. These data suggest that the obesity-sensitizing effects on muscle atrophy could be related to elevations of FOXO3 and these two atrogenes.

### Obese Dexamethasone-Treated Mice are Insulin Resistant After Adjusting for Muscle Mass

Since obesity can enhance steroid-induced skeletal muscle atrophy, I next evaluated insulin sensitivity in these mice as the majority of all postprandial glucose uptake occurs within the muscle (8). In lean animals, there was no significant change in fasting blood glucose with a reduction of 6.2% between treatment groups however there was a 44% increase in fasting blood glucose in obese animals given dexamethasone (pinteraction=0.033; Figure 4A), consistent with our previous report (11). In order to evaluate whether the dexamethasone-treated animals were insulin resistant after accounting for reductions in lean mass, I treated lean and obese mice with insulin at doses relative to their lean mass that day. 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). In these experiments 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 we demonstrate that dexamethasone treatment in concert with pre-existing obesity caused elevated reductions in muscle strength, size and insulin sensitivity in mice. Muscle weakness is a common side effect of exogenous glucocorticoid consumption as well as continually elevated levels of endogenous glucocorticoids (9, 35). For example, adults who had elevated salivary cortisol had a significantly higher risk of loss of grip strength than their peers (27). This work could be particularly important because those with obesity are more likely to have reduced muscle function (1, 16, 24, 45). Notably people with obesity are also more likely to have elevations in endogenous glucocorticoid levels (31, 42).

We used exogenous glucocorticoid treatment in the form of dexamethasone, a fluorinated synthetic glucocorticoid. Our dose of dexamethasone treatment is equivalent to a human dose of 0.081 mg/kg/d, which is comparable to a high therapeutic dose administered to human patients which usually can range from 0.002-0.2mg/kg/d (4, 22, 23, 25) Our data agrees with prior research my our group that has shown that obese mice drank slightly more dexamethasone water, which this is a limitation to our findings (11).

Glucocorticoids induce muscle atrophy in a muscle and fiber-type specific manner. Specifically, consistent with our findings, type II fibers are more prone glucocorticoids (6, 9, 20, 32, 35). It is plausible that a loss in non-oxidative fibers could reduce a human’s ability to use short bursts of energy, make rapid postural changes or lift heavy objects (43).

The mechanisms underlying increased responsiveness to dexamethasone in obese animals occurs is not currently understood. One possibility is that obesity remodels the chromatin landscape, allowing for easier GR access. Obesity alters the packing and accessibility of DNA in adipocytes. (11, 15, 19) Obesity may have a similar effect in muscle in which Glucocorticoid Response Elements or other transcription factors may more easily bind to receptors causing increased glucocorticoid action. Another theory is that the effects of GR-dependent signaling is promoted by insulin resistance. Obesity promotes insulin resistance and could enhance the glucococorticoid-induced cycle of muscle breakdown. It may also be a combination of these two ideas in which drugs could directly affect chromatin remodeling thereby increasing insulin sensitivity (19). These data are also consistent with a recent report showing that glucocorticoids given simultaneously with HFD enhances muscle decay and exacerbated induction of atrogenes (2).

Glucocorticoids and obesity both have deleterious health effects. These effects include loss of skeletal muscle which may result in reduced motor function, coordination, and energy production (16, 24, 45). Insulin resistance is an additional negative effect associated with both elevated glucocorticoids and excess adiposity in the body (11, 13, 28). The process by which these factors induce insulin resistance is not yet fully understood. In this study, we 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 of dexamethasone treatment (n=4-8 per group). Force generated by nerve stimulation (C) and by direct muscle stimulation (gastrocnemius; D) in lean and obese mice treated with dexamethasone for 15-21 days. Force plotted relative to whole muscle cross-sectional area (gastrocnemius; 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.** A) Lean mass determined via EchoMRI. Gastrocnemius weights (C) and cross-sectional area (D) in lean and obese mice treated with dexamethasone (n=5-8 per group). H&E stained section of muscles (quadriceps; E) Average fiber cross-sectional area (F) averaged from 200 fibers per section (quadriceps; n=4 mice per group). NADH-NBT stained section of muscles (quadriceps; G) from mice treated with vehicle (water) or dexamethasone for six weeks. Percent of slow-oxidative or Type I fibers to total fibers (H; n=4 sections per group). Average fiber cross-sectional area separated by NADH-NBT staining density with dark fibers indicating slow-oxidative or Type I muscle fibers (quadriceps; 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 mice (quadriceps) treated for six weeks with vehicle (water) or dexamethasone. Asterisks indicate significant interaction between diet and dexamethasone treatment 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). Asterisks indicate significant interaction between diet and dexamethasone treatment 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.Asterisks indicate significant interaction between diet and dexamethasone treatment by two-way ANOVA.

**Table 1: Primers used in this manuscript.** Key atrophy transcripts, *Fbxo32* and *Trim63*, (Atrogin-1 and MuRF1 respectively) and their upstream regulators, *Foxo1* and *Foxo3. Rplp13* was used a control gene.

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

**Table 2. Body Weight, fat mass and Food and Treatment Intake.** Asterisks indicate significant interaction between diet and dexamethasone treatment by two-way ANOVA. n=6-8 mice per group.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **NCD, Water** | **NCD, Dexamethasone** | **HFD, Water** | **HFD, Dexamethasone** |
| Body weight at dexamethasone treatment (g) |  |  |  |  |
| Fat mass at dexamethasone treatment (g) |  |  |  |  |
| Percent fat mass dexamethasone treatment |  |  |  |  |
| Body weight at sacrifice (g) | 31.5 ± 7.5 | 29.2 ± 1.5 | 46.5 ± 9.8 | 34.2 ± 1.6 \* |
| Fat mass at sacrifice (g) | 3.1 ± 0.6 | 3.6 ± 0.5 | 16.0 ± 1.3 | 11.6 ± 1.6 \* |
| Percent fat mass at sacrifice | 9.8 ± 1.7 | 12 ± 1.2 | 34 ± 2.0 | 33 ± 3.2 |
| Food intake per mouse per day during dexamethasone treatment (g) | 3.5 ± 0.09 | 3.7 ± 0.21 | 2.1 ± 1.0 | 3.6 ± .31 |
| Calorie intake per mouse per day during dexamethasone treatment (kcal) | 10.1 ± 0.26 | 10.8 ± 0.61 | 9.9 ± 4.7 | 17.0 ± 1.5 \* |
| Fluid intake per mouse per day during dexamethasone treatment(mL) | 6.2 ± 1.1 | 7.4 ± 0.91 | 4.2 ± 0.43 | 11.3 ± 3.7 |