# Introduction

The estimated prevalence of oral glucocorticoids usage in the United States is 1.2% for a variety of health concerns including asthma, chronic obstructive pulmonary disease, COPD, and a range of autoimmune disorders (6). Elevated levels of glucocorticoids within the human body have shown to cause skeletal muscle atrophy. This muscle atrophy stems from an upregulation of atrogenes, or genes that cause muscle atrophy (11) (Peiera et al., 2011, Schakman et al., 2013, Sandri et al, 2004).

Obesity is chronic disease that affects approximately 40% of the US population (2). 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 lipogensis (Chapman et al., 1985) Obesity can be 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 (Lecker at al., 2004). One causal factor in muscle loss is elevated glucocorticoids. Glucocorticoids are steroid hormones that function through a Glucocorticoid Receptor (GR, encoded by *Nr3c1*) to alter tissue-specific gene expression . (Patel et al. 2014) Exogenous glucocorticoids induce muscle atrophy through increased muscle proteolysis and inhibition of protein synthesis in lean mice (12). 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 (5). 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 (12).

Preliminary work from our group and others have demonstrated that glucocorticoids promote altered adipocyte gene transcription lipolysis, glucose production and insulin resistance in obese, relative to lean animals (3, 4). 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 *Foxo3a*. 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 cages of 4 animals/cage. All animals were on a light/dark cycle of 12:12 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.

## 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 torque (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 torque (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 (NEED TO FIND) 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 identified by hematoxylin and eosin (H&E staining) and for fiber-type, muscles were stained using NADH-NBT staining. 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 averaging them using ImageJ.

## 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 250nm 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 |

## Statistics

All results are represented as mean ± SEM. Two-Way ANOVA analyses 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. Any p-value under 0.05 was considered significant. All statistical tests were conducted using R version 3.5.0 (8).

## Protein- ARE WE INCLUDING IDK

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, 10 mM sodium pyrophosphate and 1x protease inhibitor) and 1x protease inhibitor using a TissueLyser II (Qiagen) and centrifuged at 14,000 rpm for 10 minutes at 4°C. Loading buffer was added to the lysates and heated at approximately 85°C. Proteins were separated by SDS-PAGE gel electrophoresis (Life Technologies) and gels were transferred onto nitrocellulose overnight. Nitrocellulose blots were stained with Revert Total Protein Stain (catalog no. 926-11011; Li-Cor Biosciences) for total protein following product instructions. Anti-mouse and anti-rabbit fluorescent-conjugated antibodies (Invitrogen) were used to detect secondary antibodies.

Antibody list...

A Odyssey CLx image scanner (Li-Cor Biosciences) was used to visualize the images. Quantification of proteins was done using Image Studio version 5.2 (Li-Cor Biosciences) and normalized to Revert Total Protein stained blots.

# 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). This result is consistent with studies in adult humans in which elevated salivary cortisol had a significantly higher risk of loss of grip strength than their peers (Peeters et al., 2008). Obese dexamethasone-treated mice had greater overall losses in grip strength when compared to the lean animals. For mean grip strength, we saw a -0.4% reduction in lean animals (p=0.007) but a -0.9% 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 the XX nerve and by direct electrical stimulation of the muscle. In NCD animals, the force generated by nerve stimulation was reduced X% when treated with dexamethasone. However in HFD animals, force generated by nerve stimulation was reduced X% when treated with dexamethasone, with a significant interaction between pre-existing obesity and dexamethasone treatment treatment (p=.009 Figure 1C). These results are concordant with direct muscle stimulation. In NCD animals, force generated by direct muscle stimulation was reduced X% when treated with dexamethasone. While in HFD animals, the force generated by direct muscle stimulation was reduced X% 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 XXX and YYY percent of the variance in force. As cross-sectional area declined muscle force by both stimulations decreased in proportion. Pre-existing obesity did not modify this force-area relationship (p=). 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 interacts with long-term glucocorticoid treatment to induce losses in muscle mass, we treated lean and obese male mice with dexamethasone for five weeks. Dexamethasone caused a reduction in total body lean mass in both lean and obese mice. Consistent with losses in strength, obese-dexamethasone treated mice had greater losses in lean mass as assessed by echoMRI(Figure 2a). This loss in lean mass is consistent with previously reported effects of glucocorticoids on muscle atrophy (7). This loss was also observed in specific hindlimb muscles. At sacrifice, the NCD animals quadricep and tricep surae weights were smaller by X percent in the dexamethasone treated. While in HFD animals, quadricep and tricep surae weights were smaller by X percent in the dexamethasone treated (Figure 2b).

We then evaluated short-term dexamethasone treated animals by placing male mice on vehicle or dexamethasone for two weeks. These are the same animals and muscles used for all isometric force testing. The obese-dexamethasone treated animals had the smallest gastrocnemius weights and whole-muscle CSA (Figure 2c-d). At sacrifice, the NCD animals gastrocnemius weights were smaller by X percent in the dexamethasone treated. While in HFD animals, gastrocnemius weights were smaller by X percent in the dexamethasone treated. There was a significant interaction between diet and treatment, P=.021.

## Obesity and Dexamethasone Treatment Resulted in Muscle Fiber Degradation

In order to assess changes in muscle size at the fiber-level, we sectioned the 5-week dexamethasone-treated mice quadriceps at the mid-belly and H&E stained the sections(Figure 2e). The NCD animal’s muscle fibers were smaller by X percent in the dexamethasone treated and in HFD animals muscle fibers were smaller by X percent in the dexamethasone treated. There was a significant interaction between diet and treatment, P=.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. These are the same section from the quadriceps used ….. Mice skeletal muscle is made up Type I, Type IIa, Type IIb, and Type IIx fibers. Oxidative fibers or Type I fibers stained 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. (Figure 2h) This result may be different in other types of muscles within the hindlimb because quadriceps have fairly high oxidative capacity due their mitochondrial content (Jacobs et al., 2012).

## Dexamethasone Reduced Type II Fiber CSA

Though we did not see changes in composition of fiber types, we wanted to investigate changes in fiber-type size. In order to evaluate fiber-type specific atrophy, we labeled fiber types based on their stained color and measured their CSA. Type I fibers stained darkest, Type IIb stained intermediate, and Type IIa stained the lightest (Figure 2g). Dexamethasone-treatment reduced Type IIa fibers CSA in lean and obese mice by X% and X% respectively. Dexamethasone-treatment also reduced Type IIb fibers CSA in lean and obese by X% and X% respectively. As for Type I Fibers, dexamethasone treatments only reduced fiber CSA in NCD animals. There was no significant effect of dexamethasone on Type I fibers in obese mice (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 (10).

## 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 (5, 11, 13). We chose to treat C2C12 myotubes with dexamethasone overtime in order to assess the expression of Foxo1, Foxo3, and well-established atrogenes, Murf1 and Atrogin-1 (encoded by *Trim63* and *Fbxo32* respectively) *in vitro.* Relative expression of all genes were elevated after 2 hours of treatment with dexamethasone (Figure 3a). Only Murf1 and Atrogin-1 reached peak expression at the end of the time-course at 24 hours of dexamethasone.

To evaluate the molecular effects of dexamethasone *in vivo*, 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 (Figure 3b). The expression of *Trim63, Fbxo32,* and *Foxo3* was elevated by approximately X%, X% and X% respectively in obese mice than their lean counterparts*.* However we need not see an increase in Foxo1 or Ncr31, glucocorticoid receptor.

### Obese Dexamethasone-Treated Mice are Profoundly Insulin Resistant

Since we have highlighted that obesity can enhance skeletal muscle atrophy generated by glucocorticoids, we wanted to take a look at the role of insulin and insulin sensitivity in this muscle atrophy model. Insulin is central to glucose-uptake in skeletal muscle, where the majority of all glucose uptake occurs within the body. Both dexamethasone and obesity are known to cause insulin resistance (references) In order to measure insulin sensitivity during dexamethasone-treatment, we treated lean and obese mice with dexamethasone and measured their fasting blood glucose(FBG). In lean animals, there was no significant change in FBG between treatment groups however there was a X% increase in obese animals given dexamethasone (Figure 4a). There was a synergistic effect of obesity and dexamethasone on fasted blood glucose, P=.033.

In order to evaluate whether the dexamethasone-treated animals were insulin resistant, we treated the same lean and obese mice with insulin and monitored with blood glucose overtime. Insulin doses were given dependent on their lean mass composition to account for their difference in size between chow-fed and high-fat diet fed mice. In both NCD and HFD animals, dexamethasone induced insulin resistance (Figure 4b). This is consistent with prior research in which insulin-stimulated glucose-uptake is significantly reduced in lean and obese dexamethasone-treated muscles when compared to controls.

# Discussion

Here we show that dexamethasone-treatment in concert with pre-existing obesity caused elevated reductions in muscle strength and size and insulin resistance. Muscle weakness is a common side effect of exogenous glucocorticoid consumption as well as continually elevated levels of endogenous glucocorticoids . This research could be particularly important because those with obesity are more likely to have reduced muscle function (Maffiuletti et al. 2007; Blimkie, Sale, and Bar-Or 1990; Hulens et al. 2001; Zoico et al. 2004). Importantly people with obesity are also more likely to have elevations in endogenous glucocorticoid levels (9, 14).

We show, consistent with previous reports that glucocorticoid-dependent reductions are more dramatic in type II muscle fibers (Falduto et al., Rouleau et al., 1987). 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 (Nicks et. al., 1989, Rouleau et al., 1987, Trappe et al., 2004). While this targets a different fiber type, these changes are consistent with our results in that we saw reductions in whole muscle mass, CSA and muscle fiber CSA, but no change in the proportion of fibers. Disuse atrophy has also been shown to generate fiber plasticity where type I fibers or oxidative fibers take on characteristics of fast-twitch, non-oxidative fibers or change completely to type II fibers . (Debré et al., 2016, Stevenson et al., 2003) Even though we saw no change in fiber composition when treating animals with dexamethasone, an other study has shown that dexamethasone reduces both that quantity and size of Type 2 fibers in muscles in rats (Ma et al., 2003). This discrepancy could be due to quantity of dexamethasone provided to the animals; our dose which is consistent with human under constant stress or taking a high prescription dose. The discrepencey could also be due to the specifc muscle chosen to test.

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 (1) (Falduto et al., 1990, Kelly and Goldspink, 1982, Livingstone et al., 1981), so it is possible that muscles with higher concentrations of type II fibers may be more vulnerable to 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 (10). We evaluated fibers within the mouse quadriceps. It also possible that we would see more dramatic changes in the forces generated by other muscles, than the gastrocnemius we tested, that have higher in type II fiber content. 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 (Mänttäri et al., 2005). It is possible that glucocorticoids function to reduce contractile force in muscles with greater type II fiber content by reducing fiber size.

This work shows that how 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 glucocorticoids increase sensitivity to pro-inflammatory cytokines and separately glucocorticoids and excess adiposity exacerbate levels of these cytokines circulating in the body and act upon skeletal muscle. Pro-inflammatory cytokines have catabolic effects on protein metabolism and anabolic effects such as reduced *de-novo* protein synthesis. tumor necrosis factor alpha(TNFa) has been shown to directly act on muscle cells to induce protein degradation in C2C12 myotubes(Lee and Reid 2000). It is possible that glucocorticoids and obesity could coregulate genes associated with atrophy and make muscle more susceptible to breakdown. For example leptin-receptor deficient, obese mouse mice were found to have increase in interleukin 1beta and TNFa in the brain and a lowered threshold for release of pro-inflammatory cytokines. (Dey at al. 2014)

Lastly there are deleterious health effects associated with loss of skeletal muscle.

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# Acknowledgements

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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. \*=Significance identified by Student’s T-Test. Force (mN) 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 by whole gastrocnemius CSA (e-f). N=5-8 per group. \*=Diet-Treatment interaction identified by Two-Way ANOVA.

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

Lean mass 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 CSA (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. N=4 quadricep sections per group. Average fiber CSA (f) from 200 fibers per quadricep section. 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 mouse fibers (h). N=4 quadricep sections per group. Average fiber CSA by NADH-NBT staining density (i). \*=Diet-Treatment interaction identified 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.