STATIN-ASSOCIATED SKELETAL MUSCLE DAMAGE AND ITS INTERACTIONS WITH NOVEL OR ACCUSTOMED EXERCISE

BY

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DISSERTATION

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ABSTRACT

Statin drugs are a very commonly prescribed medication, and their most common side effect is some degree of skeletal muscle myopathy. Unfortunately, this side effect is more likely/severe in statin users who are also exercisers. **Purpose:** To examine the previously unaddressed interactions of statin treatment with novel vs. accustomed exercise, as well as to examine cellular markers of the stress response—heat shock proteins (Hsps)—and apoptosis—activated caspases. **Methods:** C57 mice were treated with daily cerivastatin (1/mg/kg/day) or saline for two weeks, with/without concomitant wheel running (RW) (Novel & Sedentary groups). Additional groups also performed two weeks of RW activity prior to the initiation of statin treatment (Accustomed groups). RW activity was tracked daily, and hindlimb plantarflexor maximal force and fatigue was measured at the end of the intervention. Hsp25, αB-Crystallin, Caspase-3, and Caspase-9 were measured by western blot, plasma creatine kinase (CK) was assessed by activity assay. **Results:** Statin treatment did not significantly impact RW activity, however both sedentary and novel-exercise groups showed decrements in muscle force and fatigability with statin treatment. The effect on maximal force was more severe in the novel-exercise group, while accustomed exercise mice were protected from this decrement. Plasma creatine kinase levels did not correlate with functional outcomes. No significant effect of statin treatment was found for hsp25 or αB-Crystallin expression, though both proteins were increased by both the novel and accustomed exercise interventions. A significant injection by activity interaction was found for active caspase-9 expression, with statins increasing expression in the sedentary and novel groups, but decreasing expression in the accustomed groups. Active caspase-3 was not detectable in any group. **Conclusions:** These results indicate that exercise training prior to statin treatment can protect against myopathy, rather than exacerbate it, as seen with novel exercise. Additionally, accustomed exercise was able to reverse statin treatment’s activation of caspase-9, though the physiological significance of this is unclear, as the lack of caspase-3 expression indicates that apoptosis did not in fact occur. An upregulation in Hsp
expression with exercise may have contributed to the preservation of muscle force and decreased caspase-9 expression in the accustomed groups.
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GLOSSARY OF KEY TERMS

Caspase-3: Cysteine Aspartic acid Protease 3. Important to the execution of cellular apoptosis, especially so in skeletal muscle, as it is likely the rate-limiting protease in actinomyosin complex breakdown.


Cerivastatin (Baycol): Synthetic statin marketed by Bayer. Withdrawn from the market in 2001 due to an unusually high incidence of fatal rhabdomyolysis.

Creatine Kinase (CK): Enzyme catalyzing the conversion of creatine to phosphocreatine, expressed at especially high levels in striated muscle. Damage to muscle membranes allows leakage into the blood, leading to CK’s use as a marker of muscle damage.

Familial Hypercholesterolemia: Genetic disorder characterized by exceptionally high LDL levels. Typically due to mutations in the genes encoding for LDL receptors or apolipoprotein B.

F-Box Protein: Family of proteins containing one or more F-box structural motifs. Importantly, a component of a ubiquitin proteasome complex (SCF complex) which targets proteins for ubiquitin-tagging and subsequent degradation in the proteasome. F-box proteins provide ligase specificity to this complex.

GTPase: Large family of enzymes able to bind and hydrolyze GTP that are involved in cell signaling and protein synthesis. Parent family to the Ras superfamily of small GTPases. Small GTPases typically require prenylation before participating in cell signaling.
**Heat Shock Protein (Hsp):** Family of highly conserved proteins involved in both basal cellular processes and cellular protection and stress response. Both protective against and activated by a wide variety of cellular stressors, such as inflammation, oxidative stress, toxins, and exercise.

**Myoblasts:** Embryonic progenitor cell that can develop into a myocyte.

**Myopathy:** General term for muscle disease leading to weakness or other dysfunction.

**Myosin Heavy Chain (MHC):** Component of the myosin II protein responsible for the development of contractile force in muscle. Different MHC isoforms demonstrate differing contractile characteristics, and are therefore a common method of fiber-typing.

**Myotubes:** Developing skeletal muscle cells grown in culture, formed by the fusion of multiple myoblasts. So named due to their tubular appearance, which is quite distinct from myoblasts.

**Prenylation:** Attachment of one or more lipid groups (i.e. a terpenoid) to a protein. Affects the lipophilic properties of the protein, importantly, impacts membrane attachment.

**Rhabdomyolysis:** Potentially fatal condition of uncontrolled muscle breakdown which releases muscle cell content into the bloodstream. Several released compounds have toxic effects outside the muscle, and cause disruptions in renal function, electrolyte balance, and blood clotting.

**Statin:** HMG-CoA reductase inhibitor. Class of drugs used in the reduction of blood cholesterol via the inhibition of the mevalonate pathway at HMG-CoA reductase, though their reductions in cardiovascular disease risk go beyond these effects. Some degree of myopathy or myalgia is the most common side effect.
**Squalene:** Downstream compound of the mevalonate pathway necessary to the biosynthesis of cholesterol. Squalene’s position in the pathway downstream of most intermediate compounds but upstream of cholesterol positions it as a useful compound for investigating the impact of cholesterol reductions (by inhibiting squalene synthase) independent of statins.

**Terpenoids:** Extremely large group of lipid compounds. Important to the current topic due to their being produced via the mevalonate pathway and their importance to the process of prenylation.

**Ubiquinone:** Also known as coenzyme Q10. Involve in the electron transport chain and aerobic respiration, and therefore mostly expressed in the mitochondria. Ubiquinone biosynthesis occurs via the mevalonate pathway.

**Ubiquitin Proteasome:** Also known as the 26S proteasome. Proteasome responsible for the breakdown of proteins that have been ubiquitin-tagged by the SCF-complexes (see F-box protein).
INTRODUCTION

HMG-CoA reductase inhibitors (statins) are a common and effective pharmaceutical treatment for elevated blood cholesterol levels and high coronary heart disease risk. While they are a safe class of drugs overall, they do have side effects, the most common among these being some degree of skeletal muscle myopathy or myalgia. The risk for these side effects has not been conclusively determined, however it is judged to range between 1 and 10% of all statin users. The primary concern of the current work is that this risk is considerably elevated in those statin users who are also exercisers, reportedly as high as 25%. Unfortunately, while the mechanisms behind statin-associated myopathy in general are not well understood, the mechanisms involved in this exacerbation by exercise are neither well understood nor well-examined.

The vast majority of the available literature has examined the response to a single bout of exercise after a period of statin treatment, and has therefore examined a statin-potentiated exercise-damage response, and not an exacerbation of statin-associated myopathy by exercise. However, the true distinction between these two effects is impossible to discern based on current literature, as it is currently unknown whether exercise-exacerbated myopathy is the result of cumulative exaggerated damage responses to single exercise bouts, exercise-induced exacerbation of damage pathways active in sedentary statin-treated muscle, or exercise-induced activation of an otherwise dormant statin-induced damage pathway. In any case, the majority of this literature has not been mechanistic in nature, and examinations of the actual mechanisms involved in statin/exercise interactions is extremely sparse.

Unfortunately, investigation of this interaction is further complicated by the adaptations that occur in skeletal muscle in response to an exercise bout or to exercise training. As noted, the
majority of the prior literature examines the response to a single exercise bout, and there exists only one prior study investigating multiple exercise bouts. None of these studies have allowed exercise accustomization to occur prior to the initiation of statin treatment. Therefore, since all prior research has utilized an exercise stimulus that is novel—and therefore unusually damaging so far as the subject is concerned—none of this prior research accounts for the adaptations that occur in muscle in response to as little as a single exercise bout. As a result, this research is not necessarily translatable to an exercise-trained individual, or even to an individual who might not be considered “trained”, but who does not change their physical activity routines after beginning statin treatment.

Finally, an additional issue exists in the quality of the damage measures used to assess myopathy. Literature examining exercise and statin interactions commonly use blood creatine kinase (CK) in the examination of damage responses, and only a few studies have examined muscle damage though histological or other methods. Creatine kinase is not a reliable or repeatable measure of exercise-induced muscle damage, and the use of CK is especially problematic in examining the combination of exercise and statins because CK levels have also been repeatedly shown to be an insensitive marker of statin-associated myopathy. Therefore the interactions of statins and exercise have commonly been examined through a measure that is a poor marker for both components of the interaction individually, and therefore cannot be expected to be a reliable measure of them in combination.

The current work seeks first to address the issue of exercise training prior to the initiation of statin treatment, and whether statins’ interaction with accustomed exercise is the same as that with novel exercise. Furthermore, in addition to increasing the overall validity of the study, the use
of a functional and reliable measure of muscle damage—force-production ability—allows the examination of CK’s utility as a measure for exercise/statin interactions.

Finally, mechanisms of the exercise/statin interaction will be examined. Specifically, the effect of statin treatment on skeletal muscle heat shock protein expression has not yet been investigated, though heat shock protein expression presents a potential mechanism affecting the interactions between statins and repeated exercise. Additionally, markers for apoptosis will be examined, as there is strong in vitro evidence indicating that statins are able to initiate apoptosis in numerous cell types, including skeletal muscle cells. In vivo research has indicated that skeletal muscle apoptosis does not in fact occur, however this has not been examined under the potentially more damaging condition of combined statin and exercise treatments.

**SPECIFIC STUDY AIMS**

**Aim 1: Examine the effects of accustomed exercise versus novel exercise on statin-induced myopathy, and how either exercise treatment compares to sedentary mice.**

Hypothesis: We hypothesize that two weeks of running wheel activity prior to the commencement of statin therapy will be protective against statin-induced myopathy during the subsequent two weeks of concomitant wheel-running and statin treatment. We expect the sedentary, statin-treated mice to show decrements in maximal force production ability, and that the novel-exercise mice will show an even greater deficit.

**Aim 2: Examine the validity of using CK measures as a marker for statin-associated myopathy under both sedentary and exercise conditions.**
Hypothesis: We expect that statin treatment may increase blood CK levels in the sedentary and novel-exercise mice at the level of a statistical main-effect. However, we do not expect significant within-subject correlations to exist between CK levels and functional muscle measures, which would contraindicate CK’s sole use as a clinical measure for statin-associated myopathy.

**Aim 3: Examine molecular markers for apoptosis (caspase-3, caspase-9) and the stress response (Hsp25, αB-Crystallin) under all combinations of statin and exercise treatment.**

Hypothesis: We expect two weeks of running wheel activity to upregulate heat shock protein expression in the novel-exercise mice, and for this upregulation to be associated with the preservation of muscle function during statin treatment. Further, we hypothesize that statin treatment in the novel-exercise mice will block this upregulation. Finally, while apoptotic markers are not expected to be elevated in the sedentary mice, we hypothesize that the combination of novel exercise and statin treatment may lead to the expression of caspase-9 and caspase-3 in skeletal muscle.

**Significance**

While statins do not have unusually high incidences of side effects, the large population of individuals on statin therapy lends considerable weight to any advancement that can decrease the incidence of harmful side effects and improve quality of life. The current work seeks to contribute to such advancements through several avenues. The first is to address the issue of novel versus accustomed exercise and statin treatment. This topic has not previously been examined, but it potentially presents a subject of great clinical importance. If it were discovered that certain exercise modalities or scheduling regimens are able to prevent or alleviate the occurrence of statin-
associated myopathy clinicians would have an entirely new set of tools with which to address
exercise and statins’ deleterious interactions. This research makes the first inroads towards
developing a body of research to hopefully make such interventions possible.

Secondly, as previously noted, investigations into the mechanisms behind exercise and
statins’ interactions are extremely limited. The current investigations of heat shock protein and
caspase expression will expand the available mechanistic data on novel exercise, as well as provide
the first mechanistic data in the examination of statins and accustomed exercise. While it may not
necessarily be required in the development of the type of exercise regimens mentioned above, it is
unlikely that any improvement in statin-associated myopathy will be achieved through
pharmacological, nutritional, or similar means without a further understanding of the mechanisms
involved.

Finally, a more society-based, less scientific, and perhaps overly optimistic goal of the
current work is to encourage further research into this remarkably barren area. Ultimately, a
mature literature base covering diverse combinations of exercise interventions and cellular
mechanisms is likely to be needed before any significant breakthroughs can be made. As the
current literature base is far from being well developed, there is little expectation that the current
work will lead to any such breakthrough. Therefore this study was conceived with the intention of
challenging the prior research designs all utilizing similar exercise interventions and measures in
the hope that others will take an interest in expanding this area of research.
CHAPTER 1: LITERATURE REVIEW

Statin-Associated Myopathy and its Exacerbation with Exercise*

* This manuscript has been previously published and can be accessed by the below reference. Its current use has been permitted by the original publisher.

**INTRODUCTION**

HMG-CoA reductase inhibitors (statins) are currently the most effective lipid-lowering drugs available. Due to their high efficacy and the fact that they are generally very well-tolerated, they are widely used, with over 100 million prescriptions filled for over 14 million patients in the year 2004 \(^1\). Despite this, many consider statins to still be under-prescribed \(^2\text{-}^6\), and their use is expected to continue to grow alongside the increasing elderly population and ongoing discoveries of pleiotropic effects of statins \(^1\).

Overall, statins are a relatively safe family of drugs. However, statin therapy significantly elevates the risk of myopathy, which can range from muscle weakness, aches, and fatigue to rhabdomyolysis. It has been estimated that rhabdomyolysis affects as many as 0.1\% of all statin users, and the occurrence of general myopathies has been estimated to range from 1\%-10\% \(^1,^7\text{-}^9\). While this presents a relatively low risk of complications and almost certainly does not outweigh the benefits in cardiovascular risk modification, the sheer number of patients prescribed statin medication leads to a large absolute number of myopathic complications. Additionally, while low-density lipoprotein (LDL) levels and cardiovascular disease risk decrease with higher statin doses \(^10\), the risk of statin-associated myopathy may increase in a dose-dependant manner \(^11,^7\). Unfortunately, this puts lipid profiles and muscle function at odds, and considering that the majority of adults are sedentary \(^12\), it is not surprising that the recommended doses favor optimal lipid profiles over optimal muscle function \(^10,^13\).

The risk/benefit analysis of statin therapy and dosing is further complicated by the common finding that exercise seems to increase the risk for statin-induced muscle myopathy. The prevalence of myopathy dramatically rises to as high as 25\% among statin users who are also exercisers \(^1,^14,^13\), and may be over 75\% in statin-treated athletes \(^15\). Clearly, this presents an even
greater paradox, as certainly it is not desirable for physical inactivity to ever be a necessary prerequisite to successful and complication-free statin treatment. Numerous studies have shown that physically active individuals have a significantly reduced risk of cardiovascular disease as compared to less active individuals \(^{16-21}\), therefore, the potentially antagonistic effects of statin therapy and physical activity on muscle function present a challenge in achieving the best possible reductions in cardiovascular risk. Identification of the circumstances in which physical activity exacerbates statin-induced myopathy and the mechanisms through which these effects are mediated is needed to overcome this antagonism and appreciate the full benefits of both statin therapy and physical activity.

Several reviews address the general issues of statin-related myopathies \(^{1,22,23,13}\), and thus this review focuses primarily on the exacerbation of myopathy incidence or severity by the combination of physical activity and statin treatment. Our goal is to outline the relevant research and to highlight topics in which further examination is needed in this sparsely researched area.

**M E C H A N I S M S O F S T A T I N - I N D U C E D M Y O P A T H Y**

The mechanisms behind statin-induced myopathy are currently not well understood. Furthermore, whether the pathways behind myopathy in sedentary individuals are also the pathways responsible for exacerbated myopathy with exercise is currently unknown. However, in the absence of mechanistic literature regarding statins and exercise, pathways implicated in sedentary myopathy present a logical starting point for investigating exercise interactions, and in the interest of highlighting directions for future research we have made note of those pathways which may be candidates for exercise exacerbation.
Statins have been shown to induce apoptosis in numerous cell types, including cardiac myocytes, smooth muscle cells, pericytes, and cancer cells\textsuperscript{24-33}. While these apoptotic actions have indicated a potential role for statins in anti-cancer treatments\textsuperscript{28} and the attenuation of cardiac hypertrophy\textsuperscript{34}, in skeletal muscle they may lead to myopathy. Specifically, statin therapy has been shown to induce apoptosis in myotubes and myoblasts, as well as differentiated primary human skeletal muscle cells\textsuperscript{35-38}.

Multiple pathways exist to activate apoptosis in skeletal muscle, both intrinsic to the muscle fiber itself—such as elevated intracellular calcium or reactive oxygen species—and extrinsic—such as Tumor Necrosis Factor-alpha signaling. Additionally, it has been shown that statin-induced apoptosis proceeds through the caspase-dependant pathway, as it leads to an increase in caspase-9 and caspase-3 activity\textsuperscript{35,38}. In muscle, the ubiquitin-proteasome (UP) system cannot break down actinomyosin complexes. However, caspase-3 is likely the rate-limiting protease for myofilament release, and as such is strongly implicated in both accelerated contractile protein breakdown\textsuperscript{39,40} and muscle weakness\textsuperscript{41}.

Unfortunately, the \textit{in vitro} evidence for statin-induced apoptosis has not been confirmed \textit{in vivo}, and no studies have investigated if exercise exacerbates the potential apoptotic effects of statins. To date, apoptotic markers have only been quantified in two studies. In 11 hypercholesterolemic clinic patients with statin-associated myopathy no evidence of increased apoptosis was observed in several apoptotic markers including TUNEL staining, Bax, Bcl-2, and caspase-3\textsuperscript{42}. In rats receiving a high dose of cerivastatin (1mg/kg/day) no differences in activated caspase-3 expression were observed following 14 days of statin treatment\textsuperscript{43}. While this intervention corresponds temporally to the ~2-week time-points at which statin-induced damage has been confirmed\textsuperscript{44,43}, the authors suggest their measurement of activated caspase-3 at 24h post-
statin administration may have missed a potential rise in active caspase-3, since it is an early measure of apoptosis. Information on the temporal relationship between muscle biopsies and statin administration in the aforementioned human patient study was not given. Whether statins induce elevations in caspase-3 activity in vivo prior to the previously examined 24-hour time-point is currently unknown, and evidence is lacking regarding the mechanism through which statins may activate apoptotic pathways in muscle fibers.

Another possible contributor to statin-associated myopathy is a reduction in muscle membrane fluidity due to reduced cholesterol content, which has been a concern with other lipid-lowering medications such as niacin and clofibrate. This would also help explain an elevated risk with exercise, in which the muscle can be exposed to high levels of mechanical loading and stretch. However—despite being the major clinical target of statin prescription—reductions in cholesterol levels do not appear to be a direct contributor to myopathy. Inhibition of the cholesterol synthesis pathway downstream of HMG-CoA—by inhibition of the enzyme squalene synthase—does not trigger myopathy, as would be expected if a shift in membrane composition was a major contributor to myopathy. These data also indicate that at least some of statins’ myotoxic effects are downstream of mevalonate, but not of squalene synthase (Figure 1.1). However, since membrane composition or fluidity has not been examined under the additional stress of exercise, it is possible that the increased mechanical load/stretch imposed on a muscle fiber with exercise would bring to light subtle shifts in membrane composition or reductions in fluidity that could lead to damage.

One of the possible mediators fitting the requirement of being upstream of squalene synthase but downstream of mevalonate are the terpenoids, and indeed it has been consistently shown that reduced terpenoid levels induce apoptosis in various cell types.
Terpenoids are a highly diverse class of lipid molecules which are precursors to many compounds, and—importantly—can also modify proteins by adding lipid groups in a process called prenylation. Several studies have demonstrated that decreased geranylgeranyl pyrophosphate and farnesyl pyrophosphate levels—primary precursors to terpenoid production—result in the expected downstream effect of terpenoid depletion and can, contribute to statin-induced apoptosis. Among the proteins affected by reductions in prenylation would be the important GTPases Ras, Rho, and Rac, which require prenylation by terpenoids before membrane association, where they participate in cell signaling. If prenylation is inhibited, GTPases will remain cytosolic and inactive (Figure 1.1). Therefore, as GTPases are involved in a wide range of cell signaling pathways, a reduction in terpenoid levels has the potential to impair cell signaling in a wide variety of pathways, including apoptotic and MAPK signaling. Since the MAPK pathway is rapidly activated by muscle contraction, the effects of decreased terpenoids on this signaling pathway may present another candidate mechanism for exacerbation of statin-induced myopathy with exercise (Figure 1.2). However, it should be noted that these results are often correlative and not mechanistic in nature. Additionally, the pathways involved are highly dependent on cell type, and have not yet been examined in differentiated skeletal muscle.

Alternatively, it has been theorized that downregulation of the mevalonate pathway and reductions in terpenoid availability could lead to a reduced muscle content of ubiquinones (Figure 1.2). However, this theory is not well-supported by the literature, as both in vitro and in vivo data has been inconsistent. Furthermore, in vitro data has indicated that the changes that may occur in ubiquinone content do not correlate with an increase in apoptosis. Currently, the data on ubiquinone levels is deemed insufficient to justify the recommendation of ubiquinone supplementation to statin patients as a therapy to reduce myopathy.
Figure 1.1: Simplified schematic of the mevalonate pathway in which inhibition of the enzyme HMG CoA Reductase by statins inhibits the production of mevalonate and its downstream compounds. Studies of statin-induced myopathy independent of exercise suggest the following: (A) pathway inhibition at squalene synthase is not associated with myopathy; (B) ubiquinone’s role is less clear, but there is evidence indicating reductions do not induce myopathy; and (C) terpenoid depletion and subsequent reductions in prenylated proteins are associated with greater myopathy. No studies to date have investigated the potential contribution of these pathways in the exacerbation of statin-associated myopathy with exercise.
Finally, it has been suggested that statin-associated myopathy may be related to mitochondrial dysfunction \(^{59,60}\). An elevated lactate/pyruvate ratio \(^{59}\), intramuscular lipid-droplet accumulation \(^{60}\), and impaired β-oxidation \(^{61}\) have all been found in statin-treated muscle. Furthermore, transmission electron microscopy of statin-treated muscle fibers has shown that mitochondrial damage is present even in fibers which appear otherwise unaffected \(^{43}\). Because exercise dramatically increases oxygen consumption and consequently the activity of a muscle’s metabolic pathways—of which mitochondria are an essential component—this presents an obvious route through which exercise may exacerbate statin-associated myopathy (Figure 1.2). However, this possibility has been examined only indirectly in the current research, with conflicting results \(^{44,43}\).

**Potential Role for Exercise: Directed Research**

Unfortunately, while the literature addressing the mechanisms behind statin-associated myopathy is limited, literature specifically addressing the interactions of statin treatment and exercise is even more so. While contributory, a large proportion of this literature is based on blood markers of muscle damage, and currently less than half a dozen articles are available in the literature that could be considered controlled, mechanistic investigations of the effects of statin use on exercise associated muscle damage. Also, before addressing this literature it is important to note the distinction between an exacerbation of statin-associated myopathy by exercise and an exacerbation of exercise-induced damage by statins. Literature using blood creatine kinase (CK) generally addresses only the latter, and does not determine a change in myopathy risk with exercise. In fact, this is true of the vast majority of literature addressing statins and exercise, which examine the response to a single exercise bout. However, the line between exacerbated statin-
associated myopathy by exercise and exacerbated exercise-induced damage by statins is currently unclear, as no literature currently exists to determine whether elevated myopathy risk with exercise is the result of cumulative elevations in the damage response to single exercise bouts, or if distinct pathways for statin-induced damage are activated by exercise, or both.

**Creatine Kinase**

In the early 1990’s numerous studies were conducted addressing the combination of exercise and statin treatment. Until that time, both exercise and statin therapy had been shown to independently increase CK levels, but the interactions between the two had not been examined. Two studies investigating the interaction of simvastatin or lovastatin treatment and exercise found that statins exacerbated the CK response to a single bout of cycling and downhill walking, respectively. Alternatively, two studies, Reust et. al. and Thompson et. al. examined the effect of 4 weeks of Lovastatin treatment (40mg/day and 20mg/day, respectively) on the serum CK response to a single bout of eccentric or concentric treadmill exercise, respectively, and neither research group found evidence that Lovastatin treatment significantly elevated the CK response to exercise. However, while methodologically sound, the studies reporting no interaction between statins and exercise on the CK response both had small sample sizes, with 10 and 14 subjects for Reust et. al. and Thompson et. al., respectively.

Thompson et. al. later completed a similar investigation including a greater number of subjects who performed both resistance exercise and treadmill exercise. Forty-nine subjects with LDL-C levels above 130mg/dL (aged 18-85 years) were randomly assigned to five weeks of Lovastatin (40mg/day) or placebo treatments. After 4 weeks of treatment, subjects performed a downhill walking exercise consisting of three 15-minute intervals on a 15% decline. One week
later, the subjects performed a bicep curl exercise, consisting of 4 sets of 10 repetitions at 50% of their 1-repetition maximum. Serum CK measures were taken immediately prior to each exercise intervention, and subsequent measures were taken every 24 hours extending to 96 hours post-treadmill and 120 hours post-bicep curl. Following the bicep curl exercise there were no significant differences in CK levels between Lovastatin and placebo groups. In contrast, after the downhill treadmill exercise the Lovastatin-treated group had higher CK levels than the placebo group at all time points, reaching statistical significance at 24 and 48 hours post-exercise. These finding suggest that the mode of exercise, type of muscle action, and muscle group may all contribute to the interaction of statin-induced damage and exercise. Specifically, downhill walking has a large eccentric component and involves large muscle groups (i.e. quadriceps) and eccentric or lengthening exercise is a well-established model to induce muscle damage as compared to concentric or isometric exercise.

While eccentric exercise in combination with statin treatment may be associated with greater muscle damage as assessed by CK levels, increased statin dosage may not exacerbate the damage. Kearns et al. investigated the relationship between atorvastatin dosage and the serum CK and muscle soreness response to a single downhill treadmill exercise bout. Their results suggested that the degree of elevation in CK levels and soreness after a damaging exercise bout may not be related to the prescribed statin dosage. Specifically, groups treated with 10mg/day or 80mg/day both showed similar responses. Additionally, the damage outcome measures—serum CK and muscle soreness—were not significantly correlated within subjects at either dose. Unfortunately, no placebo group was included in this study, leaving the results open to multiple interpretations. If maximal damage occurred at a dose of 10mg/day, this could explain the absence of an exacerbated damage responses at 80mg/day. Alternatively—since no placebo was included—
it is possible that neither group experienced any further damage due to statin treatment, i.e., had a placebo group been included similar CK and soreness responses may have been observed with placebo and 10 or 80 mg/day of atorvastatin. Consequently, the relationship between statin dose and exercise-exacerbated myopathy remains unresolved. Further, in non-exercising statin users the relationship between myopathy and statin dose is also conflicting, reporting that myopathy may 11,7 or may not 70,71 be dose-responsive.

The lack of correlation between muscle soreness ratings and CK levels in the study by Kearns et al. highlights a considerable issue with this body of research. The serum CK response to exercise is unreliable and does not necessarily correlate with more robust measures of muscle damage 72-74. Furthermore, a large number of studies have reported manifestations of statin-induced myopathy without corresponding elevations in serum CK levels 75,76,60,77,7. Taken together, this contributes an additional element of uncertainty to the literature, as the utilization of serum CK as a marker of muscle damage may underestimate the prevalence and/or severity of statin-associated myopathy, especially in combination with exercise. Consequently, studies mechanistically examining this interaction through more reliable and descriptive measures are needed.

**Potential Mechanisms Contributing to Statin-Induced Myopathy with Exercise**

As discussed, there is a reasonable body of research reporting potential cellular mechanisms underlying statin-induced myopathy independent of exercise, and the serum CK response to a single exercise bout after a period of statin therapy has been examined using several modes of exercise. However, research investigating the cellular mechanisms behind the apparently
agonistic interactions between physical activity and statin treatment is extremely limited, as evidenced by only two studies to date which have examined this important subset of statin-associated myopathies.

A study by Urso et. al. quantified skeletal muscle gene expression eight hours after an eccentric exercise bout in human subjects receiving four weeks of Atorvastatin (80mg/day) or placebo. They found significant statin-induced alterations in genes involved in the ubiquitin proteasome pathway (UPP), protein catabolism, inflammation, and apoptosis. Unfortunately, this study did not measure protein levels, and thus it is unknown whether these mRNA changes were observed at the functional protein level. However, animal models investigating catabolic stimuli such as muscle damage, disuse, or denervation have shown that up-regulation of UPP genes is associated with increases in protein catabolism. Interestingly, while previous research suggests that statin-induced myopathies may be partially due to apoptosis, this study reported that genes involved in catabolism and the UPP were up-regulated, while genes involved in apoptosis and inflammation were down-regulated after exercise in statin-treated compared to placebo-treated subjects. Among the genes with altered expression, the authors identified the UPP gene FBXO3—which showed a four-fold increase in expression—as the strongest candidate contributing to the exacerbation of exercise-induced myopathy with statin treatment. While the exact role for FBXO3 is unknown, as an F-box-motif protein, FBXO3 likely selects specific proteins for ubiquitin tagging and subsequent degradation in the proteasome. However, which particular protein or proteins FBXO3 has an affinity for is not presently known. If FBXO3 targets important structural or contractile proteins its upregulation could clearly contribute to statin-associated muscle damage. Thus, further investigation is needed into the potential physiological actions of FBXO3 and other UPP proteins during exercise-induced myopathy in statin users.
A review of the literature finds only a single study investigating the effects of exercise on statin-induced muscle damage beyond performance of a single exercise bout. This is clearly important, as a consistent exercise training program—not a single exercise bout—is necessary to observe positive changes in lipid profiles. Seachrist et. al. examined both serum and muscle measures after two weeks of concomitant treadmill running and statin treatment in rats receiving various cerivastatin doses. While exercise alone or cerivastatin alone at doses of 0.5 and 1mg/kg significantly elevated serum CK levels, these levels were not further elevated by the combination of Cerivastatin and exercise. Serum levels of the muscle and liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were increased and blood glucose was decreased by high-dose cerivastatin treatment; however, none of these measures were affected by the combination of cerivastatin and exercise. Further, intramuscular cerivastatin concentrations were reflective of plasma concentrations following repeated administration, indicating that cerivastatin may not accumulate in muscle. Muscle concentrations of cerivastatin were not affected by the addition of exercise, suggesting that muscle contraction per se does not lead to a potentially damaging accumulation of the drug in the muscle. However, it should be noted that while there has been no indication of the various statins causing myopathy through different pathways, they are well known to have different lipophilic and hydrophilic properties. Differences in these properties affect membrane permeability, and therefore these findings on cerivastatin’s muscle pharmokinetics may not necessarily translate to other statins.

In contrast to the aforementioned blood measures, an interaction effect of cerivastatin and exercise was found in numerous muscle measures. Quadriceps muscle weight was reduced in exercising mice receiving statin treatment at 1mg/kg compared to exercising mice receiving placebo. Histological examination of muscle from sedentary rats treated at 1mg/kg showed
fragmented sarcoplasm and vacuoles, centrally-located nuclei, and an absence of cross-striations. Importantly, the addition of exercise resulted in increased severity of these damage markers. Statin-induced damage was seen in all muscles studied with the exception of the soleus, and the damage was exacerbated by exercise in the gastrocnemius, quadriceps, psoas, and extensor digitorum longus (EDL). In addition to the aforementioned damage markers, muscles that were subjected to both exercise and statin treatment had increased infiltration of inflammatory cells and mineralization in degenerated areas.

Using Transmission Electron Microscopy, statin (0.5 mg/kg)-associated damage was detected in subsarcolemmal mitochondria in psoas and EDL fibers that appeared otherwise unaffected, but this was not exacerbated by exercise. At 1mg/kg however, exercise increased the degeneration of mitochondria and other subsarcolemmal organelles, as well as caused inflammatory cell infiltration and glycogen accumulation. Structural changes included disorganized myofiber structure, missing z-bands, and fragmented myofibrils. These changes were not seen in muscles that were only statin-treated.

Consistent with the literature\textsuperscript{44,83,84}, degeneration induced by statin treatment was specific to muscles composed of predominantly type II myosin heavy chain (MHC), leaving muscles composed of predominately type I MHC—such as the soleus—unaffected. Additionally, it was shown that the increased propensity for damage to type II MHC fibers does not appear to be the result of different statin pharmacokinetics between MHC fiber types, as both type I and type II fibers had similar intramuscular cerivastatin concentrations\textsuperscript{43}.
Figure 1.2: Potential pathways for the exacerbation of statin-associated myopathy with exercise. Increased Ubiquitin Proteasome Pathway (UPP) activity and elevated mitochondrial damage have both been shown to occur with the addition of exercise in statin users. The remainder of the illustrated pathways currently remain speculative, however exercise increases energy flux, which would likely impact damaged mitochondria. Exercise also activates the MAPK pathway and can lead to glycogen depletion.

INDEPENDENT ROLE FOR HYPERCHOLESTEROLEMIA

In 1995 the myoglobin (Mb) and CK response to exercise in two equicholesterolemic groups of male patients with heterozygous familial hypercholesterolemia—with one group receiving simvastatin and the other a lipid-lowering diet—was investigated. To assess the independent role of cholesterol, results were also compared to a normocholesterolemic control group. All groups were matched for age, body mass index (BMI), and physical activity, and the two hypercholesterolemic groups were matched for LDL cholesterol. There were no differences in
serum Mb or CK responses to exercise between hypercholesterolemic groups, but both showed elevated responses compared to healthy controls. These results suggest that myopathy attributed to statin therapy in combination with exercise may be due in part to high cholesterol levels, and not statin treatment *per se*. In contrast, other controlled trials in subjects without heterozygous familial hypercholesterolemia that were normocholesterolemic at the time of exercise 43,66,78 found that statins did play an independent role in the exacerbation of myopathy with exercise. These studies suggest that hypercholesterolemia is clearly not the only factor in greater statin-induced myopathy with exercise.

Individuals with a wide range of cholesterol values have been used to examine potential interactions between statin treatment and exercise in contributing to myopathy. Interestingly, positive interactions between statins and exercise have generally been found at low cholesterol values—i.e. healthy rats 43, and humans w/LDL below 110mg/dL 66,78—while subjects with higher LDL values (>190mg/dL) tended to show no adverse interactions 64,85. Downhill walking has been used in studies finding both positive 66 and negative interactions 85. Therefore, directed research is needed to determine whether this is an artifact of the literature or whether cholesterol below a certain level could potentially predispose individuals to an elevated level of myopathy with the combination of exercise and statins that is either masked or not present in individuals with higher cholesterol levels. It would also be of interest to examine whether the time-course of cholesterol reductions with statin therapy is temporally related to changes in the incidence of myopathy.

**Muscle Glycogen and Fiber Type**

It has been reported that glycogen-depleted muscle fibers are more susceptible to statin-induced damage than glycogen-rich fibers 86. Since certain types of exercise result in significant
glycogen depletion in the exercising muscle \cite{87}, this suggests another potential mechanism underlying the elevated risk for statin-associated myopathy with exercise. Interestingly, diabetes has been associated with reduced baseline glycogen synthesis \cite{88,89} and diabetic individuals are more susceptible to statin-associated myopathy \cite{90,23}. Additionally, insulin resistance may impede the ability to restore muscle glycogen after glycogen-depleting resistance exercise \cite{91}. These data present an interesting potential contribution of exercise-induced muscle glycogen depletion to the exacerbation of myopathy associated with exercise and statin use.

Alternatively, several studies have shown that muscles composed of predominately type II MHC fibers are more susceptible to statin-induced damage than predominately Type I MHC muscles \cite{44,43,83,84}. However, depletion of muscle glycogen is greater in Type I fibers at exercise intensities below \( VO_2 \text{max} \), and depletion may not be higher in Type II fibers until very high exertion levels \cite{87}. Paradoxically, available literature examining statins and exercise has used submaximal exercise intensities, which would not be expected to deplete glycogen in the more myopathy-prone Type II fibers. Research examining both a resistance and an aerobic bout of exercise while receiving statin treatment found that the aerobic exercise (downhill walking for 45 min at 65\% maximum heart rate)—which could reasonably be expected to reduce glycogen levels in type I fibers—was associated with an elevated CK response to exercise when receiving statin treatment. In contrast, low-intensity (50\% 1-repetition maximum) resistance exercise—which would not be expected to appreciably reduce glycogen levels in either fiber type—showed no effect on CK levels with the combination of exercise and statin treatment \cite{66}. Additionally, some research has implicated mitochondrial dysfunction and impaired \( \beta \)-oxidation in myopathy \cite{43,61,60}, which—if present—may further complicate this issue by altering glucose/glycogen kinetics from that expected in healthy muscle and making glycogen depletion even more likely.
Taken together, these findings present an intriguing and perplexing set of data. As such, directed research should be aimed at resolving the potential role of glucose/glycogen kinetics during and after exercise in statin-associated myopathy and its interaction with exercise. Further, based upon the differences in glycogen storage and utilization among fibers with different MHC fiber types, future studies should address potential interactions between glycogen kinetics and muscle MHC fiber type profile.

**Exercise Training vs. a Single Exercise Bout**

Currently, little information is available addressing how exercise frequency, intensity, or modality may affect the risk of statin-related myopathy. While not the rule, the majority of current exercise and statin investigations commonly utilize a single exercise bout and indirect measures (i.e. serum CK) of muscle damage. While several human studies have shown that statin treatment increases muscle damage in response to a single exercise bout, only a single animal study has directly examined multiple exercise bouts in conjunction with statin treatment. However, all exercise groups in this study followed identical protocols for incomplete accustomization to the exercise before the initiation of statin treatment. Therefore, no comparisons between unaccustomed or fully-accustomed animals were possible.

To our knowledge, no study has yet addressed chronic exercise training prior to statin treatment. In the current literature there is generally accustomization to the statin treatment prior to exercise, but never accustomization to exercise before beginning statin treatment. As such, it is not possible to account for the well-established differences in the muscular response to an unaccustomed exercise bout as compared to the response to an acute exercise test after a period of training. In response to a novel exercise stimulus, rapid adaptive responses occur within the muscle
such that as little as one bout of novel exercise can be sufficient to protect against damage during subsequent bouts. Additionally, exercise training induces further well-established metabolic and structural adaptations within the muscle beyond those stimulated by a single novel stimulus. For instance, endurance training is associated with significant increases in the expression of numerous enzymes related to respiratory capacity, and improvements in subsarcolemmal mitochondria with training may increase the ability to maintain cell membrane integrity. Resistance training, on the other hand, can increase overall strength, improve peak rates of force development, and increase the ability to recruit high-threshold motor units. Because of these and other considerable adaptations made to both an initial, novel exercise bout and to subsequent exercise training the responses evoked by a single exercise stimulus while on statin therapy such as predominate the literature may not translate to muscles after chronic training.

Furthermore, there are numerous potential mechanisms whereby exercise training might be successful in attenuating statin-induced muscle damage. Possible mechanisms for this include the up-regulation of protective heat-shock proteins (Hsp), suppression of apoptotic factors such as caspase-3, and increases in muscle ubiquinone content.

A primary focus of our laboratory has been the potential protective role of Hsps during muscle adaptation. The protective effects of Hsp70, Hsp25 and αB-crystallin have been documented in many cell types, but growing evidence supports their important role in maintaining skeletal muscle structure and function. In muscle, the Hsps have been shown to reduce oxidative stress, inhibit apoptosis and potentially attenuate muscle damage. Interestingly, Hsp70, Hsp25 and αB-crystallin are more highly expressed in fibers composed of predominately type I MHC, which is the type of muscle fiber that appears to be least susceptible to statin-induced myopathy. Currently, data on statin treatment and Hsp expression is conflicting, showing either...
increases or decreases in expression depending on the tissue examined. It is likely that—similar to statin’s apoptotic actions—their effects on Hsp expression are cell-type dependent, and future studies are necessary to investigate the effects of statin therapy on Hsp expression in skeletal muscle.

In addition to stabilizing muscle structure and potentially reducing the risk for muscle damage, the Hsps can inhibit both the intrinsic and extrinsic apoptotic cell death pathway at unique as well as common points. Additionally, Hsp25 protein levels are positively associated with reductions in activated caspase-3 activity in vivo. Since statin-induced apoptosis is associated with increases in caspase-3 and -9 expression levels in vitro, if muscle apoptosis is induced in vivo any intervention to block these pathways could reduce the incidence of statin-associated myopathy. Importantly, chronic increases in muscle activation and loading (i.e. training) are associated with increased muscle Hsp content, and could therefore potentially attenuate statin-associated muscle damage.

Finally, several studies have shown that exercise training increases ubiquinone levels in skeletal muscle. While the role for ubiquinone levels in statin-induced myopathy is not clear, if reductions in ubiquinone are a factor in myopathy, this presents yet another pathway through which exercise training could benefit individuals seeking to improve lipid profiles through both exercise and statin treatment. However, there is some indication that increases in ubiquinone levels with exercise occur only in the already myopathy-resistant type I fibers.

While no human studies have directly investigated the effects of an exercise training intervention on statin-induced myopathy, a cross sectional study of athletes has been reported. Sinzinger and O’Grady examined how professional athletes with familial hypercholesterolemia would tolerate statin therapy. They reported that of 22 athletes, only 6 were able to tolerate statin
treatment, and only 3 of these 6 tolerated the first statin prescribed (athletes were tried on atorvastatin, fluvastatin, lovastatin, pravastatin, and simvastatin before determining intolerance). These athletes participated in fourteen different athletic disciplines and showed no indication of varying tolerance based on modality. However, these data must be interpreted carefully, as the recent intensity, duration, or frequency of these athletes’ training regimens was not reported, and thus their training state at the initiation of statin therapy—and beyond—is not known. Additionally, the interpretation of “tolerate” was not defined, as the presenting symptoms were neither quantitatively nor qualitatively defined. Finally, and perhaps most importantly, no placebo treatment was included in the drug rotation. Therefore, while data indicating statin intolerance in professional athletes would argue against exercise training being protective, it cannot in fact be determined from the published data whether these athletes experience the same or lower levels of myopathy as less active individuals but are more sensitive to changes or perceived changes in performance level or pain, or whether high levels of athletic training may make an individual more susceptible to muscular complications.

**CONCLUSIONS AND FUTURE DIRECTIONS**

Statins are currently the most effective and common cholesterol-lowering drug, and are overall quite safe. The most common complication is some degree of skeletal muscle myopathy, generally seen in only a small percentage of patients. However, this complication is exacerbated by physical activity, and therefore presents an unfortunate obstacle to the minimization of cardiovascular disease risk and to optimal physical activity levels that has not been adequately addressed in the literature.
Pathways underlying statin-associated myopathy are not well understood, though several potential mechanisms have been identified, including the induction of apoptosis, mitochondrial dysfunction, and—especially as it pertains to the prenylation of small GTPase signaling molecules—terpenoid depletion. How these potential mechanisms may interact negatively with physical activity is even more uncertain. However, mechanistic research has shown that the combination of exercise and statins may exacerbate mitochondrial damage and degrade muscle proteins via up-regulation of ubiquitin proteasome pathway activity.

We have highlighted several areas which may warrant future investigation; A) we propose that glucose/glycogen kinetics during exercise may play a key role in the interaction between statins and exercise. Glucose/glycogen utilization and re-synthesis are significantly impacted by a single bout of exercise and—importantly—glycogen utilization changes with chronic exercise training (i.e. training is associated with greater lipid utilization at a given absolute intensity). For example, future studies could utilize dietary interventions and pre/during/post-exercise glucose supplementation to manipulate muscle glycogen levels and quantify statin-associated muscle damage under acute exercise and exercise-trained conditions. This type of information would help clinicians/dieticians provide dietary recommendations that could potentially reduce the occurrence of statin-associated muscle damage with exercise. B) The role of hypercholesterolemia per se has not been well examined, either in relation to general statin-associated myopathy or with the interaction of exercise, though the literature suggests it may play an independent role. It would be of interest to complete an intervention study to determine if changes in statin associated muscle damage are correlated to changes in lipid profiles. Further, an exercise training intervention would suggest if any corresponding changes in damage and cholesterol are due to training induced changes in muscle, improved lipid profiles, or a combination of both factors. C) All physical
activity cannot be treated equally in the examination of statin-associated myopathy. Research differentiating between an exercise bout and exercise training—as well as between different modalities, namely resistance and aerobic, and shortening vs. lengthening muscle actions—is needed. Specifically, there is a clear need for controlled intervention studies to investigate if prior exercise training alters myopathy risk or statin-associated elevations in exercise-induced damage, and whether these interactions are dependant on exercise modalities. If future research finds that exercise frequency or modality affects statin-associated myopathy it would present data of immediate importance and use to clinicians. D) Serum CK is most commonly used to evaluate the exacerbation of myopathy with exercise and statin use; however, it is indirect and is not a consistently reliable marker of exercise-induced damage or statin-associated myopathy. Research utilizing more direct muscle assessments is needed, as the utilization of serum CK has likely contributed to an under-appreciation of the prevalence of statin-associated myopathy with exercise. Animal models lend themselves well to both functional and direct damage assessments, and functional muscle testing and limited biopsy research is practical in human subjects. Further, while it may not contribute mechanistic data, muscle strength presents perhaps the most reliable measure of muscle function, and further, provides insight into whether statin-associated muscle damage actually results in physiological performance decrements. We would suggest that in the process of examining exercise/statin interactions, future research efforts should also work towards building a reliable literature base of both functional muscle outcomes and direct assessments of muscle damage.
CHAPTER 2: EXPERIMENTAL METHODS
**Overview.**

These experiments used C57BL/6J male mice (Jackson Labs) for all procedures. All methods involving live animals were approved by the Illinois Institutional Animal Care and Use Committee.

The experimental design encompassed three treatment conditions: activity during days 1-14 (either running wheel access or no wheel access), activity during days 15-28, and saline or statin treatment during days 15-28. With the exclusion of groups having access to the running wheel for the first two weeks but not the second, this creates six groups, as shown below. Each group required 10 mice (see power analysis, statistical methods section), for a total of 60 mice. All mice were 10 weeks old at the initiation of statin or saline injections. The two week time-points were chosen in light of preliminary data from our own laboratory showing significant alterations in Hsp and Caspase-3 expression by running wheel exercise at two weeks, as well as data from other researchers indicating that the current statin treatment induces myopathy within two weeks.\textsuperscript{123,44,43}

Treatment groups were organized as shown in table 2.1. To reduce the total number of animals necessary, groups Sed/Saline and Sed/Statin were used as the control groups for Aims 1, 2, & 3.

<table>
<thead>
<tr>
<th>Group ID</th>
<th>Days 1-14 Treatment</th>
<th>Days 15-28 Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sed/Saline</td>
<td>Sedentary</td>
<td>Sedentary, Saline inj.</td>
</tr>
<tr>
<td>Sed/Statin</td>
<td>Sedentary</td>
<td>Sedentary, Statin inj.</td>
</tr>
<tr>
<td>Novel/Saline</td>
<td>Sedentary</td>
<td>Running Wheel, Saline inj.</td>
</tr>
<tr>
<td>Novel/Statin</td>
<td>Sedentary</td>
<td>Running Wheel, Statin inj.</td>
</tr>
<tr>
<td>Accust/Saline</td>
<td>Running Wheel</td>
<td>Running Wheel, Saline inj.</td>
</tr>
<tr>
<td>Accust/Statin</td>
<td>Running Wheel</td>
<td>Running Wheel, Statin inj.</td>
</tr>
</tbody>
</table>

*Table 2.1:* Treatment groups formed by the combinations of days 1-14 activity, days 15-28 activity, and days 15-28 injections.
All mice were housed individually, fed food (standard chow diet) and water *ad libitum*, and kept on a 12/12 light/dark cycle. The running wheel mice had constant access to an 11.5 cm running wheel (Mini-Mitter) in their cage. These running wheels were equipped with magnetic reed switches and a small bicycle computer (Sigma), which allowed daily tracking of distance covered and time spent in the wheels. Running wheel activity was recorded every twenty-four hours during the light cycle.

Starting on day 15, mice in the novel exercise groups were moved into running wheel cages, and all mice began to receive daily injections of either saline or cerivastatin. Cerivastatin (Sequoia Research Products) injections were given intraperitoneally at a dosage of 1mg/kg, delivered as a suspension at 0.25mg/ml in sterile saline. Previous reports have confirmed this dosage and duration to consistently cause myopathy, but not to the extent that the animals will not exercise. As the mice perform the majority of their running wheel activity during the dark cycle, injections were given towards the beginning of the light cycle in order to minimize any direct effects of the injections themselves on activity. Running wheel activity continued to be monitored daily up until day 28.

**DATA AND TISSUE COLLECTION:**

**Force Measurement:**

**Setup:** On day 28, all mice had measurements taken for the maximum isometric contraction strength of the hindlimb plantar flexors. The force-measurement apparatus consists of a servomotor and analog control unit (model 305C-LR, Aurora Scientific), a square-wave stimulator (model 2100, A-M Systems), and a PC running a customized LabView 8.2 program to control both
the servomotor and the stimulator. The servomotor system was calibrated by placing a series of known weights at a distance of 20mm from the axis and creating a torque/voltage standard curve.

For force measurement, mice were first weighed, then fully anesthetized by the administration of 90-120mg/kg Ketamine, 9-12mg/kg Xylazine IP. Surgical-plane anesthesia was confirmed by pinching the pad of the hind foot. Once the anesthesia took full affect, the mice were placed on a table warmed to 37°C, where all further surgical procedures were performed.

**Surgery:** An incision was first made on the lateral thigh of the mouse, parallel and superficial to the femur. At this point the illiotibial band is visible, and the hamstring and quadriceps muscle groups were dissected apart, allowing access to the sciatic nerve, located between the hamstrings and femur. Further dissection into the popliteal fascia allowed the split of the sciatic into the tibial and common peroneal nerves to be visible. The common peroneal branch was identified visually and confirmed by a dorsiflexion muscle twitch upon gentle pinching with a forceps. At this point the common peroneal nerve was severed, ablating innervation to the anterior compartment of the lower limb and ensuring stimulation of only plantarflexor muscles upon stimulation of the sciatic.

The ankle of the mouse was then fixed in the servomotor’s footplate, with the talus (the rotational center of the ankle joint) aligned with the servomotor axis. The mouse was then positioned so as to place the knee joint at 90° and the ankle at 90°, where the knee was immobilized with an alligator-clip style clamp about the femoral condyle.

Two hooked-needle electrodes were then placed proximal to the knee on the sciatic nerve, with the two electrodes approximately 1mm apart and contacting only the sciatic and not adjacent muscle tissue. The sciatic nerve was then stimulated at 250hz for 1.5 seconds to evoke a maximal contraction, during which time the servomotor held the ankle to 90° in order to measure isometric
force production. This stimulation was repeated 9 times with a recovery period of 5 seconds between contractions (6.5 seconds from contraction 1’s start to contraction 2’s start), for a total of 10 measurements. The highest of the 10 acquired data points was taken as the maximum. The final contraction was compared to the maximal contraction and expressed as a percentage of maximal force to provide a measure of fatigability. Data were recorded by a customized LabView program (National Instruments) to provide a plot of ankle-torque/time.

**Tissue Collection:**

The mice were euthanized immediately after the completion of the force-measurement protocol. At this time, blood was drawn from the inferior vena cava into EDTA treated syringes and deposited in EDTA treated microcentrifuge tubes on ice. Subsequently, the gastrocnemius were isolated from the hindlimbs and immediately snap-frozen in isopentane chilled by liquid nitrogen, then stored at -80°C for subsequent analysis. For each mouse, muscles from the left limbs were used for analysis, as this is the limb that was not subjected to the force-measurement protocol. After tissue collections whole blood was centrifuged at 1200g for 10 minutes at 4°C. Plasma was then extracted and frozen at -80°C until analysis.

**Protein Isolation:**

Pre-weighed muscle samples (~40 mg) were homogenized in 10 volumes of an ice-cold buffer containing 50mM Tris _HCl (pH 7.8), 2 mM potassium phosphate, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 3 mM benzamidine, 1 mM sodium orthovanadate, 10 mM leupeptin, 5 mg/ml aprotinin, and 1 mM 4-[(2-aminoethyl) benzenesulfonyl fluoride], and supplemented with protease and phosphatase inhibitors (Sigma) using a motor driven glass pestle.
in a textured glass test tube. Homogenates were immediately centrifuged at 12,000g for 12 min at 4°C and the supernatant removed as the detergent-soluble fraction. Protein concentrations were then determined by Bradford assay (Biorad) using bovine serum albumin for the standard curve, after which samples were saved immediately in aliquots at –20°C for subsequent use in Western blotting.

**Western Blot Analysis:**

The necessary volume for loading 30μg of total protein was determined from protein assay data, and the sample was boiled in loading buffer for 5 minutes at 95°C. Proteins were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% acrylamide) and transferred to PVDF membranes (Millipore). After transfer, successful equal loading, electrophoresis, and transfer were confirmed by reversible Ponceau-S staining. Only membranes showing equal loading across all samples were used for subsequent antibody staining and analysis. Ponceau-S staining was removed by water rinse and a 5 minute wash in TBS/0.15% Tween, after which the membranes were blocked for a minimum of one hour in Tris-buffered saline (TBS) containing 5% powdered dry milk. Following blocking, the membranes were incubated overnight at 4°C in primary antibodies (Hsp25 & αB-Crystallin, StressGen, 1:5000. Caspase-3, Upstate/Millipore, 1:1000. Caspase-9, Cell Signaling Technologies, 1:1000) in TBS w/2.5% bovine serum albumin (BSA). After primary antibody incubation, membranes were washed three times with TBS/0.15% Tween, then incubated in anti-rabbit IgG secondary antibody (Amersham Biosciences) at 1:5000 for one hour at room temperature. Following the secondary antibody, the membranes were again washed three times in TBS/0.15% Tween. Membranes were then developed using enhanced chemiluminescence (Amersham Biosciences) detected by a computer-
controlled charge-coupled device (CCD) camera (Fujitsu). Developed images were analyzed using ImageQuant software (GE Healthcare).

**Plasma Creatine Kinase:**

Plasma aliquots were thawed to 4°C, then analyzed by a CK activity assay (BioAssay Systems) as per the manufacturer’s instructions. Briefly, this assay provides the substrates necessary to allow CK to convert creatine phosphate and ADP into creatine and ATP, and for the created ATP to ultimately be used to oxidize NADP to NADPH. The created NADPH can then be quantified by spectrophotometry.

**STATISTICAL METHODS:**

**Power Analysis/Justification of Sample Size:**

All power analysis calculations were done using the Power and Precision (Biostat) statistical software. Parameters for the power analysis were an alpha level of .05 and power of .80. Anticipated effect sizes for each effect are described below.

At this time, data on muscle force production capabilities with the combination of statin treatment and exercise does not exist in the literature. Additionally, available histology data addressing these treatments has been collected on subjective and discontinuous scales that do not lend well to power analysis. Therefore, we took available data from our own laboratory regarding force production measurements to perform this analysis, and calculated the necessary number of mice per group to detect what would be a clinically relevant outcome. The primary outcome of interest was the interaction effect between exercise and statin treatment in a 2x3 Regression/ANOVA model. However, this effect was found to be severely underpowered at any
reasonable group size. Therefore, analysis was performed on the primary group differences of interest: between the Sed/Statin and Sed/Saline groups, and the Novel/Statin and Accust/Statin groups. Previous data from our laboratory indicates that the group mean for the Sed/Saline group should be ~40 grams of force, and that the standard deviation within groups is ~4.0 grams. A 15% decrement in force-production capability was deemed a clinically relevant outcome, which equates to a 6 gram decrement. With Sed/Saline and Sed/Statin group means at 40±4 and 34±4 grams, respectively, the predicted power for 8 mice per group was 0.78. Since group differences in the two exercise-treated groups were hypothesized to be greater than the differences found in the sedentary mice, this group size was deemed appropriate for all groups. To account for unexpected mortality, non-voluntary exercisers, and potential statin nonresponders, a final group size of 10 mice was decided.

**Data Analysis:**

All analyses were performed using SPSS version 15 statistical software, setting alpha to reject the null hypothesis at 0.05. There were two treatment conditions (statin/saline), and as there were no groups exercising for the first two weeks but not the second two, the activity conditions (sedentary, novel, accustomed) were analyzed as 3 distinct treatments, rather than two distinct day 1-14 treatments and two distinct days 15-28 treatments. Therefore, each outcome measure—with the exception of running wheel activity (see below)—was submitted to a separate 2x3 multiple regression model including a term for the injection*exercise interaction. First order autocorrelation was examined by Durbin-Watson’s D and normality by Shapiro-Wilk. Equality of variance was tested by Spearman’s Correlation Coefficient for correlations between model-predicted values and the absolute values of the residuals. Transformations were performed if necessary to satisfy these
model-assumption tests. Post-hoc analyses were performed only if significant main or interaction effects were found. In the case of significant main effects, group differences were only examined across the treatment for which the main effect existed. If within-group variances were equal across groups, post-hoc analysis was performed by Tukey post-hoc test. If homogeneity of variance did not exist, post-hoc analysis was run by repeated t-tests. Running wheel distances were examined as a possible regression covariate for both the force measurements and the CK measures.

**Outcome Correlations:**

Pearson correlation tests were performed to examine relationships between muscle force-production ability, plasma CK levels, and running wheel activity. Significant correlations were accepted at p<0.05.

**Running Wheel Data:**

Running wheel data was analyzed as a set of time-points of interest by independent-samples t-tests. 3-day activity averages were calculated for each individual mouse for days 12-14, 15-17, and 26-28. Comparisons were only made within time-points between saline and statin treatments, and not between novel and accustomed exercise groups.

**Western Blotting Analyses:**

In order to allow the most accurate possible analyses, each gel was run with one animal from each group. The cumulative absorbance from each of the 6 samples per gel for the protein of interest in the gel was then measured, and each sample’s relative contribution to the total gel absorbance was calculated and expressed as a percentage of the total gel absorbance. Relative
contributions within groups across multiple gels were then pooled, allowing for statistical analysis with the full experimental group sizes, instead of only within a single gel. This procedure prevents the systematic limitation in comparable group sizes that commonly confounds western blot analyses.
CHAPTER 3: STATINS AND MUSCLE FUNCTION

Statins and Novel or Accustomed Exercise: Impacts on Skeletal Muscle

Strength and Fatigue
INTRODUCTION:

HMG-CoA reductase inhibitors (statins) are a well-known class of drugs that are highly effective in reducing low-density lipoprotein levels and cardiovascular disease risk. They are also well-tolerated, with a low overall incidence of side effects, and this combination of efficacy and safety has led to common prescription, which is expected to continue increasing in the future alongside a growing elderly population and the ongoing discovery of their pleiotrophic effects \(^1\). Unfortunately, however, statin treatment is not without risk, and the most common side effect is some degree of skeletal muscle dysfunction, ranging from mild soreness or weakness to potentially fatal rhabdomyolysis. While the prevalence of myopathy in the general statin-treated population may be up to 10\(^\%\) \(^1,7-9\), the risk of myopathy is estimated to range up to 25\(^\%\) in statin users who also exercise \(^1,14,13\), and perhaps above 75\(^\%\) in statin-treated athletes \(^15\).

The pathways underlying statin-associated myopathy in general are not yet understood, and the literature directly examining the pathways responsible for the exacerbation by exercise is extremely limited. Existing research has indicated that statin treatment prior to a damaging exercise bout can increase genes involved in protein catabolism in humans \(^78\), while two weeks of concurrent statin treatment and treadmill running in rats has been shown to increase mitochondria and organelle damage, inflammatory cell infiltration, and structural damage \(^43\).

With only a single exception \(^43\), the current literature regarding statins and exercise has examined the impact of statin treatment on the muscle damage induced by a single bout of exercise \(^78,69,66,62-65\). These studies commonly use 4 or more weeks of statin treatment prior to the exercise bout, with creatine kinase (CK) as a common measure of the damage response. Examination of the available literature reveals no investigations of deliberate exercise training or multiple exercise bouts prior to the initiation of statin treatment. This presents a significant void in the literature, as
many rapid and long-term adaptations occur within skeletal muscle in response to both acute and chronic exercise stimuli \(^{92-94}\). Consequently, the current literature cannot be assumed to translate to situations where exercise accustomization occurs prior to statin treatment.

Furthermore, many adaptations that occur in muscle with exercise training—such as reduced ultrastructural damage, increased heat shock protein (Hsp) expression, and improved metabolic function \(^{95,96,123,92-94}\)—could be expected to protect against statin-induced muscle damage. Therefore, we hypothesized that the exacerbation of statin-induced myopathy by exercise may be specific to untrained muscle, and that exercise training prior to the commencement of statin treatment may be protective against subsequent damage. As such, the current study was designed to investigate the muscle damage associated with statin use in response to either novel and accustomed exercise. Additionally, muscle contractile function measures were used for the evaluation of muscle damage, presenting the first use of a physiological measure of muscle function in a randomized, controlled trial examining statins and exercise. Furthermore, as a reliable measure of muscle damage, muscle force measures were used to examine the usefulness of blood CK levels as an indirect marker of statin-induced myopathy.

**METHODS:**

**Overview:** Eight-week old, male C57BL/6J mice (Jackson Labs) were used for all procedures. The intervention was designed to examine statin treatment in combination with novel or accustomed exercise, and to compare these conditions to sedentary mice. Voluntary running wheel activity was used as the exercise model, and cerivastatin (1mg/kd/day) was used for statin treatment. Previous rodent research has shown this drug regimen to cause skeletal muscle myopathy at two weeks \(^{44,43}\). Additionally, research from our own lab and others has shown two
weeks of running wheel activity to elicit protective adaptations in murine skeletal muscle \cite{123,127}.

Fourteen days was therefore chosen as the duration for both statin and exercise treatments.

To accommodate both novel and accustomed exercise treatments, mice were subjected to a 28-day intervention. During the first 14 days the mice received no statin or saline injections, and only the accustomed exercise groups had access to running wheels. Starting on day 15, mice in the novel exercise groups were given access to running wheels, and all mice began receiving daily injections of either cerivastatin or saline. Daily injections and 24h running wheel activity tracking continued until day 28. These treatments yield six distinct treatment groups (See Table 2.1).

**Statin Injections:** Cerivastatin (Sequoia Research) was suspended in sterile saline at .25mg/ml and injected intraperitoneally daily at a dosage of 1mg/kg/day. Injections were given towards the beginning of the mice’s light (inactive) cycle to minimize the disturbance of running wheel activity.

**Running Wheel Activity:** Mice were individually housed in cages equipped with running wheels (MiniMitter). These wheels are equipped with magnetic reed switches which were connected to bicycle computers (Sigma), allowing distance and time to be tracked continuously. Running wheel activity was recorded every 24 hours during the light cycle. Novel exercise group mice were transferred to running wheel cages on day 15 near the end of their light cycle, and so received their first statin injections ~8 hours before their first dark (active) cycle with running wheel access.

**Force Measurement:** Measurements of hind-limb plantarflexor force-production ability were made on day 28, approximately 24 hours after the previous statin injection. To perform these
measures, mice were fully anesthetized under ketamine/xylazine, and the sciatic nerve was dissected through the lateral thigh. The common peroneal branch was severed, allowing isolated activation of the plantarflexors by stimulation of the sciatic. The hindfoot was then placed on a plate attached to a servomotor able to measure applied torque and control ankle rotation (305C-LR, Aurora Scientific). The sciatic nerve was then hooked by electrodes to a square wave stimulator (A-M Systems) and stimulated at 250hz for 1.5 seconds to evoke a maximum-force contraction. The servomotor was set to maintain position, ensuring that the contraction was isometric. With 5-second delays between stimulations, the contraction was repeated 9 additional times in order to examine muscle fatigability. As a measure of fatigue, the 10th contraction was compared to the maximum contraction, and expressed as a percentage of maximal force.

**Creatine Kinase:** Immediately after force measurements, blood was drawn into EDTA-treated tubes, then centrifuged for 10 minutes at 1,200g and 4°C to extract plasma. Creatine kinase levels were measured by an activity assay (BioAssay Systems) as per the manufacturer’s instructions.

**Statistical Analyses:** Running wheel activity was analyzed by the comparison of important timepoints. 3-day activity totals were calculated for each mouse for days 12-14, 15-17, and 26-28 and compared between saline/statin treatments by independent-samples t-test. Additionally, total Area Under the Curve (AUC) was also compared between saline/statin treatments.

Muscle force and plasma CK data were analyzed by separate multiple regression models including main effect terms for injection and exercise, as well as a term for injection*exercise interactions. Running wheel distances were also examined as a possible covariate for the muscle
force and plasma creatine kinase regression models. Tukey post-hoc analysis was performed to examine specific group differences only if a significant interaction or main effect was found.

Finally, Pearson correlation tests were performed to examine correlations between running wheel activity, muscle strength, and plasma CK within activity groups. All analyses were performed with SPSS v.15 statistical software (IBM) with alpha set at 0.05.

**RESULTS:**

**General:** No significant group differences were found at the end of the intervention for bodyweight. One mouse in the Novel/Saline group died due to unknown causes, leaving n=9 for this group and n=10 for all other groups.

**Running Wheel Activity:** No significant differences in wheel activity were found between saline and statin-treated groups for any of the three 3-day totals or for activity AUC (Figure 3.1).

**Muscle Force:** A significant regression model ($f(3,55)=19.4$, $p<0.000$, $R=0.72$) was found for muscle force, and a significant injection by activity interaction effect ($p<0.05$) was found for maximal isometric force-production. Statin-treated mice showed reduced isometric force as compared to their saline controls in both the sedentary and novel exercise groups (15% and 27% reduction, respectively), while the statin-treated accustomed exercise group showed no reduction in force (Figure 3.2). A significant model ($f(3,55)=4.9$, $p=0.004$, $R=0.46$) was also found for muscle fatigability, including a significant injection by activity interaction ($p<0.05$), with both the sedentary and novel exercise statin-treated groups showing reduced isometric force-production on the 10th contraction (51 and 54% maximum-force, respectively) as compared to their respective
saline-treated groups (62 and 70% maximum, respectively). Mice in the accustomed exercise
groups finished at 67 and 69% maximal isometric force for the saline and statin groups,
respectively. No significant group differences were found for gastrocnemius muscle weights.
Inclusion of running wheel activity as a covariate did not significantly affect the regression model
for maximal force production or fatigability.

**Creatine Kinase:** No significant main effects for injection or activity were found for plasma CK
(Table 3.1).

<table>
<thead>
<tr>
<th>Injection</th>
<th>Sedentary</th>
<th>Novel Ex.</th>
<th>Accust. Ex.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>142.8±14.1</td>
<td>131.1±19.5</td>
<td>146.8±13.3</td>
</tr>
<tr>
<td>Statin</td>
<td>133.4±17.0</td>
<td>126.8±17.6</td>
<td>145.6±16.7</td>
</tr>
</tbody>
</table>

Table 3.1: Plasma creatine kinase levels by group. No significant effects of injection or activity
were found.

**Outcome Correlations:**

Between the three running wheel time-points, isometric muscle force, and plasma CK, no
significant correlations were found.
Figure 3.1: Average daily voluntary running wheel activity (+/- SE) in Accustomed (A) and Novel (B) exercise groups. Analysis of 3-day distance totals for days 12-14, 15-17, and 26-28 revealed no significant group differences for either activity treatment.
**Figure 3.2: Maximal Force Production**

**Discussion:**

A variety of previous reports have confirmed that statin-induced myopathy is exacerbated by physical activity or exercise, with activity raising the risk for myopathy by greater than 100% \(^1,^{14,13}\). We investigated the hypothesis that statin-associated muscle damage is greater in response to novel exercise compared to accustomed exercise. Specifically, training–induced muscle adaptations may protect muscle from the deleterious interactions between statin treatment and exercise. This study is the first known examination of exercise accustomization prior to the commencement of statin treatment, contributing significant new knowledge regarding
statin/exercise interactions in regards to myopathy. Additionally, a reliable and functional measure of muscle damage—isometric force—was used for the first time in a controlled statin/exercise intervention setting, allowing improved validity and physiological relevance in myopathy assessment as compared to indirect markers such as blood creatine kinase levels.

As predicted, the statin-treated sedentary mice showed reduced isometric force production compared to the saline-treated controls, indicating that cerivastatin at 1mg/day causes physiologically relevant myopathy in sedentary mice, which is consistent with previous histological reports of this same intervention in rats. Additionally, statin treatment was associated with even greater decrements in isometric force in the novel exercise group, demonstrating exacerbation of statin-induced myopathy in response to novel exercise. This is also consistent with previous histological evidence of exacerbated myopathy in rats undergoing two weeks of treadmill running while receiving cerivastatin treatment. Specifically, the muscles of rats receiving cerivastatin while running showed elevated mitochondrial damage, increased inflammatory cell infiltration, and disorganized myofiber structure. Most significantly, our accustomed exercise group showed no decrements in muscle force with the introduction of statin treatment, presenting the novel finding that two weeks of prior running wheel activity is protective against both statin-associated decrements in muscle function and its exacerbation by exercise.

While the daily average wheel-running distances for the novel/statin group were consistently lower than the daily distance for the novel/saline group, no significant statistical differences were found between these groups due to high individual variability (Average SD for 24h distances: 2290 and 1960 meters for novel/saline and novel/statin groups, respectively). However, based on previous literature examining differences in susceptibility to statin-induced damage between type I and type II muscle fibers, these results are not unexpected. Prior reports
have shown that type II fibers—the greatest contributors to maximal muscle force—are susceptible to statin-induced damage, while Type I fibers—which would be most important for running wheel activity—are protected 44,83,84,43.

Due to these apparent differences in susceptibility between fiber types, it was not expected that the statin-treated mice would show an increase in fatigability—when expressed as a percentage of maximum—as type I fibers would likely represent a greater percentage of initial force in myopathic muscle. It is interesting to note, however, that the sed/statin and novel/statin groups showed significant increases in fatigability, which indicates potentially severe inabilities to cope with the stresses of repeated activation, likely in type II fibers. Hypothetically, given two muscles of equal size and fiber-type distribution, if one muscle suffers type II fiber damage and dysfunction, this muscle could be expected to complete the current isometric protocol at a higher percentage of initial contraction than the undamaged muscle, though at a lower absolute strength. This would assume that the damaged type II fibers either do not contribute to force production, or contribute but fatigue at a relative rate similar to that of healthy fibers. Up to a point, increased fatigability in damaged type II fibers would only serve to increase overall fatigability in this damaged muscle back towards that of the undamaged muscle. Therefore, the elevated fatigability in the sed/statin and novel/statin groups indicates that either type I fibers have been affected, or that damaged type-II fibers are still contributing to force production, but their ability to recover from contractions has been significantly compromised.

Despite the significant reductions in isometric force observed with statin treatment in both the sedentary and novel exercise groups, plasma creatine kinase levels were not elevated by statin treatment. In addition, plasma CK levels did not significantly correlate with running wheel activity or isometric force. A number of prior reports have demonstrated that statin-associated
myopathy can manifest without increases in CK levels, and the current findings in the sedentary groups support this. Additionally, the data from the novel exercise groups indicates that CK levels are also not sensitive to the exacerbation of statin-associated myopathy with exercise. Together, these findings present a strong case against the sole use of CK as a measure of statin-induced myopathy—whether examined in combination with exercise or not—and indicate that literature using CK as a marker may underestimate the prevalence of myopathy.

The mechanisms underlying statin-associated skeletal muscle myopathy are not well understood, however several implicated pathways could be exacerbated by exercise. Mitochondrial dysfunction has been implicated in general statin-associated myopathy, and mitochondrial damage can be increased by the combination of statin-treatment and exercise. In the current findings, the increased fatigability shown with statin-treatment suggests that some metabolic dysfunction may be present. However, the short-duration, high-intensity nature of the fatigue test and the absence of differences in running wheel activity indicate that any significant metabolic disturbances induced by the current intervention are more likely to be in glycolytic or high-energy phosphate pathways, rather than mitochondrial oxidative pathways. Nonetheless, it is possible that statin-induced mitochondrial damage could have indirect effects on a short-term measure such as the current protocol. In the only other study to perform a controlled examination of statins’ effects on muscle strength, it was concluded that statin-induced mitochondrial dysfunction can lead to disruptions in calcium homeostasis, which was associated with decreases in voluntary strength and increased fatigability in the forearms of sedentary rats.

Another proposed mediator of statin-associated myopathy is a reduction in membrane fluidity due to reduced cholesterol availability, which has been shown to be a concern with cholesterol-lowering medications such as niacin and clofibrate. However, reduced membrane
fluidity has not been implicated in statin-associated myopathy, and reducing cholesterol synthesis
downstream of mevalonate—at squalene synthase—does not induce myopathy \(^{47,48}\). This suggests
that reduced cholesterol levels are not directly responsible for statin-associated myopathy under
sedentary conditions. However, membrane fluidity has not been examined with exercise, so it
remains possible that membrane fluidity is reduced with statin-treatment and that this reduction
does not cause pathology without the additional mechanical stressors of exercise. Conversely,
while membrane fluidity was not directly examined in the current study, the absence of increased
plasma CK in both sedentary and exercised statin groups indicates that membrane integrity was not
compromised, despite significant reductions in muscle contractile function.

Further investigation is required to examine the mechanisms behind the protective effects
of exercise training observed in the present study. As mentioned, skeletal muscle makes
adaptations to both acute and chronic exercise stimuli, and many of these adaptations might be
expected to protect muscle contractile function during statin treatment. For instance, our laboratory
has previously shown that the 2-week running wheel intervention used in this study is associated
with significant up-regulation of heat shock proteins in active muscles \(^{123}\). These stress proteins
have been shown to be protective against a diverse array of stressors, including mechanical
damage and reactive oxygen species \(^{97-104}\). In skeletal muscle, the Hsps have been shown to move
to the cytoskeletal fraction after muscle contraction and potentially stabilize muscle structure,
which would likely preserve contractile function. Furthermore, \textit{in vitro} data has indicated that
statins can induce apoptosis in myotubes, myoblasts, and differentiated primary skeletal muscle
cells \(^{35-38}\), and Hsp’s have been shown to inhibit apoptotic pathways \(^{99,115-117}\), as well as show an
inverse relationship to caspase-3 expression—an important marker of mitochondrially-mediated
apoptosis—in some tissues \(^{118-120}\).
Further research is also required to determine the volume, frequency, or modality of exercise required to achieve protective accustomization in humans. In this study, the mice consistently ran upwards of 5 hours per day, presenting an impractical/impossible intervention for humans. However, the statin treatment was also severe, using a high dosage of cerivastatin—a drug that was removed from the market in 2001 due to unusually high incidence of serious myopathy. Therefore, the exercise training necessary to protect against the myopathy associated with current clinical statin prescriptions cannot be determined from the current study. However, it is reasonable to expect that less myopathic, more clinically realistic statin prescriptions would require less extensive exercise accustomization.

In conclusion, the current investigation represents the first known examination of exercise training prior to the initiation of statin treatment, and presents the novel finding that exercise accustomization is protective against statin-induced muscle damage, as well as the exacerbation of contractile dysfunction with exercise. These results present the encouraging and clinically important possibility that statin-induced myopathy and its exacerbation by exercise could be alleviated through carefully regimented exercise programs. Additionally, while statin-induced damage has been shown in both sedentary and exercised muscle by histological measures, these data present the first controlled confirmation of physiologically relevant deficits in muscle function and exacerbated functional losses in muscle subjected to novel exercise. Further, creatine kinase was shown to be completely insensitive to reduced contractile function in both sedentary and novel-exercise mice. In combination with previous research also indicating a lack of CK sensitivity to statin-associated myopathy, these findings indicate that previous literature utilizing CK may have underestimated the prevalence of myopathy, and strongly suggest against the use of CK as the sole marker of myopathy in both clinical and research settings.
CHAPTER 4: CELLULAR MECHANISMS

The interactions of Statins and Novel or Accustomed Exercise and their impact on Skeletal Muscle Caspase and Heat Shock Protein Expression
Introduction:

HMG-CoA reductase inhibitors (statins) are a widely prescribed, effective, and generally safe class of drugs, used to lower cholesterol levels and reduce the risk of cardiovascular disease. With the ongoing discovery of pleiotropic effects and the increasing elderly population, their future prescription is expected to continue increasing \(^1\). The most common of these is some degree of skeletal muscle myopathy, ranging from potentially fatal rhabdomyolysis to minor aches or weakness. While the risk for myopathy has been estimated at 1-10% in statin-users overall \(^1,7-9\), the risk for myopathy is elevated to as high as 25% in those who are also exercisers \(^1,14,13\), and may be as high as 75% in statin-treated athletes \(^15\). The mechanisms responsible for statin-associated myopathy in general are not currently understood, and unfortunately, the potentially detrimental interaction with exercise has been sparsely researched.

While a number of studies have investigated the influence of statins on the skeletal muscle damage response to a single bout of exercise \(^62-66,69,78\), few studies have mechanistically examined the exacerbation of statin-associated myopathy with exercise. Additionally, to date only one study has examined the impact of repeated exercise bouts on statin-induced muscle damage \(^43\). Furthermore, there are no data on the interactions between statin-induced myopathy and exercise when exercise training was performed prior to the initiation of statin treatment, i.e., the exercise stimulus given during statin treatment has always been a novel stimulus. This is especially problematic, as a variety of structural and metabolic adaptations occur in skeletal muscle in response to repeated exercise bouts, or even to a single novel bout \(^92-94\). As a result, the literature addressing statins and exercise using novel exercise stimuli cannot be assumed to also apply to statins and accustomed exercise.
Therefore, the aim of this study was to investigate the impact of both novel and accustomed exercise on molecular stress markers in skeletal muscle during statin treatment. We chose to focus on two specific stress and damage activated markers in skeletal muscles: heat shock proteins (Hsps) and activated caspases—an apoptotic marker. The effect of statins on Heat shock protein expression in skeletal muscle has not previously been examined, and while statin-initiated apoptosis has so far been shown not to occur \textit{in vivo}, it has not yet been examined with the combined treatments of statin and repeated exercise.

Within the large family of Hsp stress proteins, the effects and statins and exercise on two small Hsps—Hsp25 and αB-crystallin—was investigated, since both of these Hsps are associated with helping maintain muscle integrity with damaging contractions\textsuperscript{101}. While acute damaging exercise increases both large and small Hsps, only one study has characterized the response of the small Hsps Hsp27 and αβ-crystallin to non-damaging exercise, and the results suggest that muscle damage may be important for increases in Hsp27 and αβ-crystallin to occur, as only Hsp72 was increased in human skeletal muscle following a bout of non-damaging exercise\textsuperscript{129}. Numerous other studies have also indicated that a single bout of non-damaging treadmill exercise can increase Hsp72 in both humans and rodents\textsuperscript{130-133,129}. Therefore, small Hsps were chosen to be examined due to their possibly unique responses to and role in muscle damage.

For indicators of statins’ impact on apoptosis, the expression of caspase-9 and activated caspase-3 were chosen. Caspases are proteases involved in both the initiation (caspase-8, -9, -12) and execution (caspase-3, -6, -7) of apoptosis. Importantly, caspase-9 is an activator of caspase-3, and the activation of caspase-3 is likely a critical rate-limiting step for myofilament release and is strongly implicated in accelerated contractile protein breakdown and muscle weakness\textsuperscript{39,40,134}, thereby positioning these as markers of especially high importance in skeletal muscle. Hsp25 or
αβ-crystallin over-expression in non-muscle tissues has been shown to significantly reduce caspase-3 activity in vivo\textsuperscript{120,118} and in vitro\textsuperscript{135-137} likely by inhibiting the cleavage of pro-caspase-3 into active caspase-3\textsuperscript{136,138}. Additionally, hsp25 has been shown to inhibit the cleavage of procaspase-9 into active caspase-9\textsuperscript{139}. Therefore, caspase-9 and caspase-3 present interesting points to examine the apoptotic cascade, due both to their position near the end of the apoptotic pathway and subsequent importance to the execution of apoptosis in skeletal muscle and to the potential for exercise and muscle damage to affect their expression via the Hsps of interest.

It was hypothesized that statins would cause a reduction in the heat shock proteins hsp25 and αB-crystallin, and may increase the apoptotic markers caspase-3 and caspase-9. Further, it was predicted that these changes would be exacerbated by novel exercise while undergoing statin treatment. Alternatively, it was predicted that exercise training prior to the initiation of statin treatment would be protective against—and perhaps reverse—the changes seen in sedentary and novel exercise groups, and that elevated hsp expression due to exercise would be associated with reductions in caspase expression.

**METHODS:**

**Overview:** This intervention was designed to compare the effects of statin treatment between sedentary, novel exercise, and accustomed exercise mice. C57BL/6J mice (Jackson Labs) were used for all procedures, and were 10 weeks old at the initiation of statin treatment. 60 total mice were used, with n=10 for all groups.

A four-week intervention, divided into two halves was used. Sedentary mice (Sed/Saline and Sed/Statin groups) remained in standard cages from days 1-28, and received statin/saline injections from days 15-28. Novel exercise mice (Novel/Saline and Novel/Statin) were in standard
cages from days 1-14, but were then transferred to running wheel cages on day 15 and received injections from days 15-28. Accustomed exercise groups (Accust/Saline, Accust/Statin) were housed in running wheel cages from days 1-28, and received injections from days 15-28 (See Table 2.1).

**Running Wheels:** Exercising mice were individually housed in running wheel cages (Mini-Mitter) with continuous access to their wheels. Accustomed exercise mice had running wheel access days 1-28, while novel exercise mice had running wheel access days 15-28. Two weeks was chosen as the accustomization and exercise period as research from our own lab and others has shown two weeks of running wheel activity to initiate exercise adaptations in skeletal muscle, including upregulations in the hsp5 of interest to the current investigation 123,127.

**Statin Injections:** Beginning on day 15, cerivastatin or saline intraperitoneal injections were given every 24 hours until day 28. Cerivastatin (Sequoia Research) was dissolved at .25mg/ml in sterile saline and injected at 1mg/kg/day via a single injection. Injections were given towards the beginning of the mice’s light cycle to minimize any direct effects on running wheel activity. Cerivastatin was the statin of choice due to its unusually high incidence of muscle side effects, and the fact that previous interventions have shown this dosing to consistently cause myopathy at 2 weeks 44,43.

**Western Blotting:** Western blots were performed to analyze muscle expression of hsp25, αB-crystallin, caspase-3, and caspase-9. The gastrocnemius was excised on day 28 and mechanically homogenized in a buffer containing 50mM Tris-HCl (pH 7.8), 2 mM potassium phosphate, 2 mM
EDTA, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 3 mM benzamidine, 1 mM sodium orthovanadate, 10 mM leupeptin, 5 mg/ml aprotinin, and 1 mM 4-[(2-aminoethyl) benzenesulfonyl fluoride], and supplemented with protease and phosphatase inhibitors (Sigma). Homogenates were immediately centrifuged at 12000g at 4°C for 12 minutes, after which the supernatant—the detergent soluble fraction—was removed. Protein concentrations were determined by Bradford assay (Biorad) using a bovine serum albumin standard curve and immediately stored at -20°C for subsequent use in western blotting. For gel loading, 30μg of total protein was boiled in loading buffer for 5 minutes at 95°C. Proteins were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%) and transferred to PVDF membranes (Millipore). After transferring, successful equal loading was confirmed by Ponceau-S staining. Only gels with equal loading were used for subsequent antibody staining and analysis. After Ponceau-S staining, membranes were blocked for a minimum of one hour in Tris-buffered saline (TBS) w/5% milk. After blocking, the blots were incubated overnight in primary antibodies (Hsp25 & αB-Crystallin, StressGen, 1:5000. Caspase-3, Upstate/Millipore, 1:1000. Caspase-9, Cell Signaling Technologies, 1:1000) in TBS w/2.5% bovine serum albumin (BSA). After primary antibody incubation, blots were washed three times with TBS/0.15% Tween, incubated in anti-rabbit IgG secondary antibody (Amersham Biosciences) at 1:5000 for one hour. After the secondary antibody the membranes were washed 3x in TBS/0.15% Tween, and developed using enhanced chemiluminescence (Amersham Biosciences) detected by CCD camera (Fujitsu). Blot images were analyzed using ImageQuant software (GE Healthcare).

**Statistical Analyses:** Western blot data for each protein was analyzed by a separate multiple regression model including main effect terms for exercise and injection and the injection by
exercise interaction. If significant main or interaction effects were found, Tukey post-hoc tests were performed to examine appropriate group differences. All tests were performed with SPSS v.15 statistical software (IBM), with alpha set to 0.05.

**RESULTS:**

**Heat Shock Protein Expression:** Regression models were significant for both Hsp25 ($f(3,55)=13.4, p<0.001, R=0.75$) and αB-Crystallin ($f(3,55)=4.63, p=0.001, R=0.55$). No significant exercise by injection interaction was found for Hsp expression, however significant main effects ($P<0.05$) for exercise were found for both Hsp25 and αB-crystallin, with both novel and accustomed exercise increasing expression as compared to the sedentary groups (Figure 4.1). No significant effect was found for statin injection.

**Caspase Expression:** A significant model was found for caspase-9 expression ($f(3,53)=3.31, p=0.026, R=0.40$), including a significant activity by injection interaction, with statin treatment increasing caspase-9 expression in the sedentary and novel exercise groups, but decreasing expression in the accustomed exercise group. Post-hoc tests did not indicate any individual group differences (Figure 4.2). Caspase-3 was not expressed at detectable levels in any group. Blots including caspase-3 positive controls (Cell Signaling Technologies) were used to confirm antibody specificity.
Figure 4.1: Expression of the heat shock proteins Hsp25 and αB-crystallin in the gastrocnemius after two weeks of statin/saline injections under sedentary, novel exercise, and accustomed exercise conditions. A significant main effect for activity was found for both Hsps, with both the novel and accustomed exercise groups showing significant elevations in...
expression. Statin treatment was not found to significantly influence expression under any activity condition.
Figure 4.2: Gastrocnemius caspase-9 expression after two weeks of statin/saline injections under sedentary, novel exercise, and accustomed exercise conditions. A significant activity*injection interaction was found, with statin treatment increasing caspase-9 expression in the sedentary and novel exercise groups, but decreasing expression in the accustomed exercise groups. No significant individual group differences were found. Active caspase-3 was not detectable in any group; positive control shows Jurkat cells treated w/cytochrome-c to activate caspase-3.

**DISCUSSION:**

To our knowledge, this study presents the first examination of the influence of exercise novelty and accustomization on the skeletal muscle stress-response to statin treatment, and complements our previous work examining muscle function following this intervention (See Chap. 3). Specifically, these data are the first *in vivo* examination of the effects of statin treatment and/or exercise on heat shock protein expression in skeletal muscle, and of exercise and statins’ influence
on apoptotic markers beyond a single exercise bout. Currently, the majority of the available literature regarding statins and exercise investigates a statin-potentiated damage response to a single exercise bout, rather than an exercise-induced exacerbation of statin-associated myopathy, though the line between the two phenomena is currently unclear. This study sought to address this shortcoming by utilizing repeated exercise bouts during statin treatment, as well as investigating the influence of exercise accustomization prior to the initiation of statin treatment, which had not been previously examined.

**Heat Shock Proteins:**

Heat shock proteins have been shown to protect against a number of cellular stressors, including oxidative stress⁹⁸ and apoptosis⁹⁹,1¹⁵⁻¹¹⁷, and may protect against mechanical damage in skeletal muscle¹⁰⁰⁻¹⁰⁴. Interestingly, heat shock protein expression is higher in muscle fibers of primarily type I myosin heavy-chain composition¹⁰⁵⁻¹⁰⁹—which have been shown to be protected from statin-induced damage—and can also be increased by exercise, including following two weeks of wheel running, as used in the current intervention¹²³. The effects of statin treatment on hsp expression have been shown to vary by tissue¹¹⁰⁻¹¹⁴, and the effect in muscle has not been previously examined. Taken together, the existing knowledge on the functions of Hsps in muscle and the fact that their expression can be modulated with exercise suggests that Hsps may contribute to the previously reported protective effects of exercise accustomization against statin-induced decrements in force-production ability (See Chap. 3).

In the current intervention, statin treatment was found to have no significant effect on hsp25 or αB-crystallin expression, regardless of activity condition, indicating that unlike many other tissues, statins do not affect small Hsp expression in skeletal muscle within the currently investigated time frame. Both novel and accustomed exercise treatment showed elevated Hsp
expression as compared to the sedentary groups, indicating an elevation in Hsp expression with exercise, though there were no differences between novel and accustomed exercise groups at four weeks, i.e. no difference between 4 weeks of running and 2 weeks. However, the elevated Hsp levels in the saline-treated, novel exercise group is of specific importance, because if the effect of saline injections on muscle Hsp expression is assumed to be negligible, this should also reflect the Hsp levels present in the accustomed exercise groups at the initiation of statin treatment. This indicates that while statin treatment does not appear to affect muscle Hsp levels, Hsp elevations present at the initiation of statin treatment may be protective against statin-associated myopathy and its exacerbation by exercise.

Further data concerning the time-courses of statins’ myopathic effects and of Hsp upregulation with running wheel exercise would be of interest in examining the novel exercise groups’ responses. The current results would indicate that while statin treatment did not prevent the upregulation of heat shock proteins during novel exercise training, this upregulation was not protective against myopathy. Further investigation is needed to determine why this is the case, though several possibilities can be hypothesized. Most significantly, it is possible that the Hsp upregulations seen with exercise—while associated with maintaining muscle integrity—are in fact not directly involved in exercise training’s protective effects. Directed investigations utilizing muscle-specific Hsp overexpression and/or deletion are needed to address this question. Alternatively, statin treatment may have caused the observed reductions in muscle force in the novel-exercise mice prior to the exercise-induced upregulation of Hsps, which would explain the current results if myopathy is preventable but not reversible by Hsp expression, or not reversible within the intervention timeframe. Currently, data addressing the time courses of exercise/statin
interactions are not available, therefore, it is impossible to determine what changes may have been seen with a shorter or longer exercise intervention.

Caspases and Apoptosis:

Statins have been shown to induce apoptosis in a variety of cell types, including smooth muscle and cardiac myocytes. Additionally, in vitro and ex vivo research has demonstrated that myotubes, myoblasts, and primary human muscle cells are also susceptible. Further, the apoptosis shown in skeletal muscle cells has been shown to progress via caspase-dependent pathways, as statin-induced apoptosis is accompanied by elevations in caspase-9 and caspase-3. Activation of caspase-3 is especially important in examining the apoptosis of skeletal muscle, as the ubiquitin-proteasome system cannot split actinomyosin complexes, and caspase-3 is likely the rate-limiting factor in myofilament breakdown and therefore likely involved in muscle breakdown and weakness.

While numerous studies have shown that statins can induce apoptosis in vitro, few studies have examined the effects of statins on skeletal muscle apoptosis in vivo, though the results have consistently indicated that in vivo apoptosis does not occur. In one examination, clinic patients with statin-associated myopathy were found to have no indication of muscle apoptosis based on TUNEL staining, Bax, Bcl-2, and caspase-3 expression. While Bax—a pro-apoptotic protein—and Bcl-2—an anti-apoptotic protein—are only intermediates in apoptotic signaling, TUNEL staining detects DNA that has been fragmented due to apoptotic processes, and therefore presents a measure of ongoing or recent apoptotic activity. In another examination of in vivo apoptosis, treatment of rats with 1mg/kg/day of cerivastatin for two weeks was not shown to increase muscle caspase-3 expression. While skeletal muscle appears to be resistant to statin-induced apoptosis in vivo, the exacerbation of statin-associated myopathy by exercise indicates that ruling out in vivo
apoptosis also requires careful examination of apoptosis under the combined treatments of statins and exercise. However, the only prior study investigating statins and apoptosis in combination with exercise was indirect, as it only examined the human gene response to a single bout of damaging exercise after four weeks of atorvastatin treatment. Interestingly, the investigators actually found reduced expression in apoptotic genes in the statin-treated group eight hours after exercise, though unfortunately the specific genes involved were not discussed 78.

*In vitro* research has shown that statins increase the expression of caspase-9 and activated caspase-3 during the apoptosis of myotubes and myoblasts, and in the current study statin treatment increased *in vivo* caspase-9 expression in the sedentary and novel exercise groups. In contrast, expression was decreased in the accustomed exercise group, indicating that exercise accustomization may block or even reverses statin’s activation of apoptotic pathways. An upregulation in Hsp expression with exercise may be responsible for this interaction, as Hsp’s—including hsp25—have been shown to inhibit the cleavage of procaspase-9 into the active subunit caspase-9, which was measured in the current study. Therefore, an elevation in muscle hsp expression after two weeks of wheel running may have inhibited any elevations in caspase-9 activity by statin treatment.

Caspase-9 is an activator of caspase-3; however, since activated caspase-3 expression was undetectable, it is difficult to say what physiological impact—if any—this reduction in caspase-9 expression with exercise may have, as the activated caspase-3 data indicates that repeated exercise during statin treatment does not lead to *in vivo* skeletal muscle apoptosis. This is in agreement with previous research showing that statins do not induce *in vivo* apoptosis in sedentary muscle or muscle exposed to a single exercise bout. However, the lack of active caspase-3 expression in the novel-exercise group is an important confirmation, as the novel exercise intervention was
previously shown to be especially damaging as compared to sedentary statin treatment (See Chap. 3), and this presents the first investigation of statin-associated myopathy and apoptosis with repeated exercise. Combined with the prior *in vivo* research, the current data indicate that statin treatment does not induce *in vivo* skeletal muscle apoptosis, be it in sedentary or exercised muscle.

In conclusion, this study presents a novel investigation into the impact of statin treatment on skeletal muscle expression of Hsp25, αB-crystallin, caspase-9, and caspase-3 in mice completing repeated novel or accustomed exercise. The results indicate that while statin treatment increases caspase-9 expression in sedentary and novel-exercise muscle, this effect is reversed in the muscle of mice undergoing exercise accustomization prior to the initiation of statin treatment. However, the caspase-3 data indicate that the combination of statin treatment and repeated exercise—even the novel exercise intervention, which has been shown to be especially damaging to skeletal muscle—does not lead to an increase in apoptosis *in vivo*. This is in agreement with previous literature indicating that statins do not induce *in vivo* apoptosis in sedentary muscle or muscle exposed to a single exercise bout. Additionally, we have found that an upregulation in Hsp25 and αB-crystallin expression after two weeks of wheel running presents a possible mechanism for the previously demonstrated protective effects of exercise accustomization, though further investigation is necessary to examine the causal role for Hsps.


51. Hancock JF, Magee AI, Childs JE, Marshall CJ. All ras proteins are polyisoprenylated but only some are palmitoylated. Cell 1989;57(7):1167-1177.


123. Huey KA, Meador BM. Contribution of IL-6 to the Hsp72, Hsp25, and {alpha} {beta}-crystallin responses to inflammation and exercise training in mouse skeletal and cardiac muscle. J Appl Physiol 2008.


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APPENDIX

Prior Work Regarding Muscle Function and Interventions to Preserve/Restore Reduced Function
Abstract:

Purpose: To test the hypothesis that acute Glutamine (GLN) supplementation can counteract skeletal muscle contractile dysfunction occurring in response to inflammation by elevating muscle heat shock protein (Hsp) expression and reducing inflammatory cytokines. Methods: Mice received 5mg/kg Lipopolysaccharide (LPS) concurrently with 1g/kg GLN or vehicle treatments. Plantarflexor isometric force-production was measured 2 hours post-injection. Blood and gastrocnemius muscles were collected, and serum and muscle Tumor Necrosis Factor-alpha (TNFα) and Interleukin-6 (IL-6) and muscle Hsp70 and Hsp25 were quantified. Results: Saline/LPS-treatment was associated with a 33% reduction in maximal force and elevated serum TNFα and IL-6. GLN completely prevented this force decrement with LPS. GLN was found to reduce muscle Hsp70 and IL-6, but only in the presence of LPS. Conclusion: GLN supplementation provides an effective, novel, clinically applicable means of preserving muscle force during acute inflammation. These data indicate that force preservation is not dependent on reductions in serum cytokines or muscle TNFα, or elevated Hsp levels.

* This manuscript has been previously published and can be accessed by the below reference. Its current use has been permitted by the original publisher.

Introduction

Chronic and acute inflammatory diseases—such as chronic cardiopulmonary disease, cancer, transplant rejection, and sepsis—are often associated with respiratory and skeletal muscle weakness [141-145], which typically correlates with a loss of muscle protein [141,146,142,147]. The decrements in muscle function under these conditions are believed to be mediated in part by elevation of the catabolic cytokines Tumor Necrosis Factor alpha (TNFα) and interleukin-6 (IL-6) [144]. However, it has also been demonstrated that inflammatory disease can cause reductions in muscle strength without muscle protein catabolism [148-151]. The mechanisms behind contractile dysfunction in the absence of muscle wasting are not well understood, though some loss of contractile regulation has been indicated [144]. Both with and without muscle wasting, TNFα—which is known to be elevated in several inflammatory diseases [152-158] and has been shown to independently, acutely reduce contractile function [159-161,144,162,163]—is likely a key cytokine involved in contractile dysfunction. Additionally, IL-6 has been implicated in some chronic conditions of muscle atrophy and strength loss such as chronic cardiopulmonary disease and cancer, where muscle wasting is a significant factor [164-169]. Regardless of the etiology, contractile dysfunction presents a clear hindrance to physical function in individuals with these chronic diseases, and it is a potential obstacle to rapid recovery and rehabilitation after surgical complications of infection or even sepsis.

In this study we examined the potential role for glutamine (GLN) supplementation in counteracting contractile dysfunction during an inflammatory insult. GLN is a non-essential amino acid that plays critical roles in immune system function and skeletal muscle protein turnover. During stressful events, such as systemic sepsis, the body’s natural ability to synthesize GLN is
impaired, which has led to it being known as a conditionally essential amino acid. GLN supplementation has been found to improve clinical outcomes during sepsis, and these improvements have been related to increases in heat shock protein (Hsp). Hsp70 expression is increased in non-skeletal-muscle tissues such as the lungs, heart, kidney, and colon, and increases in Hsp25 expression in the heart, liver, and colon, as well as reductions in systemic inflammatory mediators, namely NF-KB and TNF-α are also found. GLN’s effects on Hsp expression in skeletal muscle during an inflammatory insult have not been examined. It has been shown that GLN supplementation can increase Hsp70 expression in myotubes exposed to heat stress. If GLN has similar affects in mature skeletal muscle as in these other tissues, it would have the potential to upregulate expression of Hsp25 and Hsp70. Such an effect in response to a contractile dysfunction-inducing inflammatory insult would have the potential to help preserve muscle function, as these Hsp’s have been shown to have protective effects against heat stress, oxidative stress, apoptosis, and muscle damage.

Since skeletal muscle is by far the largest contributor to plasma GLN levels, the “conditionally essential” classification of GLN would indicate that these systemic inflammatory events drastically affect skeletal muscle protein metabolism. Still, to our knowledge, the effects of GLN supplementation on skeletal muscle during an inflammatory insult have not been examined. The purpose of this study is to examine the effects of GLN supplementation on muscle strength, as well as serum and muscle cytokine levels and muscle Hsp70 and Hsp25 expression during acute inflammation induced by lipopolysaccharide (LPS) administration. It is hypothesized that, in response to an acute inflammatory insult, GLN supplementation will preserve muscle strength and endurance and that this will be associated with reduced levels of muscle and serum inflammatory cytokines, primarily TNF-α, and increased expression of Hsp70 and Hsp25 in muscle.
Methods

A total of 29 C57BL/6J mice (Jackson Labs), aged 8-10 weeks were used in these studies. For the first set of experiments, 21 mice were randomly assigned to one of the following groups: Saline/Saline (n=5), Saline/LPS (n=5), GLN/Saline (n=5), and GLN/LPS (n=6). Saline was the vehicle for both GLN and LPS. LPS-group mice received I.P. injections of 5mg/kg E. Coli LPS (Sigma, serotype 0127:B8) in a 0.5mg/ml saline solution. Dose-response experiments with LPS (1 mg/kg, 2.5mg/kg, 3.5mg/kg, & 5mg/kg) found that a dose of 5mg/kg was necessary to induce reductions in muscle force without inducing sepsis. The GLN groups received I.P. injections of 1g/kg GLN (HyClone) (.05g/ml) in saline suspension. The dose of GLN was chosen based upon previous studies that showed positive effects when 1g/kg or higher of I.P. GLN were used. Saline or LPS and Saline or GLN injections were given concurrently. Concurrent injections were chosen over pre-treatment with GLN to increase the clinical applicability of the intervention. Force measurements were taken 2 hours post-injection, and muscles and blood were immediately isolated as described below.

A later time-point (24h) utilizing a lower dosage (1mg/kg) was examined using 8 additional mice divided into three treatment groups: Saline/Saline (n=2), Saline/LPS (n=3), and GLN/LPS (n=3). These mice were used to examine time-dependent changes in Hsp expression. They received a lower LPS dosage, as the 5mg/kg LPS dose used at the 2-hour time-point corresponds to approximately LD50 in this strain of mice. LPS mortality occurs ~18-22 hours post-injection, therefore the lower dosage was selected to avoid LPS-induced mortality at the 24-hour time-point. Force was not measured in these groups, as preliminary experiments found that this lower dose of LPS was not associated with any significant decrements in muscle isometric force. These
experiments were approved by the Animal Use Committee at the University of Illinois and followed the American Physiological Society Animal Care Guidelines.

**Force Measurement:** An *in vivo* force system similar to previously described models was developed for these measurements. Specifically, all mice had measurements taken for the maximum isometric contraction strength of the hindlimb plantar flexors. Force was measured using a dual-mode lever system, which controlled both length and force/speed (Aurora Scientific, 305C-LR). The servomotor system was calibrated by placing known weights 20mm from the rotational axis and creating a torque/voltage standardized curve.

Mice were fully anesthetized by the administration of 100mg/kg Ketamine/10mg/kg Xylazine IP. Mice were then placed on a table warmed to 37°C, where all further procedures were done. An incision was made in the lateral thigh, parallel and superficial to the femur, through which the sciatic nerve branch innervating dorsiflexors was transected to allow isolated contraction of the plantarflexors. Two electrodes were placed proximal to the gastrocnemius complex on the sciatic nerve, with the two electrodes approximately 1mm apart. The ankle of the mouse was fixed in the servomotor’s footplate, with the talus (rotational center of the ankle joint) aligned with the servomotor axis. The mouse was positioned to place the knee joint at 90° and the ankle at 80°, where the knee joint was immobilized with an alligator-clip at the femoral condyle. The sciatic nerve was stimulated at 200hz for 1.5s to evoke a maximal contraction. This stimulation was repeated nine times with a recovery period of 5s, for a total of 10 measurements. The highest acquired data points were taken as the maximum. Fatigue was quantified by comparing the final contraction force to the maximal contraction and calculating a percent reduction. The exact timing of the force measurement in relation to the LPS and GLN injections (time-post) was recorded for
statistical analysis purposes. Data were recorded by a customized LabView program (National Instruments) to provide a plot of ankle-torque/time.

Mice were euthanized immediately after the completion of the force-measurement protocol. Blood was drawn from the inferior vena cava, and the plantarflexors were isolated from the hindlimbs. The gastrocnemius was isolated from the soleus and immediately frozen on dry ice and stored at -80°C. Muscles from the stimulated and non-stimulated limbs were divided for separate analysis. Unless otherwise noted, the muscle from the non-stimulated limb was used for analysis to ensure that stimulation itself did not affect variable outcomes. Whole blood was centrifuged at 1500rcf for 10 minutes, and the serum was subsequently extracted and immediately stored at -80°C.

**Protein Isolation:** Pre-weighed muscle samples (~40 mg) were homogenized in 10 volumes of an ice-cold buffer containing 50mM Tris HCl (pH 7.8), 2 mM potassium phosphate, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 3 mM benzamidine, 1 mM sodium orthovanadate, 10 mM leupeptin, 5 mg/ml aprotinin, and 1 mM 4-[(2-aminoethyl)benzenesulfonyl fluoride], and supplemented with protease and phosphatase inhibitors (Sigma) using a motor driven glass pestle. Homogenates were immediately centrifuged at 12,000 g for 12 min at 4°C, and the supernatant was removed as the detergent-soluble fraction. Protein concentration was determined with the Biorad Protein Assay using bovine serum albumin for the standard curve, after which samples were saved immediately in aliquots at −80°C for subsequent use in ELISA analysis.

**Cytokine Measurements:** TNF and IL-6 measurements were performed on serum and muscle homogenate from the non-stimulated gastrocnemius using the Endogen ELISA kits (kits EMTNFA and EM2IL6, respectively), according to the manufacturer’s protocol as described
previously. Cytokine concentrations were expressed as pg/mg protein or pg/ml for muscle and plasma, respectively.

**Heat Shock Protein Measurements:** Western blot analysis was used to determine Hsp25 and Hsp72 protein levels in skeletal muscle. Twenty-five μg of detergent-soluble proteins were boiled (5 min at 95°C) and separated by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%) and transferred to nitrocellulose membranes (pore size 45 μm). After protein transfer, the membranes were blocked for 1 hr in Tris buffered saline (TBS)/5% milk. Following blocking, the membranes were incubated overnight at 4°C with antibodies to detect Hsp25 (1:5000) or Hsp70 (1:5000) antibody in TBS/2.5% BSA. All antibodies were purchased from Assay Designs (Ann Arbor, MI). Blots were washed 3 times in TBS/0.1%Tween and incubated with anti-rabbit secondary antibodies (Amersham Biosciences, Piscataway, NJ, USA) at 1:5,000 for 1 hr at room temperature. Blots then were washed in TBS/0.1%Tween, and the bound antibodies were detected with enhanced chemiluminescence (Amersham Biosciences). Quantification of the bands was performed using ImageQuant Analysis software. All the necessary controls for Western blots were performed to assure antibody specificity. Equal loading was quantified with Ponceau S by densitometric analysis of 3 non-specific bands prior to blocking and protein detection. Only gels with equal loading were used for subsequent quantification of specific proteins.

**Statistical Methods:** A separate 2x2 factorial ANOVA was performed for each variable of interest. The exact time of the force measurements in relation to the GLN and LPS injections was used as a covariate for all analyses. If a significant main effect or interaction was detected, post-hoc analysis was performed by the LSD test to examine group differences. All calculations were performed with SPSS statistical software (v. 15), with alpha = 0.05.
Results:

No significant group differences in body weight at the time of force measurement were found 25.4±0.1, 25.6±1.0, 24.2±0.8, & 24.0±0.6 for Saline/Saline, GLN/Saline, Saline/LPS, & GLN/LPS groups, respectively. Additionally, while time-post varied slightly from animal-to-animal due to anesthesia and surgery times, it ranged between 119 and 127 (mean 121.5) minutes, and was not significantly different between any groups.

Force Measures: A significant Saline/GLN*Saline/LPS interaction effect was found for muscle force normalized to body weight (p=.001). Post-hoc analysis showed a significantly lower average force in the Saline/LPS group as compared to all three of the other groups by approximately 33% (Fig. 1). However, there was no significant difference in force outputs between any of the other three groups. Additionally, fatigue levels were not significantly different across groups (Fig. 1).

Cytokine Levels: Significant main effects of LPS were found in both the serum and muscle for TNFα, while a significant main effect for LPS on IL-6 was found only in the serum (all p<0.05). Serum TNFα and IL-6 were found to be significantly elevated in the LPS groups as compared to the corresponding saline groups (Table 1). In muscle, TNFα levels were lower in the LPS groups as compared to the corresponding saline group. A significant Saline/GLN*Saline/LPS interaction was found for muscle IL-6 levels, with IL-6 in the GLN/LPS group found to be significantly lower than all other groups.

Heat Shock Protein Measures: For the 2h time-point, no differences in the expression of Hsp-25 were found for any group. However, Hsp-70 showed a significant main effect for GLN treatment and a significant 47% reduction in the GLN/LPS group as compared to all other
groups. At 24h, Hsp-25 showed a significant 29% reduction in the GLN/LPS group as compared to the Saline/LPS group (0.11±0.02, 0.15±0.01, & 0.11±0.01 arbitrary units for the Saline/Saline, Saline/LPS, and GLN/LPS groups, respectively) (Figure 2).
**Figure A.1:** Changes in average maximal isometric plantarflexor force 2 hours after administration of LPS with or without glutamine (GLN). Saline (Sal) was the vehicle control for both LPS and GLN. Total column height denotes maximal isometric force. White inner columns denote contraction force at repetition 10, with the percentage of initial force at 72, 72, 71, and 77% in the Sal/Sal, GLN/Sal, Sal/LPS, & GLN/LPS groups, respectively. *p<0.05 versus all other groups for maximal contraction force. Data are presented as means ± SE.

**Table A.1:** Cytokine expression in muscle and serum

<table>
<thead>
<tr>
<th></th>
<th>Sal/Sal</th>
<th>GLN/Sal</th>
<th>Sal/LPS</th>
<th>GLN/LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Serum</td>
<td>0.8 ±.7</td>
<td>2.6 ±1.9</td>
<td>261.2 ±85.4*</td>
<td>347.5 ±192.1*</td>
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<tr>
<td>Muscle</td>
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<td>12.8 ±5.2</td>
<td>9.0 ±3.8*</td>
<td>7.2 ±2.7*</td>
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<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Serum</td>
<td>13.5 ±4.7</td>
<td>17.0 ±7.7</td>
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<td>728.1 ±539*</td>
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<tr>
<td>Muscle</td>
<td>325.1 ±81.6</td>
<td>336.4 ±67.8</td>
<td>311.8 ±158</td>
<td>160.0 ± 91.8†</td>
</tr>
</tbody>
</table>

*Table A.1: TNFα and IL-6 protein levels in serum and skeletal muscle 2 hours after administration of LPS with or without glutamine (GLN). Saline (Sal) was the vehicle control for both LPS and GLN. * p<.05 versus corresponding saline group. †p<.05 versus all other groups.
Figure A.2. Changes in skeletal muscle Hsp25 (A) and Hsp70 (B) protein levels 2 hours after administration of LPS with or without glutamine (GLN). Saline (Sal) was the vehicle control for both LPS and GLN. *p<0.05 as compared to sal/sal control. Data are presented as means ± SEM.
Discussion

Inflammatory diseases and acute inflammatory insults are often associated with skeletal muscle weakness, either as a result of muscle wasting or a more direct mechanism of contractile dysfunction. In this study we investigated a potential therapy for these conditions, with the design following from the fact that similar effects are seen in animal models of endotoxin exposure. Our data confirms the hypothesis that GLN supplementation preserves muscle force output during an acute inflammatory insult; the group that received LPS without GLN showed a significant, 33% reduction in force as compared to the two saline groups. In contrast, concurrent injection of LPS and GLN was associated with no reductions in maximal isometric force and a significant reduction in muscle levels of IL-6. Surprisingly, the preservation of force with concurrent GLN and LPS administration was not associated with a reduction in muscle or serum TNFα.

A large body of previous research has found elevated TNFα levels to be correlated with a decrease in force production ability in the diaphragm, and GLN administration has been shown to reduce circulating TNFα during sepsis. As such, a primary hypothesis of the current study was that GLN would preserve force under inflammation in skeletal muscle, and this would be associated with a reduction in TNFα levels. However, muscle TNF levels were significantly lower in the LPS-treated groups, though the absolute levels were very low and it is unlikely that the ~2pg/mg reduction caused by GLN had any physiological impact. In perspective, previous data from our lab found basal levels of TNFα to be as high as ~75pg/mg. Additionally, while serum TNFα was elevated by LPS administration as expected, no significant effect on TNFα levels was found for GLN supplementation. These findings suggest that GLN’s force-preserving properties are downstream of any effects TNFα may have on the muscle. Furthermore, the
literature on TNFα and skeletal muscle force is not as definitive as that regarding the diaphragm; the studies that have been conducted are not all in agreement on the effects of TNFα. Some show a decrease in force \(^{159}\) and others show no effect \(^{161}\). Indeed, the findings of our study support the notion that at least circulating TNFα levels do not decrease force in hindlimb skeletal muscle in this experimental model.

While LPS administration significantly elevated IL-6 levels in the serum, these changes were not observed at the muscle level. This is in agreement with previous work that showed LPS-induced increases in muscle IL-6 mRNA occurred 2 hours post-injection and peaked at ~4 hours \(^{194}\). Further, we have shown that lower doses of LPS are associated with significant increases in both skeletal and cardiac muscle 4 hours following LPS injection \(^{195,189}\). Therefore, the expectation in this study was that the more rapidly responding serum, not muscle, IL-6 would play a role. However, our data shows that muscle IL-6 was significantly reduced in the GLN/LPS group as compared to all other groups. Interestingly, GLN was associated with reductions in IL-6 below basal levels, indicating that, in the presence of an inflammatory stressor, GLN is able to reduce IL-6 levels in muscle, rather than attenuating increased expression. The direct impact of this reduction on force production is difficult to determine, as IL-6 has been implicated in chronic muscle wasting and atrophy, rather than acute functional decrements \(^{164-169}\). IL-6 has, however, been shown to augment acute proteolysis, but only when other inflammatory mediators such as TNFα are present \(^{165}\). This property may explain why the saline/LPS group experienced a force decrement despite the fact that IL-6 levels were not elevated in comparison to the saline treated groups; the saline treated groups did not have the elevated serum TNFα levels necessary for IL-6’s augmentation of proteolysis. Conversely, the GLN/LPS group had elevated serum TNFα, and so it may have benefited from a reduction in muscle IL-6.
Interestingly, concurrent administration of GLN and LPS was associated with significant reductions in Hsp70, but no increase in Hsp70 was seen with the administration of LPS alone. While this does not support our hypothesis and is in contrast to the GLN-associated increases in Hsp expression shown in non-muscle tissues, it is possible that GLN administration during this inflammatory insult significantly reduced the stress experienced by the muscle, thereby attenuating expression of the stress protein Hsp70. This is further supported by the finding that at 24 hours following concurrent administration of LPS and GLN, Hsp25 was also reduced in skeletal muscle. While these findings are in contrast to the literature regarding GLN and Hsp expression in several non-muscle tissues\(^\text{170,173}\), they suggest that the primary role of skeletal muscle in maintaining GLN levels in the blood positions it to be uniquely affected by supplementation. Furthermore, the Hsp data mirrors the IL-6 findings in that both measures were reduced below baseline levels by GLN supplementation, but only in the presence of LPS. These Hsp reductions may be a direct result of the IL-6 reductions, as IL-6 has been found to mediate increases in Hsp70 in human skeletal muscle\(^\text{196}\) and increases in both Hsp25 and Hsp70 in human hepatoma cells\(^\text{197}\). Therefore, the reduced IL-6 protein levels in the GLN/LPS treated mice may have led to a reduced stimulus for Hsp expression.

While the GLN dose utilized in the present study (1g/kg) may not be suitable for long-term therapy in humans, it would be a very possible intervention during a more severe and acute inflammatory insult such as sepsis. However, lower doses of GLN may also be effective, as in a cecal ligation and puncture (CLP) model of sepsis in rats, a 0.75g/kg dose of GLN given 1 hour post-CLP effectively improved clinical outcomes\(^\text{172}\). Among the outcomes of interest was the development of Acute Respiratory Distress Syndrome (ARDS), which was reduced in the GLN-supplemented animals, and this reduction was associated with lower inflammatory cytokine release.
and reduced tissue damage in the lungs. Our novel findings suggest that there may also be a role for GLN in diaphragm function during sepsis, as endotoxemia has been shown to decrease diaphragm function, thus potentially contributing to respiratory distress. Since the diaphragm shares the striated muscle phenotype with the gastrocnemius it could also potentially benefit from GLN supplementation through similar mechanisms.

These results demonstrate that GLN administration has the ability to completely restore acute inflammation-induced reductions in muscle force, and that as such it presents a clinically relevant intervention for a variety of inflammatory insults. The cytokine and Hsp data indicate that the preservation of muscle force by GLN administration is not dependant on a reduction in TNFα or an increase in Hsp expression, but rather it was associated with a reduction in muscle IL-6 and Hsp70 levels. Additionally, the unique responses of the stress-induced Hsp proteins and IL-6 in skeletal muscle indicate that GLN supplementation may play a significant role in reducing the stress experienced by skeletal muscles during an acute inflammatory insult. Clinically, this may greatly improve recovery following an inflammatory insult, as skeletal muscle comprises 40-50% of body mass and is critical for performing activities of daily living.
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