GLUCOCORTICOID-INDUCED MYOPATHY IS MEDIATED BY IMPAIRED NITRIC OXIDE SYNTHESIS

By

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CAT:	Cationic amino acid transporter
MAFbx:	Muscle Atrophy F-box
NO:	Nitric oxide
eNOS:	Endothelial nitric oxide synthase
iNOS:	Inducible nitric oxide synthase
nNOS:	Neuronal nitric oxide synthase

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Glucocorticoid drugs are potent and widely prescribed anti-inflammatory agents. However, glucocorticoid-induced skeletal muscle wasting severely limits the efficacy of these drugs, especially in chronic treatment situations. Understanding the mechanisms behind this muscle-wasting side effect will lead to more effective countermeasures, thus improving quality of life in various patient populations.

The systemic effects of glucocorticoids are mediated, in part, by inhibition of inducible nitric oxide synthase (iNOS). Nitric oxide (NO) production by muscle serves important signaling functions. Therefore, NOS down-regulation in skeletal muscle may contribute to glucocorticoid-related myopathy. Based on this, we hypothesized glucocorticoid use inhibits NO production in skeletal muscle, and that this inhibition is partly responsible for the atrophy noted with glucocorticoid use. Furthermore, we hypothesized that addition of L-Arginine (L-Arg), a NOS substrate, and DETA-NONO, an NO donor would attenuate the glucocorticoid effects.

We chronically (8wk) treated mice with prednisolone or saline at a concentration of 2.1mg/kg of body weight. At the end of the treatment period the animals were euthanised and their gastrocnemius removed for either Western Blot analysis or Single Fiber Isolation and culture. We demonstrated chronic exposure of mice to glucocorticoids decrease muscle satellite

cell activity and NOS expression. In vitro treatment with the nitric oxide donor, DETA-NONO, restored satellite cell activity in myofibers isolated from glucocorticoid-treated mice to control levels. Additionally, we cultured L6 myotubes to study the acute effects of glucocorticoids. After 24h of treatment (100μM dexamethasone), the myotubes displayed 6-fold increased expression of MAFbx, a muscle specific ubiquitin ligase. Co-treatment with DETANONO or L-Arg blunted the effect. Following 48h of treatment myotubes dimensions were assessed. The glucocorticoid treatment reduced myotube area by over 50%. When DETANONO or L-Arg was added, myotube atrophy was significantly attenuated. Thus, NO may be a useful therapeutic target for opposing the negative effects of both acute and chronic glucocorticoid use.

CHAPTER 1
INTRODUCTION

Muscle mass is determined by a delicate balance between protein synthesis and breakdown, which is keenly sensitive to active muscle tension and the pattern of muscle recruitment. Skeletal muscle atrophy, denoted by a decrease in muscle mass and fiber size, can arise from various etiologies including denervation, disuse, sepsis, cancer, AIDS, and chronic exposure to high levels of glucocorticoids. The latter could arise from adrenal hyperactivity (Cushing's Disease) or pharmacological administration of glucocorticoids as anti-inflammatory agents. Regardless of the initiating factor, atrophy is characterized by a decrease in protein synthesis and an increase in protein degradation, with the latter having a larger impact. And, rather than simply an absence of growth or maintenance signals, muscle wasting is an active remodeling mechanism by which skeletal muscle myofibrils are dismantled and removed. Understanding this mechanism will lead to more effective strategies to attenuate muscle atrophy, thus improving quality of life in various patient populations.

Glucocorticoid administration is linked to skeletal muscle atrophy (33, 34, 75) and increased expression of genes involved in proteolysis (17, 19, 37, 105). Additionally, the inhibition of calcium-activated proteases (calpains) in glucocorticoid treated myotubes attenuates the usual glucocorticoid-induced protein loss and decrease in myotube size (23). Therefore, glucocorticoids have the capacity to affect multiple components of the atrophy program.

Three major proteolytic systems have been identified in skeletal muscle: the lysosomal, calpain, and ubiquitin-proteasome pathways. The lysosomal system is unlikely to be involved because it does not degrade myofibrillar proteins (24, 54, 111). It has been shown in multiple catabolic situations that contributions to atrophy via the lysosome are minimal (91, 99, 102).

However, calpain and ubiquitin-proteasomal pathways seem to be intimately involved with the protein degradation associated with atrophy.

The ubiquitin-proteasomal pathway is primarily responsible for degrading myofibrillar proteins into component amino acids. However, it does not degrade intact myofibrillar proteins (46, 84). Thus, myofibrillar loss initially is dependent on disassembly of myofibrils, which can occur via calpain activity. The dismantled myofibrils are then targeted to the proteasome for degradation, resulting in protein loss. Glucocorticoids activate both the calpain and the proteasome pathways (22, 23, 76, 100).

Nitric oxide, a soluble gas product of L-arginine metabolism via nitric oxide synthase (NOS) activity, has rather global impacts on skeletal muscle. It is necessary for muscle differentiation (53), myoblast fusion (50, 67), satellite cell activation (2, 3, 93, 94), growth (78), and maintenance of muscle mass (44, 106). In fact, calpain activity, which initiates protein degradation, is inhibited by basal NO production (44). Attenuation of endogenous nitric oxide production in skeletal muscle may be an important mechanism of glucocorticoid-induced myopathy since NO production and expression of NOS enzymes are known to be downregulated in the presence of glucocorticoids (7, 41, 43). In preliminary studies, we have found a dramatic deficit in satellite cell number and responsiveness to mechanical activation in muscle fibers isolated from glucocorticoid-treated mice. Furthermore, 24h of treatment with DETA-NONO (an NO donor) can attenuate this satellite cell deficit (Betters et al., unpublished data). Therefore, the loss of NO production in skeletal muscle may initiate proteolysis via calpain and/or proteasome activation. Concomitantly, loss of NO may compromise the ability of skeletal muscle to maintain muscle mass in the long-term via a loss of satellite cell number and/or activity.

Cellular availability of nitric oxide or its biochemical precursors may offer a convenient and effective target for treatment and prevention of glucocorticoid-induced myopathy.

Therefore, this project will employ two experimental models, daily injection of prednisolone for 8 wks in adult mice and 24 or 48h treatment of cultured C2C12 mouse or L6 rat myotubes with dexamethasone, to examine the effects of glucocorticoid treatment on skeletal muscle NOS expression, calpain activity, expression of the muscle-specific ubiquitin E3 ligase, MAFbx, and abundance and activity of satellite cells. Further, treatment of cultured adult myofibers and L6 myotubes with the NO donor, DETA-NONO, and the NOS substrate, L-arginine will test the postulate that glucocorticoid-induced myopathies are related to compromised NO production. The potential for NO supplementation to ameliorate the negative effects of glucocorticoids on skeletal muscle will be assessed.

Questions to be Addressed

1. What is the effect of glucocorticoid treatment on NOS protein expression and cationic amino acid transporter (CAT) mRNA expression in mouse skeletal muscle?
2. What is the effect of glucocorticoid treatment on NO production (nitrate accumulation), NOS protein expression, and CAT and MAFbx mRNA expression in myotubes?
3. What is the effect of glucocorticoid treatment on calpain activation?
4. What is the effect of glucocorticoid treatment on satellite cell activation?
5. Can nitric oxide supplementation in glucocorticoid treated myotubes inhibit MAFbx expression, calpain activity, and atrophy?
6. Can L-arginine supplementation ameliorate glucocorticoid-induced MAFbx expression, calpain activity, and atrophy in myotubes, due to enhanced nitric oxide production?

Hypotheses

In Vivo

1. Daily injections of prednisolone for 8 wks will decrease NOS protein and CAT mRNA expression in the gastrocnemius muscle of mice.

2. Prednisolone treatment (8 wks) will reduce the number and activation/proliferation of satellite cells in single myofibers isolated from the mouse gastrocnemius muscle.

3. Treatment of isolated myofibers from prednisolone-treated mice with the NO donor, DETA-NONO, will increase the activation/proliferation of satellite cells in response to a mechanical stimulus.

In Vitro

4. NO or L-arginine supplementation in dexamethasone-treated myotubes will increase NOS protein and CAT mRNA expression, limit MAFbx expression, and inhibit atrophy.

5. NO or L-arginine supplementation in dexamethasone-treated myotubes will decrease the abundance of calpain-specific proteolytic fragments of α-spectrin.

Significance

The debilitating consequences of glucocorticoid-induced myopathies are of clinical relevance. Although the pathological overproduction of cortisol (i.e. Cushing's Disease) is relatively rare, synthetic glucocorticoids are widely used pharmacologically for their dramatic anti-inflammatory effects. The loss of skeletal muscle can lead to loss of independence, falls or, potentially, respiratory failure. Thus, it is critical we understand the mechanisms that cause atrophy in response to chronic glucocorticoid treatment so that better countermeasures can be designed. Clearly nitric oxide is involved in normal skeletal muscle development, function, and hypertrophy (2, 67, 78, 83), but its role in attenuating atrophy is unclear. The attenuation of endogenous nitric oxide production due to glucocorticoid-induced loss of nNOS protein may represent an important signal initiating the atrophy process. If so, supplementation with the NOS substrate, L-arginine or with a pharmacological nitric oxide donor could be an effective strategy to ameliorate glucocorticoid-induced skeletal muscle myopathy. This study is designed to examine this possibility and contribute to our understanding of the mechanisms involved in muscle wasting.

CHAPTER 2
REVIEW OF LITERATURE

Skeletal muscle is a highly adaptive tissue. It is sensitive to not only changes in use, but also to various common pharmacological treatments. Muscle mass is determined by a delicate balance between protein synthesis and breakdown, and is capable of responding to increased or decreased loading by increasing (hypertrophy) or decreasing (atrophy) mass accordingly. Unfortunately, certain disease states (e.g. cancer, sepsis) and/or pharmacological agents (e.g. glucocorticoids) can induce rapid and severe muscle wasting. This skeletal muscle atrophy, denoted by a decrease in muscle mass and fiber size, is characterized by a decrease in protein synthesis and an increase in protein degradation, with the latter having a more prominent role. It is not wholly clear how glucocorticoids exert their effects, but their administration has been linked to increased expression of genes involved in proteolysis (17, 19, 37, 105). Therefore, delineating the mechanism of glucocorticoid-induced atrophy is key to developing countermeasures to this potentially debilitating condition.

Glucocorticoids and Muscle Remodeling

Glucocorticoids are a group of steroid hormones that have receptors throughout the body, thus their effects impact a number of physiological systems. The most widely understood effects are on carbohydrate metabolism and immune function. This class of hormones stimulates gluconeogenesis in the liver and increases amino acid mobilization from non-hepatic tissues. Additionally, they inhibit glucose uptake in muscle, and stimulate lipolysis in adipose tissue. Clearly these hormones elicit a catabolic effect in skeletal muscle. Despite the deleterious effects of chronic exposure to glucocorticoids, they are widely used pharmacologically for their anti-inflammatory and immunosuppressant qualities.

Glucocorticoids have been shown to cause proteolysis in several situations (59). Treatment of C2C12 cells with dexamethasone, a synthetic glucocorticoid, not only inhibits proliferation (96), but also induces atrophy in terminally differentiated myotubes (7, 75, 89). In addition to protein degradation, dexamethasone administration induces MAFbx and MuRF1 expression (59, 75, 105). The up-regulation of these transcription factors was blocked by a pharmacologic inhibitor of glucocorticoids (113). Furthermore, glucocorticoid-induced MAFbx and MuRF1 expression seems to be operating through FOXO activation (76, 87), which is directly related to the PI3K/Akt pathway.

In addition to the increase in components of the ubiquitin-proteasome pathway, dexamethasone increases intracellular concentrations of calcium in myocytes (Evenson et al. 2005, unpublished observation). This could in turn lead to increased calpain activity and may be one of many ways glucocorticoids exert their wasting effects on skeletal muscle. Another potential path is via inhibition of IGF-1 signaling. In the absence of IGF-1, Akt is dephosphorylated which leads to FOXO activation (76), thus promoting atrophy. Additionally, it should be noted that diminished Akt signaling promotes the cleavage and activation of caspase-3 (85). IGF-1 has been shown to attenuate the effects of glucocorticoids on L6 cells (52). In fact, Latres et al. (48) demonstrated an inverse regulation of MAFbx dependent on IGF-1 presence and determined that both the Akt/FOXO and Akt/mTOR pathways are needed for transcriptional changes induced by IGF-1.

Proteolytic Systems Involved in Skeletal Muscle Remodeling

Three major proteolytic systems have been identified in skeletal muscle: the lysosomal, calpain, and ubiquitin-proteasome pathways. The lysosomal system is unlikely to be involved in atrophy because it does not degrade myofibrillar proteins (24, 54, 111). It has been shown in multiple catabolic situations that contributions to atrophy via the lysosome are minimal (91, 100,

102). However, calpain and ubiquitin-proteasomal pathways seem to be intimately involved with the protein degradation associated with atrophy.

The calpains (calcium-activated proteases) are comprised of a family having at least 14 members. Some of these are ubiquitous enzymes, such as μ- and m-calpain, while others are tissue specific proteins, such as the muscle specific calpain 3, also called p94 (28, 86, 90). The μ- and m-calpain isoforms are named based on the calcium concentration needed to attain their half-maximal proteolytic activation. The μ-calpain form requires μM concentrations and the m-calpain requires mM concentrations (5, 16, 20). Both levels of calcium are supraphysiological (16, 55), so some mechanism exists to activate the normally inactive calpains. This is evident in that calpains are relevant in physiological systems (55), as demonstrated by the work showing that *in vivo* calpain inhibition has a protective effect on skeletal muscle (70, 101).

The calpains are activated in skeletal muscle during reduced use (79, 91). When activated these proteases target structural proteins within the muscle cell such as talin, a linker protein in the cell membrane (44). The dismantling of the cytoskeleton via calpain activation seems to be a critical event in cellular protein degradation (27, 39).

The regulation of calpain activity is complex. The most important activator of calpain is calcium (28). Cytoplasmic calcium is increased in glucocorticoid treated cell cultures (8, 48, 103). Furthermore, Wei et al. (109) has shown that calpain inhibitor (calpeptin) administration blocks dexamethasone-induced proteolysis. Thus, one potential role dexamethasone plays in the atrophic response is to increase cytosolic calcium with subsequent calpain activation.

Calcium is not the only regulator of calpain-mediated proteolysis. Calpastatin (an endogenous inhibitor of calpains) and nitric oxide may play a regulatory role (44). Calpastatin prevents calpain enzymatic activation and the expression of catalytic activity (27). Nitric oxide

has been shown to regulate sarcoplasmic reticulum function and therefore intracellular calcium. This action inhibited calpain action in ischemia-reperfused rat hearts (14). Zhang, Kraus, and Truskey (114) showed NO inhibited calpain-mediated proteolysis of talin in C2C12 myotubes. Thus, calpains are regulated by a number of factors.

Calpain activity is likely not the sole contributor to atrophy in the presence of elevated glucocorticoids. The role of the ubiquitin-proteasome pathway in atrophy cannot be discounted. The cellular events involved in this method of protein breakdown are highly coordinated. First, the protein to be degraded is marked by attaching ubiquitin to it. This is accomplished via a trifecta of proteins: E1, the ubiquitin-activating enzyme, E2, the ubiquitin-conjugating enzyme, and E3, the ubiquitin-protein ligases (e.g., MAFbx or atrogin-1 and MURF1) (40). E1 activates ubiquitin and transfers it to E2. Then, E3 transfers the ubiquitin from E2 to the target protein. This occurs multiple times until the protein to be degraded is marked by a chain of ubiquitin molecules. Once polyubiquinated, the targeted protein is rapidly degraded by the proteasome (26, 58, 98).

Atrophy is partly a result of the ubiquitin-proteasome pathway. In both the skeletal muscle of immobilized rat hindlimb and atrophied cardiac muscle, mRNA for MAFbx and MuRF1 were increased (47, 71). Ikemoto et al. (40) demonstrated an increase in ubiquinated proteins within skeletal muscle during reduced use. There is a concomitant increase in proteasome enzyme activity (79). It has been demonstrated by a number of authors that this system accounts for most of the elevated muscle proteolysis in a variety of atrophic situations (36, 91, 95) including glucocorticoid use (38, 112).

IGF-1 Pathway and Muscle Remodeling

IGF-1 (insulin-like growth factor-1) is a growth factor that initiates a cascade of intracellular signaling events leading to hypertrophic and antiproteolytic responses. Two main pathways stimulated by IGF-1 are the Ras-Raf-MEK-ERK pathway and the PI3K/Akt pathway. The Ras-Raf-MEK-ERK signaling events affect fiber type composition, but not myofiber size (62), thus this pathway is likely unimportant in causing hypertrophy or atrophy. However, the PI3K/Akt pathway seems to cause muscle hypertrophy ultimately by stimulating translational events via the mTOR and GSK kinases (12).

mTOR, one target of Akt, when phosphorylated will activate p70S6 kinase. This kinase increases muscle protein translation, ultimately supporting hypertrophy. Recently Thompson and Gordon (97) demonstrated that older rats subjected to skeletal muscle overload had less phosphorylation of mTOR and its downstream target p70S6K that correlated with diminished total protein content. Further evidence of a hypertrophic role of mTOR was shown by rapamycin blocking myotube hypertrophy in a C2C12 model of terminally differentiated myotubes (63, 65). The Park et al. group further demonstrated the necessity of mTOR for hypertrophy by stably expressing in C2C12s rapamycin-resistant forms of mTOR and p70S6K. The rapamycin-resistant cultures underwent hypertrophy with rapamycin treatment, while the control cultures did not hypertrophy. This was assessed by image analysis. An additional role mTOR plays in muscle remodeling is through inhibition of FOXO (forkhead box O), which targets at least one of the E3 ligases. Based on the evidence presented here, mTOR, when phosphorylated by Akt will cause hypertrophy and inhibit atrophy (25, 49).

Akt also directly targets the forkhead box O (FOXO) family of transcription factors, which target at least one of the E3 ligases. Phosphorylated Akt inactivates (phosphorylates) FOXO, thus hindering protein degradation. FOXO, when active, translocates to the nucleus and

increases transcription of a key element of the ubiquitin-proteasome pathway, the ubiquitin ligase atrogin-1 or MAFbx. The functional importance of this protein being expressed during the atrophy process was demonstrated by using MAFbx–/– mice. Mice lacking the MAFbx gene had less atrophy following denervation (11). This was the same for the other E3, MuRF1 in a MuRF1-/- knockout mouse model. Furthermore, because MuRF1 and MAFbx have been shown to be upregulated in other models of atrophy, they are widely accepted as reliable markers of the atrophy process (11, 12, 29, 51, 113). So, under the influence of IGF-1 through Akt signaling, the activity of FOXO is repressed while that of mTOR is increased (12). This causes an increase in cell size through a concomitant increase in protein synthesis and a decrease in protein degradation. Alternatively, in the absence of IGF-1, there is an increase in proteolysis and the expression of atrophy-related ubiquitin ligases such as MAFbx, correlated with FOXO dephosphorylation (activation) (76).

FOXO factors are critical in the transcription of MAFbx in myotubes under both starvation and glucocorticoid treated conditions (76). In both situations, IGF-1 signaling decreases. Thus phosphorylation of FOXO via Akt activation is minimized (13, 64, 92), and FOXO translocates to the nucleus (9, 13) to up-regulate MAFbx expression (76), and hence induce atrophy. Further support for the necessity of nuclear FOXO in atrophic conditions was established when a constitutively active form of FOXO3 (one of the three mammalian FOXOs associated with skeletal muscle) remained in the nucleus and caused atrophy (76, 82, 88).

In addition to Akt regulating protein balance in skeletal muscle via FOXO and mTOR, it also targets glycogen synthase kinase (GSK3β). GSK3β is blocked by phosphorylated (active) Akt, and leads to hypertrophy (72). However, when GSK3β is activated, it blocks transcriptional initiation factors necessary for protein synthesis (12). This would indicate GSK3β is a potential

mitigator of skeletal muscle atrophy. However, Sandri et al. (76) demonstrated that activation of GSK3β does not influence the induction of MAFbx. So, perhaps GSK3β's role in muscle metabolism is limited to its control of initiation factors involved in protein synthesis.

The majority of the evidence regarding atrophy in skeletal muscle indicates that down-regulation of IGF-1 signaling is a key event leading to proteolysis. There seem to be two parts to the atrophic response via this mechanism. First, definitive links have been established regarding nuclear localization of FOXO factors in the absence of IGF-1 signaling and also FOXO factors binding directly to the MAFbx promoter to cause an increase in this ubiquitin ligase (76). Second, Song et al. (85) showed that down-regulation of IGF-1 signaling via the Akt/mTOR pathway leads to calpain activation. It is fairly widely recognized that calpains likely dismantle the existing cytoskeleton, readying targeted myoproteins for ubiquination and subsequent degradation via the proteosome.

Satellite Cells and Skeletal Muscle Remodeling

Adult skeletal muscle fibers consist of post-mitotic, terminally differentiated nuclei. Each of these nuclei govern a finite volume of cytoplasm, the size of which seems to be set and tightly regulated (i.e. the nuclear domain hypothesis) (31). In order for skeletal muscle fibers to recover from injuries or adapt their size to meet functional demands, and still maintain this nuclear domain range, a source of nascent myonuclei must be available. Muscle satellite cells, which are quiescent muscle precursor cells found between the myofibers and the external lamina, provide this source. In response to growth or regeneration stimuli, these cells are activated to differentiate and join an existing myofiber or form a new myotube, thus maintaining the myonuclear domain.

Activation of satellite cells is a necessary step in adult skeletal muscle growth. Several studies have used localized gamma irradiation of rat skeletal muscles to eliminate mitotically active cells within the muscle, without compromising circulating stem cells or growth factors (66, 73). These irradiated muscles are completely incapable of regaining muscle mass following injury (60, 73) or growing in response to mechanical overload (1, 73). This suggests that addition of nascent myonuclei is required for muscle growth rather than simply following changes in fiber size.

In addition to supporting muscle growth and regeneration, satellite cells may be important for maintenance of muscle mass. Atrophied muscles from hindlimb suspended animals contain fewer satellite cells associated with isolated myofibers (61). It seems that repeated use of myogenic precursor cells for muscle repair can lead to exhaustion of the satellite cell pool (18, 108). Additionally, it appears that these same satellite cells from atrophied animals have an impaired ability to activate and proliferate (61). As myonuclei are removed during atrophy, the down-regulation of satellite cell number and activity likely prevents the addition of new nuclei to maintain the fiber size. Perhaps this de-activation of satellite cells is a primary mechanism to inhibit hypertrophy and induce atrophy.

Nitric Oxide and Skeletal Muscle Remodeling

Nitric Oxide (NO), a product of L-arginine (L-Arg) metabolism, is a free radical that is produced by nitric oxide synthase (NOS) enzymes. All three isoforms of the NOS enzyme can be expressed in skeletal muscle. nNOS is most abundant, being associated with the dystrophin complex and localized in costameres at the sarcolemma (30). eNOS is also constitutively expressed in muscle and may be associated with mitochondria (42). Lastly, iNOS is believed to be only expressed in response to an inflammatory stimulus (87). Although NO is primarily known for its vasodilatory effects, it is also an important regulatory molecule in many different

tissues, including skeletal muscle (87). Our lab has demonstrated that nitric oxide (NO) positively influences skeletal muscle hypertrophy and contractile gene expression during overload (78, 83). Anderson (2) found that NO is a primary signal for skeletal muscle satellite cell activation. Others have shown nitric oxide to be important in myoblast fusion (50, 67). Thus, it is clear that NO is involved with the hypertrophic response.

NO production increases in isolated rat glomeruli when incubated with IGF-1 (107). Additionally, an increase in Akt phosphorylation was noted. This rise in Akt phosphorylation associated with NO presence has also been demonstrated in bovine aortic endothelial cells (56). Furthermore, when an mTOR inhibitor was used in activated macrophages, NO production was reduced (110). While these studies are not in skeletal muscle, they demonstrate a potential link between NO production and the PI3K/Akt/mTOR signaling pathway.

In addition to nitric oxide's influence on or production from hypertrophic pathways, it has also been associated with components of the atrophic pathway. For example, Koh and Tidball (44) showed evidence that NO could be a regulatory molecule of calpains. They used sodium nitroprusside, an NO donor, and showed no proteolysis of structural proteins. Moreover, through zymography and an activity assay, inhibition of m-calpain in C2C12 cultures was found. Michetti et al. (57) also showed inhibition of m-calpain in a dose-dependent manner with sodium nitroprusside. Further support for a role of NO in calpain inhibition comes from Chohan et al. (14). They demonstrated that ischemia-reperfused hearts had an increase in calpain activity and a decrease in cytosolic NO levels. When L-Arg was administered, the increased calpain activity was attenuated. Thus, whether NO was directly given or synthesized via one of the NOS enzymes and L-Arg, it inhibits calpain activity, suggesting that some basal endogenous NO production may inhibit proteolysis and support maintenance of muscle mass.

Endogenous NO production is dependent upon L-Arg uptake (80). L-Arg is transported into the cells via a sodium-independent y+ transport system. This system recognizes cationic amino acids, specifically L-Arg, L-Lys, and ornithine (15). There are three known transporters for L-Arg present in skeletal muscle: CAT-1, CAT-2A, CAT-2B. CAT-1 and CAT-2B have a high affinity for L-Arg compared to CAT-2A. There is some evidence that NO production in skeletal muscle may be limited by L-Arg availability. Since plasma levels of L-Arg generally exceed the Km value for the NOS enzymes, an L-Arg limitation would implicate the CAT transporters as potential regulators of NO production.

Glucocorticoids and Nitric Oxide

Glucocorticoids, known for their atrophy-inducing qualities, suppress NO production (41, 80). In fact, much of the anti-inflammatory actions of glucocorticoids are likely due to inhibition of iNOS. Dexamethasone inhibits iNOS transcription and mRNA expression (6, 45). Further, glucocorticoids have been shown to induce proteolytic cleavage of iNOS at a specific site within the calmodulin binding domain via activation of calpain (103). This domain is conserved among the three NOS isoforms, raising the interesting possibility that glucocorticoids may globally down-regulate NO production, simultaneously compromising the signaling actions of nNOS and/or eNOS while inhibiting the inflammatory effects of iNOS. In cardiac microvascular endothelial cells, treatment with glucocorticoids completely abolishes NO production, an effect attributed to eNOS down-regulation (80). eNOS has been shown to be down-regulated by FOXO1 and FOXO3A (68). These factors bind to the eNOS promoter in endothelial cells. This causes repression of eNOS expression.

Glucocorticoids may also affect NO production via L-Arg availability. Simmons et al. (80) showed a reduction in L-Arg uptake in dexamethasone treated cells. Dexamethasone treatment prevented the induction of CAT-2B mRNA by cytokines. Furthermore, Hammermann

et al. (32) found a down-regulation of CAT-2B in rat alveolar macrophages after 20h exposure to dexamethasone. It seems that the inflammatory response increases L-Arg transport into the cells, which supports large-scale NO production by iNOS. Dexamethasone, however, is able to not only attenuate this increase in L-Arg influx by the down-regulation of CAT transporters, but also seems to suppress iNOS (81). Therefore, glucocorticoids have the ability to greatly reduce NO production by inhibiting both substrate availability and NOS enzyme expression. Given the importance of NO signaling for skeletal muscle remodeling as discussed above, inhibition of endogenous NO production may be an important mechanism for glucocorticoid-induced skeletal muscle myopathy.

Concluding Statement

Our lab has demonstrated that nitric oxide (NO) positively influences skeletal muscle hypertrophy. Koh and Tidball (44) found that NO inhibition of calpains protected cells from proteolysis. Wang et al. (106) inhibited NO production via L-NAME administration and induced atrophy. Moreover, glucocorticoid use can decrease NO production, increase intracellular concentrations of calcium and induce expression of MAFbx. Taken together, it is not unreasonable to believe that glucocorticoid-induced skeletal muscle myopathy is due, at least in part, to compromised NO production. An NO-donor and/or supplementation with the NOS substrate L-Arg could attenuate glucocorticoid-induced atrophy. This study explored the effects of glucocorticoid treatment on NOS and CAT expression in skeletal muscle using both *in vivo* and *in vitro* models. Further, the potential for an NO donor or L-Arg supplementation to inhibit muscle atrophy via down-regulation of MAFbx expression and calpain activity, and improved satellite cell function was experimentally tested.

CHAPTER 3
MATERIALS AND METHODS

The purpose of this project was to examine the mechanism of glucocorticoid-induced skeletal muscle myopathy, and test the hypothesis that the loss of satellite cell activity and initiation of the calpain and proteasome proteolytic pathways during exposure to glucocorticoids is mediated by the loss of endogenous nitric oxide production.

In Vitro Studies

Experimental Design

The C2C12 mouse and L6 rat myogenic cell lines were used to examine the effects of exposure to the glucocorticoid, dexamethasone. Differentiated myotube cultures were treated with dexamethasone for 48h and monitored for myotube dimensions, protein content, and proteolytic cleavage of the calpain substrate, α-spectrin. Alternatively cultures were treated for 24h and assessed for expression of the ubiquitin ligase MAFbx (or atrogin-1) and nitric oxide production. Supplementation of cultures with DETA-NONO, a nitric oxide donor, or L-arginine, the NOS substrate, were performed to test for a causal relationship between compromised nitric oxide production and initiation of the atrophy program. Table 3-1 illustrates the design of this study.

Cell Culture

C2C12 and L6 cells (ATCC, Manassas, VA) were plated and proliferated in Dulbecco's Modified Eagle's Medium (DMEM) growth media (GM) containing 10% FBS and 1% penicillin/ streptomycin. At 80% confluency the GM was removed and the cells washed twice with 37°C PBS. Then differentiation media (DM) was added (DMEM supplemented with 2% horse serum and 1% penicillin/ streptomycin). Cultures differentiated for 5-7 days until there were confluent myotubes. At that point cultures were divided into 3 groups and treated for 24h

or 48h with one of the following: 10 mM L-arginine, 10μM DETA-NONO, or no supplement (control) in DM. Half of the cultures from each group were co-treated with 100μM dexamethasone.

At the time of harvest, cultures were rinsed twice with ice cold PBS and then harvested in two manners. For protein analysis via Western Blots, the cells treated for 48h were harvested on ice in 0.300ml non-denaturing lysis buffer (NDL: 20% Triton X-100; 1M Tris/ pH=7.5; 5M NaCl; 0.5M EDTA; 10mg/ml sodium azide; 4mg/ml NaF; 4ug/ml $NaVO_3$; 0.1% protease inhibitors) and then centrifuged at 500 x g for 5min to remove insoluble material. The supernatant was used for Western Blots for nNOS, eNOS, and α-spectrin. For mRNA analysis, parallel cultures treated for 24h were harvested in 1ml of ice cold TRIzol (Invitrogen, Carlsbad, CA).

Histochemistry

Parallel cultures were fixed with 3.7% formaldehyde and stained with hematoxylin and eosin at 48h. Microphotometric digital images of each culture were captured using a Zeiss Axiovert200 light microscope (Thornwood, NY) and Qimaging RETIGA EXi digital camera (Surry, BC, Canada) and software (IPLab3.6.5, Scanalytics, Rockville, MD). The images were evaluated for myotube dimensions (area and nuclei/ area) and density (myotube number/ field of view) using Scion Image imaging software. Three images of each culture were captured. Within each image 10 myotubes were analyzed for area and nuclear content by measuring the first 10 myotubes in each field of view beginning in the left-hand corner. Thus, 30 myotubes were analyzed per culture and each treatment had three cultures for a total n = 90 myotubes per treatment.

Nitric Oxide Production

Nitric oxide production by the cells was measured by assessing nitrate accumulation in media measured at 0h, 24h, and 48h. Following collection, the media was centrifuged at 5000 x g for 40min using microcentrifuge filters to remove serum, which can interfere with the kit components. The Nitrate/Nitrite Fluorometric Assay Kit from Cayman Chemical Company (Ann Arbor, MI) based on the procedures described by Misko et al. (1993) was used.

Western Blots

Myotube protein was collected as described above. Protein concentration was determined by DC assay (Bio-Rad, Rockville Centre, NY). SDS-PAGE was performed on 7% polyacrylamide gels. Equal amounts of protein were loaded into each lane. Positive controls for nNOS and eNOS were used. The gels were run at 60V for 1h and then 100V for 1h (NOS blots) or 100V for 2h (α-spectrin blots) with a 1h transfer at 500mA. The transfer was onto nitrocellulose membranes that were subsequently blocked in Odyssey blocking buffer for 1h. The membranes were then incubated overnight with primary antibody in 1:1 TBS-T (0.01%): Odyssey blocking buffer. For nNOS and eNOS, a 1:500 dilution was used (monoclonal, mouse, anti-nNOS: 611852, eNOS: 610328, BD Transduction Laboratories, San Jose, CA). For α-spectrin (Biomol), a 1:5000 dilution was used. β-actin incubation was used as a loading control (1:4000 dilution). Following the overnight incubation at 4˚C, the membranes were washed 4 times for 5min each with TBS-T. The fluorescent secondary antibody (anti-mouse HRP, Amersham, Piscataway, NJ) was applied to the α-spectrin membrane using a 1:3000 dilution, while the nNOS blot was incubated with anti-rabbit using a 1:8000 dilution. All blots were then incubated at room temperature for 35min. Then membranes were washed four times for 5min each with TBS-T and two additional washes with TBS. The membranes were scanned on the

Odyssey infrared imaging system (Li-Cor, Lincoln, Nebraska) and the relative fluorescence of the bands was quantified by densitometry using the accompanying software. Results are expressed as mean ± SEM.

RNA Isolation and Quantitative Real-Time PCR

We examined expression of mRNA transcripts for iNOS, the two cationic amino acid transporters responsible for L-Arg uptake in skeletal muscle cells (CAT-1 and CAT-2), and MAFbx.

Total RNA was extracted from cultured cells by harvesting in 1 ml of TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Concentration and purity of the extracted RNA was measured spectrophotometrically at A_{260} and A_{280} in 1X TE buffer (Promega, Madison, WI). Purified RNA was then stored at -80° C for later assay.

Reverse transcription (RT) was performed using the SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR) according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Reactions were carried out using 1 μg of total RNA and 2.5 μM oligo$(dT)_{20}$ primers. First strand cDNA was treated with two units of RNase H and stored at -80° C.

Primers and probes for CAT-1, CAT-2, iNOS, and MAFbx (assay # Mm00432019_m1, Mm00432032_m1, Mm00440485_m1, and Rn00591730, respectively) were obtained from the ABI Assays-on-Demand service and consisted of Taqman 5' labeled FAM reporters and 3' nonfluorescent quenchers. Primer and probe sequences from this service are proprietary and therefore, are not reported. Primer and probe sequences also consisting of Taqman 5' labeled FAM reporters and 3' nonfluorescent quenchers for hypoxanthine guanine phosphoribosyl transferase (HPRT) obtained from Applied Biosystems (Assays-by-Design) are: Forward, 5'-

GTTGGATACAGGCCAGACTTTGT-3'; Reverse, 5'-AGTCAAGGGCATATCCAACAACAA -3'; Probe, 5'-ACTTGTCTGGAATTTCA-3'.

Quantitative real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (ABI, Foster City, CA). Each 25 μl PCR reaction, performed in duplicate, contained 1 μl of cDNA reaction mixture. In this technique, amplification of the fluorescently labeled probe sequence located between the PCR primers was monitored in real-time during the PCR program. The number of PCR cycles required to reach a pre-determined threshold of fluorescence (called the CT) was determined for each sample. Samples were quantified relative to the CT for a normalizing gene determined separately in the same sample. This procedure is referred to as the comparative CT method as described by Bustin (2002). HPRT was selected as the appropriate normalizer since the expression of this gene in C2C12 cells is not significantly altered during differentiation and fusion ($p > 0.05$).

In Vivo Studies

Experimental Design

Adult mice were treated with the glucocorticoid, prednisolone, for 8 wks to examine the effects on skeletal muscle mass, protein content, satellite cell abundance and activity, and the potential for a nitric oxide donor to rescue prednisolone-induced deficits in satellite cell activation/proliferation in isolated, cultured skeletal muscle fibers.

Animals. Seven-month old male Swiss-Webster mice were obtained from Harlan (N=16). After a seven-day acclimation period, mice were weighed and then given a subcutaneous injection daily, six days per week in the morning for eight weeks. They received either Depo-Medrol (prednisolone; Pharmacia & Upjohn, Kalamazo, MI; n=8) or sterile saline (n=8) at a concentration of 2.1mg/kg of body weight. All injections were prepared so that each injection was approximately a 0.1ml volume. Food (normal mouse chow given *ad libitum*) was weighed

every three days for the duration of the study to account for weight loss due to reduced food consumption. Animals were weighed weekly on the non-injection day, and treatment dosage was adjusted for the next week based on the current animal weight. They were housed one to a cage in SPF-2 at ACS, University of Florida, under normal conditions (12h light/dark cycle). On the last day of the study they were anesthetized with inhaled isoflurane (2-3.5%) with oxygen as the carrier gas using an anesthesia cart (with the charcoal filter scavenger attached). Euthanasia was by exsanguination and was confirmed with cervical dislocation. Following this, the animal's skin and superficial fascia of the hind limbs was removed to expose the muscles of the hind limbs. The gastrocnemius (GN) muscle was dissected out and weighed immediately. In four animals per group, the muscle was flash frozen in liquid nitrogen for subsequent analysis of muscle protein content and Western blot procedures. The gastrocnemius from the remaining four animals from each group was digested with collagenase for myofiber isolation. (IACUC protocol #E401)

Protein Isolation and Western Blotting

Frozen muscles were thawed and homogenized in buffer (0.1M Tris Base; 0.1M $NaPO_4$; 0.01M EDTA; 30% glycerol; pH=7.8) with protease inhibitors (200mM Benzamidine; 1mg/ml Pepstatin A and Aprotinin; 5mg/ml Leupeptin; 1M DTT; 40mM phenylmethylsulfonyl) at a 1:5 dilution based on muscle weight. This was done on ice, with the dismemberator on for two bouts of 10sec each. Following homogenization, the slurry was allowed to sit on ice for 10min. Then 100ul was removed and added to 700ul of 0.05M NaOH and left overnight at room temperature for total protein versus connective tissue assay. The remainder of the slurry was centrifuged at 750 x g for 15min at 4°C. The supernatant was transferred to clean microcentrifuge tubes and stored at -80°C until used for Western Blotting.

Total protein and connective tissue protein was measured in the homogenized samples. Following an overnight digest (at least 18h) in 0.05M NaOH, total protein was assessed using the DC assay (Bio-Rad) and then the samples were centrifuged at 4000 x g for 15min to pellet the connective tissue and then the supernatant was subjected to the protein assay again. The second assessment of protein content is considered to be the protein in the non-connective tissue fraction. This measurement was used to determine equal protein loading for the Western Blots.

Western blot analysis for nNOS and eNOS in chronically treated muscle was done by methods described previously but the protein was transferred to a PVDF membrane. These membranes were blocked in 5% non-fat dry milk in TBS-T (0.01%) for 1h. They were rinsed in TBS-T and incubated overnight at a 1:500 dilution (polyclonal anti-nNOS, Cayman Chemical, Ann Arbor, MI; polyclonal anti-eNOS BD Transduction Laboratories, San Jose, CA). Secondary antibodies (anti-rabbit and anti-mouse for nNOS and eNOS, respectively; Sigma, St. Louis, MO) were applied for 1h (1:5000). Protein bands were detected with ECL+ (Amersham Biosciences, UK). The relative amounts of the bands were quantified by densiometry using ImageJ software.

mRNA Measurements

Total RNA was isolated from gastrocnemius (GN) samples after homogenization in Trizol. Reverse transcription followed by quantification of iNOS, CAT-1, and CAT-2 mRNA by quantitative real-time PCR was performed as described above for the cell culture experiment.

Single Fiber Isolation

GN muscles (N = 4 animals/group) were surgically removed by first removing the skin of the hindlimbs, exposing the Achilles tendon. Then a small incision was made behind the knee and forceps and small sharp scissors were used to cut away the upper layer of fascia and connective tissue down the length of the muscle. As much as possible of the fascia was carefully peeled away using a scalpel. Then the tendons at the knee were cut on either side at an angle.

Without pulling on the muscle, the Achilles tendon was cut and the GN pulled up while carefully trimming connective tissue on either side. This was done on both limbs. Once the GNs were removed, they were rinsed in PBS, filleted, and then immediately transferred to a 15ml falcon tube containing digest media (9ml DMEM, 1% penicillin/ streptomycin, 1ml collagenase). The digest was rocked at 37°C for 1.5h. Following the digest, the muscles were titurated three times with a wide-bore 10ml disposable pipet, then the fibers were allowed to settle. After that, the digest media was removed with a pipet down to about the 3ml mark. Then about 10ml of fresh, pre-warmed media (DMEM, 10% FBS, 1% penicillin/ streptomycin) was added to the falcon tube containing the fibers, and the tube inverted a few times. The media was removed again and the rinse repeated 1-2 more times. This diluted the collagenase. At this point 3-4ml of the digest was transferred to a series of p100 plates containing warm media. Quality fibers were removed from the surrounding dead (hypercontracted) fibers and debris by transferring to a clean plate (containing 15ml media) using a wide-bore 200µl pipet. Plates were not out of the incubator longer than 10min during this process. Once the chosen fibers were ready to plate, 1ml of warm media was added to each well of a 24-well plate and the plate was allowed to equilibrate in the incubator for 10-15min. Then a drop of matrigel was applied to each well. The fiber was added by extracting them from the clean plates in 50µl volume using a p200 pipet tip (with the end snipped-off) and another drop of matrigel was applied. After all fibers were plated, the 24-well plate was placed in the incubator for at least 15min. Then the plate was centrifuged at 1100 x g at 37°C for 30-40min to activate the satellite cells. The fibers were maintained at 37°C, 5% CO_2, for 24h. Treatments of DETA-NONO (0, 5, 10, 50µM) were added to the fibers from both control and glucocorticoid treated animals following the centrifugation step. After 24h, the cultures were fixed in 2% formaldehyde and immunohistochemistry performed for MyoD and

DAPI staining. The number of myogenic cells was quantified to assess activation or proliferation of satellite cells. Table 3-2 illustrates this design.

Statistical Analysis

Statistical analyses were performed on SPSS (version 12.0.1) using 2-way ANOVAs (glucocorticoid vs. supplement). Post hoc analyses were performed using Tukey's test. Significance was established at $p<0.05$.

Table 3-1. Cell diagram illustrating experimental design of the cell culture portion of proposed project.

	Dexamethasone (100 μM)	
	-	+
No Supplement Control	*n=6*	*n=6*
L-Arginine (10 mM)	*n=6*	*n=6*
DETA-NONO (10 μM)	*n=6*	*n=6*

Table 3-2. Cell diagram illustrating experimental design of the isolated myofiber portion of the proposed project.

	Fibers isolated from Control mice (n=4 mice)	Fibers isolated from Prednisolone-treated mice (n=4 mice)
No Supplement Control	*~100 fibers*	*~100 fibers*
5 μM DETA-NONO	*~100 fibers*	*~100 fibers*
10 μM DETA-NONO	*~100 fibers*	*~100 fibers*
50 μM DETA-NONO	*~100 fibers*	*~100 fibers*

CHAPTER 4
RESULTS

In Vitro Studies

MAFbx mRNA Expression

Dexamethasone treatment of L6 myotubes resulted in a six-fold increase in MAFbx mRNA expression compared to control (Table 4-1). Supplementation of arginine (10mM) or DETA-NONO (5μM) significantly blunted the effect of the dexamethasone, even though MAFbx mRNA did not reach control levels (Table 4-1). Treatment of the L6 myotubes with arginine alone significantly reduced MAFbx expression, whereas DETA-NONO supplementation alone showed a trend toward lower MAFbx expression but did not reach statistical significance (Table 4-1). The interaction between treatment groups was significant at all levels ($p<0.05$). Thus, the upregulation of MAFbx seen with dexamethasone use may be attenuated by L-Arg or DETA-NONO.

CAT and NOS mRNA Expression

In C2C12 cells, the mRNA expression for both transporters (CAT-1 and CAT-2) was significantly depressed in glucocorticoid treated cells ($p<0.05$) (Table 4-2). CT values for iNOS were below detectable limits.

NOS and α-Spectrin Protein Content

There was no significant difference in the cleaved/total α-spectrin protein expression normalized to β-actin with any treatment in cell culture ($p=0.818$) (Figure 4-1). nNOS and eNOS expression in both L6s and C2C12s was not detectable.

Histological Measurements

Size, nuclear number, and total myotube area per field of view were decreased in glucocorticoid-treated cells (Figure 4-2). The total myotube area per field of view dropped by

26% after exposure to dexamethasone, and this effect was attenuated with arginine treatment (only an 8.1% decrease when L-Arg was added). The dexamethasone effect was more moderate when DETA-NONO was added (23% decrease). Not only was less of the field of view covered by myotubes in the glucocorticoid-treated cells, these myotubes were also smaller (64%). When DETA-NONO or L-Arg was added to the dexamethasone-treated cells, the myotubes were only 42% smaller than controls (Figure 4-3). Nuclear number was significantly decreased by dexamethasone treatment ($p<0.05$), and this was attenuated when L-Arg or DETA-NONO was added to the medium (Figure 4-4). While confirming dexamethasone causes atrophy, these data also indicate that NO may partially attenuate the loss in size and nuclear number.

Nitric Oxide Production

Nitric oxide production was measured in vitro via 24h nitrate accumulation in the media. Media was assessed at time 0h, 24h, and 48h. Regardless of the treatment or time point, there were no differences between groups for fluorescence. So, cells treated with dexamethasone may not have compromised NOS activity.

In Vivo Studies

Body and Muscle Masses and Total Protein Content

Body mass and gastrocnemius mass did not significantly decline with 8 weeks of prednisolone treatment (Table 4-3). Total protein/gastrocnemius muscle did not significantly decrease, but there was a trend to be lower in glucocorticoid muscle compared to control.

MAFbx, NOS, and CAT mRNA Expression

MAFbx mRNA levels are expected to have reached a steady state of expression after 8 weeks of glucocorticoid treatment, thus this was not analyzed in muscle homogenate. However, CAT and iNOS expression were considered. CAT expression was insignificantly elevated in the

glucocorticoid treated animals (Table 4-4). iNOS values were below detectable limits, and thus not reported.

NOS Protein Content

The expression of nNOS and eNOS protein was reduced by over 30% in the glucocorticoid treated animals compared to controls (Figure 4-5B). Representative blots are displayed in Figure 4-5A.

Satellite Cell Emanation

MyoD+ satellite cells emanating from isolated myofibers 48 hours after centrifugation were significantly reduced with *in vivo* prednisolone treatment (54% of control fibers had emanating cells present vs. 25% of glucocorticoid fibers, Figure 4-6). However, supplementation of culture media with DETA-NONO (5-50 μM) induced satellite cell emanation in a dose-dependent manner. Fifty μM DETA-NONO eliminated the difference in centrifuge-induced satellite cell emanation between glucocorticoid and control fibers (76% of control vs. 74% of glucocorticoid). This suggests that NO bioavailability limits satellite cell activity in response to a mechanical stress. Further, exogenous NO can rescue compromised satellite cell function following glucocorticoid treatment.

Table 4-1. mRNA data for MAFbx in control and dexamethasone treated cells. Values are means ± SE. Transcripts are normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA and expressed relative to control value. Significantly different from *control and †dexamethasone (within group); $p<0.05$.

Treatment	Dex (-)	Dex (+)
Control	1.040±0.149	6.122±0.408*
L-Arg (10mM)	0.807±0.000*	4.321±0.426*†
DETA-NONO (10μM)	0.716±0.088*	3.904±0.476*†

Table 4-2. mRNA data for CAT transporters in cell culture. Values are means ± SE. Transcripts are normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA and expressed relative to control value. Significantly different from *control; $p<0.05$.

Gene of interest	Control	Glucocorticoid
CAT-1	2.534 ± 1.229	0.107 ± 0.071*
CAT-2	1.503 ± 0.3945	0.242 ± 0.129*

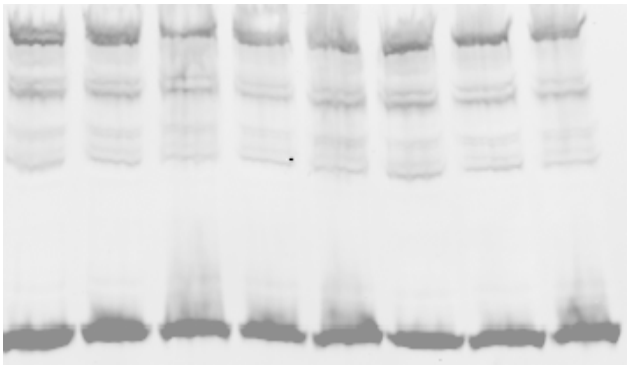
Table 4-3. Body and muscle masses and total protein content of animals. Values are means ± SE. There was no value significantly different from control; body mass $p=0.170$, gastrocnemius mass $p=0.490$, total protein/ muscle $p=0.087$.

	Control	Glucocorticoid
Body Mass (g)	36.4 ± 0.95	34.4 ± 1.18
Gastrocnemius Mass (g)	150.2 ± 5.75	154.8 ± 3.96
Total Protein (mg)/ Muscle (g)	15.81 ± 0.74	13.61 ± 0.90

Table 4-4. mRNA data for CAT transporters in animals. Values are means ± SE. Transcripts are normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA and expressed relative to control value. No value was significantly different from control (CAT-1 $p=0.221$; CAT-2 $p=0.18$).

Gene of interest	Control	Glucocorticoid
CAT-1	1.740 ± 0.699	3.146 ± 0.820
CAT-2	1.115 ± 0.281	2.002 ± 0.548

A)



B)

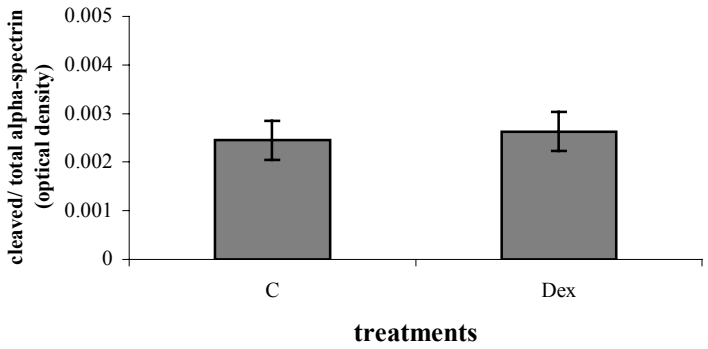


Figure 4-1. Protein analysis - α-spectrin A) representative immunoblot for α-spectrin (total 240kDa; cleaved 150kDa) and β-actin (loading control; 46kDa) in L6 myotubes treated with 24h dexamethasone or vehicle. Lanes 1 - 4 are control, lanes 5 - 8 are Dexamethasone (Dex). B) quantification of cleaved-to-total α-spectrin ratio. No value was statistically significant (p=0.818).

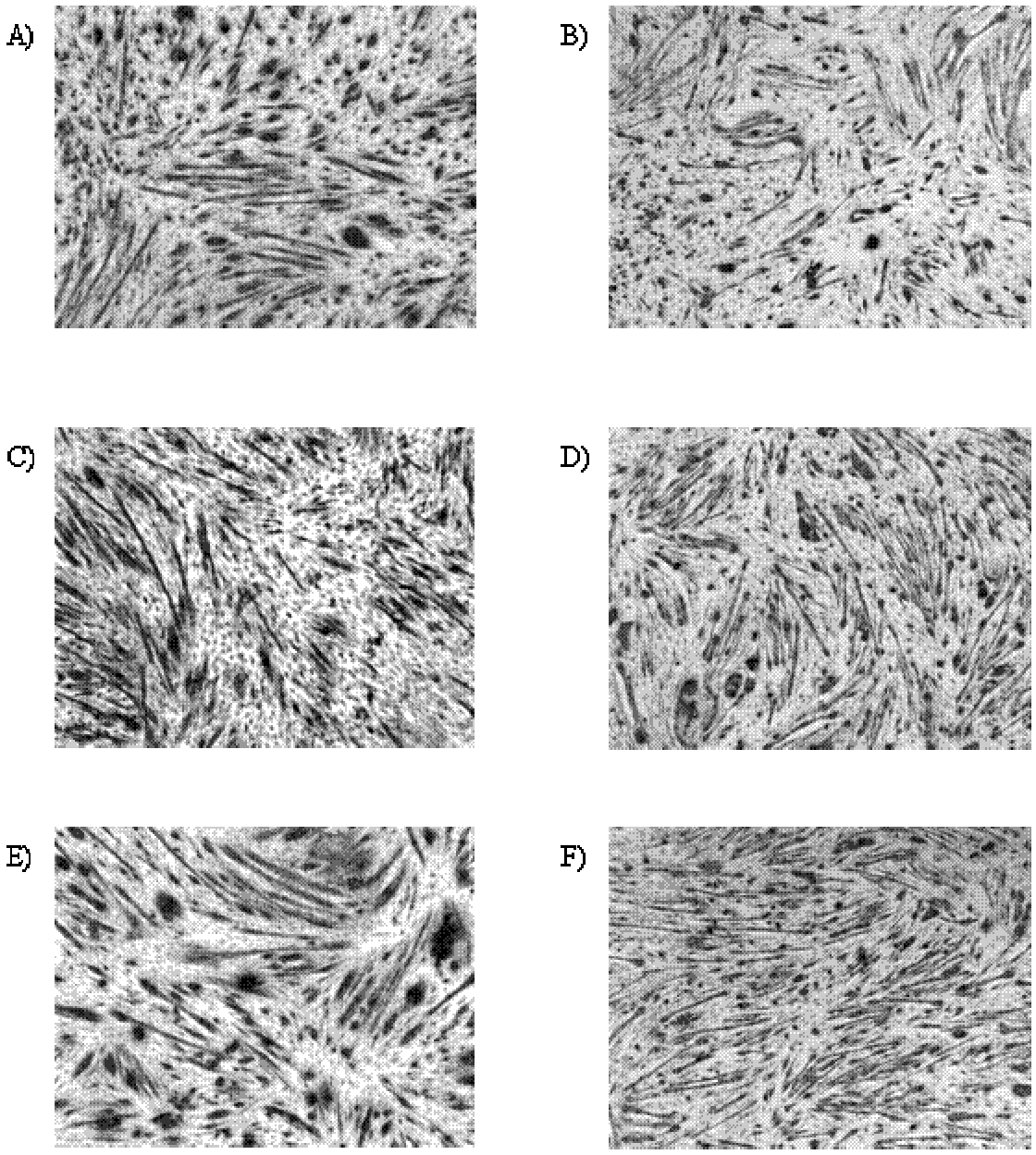


Figure 4-2. Representative images from cells fixed in 3.9% formaldehyde and H & E stained. A) control B) dexamethasone (Dex) C) 10mM L-Arg D) Dex + 10mM L-Arg E) 10μM DETA-NONO F) Dex + 10μM DETA-NONO.

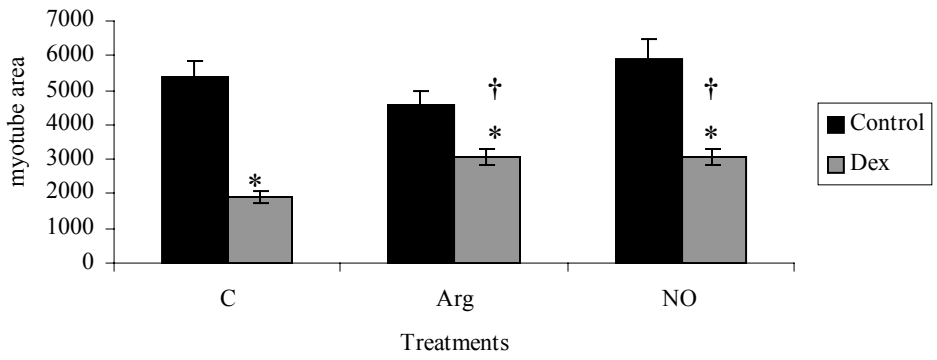


Figure 4-3. Average area of individual myotubes. Quantification of myotube area. Values are means ± SE. Significantly different from *control and †dexamethasone; $p<0.05$.

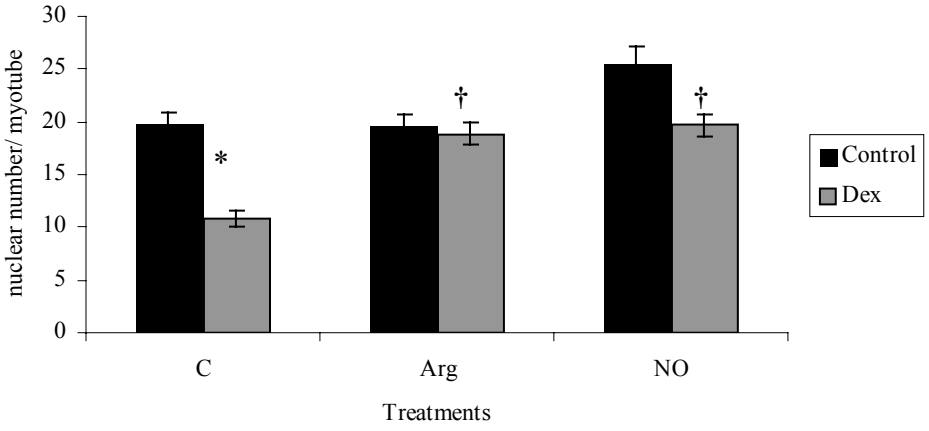
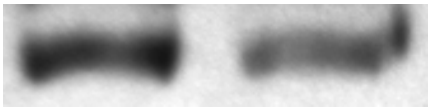
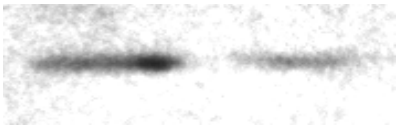


Figure 4-4. Nuclear number per myotube. Quantification of nuclear number. Values are means ± SE. Significantly different from *control and †dexamethasone; $p<0.05$.

A)

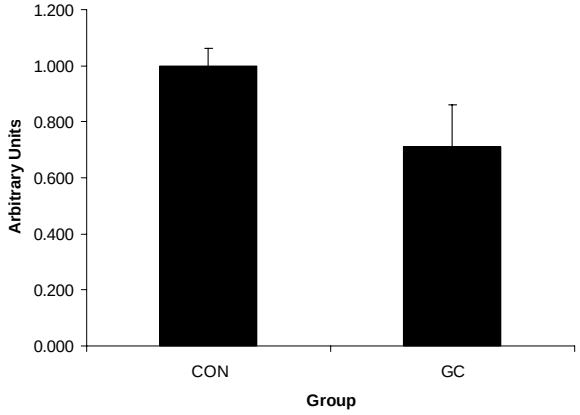


nNOS

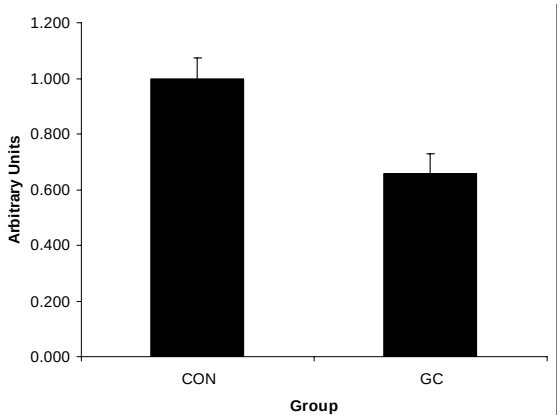


eNOS

B)



nNOS



eNOS

Figure 4-5. Protein analysis - nNOS and eNOS A) representative immunoblot for nNOS and eNOS in mouse muscle homogenate treated with 8wk prednisolone or vehicle. B) quantification of nNOS and eNOS normalized to control.

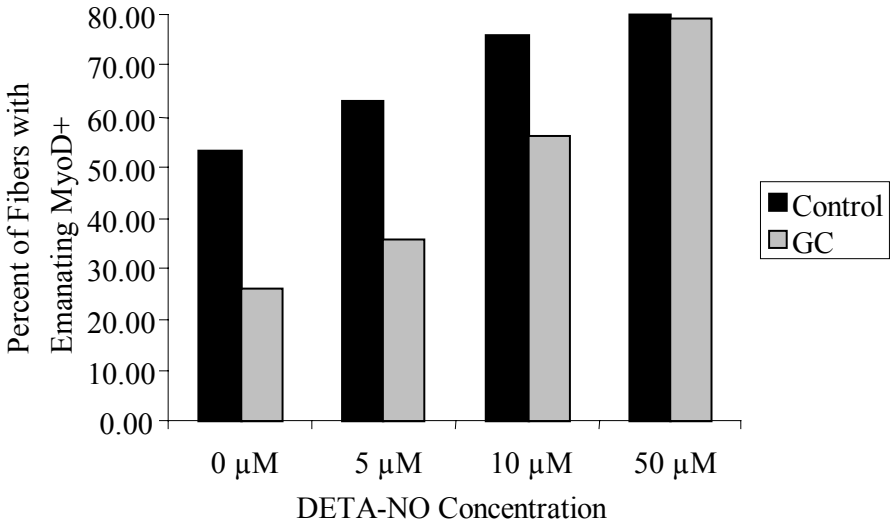


Figure 4-6. Satellite cells emanating from activated fibers. Quantification of cells emanating from activated fibers. Supplementation of culture media with DETA-NONO (5-50 µM) induced satellite cell emanation in a dose-dependent manner.

CHAPTER 5
DISCUSSION

Glucocorticoids cause substantial skeletal muscle atrophy (34, 77) and suppress NO production (42, 81). We show that 24h of treatment with glucocorticoids in cell culture increases MAFbx mRNA expression and that an NO donor attenuates the increased expression. After 48h of glucocorticoid treatment, we witness a dramatic decrease in myotube dimensions, which is partially rescued by either L-Arg or nitric oxide donation. We failed to confirm our hypothesis that NO production is compromised in glucocorticoid treated cells, as there was no change in nitrate accumulation in 24h media collection.

In addition to these acute effects observed with glucocorticoid use, we demonstrate that eight weeks of corticosteroid treatment reduces satellite cell activation/ proliferation from isolated fibers, which is necessary for skeletal muscle remodeling. Moreover, nNOS and eNOS protein content are reduced in glucocorticoid treated gastrocnemius. Interestingly, we found NO donation restores satellite cell emanation from glucocorticoid treated fibers to levels of control fibers, suggesting NO availability may be partially responsible for glucocorticoid-induced myopathy, in vivo.

Acute Effects of Glucocorticoids

Myotube Dimensions

Glucocorticoids induce protein loss and a decrease in myotube size (23). We show that 48h dexamethasone treatment reduces myotube dimensions - a decrease in size of 64%. Additionally, dexamethasone treatment resulted in less coverage of the field of view by myotubes and in fewer nuclei per myotube. We hypothesized that the atrophic myotubes were a result of reduced NO availability.

Initially to test this, we supplemented cultures with either L-Arg (10mM) or DETA-NONO (10μM). With these additions, the reduction in myotube size associated with dexamethasone treatment was attenuated by 22%. Furthermore, supplement inclusion resulted in maintenance of nuclear number. These results suggest, at least in part, that glucocorticoid-induced atrophy can be rescued with nitric oxide.

NO Availability

While the histological morphology data imply that NO availability may be compromised, these measurements fail to specifically demonstrate a reduction in NO production by glucocorticoid treated cells. In order to test this hypothesis we indirectly measured NO production by assessing nitrate and nitrite accumulation in the media. Fluorescence was constant in all samples. As we were unable to measure changes in nitric oxide production, we cannot confirm a limitation in the skeletal muscle's ability to produce nitric oxide. And, even though our attempt to verify NOS protein in L6 lysate was inconclusive, we are confident these cells possess this protein (10), and thus are capable of synthesizing nitric oxide.

Calpain Activation

Glucocorticoid administration likely induces skeletal muscle atrophy through increased expression of genes involved in proteolysis (17, 19, 38, 106). The inhibition of calcium-activated proteases (calpains) in glucocorticoid treated myotubes attenuates the usual glucocorticoid-induced protein loss and decrease in myotube size (77). And while it is generally accepted that calpain activation is a key initial event in protein degradation, we failed to show this in atrophy associated with glucocorticoid use. We showed atrophy in glucocorticoid treated myotubes versus controls can occur without a concomitant increased in cleaved versus total α-spectrin, a structural protein targeted by calpains. This is in agreement with Banik et al. (4), who

found a methylprednisolone-induced dose-dependent inhibition of rabbit muscle calpain activity. They also demonstrated enzyme inhibition with dexamethasone and prednisolone treatments. Another group (25) found a decrease in calpain activity and α-spectrin cleavage in mechanically ventilated rat diaphragm following glucocorticoid treatment. Additionally, Wang et al. (106) demonstrated only a small contribution of calcium-dependent muscle proteolysis in L6 myotube dexamethasone-induced atrophy. Furthermore, groups showing a positive correlation between dexamethasone treatment and calpain activity were actually measuring enzymatic activity. We analyzed the result of calpain activity in the living cell by measuring cleaved versus total α-spectrin protein, as the measurement of in vitro maximal enzymatic activity may not represent in vivo proteolytic activity. Further measurements will be necessary to draw firm conclusions regarding the role of calcium-dependent muscle proteolysis in glucocorticoid-induced myopathy.

MAFbx Control

MAFbx has at least three controls. It is regulated by two elements of the IGF-1/ Akt pathway and by one component of the atrophy pathway (Figure 5-1). Akt has two routes for diminishing MAFbx expression. It blocks FOXO, which is directly responsible for upregulation of MAFbx and enhances mTOR activity, which not only increases p70S6K, but also blocks MAFbx (26, 50). In addition to these events in the hypertrophy pathway that inhibit MAFbx expression, it has been shown that p38 via TNFα signaling in the atrophy pathway stimulates MAFbx expression.

We have demonstrated that 24h of treatment with glucocorticoids in cell culture increases MAFbx mRNA expression and that an NO donor attenuates the increased expression. It is not totally clear why an upregulation of MAFbx is seen with glucocorticoid use. IGF-1 and insulin both stimulate the hypertrophy pathway (Figure 5-1), and insulin resistance develops with

glucocorticoid use (70). Also it has been shown that NOS is essential for skeletal muscle hypertrophy (84). So, perhaps instead of solely increasing activity of the atrophy pathway, the hypertrophic signals are attenuated, thus removing the blockade on FOXO and MAFbx via deactivated Akt, with the ultimate effect of dramatic skeletal atrophy.

Nitric oxide's attenuation of the glucocorticoid effect on MAFbx mRNA is likely though the hypertrophy pathway, as it has been shown that NOS is essential for skeletal muscle hypertrophy (84).

Possible Mechanisms of Action for Nitric Oxide

Nitric oxide has been shown to be a key regulator of muscle hypertrophy and satellite cell activation. It has not been completely elucidated how nitric oxide is having its effect, but perhaps it is through some component of the hypertrophy pathway. This would explain its ability to attenuate glucocorticoid-induced atrophy. We have shown that adequate NO availability may be a limitation in skeletal muscle myopathy following glucocorticoid treatment.

The fact that we observed dramatic recovery of myotube dimensions and nuclear number and partial attenuation of MAFbx mRNA leads us to believe NO is potentially acting on a downstream target of the hypertrophy (IGF-1) pathway. For example, in arteries with functional endothelial cells, IGF-1 caused a concentration-dependent relaxation (78). This effect was abolished by the use of a NOS inhibitor. Futhermore, this author demonstrated formation of NO in response to IGF-1 treatment in cultured endothelial cells, and that the signaling was independent of intracellular Ca^{2+}, but involved PI3K, a direct downstream target of IGF-1. PI3K is required to phosphorylate (activate) Akt. Perhaps glucocorticoids attenuate Akt phosphorylation, which would lower activation of mTOR and activate FOXO thereby leading to atrophic signaling through MAFbx. Supplementation with exogenous L-arginine or nitric oxide reverses the atrophy by suppressing MAFbx activation.

Furthermore, MAFbx may be directly upregulated with glucocorticoid use due to FOXO activation. GSK3β is blocked by phosphorylated (active) Akt, and leads to hypertrophy (73) and FOXO inactivation (Figure 5-2). Sandri et al. (77) has shown that this same pathway, when inhibited at the IGF-1 level, will increase FOXO activation. We show the upregulation of MAFbx seen with glucocorticoid treatment was moderately attenuated by use of L-arginine, a NOS substrate, and NO donation (via DETA-NONO). Thus, the mild attenuation of MAFbx upregulation seen with the treatment of NO or L-Arg could be a result of any one of multiple elements in the IGF-1 pathway. Due to the lack of apparent calpain activation, which seems to not involve PI3K, it would be more reasonable to think that NO is working at least at the Akt level, if not further downstream. Further studies are necessary to determine if Akt and or FOXO are directly involved in NO action in skeletal muscle maintenance.

CAT Expression in Cultured Myotubes

The reduced expression of the CAT transporters seen in cell culture agrees with Simmons et al. (81) who showed a reduction in L-Arg uptake in dexamethasone treated cells. Dexamethasone treatment prevented the induction of CAT-2B mRNA by cytokines. In further agreement with our findings, Hammermann et al. (33) found a down-regulation of CAT-2B in rat alveolar macrophages after 20h exposure to dexamethasone. It seems that the inflammatory response increases L-Arg transport into the cells, which supports large-scale NO production by iNOS. Dexamethasone, however, is able to not only attenuate this increase in L-Arg influx by the down-regulation of CAT transporters, but due to its anti-inflammatory characteristics also seems to suppress iNOS (82). Our evidence supports this in that CAT-2 mRNA expression was down-regulated with dexamethasone treatment, however we were unable to measure changes in NOS. Therefore, glucocorticoids have the ability to reduce NO production by inhibiting

substrate availability through lower arginine transport into the cell. Given the importance of NO signaling for skeletal muscle remodeling as discussed above, inhibition of endogenous NO production may be an important mechanism for glucocorticoid-induced skeletal muscle myopathy. Futhermore, if the mechanism to synthesize NO is compromised with glucocorticoid treatment, then an NO donor should attenuate the effects of the glucocorticoid better than substrates for NO synthesis. This phenomenon may explain how DETA-NONO was a superior to L-Arg for inhibiting MAFbx expression. L-Arg only decreased the glucocorticoid-induced MAFbx mRNA expression by 1.8 fold, while DETA-NONO had a marginally better effect of a 2.3 fold decrease in expression. Without as much NOS or CAT present, physiological levels of L-Arg might not have been sufficient. But, bypassing the NOS enzyme, as was done using the NO donor DETA-NONO, a greater effect was observed.

Chronic Effects of Glucocorticoids

NOS Expression in Mouse Skeletal Muscle

After 8 weeks of prednisilone administration, we report a drop in both eNOS and nNOS expression in mouse skeletal muscle. This suggests that corticosteroids actually lead to lower amounts of the enzyme, which could result in reduced nitric oxide production and possible changes in both hypertrophic and atrophic signaling pathways.

eNOS down-regulation seems to be the cause of the lack of NO production observed with glucocorticoid treatment in cardiac microvascular endothelial cells (81). eNOS has been shown to be down-regulated by FOXO1 and FOXO3A (69). These factors bind to the eNOS promoter in endothelial cells. This causes repression of eNOS expression, and subsequent NO generation. So, the acute effects of glucocorticoids may be upregulation of MAFbx expression via FOXO action, with FOXO being the culprit of the long-term effect of decreased NOS expression.

CAT Expression in Mouse Skeletal Muscle

NOS presence is not the sole factor in determining NO production. L-Arg, a substrate for NOS, must be transported into the cell via the CAT transporters. These transporters had greater mRNA expression in the animals. Perhaps the increase in expression seen in the animals was due to a more chronic exposure to the corticosteroid. As NOS expression continued to decline over the 8 weeks, an upregulation in the cationic transporter was possibly an attempt to counter the decrease in eNOS and nNOS and restore NO levels. Since we did not directly measure NO production, we cannot confirm the net effect of decreased NOS expression and increased CAT transporter expression. Nevertheless, the efficacy of NO supplementation to isolated myofibers implies that endogenous NO production may have been compromised in the GC group.

Satellite Cell Activity in Single Muscle Fibers

In addition to NO inhibiting the synthesis of MAFbx and thus diminishing the initial atrophy effect, it can also be used to rescue satellite cells from glucocorticoid treated animals. It has been shown by numerous labs that activation of satellite cells is a necessary step in adult skeletal muscle growth and regeneration (1, 61, 67, 74). Futhermore, our work confirms these cells may be critical for maintenance of muscle mass. Other labs have demonstrated this in atrophied muscles from hindlimb suspended animals. Isolated myofibers from these animals contained fewer satellite cells and these cells had an impaired ability to activate and proliferate (62). This de-activation may be yet another factor in atrophy. In our study, we found a significant reduction in satellite cells emanating from glucocorticoid-treated fibers in response to mechanical stimulation. Approximately half of the fibers from control animals had at least one emanating myoD+ mononuclear cell, while only one-fourth of the fibers from glucocorticoid treated animals had emanating satellite cells.

Not only did we confirm that satellite cell function is compromised with chronic glucocorticoid treatment, but we also show exogenous NO treatment can ameliorate the glucocorticoid-induced deficit in satellite cell activation/ proliferation. In other models this has been a successful treatment for satellite cell activation (2, 3, 94, 95). Our findings suggest that NO production may be reduced with prolonged glucocorticoid exposure, and this is what reduces satellite cell activity.

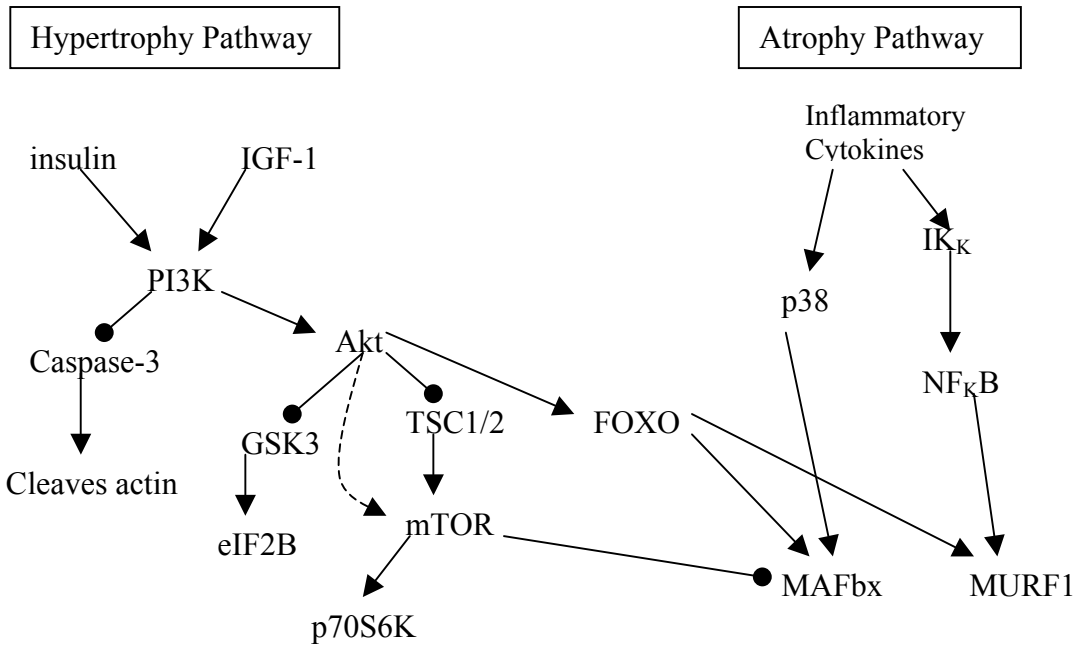


Figure 5-1. Illustration of the muscle maintenance pathways

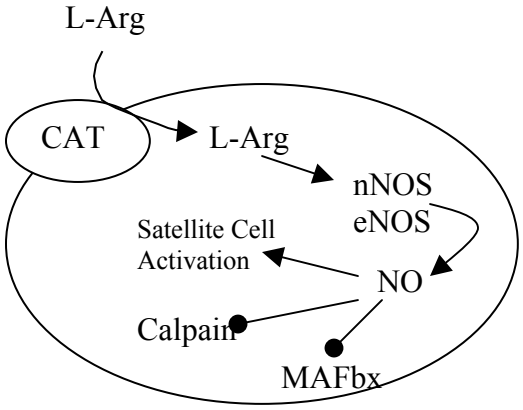


Figure 5-2. Illustration of potential effects of nitric oxide.

CHAPTER 6
CONCLUSION

Glucocorticoid treatment both *in vitro* and *in vivo* has detrimental effects. Acutely, we confirmed they cause upregulation of a key element of the Ub-prot pathway (MAFbx). Chronic treatment decreases constitutive NOS, thus potentially compromising NO production. NO has been shown to be expressed in conjunction with activation of the IGF-1 pathway and critical to satellite cell activity, both of which play a role in muscle mass maintenance. We demonstrate that NO attenuates the expression of MAFbx with acute glucocorticoid treatment. Furthermore we show that NO can rescue satellite cell activity from muscle chronically treated with glucocorticoids. So, NO may impact both the atrophic and hypertrophic pathways in its role in muscle mass maintenance. NO supplementation to cultures did have positive effects on maintaining myotube dimensions and increasing satellite cell activity. So, even if it does not directly inhibit a component of the atrophy pathway, NO may be a viable treatment option by augmenting muscle mass maintenance from the hypertrophy side via satellite cell activity and fusion with the existing myotubes. Thus, enhancing NO production may be important in attenuating the atrophic effects associated with both acute and chronic glucocorticoid use.

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BIOGRAPHICAL SKETCH

Jodi Heather Dixon Long was born in DuPage County, Illinois. The older of two children she grew up mostly in Carol Stream, Illinois, but moved with her family to St. Simons Island, Georgia in 1985 where she graduated salutatorian of the class of 1989 from Frederica Academy. In 1993, she earned her B.A. in mathematics, with a minor in secondary education from Erskine College in Due West, S.C. Upon baccalaureate graduation, Jodi served as a middle and high school math and science teacher and taught group exercise.

In 1997, Jodi returned to school to earn a M.S. in exercise physiology from Georgia Southern University. She was a graduate assistant in the physical education program while earning this degree. Following graduation in December of 1998, Jodi returned to a teaching career. She taught physical activity classes and anatomy and physiology at Coastal Georgia Community College in Brunswick, GA. In the fall of 1999, Jodi moved to a full time instructor position at her alma mater of Georgia Southern University where she taught anatomy and physiology, exercise physiology and health survey courses.

Once again, Jodi returned to school to complete her terminal degree. In the fall of 2002, she began studies at the University of Florida under the direction of David S. Criswell. While studying at UF, Jodi also taught anatomy and physiology at Santa Fe Community College in Gainesville, FL, eventually becoming a full time faculty member.

Following graduation from the doctor of philosophy program in the College of Health and Human Performance (Department of Applied Physiology and Kinesiology), Jodi plans to continue to educate college students in anatomy and physiology at Santa Fe Community College. She also continues to teach group fitness.

In addition to her professional and academic achievements, Jodi was married in 1991 to George W. “Trey” Long, III of McCormick, SC and they have two children, Sam (6yr) and Alex (4yr).