

THE IMPACT OF PHYSICAL ACTIVITY ON STATIN-ASSOCIATED  
SKELETAL MUSCLE MYOPATHY

BY

HAE RYONG (HARRY) CHUNG

DISSERTATION

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Doctoral Committee:

Associate Professor Kenneth Wilund, Chair

Professor Jeffrey Woods

Associate Professor Marni Boppart

Assistant Professor Jacob Haus, University of Illinois at Chicago

## ABSTRACT

**BACKGROUND:** HMG-CoA reductase inhibitors (statins) are a common and effective pharmacological means of treating hypercholesterolemia and decreasing cardiovascular risk. The most common side effect of statins is skeletal muscle myopathy, which appears to be exacerbated by exercise.

**PURPOSE:** The purpose of this study was to examine the effects of statin treatment with novel or accustomed exercise in hypercholesterolemic (ApoE<sup>-/-</sup>) or wild type (WT) mice on muscle function (grip strength, isometric force, and daily activity level), markers of mitochondrial content (mtDNA and PGC1- $\alpha$ ), skeletal muscle oxidative stress (4HNE), and protein degradation (atrogin-1).

**METHODS:** Mice were divided into three different activity groups, sedentary (Sed), novel (Nov), accustomed (Acct) (n=60). Mice in all groups received daily injections of either simvastatin (20mg/kg) or saline for the later 2 weeks with (Acct)/without (Nov) the prior 2 weeks of wheel running (WR). Daily WR distance was recorded. At 4 weeks, plantarflexor isometric force and grip strength were measured. mtDNA, 4HNE, and atrogin-1 and PGC1- $\alpha$  were measured as markers of mitochondrial content by real time PCR, ELISA, real time RT-PCR, respectively.

**RESULTS:** Two weeks of statin treatment decreased running wheel activity, isometric force, and grip strength regardless of exercise groups. In saline-injected animals, both Nov and Acct EX increased mitochondrial content and PGC1- $\alpha$  levels, but this effect was blunted by statins. There was an interaction effect on muscle 4HNE, and atrogin-1 gene expression between statins and EX. Specifically,

there was an overall trend for a decrease in these markers when statins were provided to sedentary mice, though both novel and accustomed exercise increased their levels.

**CONCLUSION:** These results indicate that statin treatment had a negative impact on muscle function and cellular regulation in muscles in hypercholesterolemic mice. The combination of exercise and statin treatment decreased mitochondrial content and the expression of PGC1- $\alpha$ . Additionally, muscle oxidative stress and the protein degradation were increased by the combination of exercise and statins while exercise alone elicited mitochondrial and antioxidant benefits.

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## TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION .....	1
CHAPTER 2: BACKGROUND AND LITERATURE REVIEW .....	5
CHAPTER 3: RESEARCH DESIGN AND METHODS.....	25
CHAPTER 4: RESULTS .....	32
CHAPTER 5: DISCUSSION.....	48
CHAPTER 6: CONCLUSION .....	62
REFERENCES .....	64

## CHAPTER 1

### INTRODUCTION

HMG-CoA reductase inhibitors (statins) are a common and effective pharmacological means of treating elevated cholesterol levels and decreasing cardiovascular risk. Overall, statins are considered a very safe class of drugs, however, they carry a significantly elevated risk of myopathy, ranging from weakness and fatigue to rhabdomyolysis, a potentially fatal condition. It has been estimated that general myopathy occurs in as many as 10% of all statin users<sup>1-4</sup>.

The biological basis for statin-induced myopathy is not well understood. *In vitro* and *in vivo* studies have suggested that several factors, either individually or in combination, may contribute to this condition, including mitochondria dysfunction, apoptosis<sup>5-7</sup> and reduced isoprenoid levels<sup>6, 8</sup>. Statins may cause mitochondrial dysfunction, disturbing membrane depolarization<sup>9</sup> and/or adversely affecting mitochondrial respiration<sup>10</sup>. Statins may directly block the formation of ubiquinone, or co-enzyme Q10, which plays an important role in cellular respiration as one of the electron transporters in the inner mitochondrial membrane<sup>1, 11</sup>. The reactive oxygen species created by oxidative stress can increase FOXO dephosphorylation<sup>12</sup>, FOXO nuclear translocation, and subsequent upregulation of atrogen gene expression<sup>13</sup>, an essential component of the ubiquitin proteasome pathway (UPP)<sup>14</sup>. Thus, oxidative stress may increase myofibrillar protein degradation, providing a potential basis for muscle weakness and fatigue with HMG-CoA reductase inhibition.

Clinical case studies suggest that physical activity increases the risk of statin-induced muscle myopathy. Among the subgroup of statin users who are also exercisers, the prevalence of myopathy is estimated to be as high as 25%<sup>1, 15, 16</sup>. In highly trained athletes, this frequency may reach 75%.<sup>17</sup> Unfortunately, well-designed longitudinal studies that evaluate the effect of exercise training on statin-induced myopathy have yet to be conducted.

Members of our research group have recently investigated the effect of exercise training on statin-associated myopathy in mice.<sup>18</sup> The results indicated that two weeks of exercise training prior to statin treatment is protective against myopathy. However, introduction of exercise concurrent with statin administration exacerbated the myopathy. The extent to which exercise-induced changes in mitochondrial number and/or function were responsible for these observations was not determined.

Unfortunately, several shortcomings were apparent in this preliminary research, one of which was the use of normocholesterolemic mice. Hypercholesterolemia has been shown to play an independent role in skeletal muscle myopathy. Hypercholesterolemia *per se* can induce myopathy<sup>19</sup>, and it is currently unknown how hypercholesterolemia-induced myopathy interacts with statin-induced myopathy. It has been noted that this role for hypercholesterolemia may well complicate the entire body of literature addressing statins and exercise, as the importance of normo- vs. hypercholesterolemia is not generally accounted for in the interpretation of statins' myopathic effects<sup>20</sup>. Conversely, this effect of cholesterol may also contribute to the disparate results observed when investigating the roles for statins and exercise in myopathy. Studies using subjects with high cholesterol levels tend to find no

deleterious effects of statins on exercise-induced myopathy, while studies using subjects with normal cholesterol levels tend to observe myopathic effects of statins and an exacerbation of these effects with exercise.<sup>19, 21</sup> However, these have been observational results that need further testing.

The purpose of this study was to examine the interaction between statin treatment and novel and accustomed exercise on mitochondrial number and function, as well as resultant myopathy, in a mouse model of hypercholesterolemia (ApoE<sup>-/-</sup>). The study will include the following conditions/treatments: A) saline and statin treatment and B) sedentary, novel exercise, and accustomed exercise.

**Objective 1:** Determine the effects of novel and accustomed exercise on statin-induced myopathy in ApoE<sup>-/-</sup> mice.

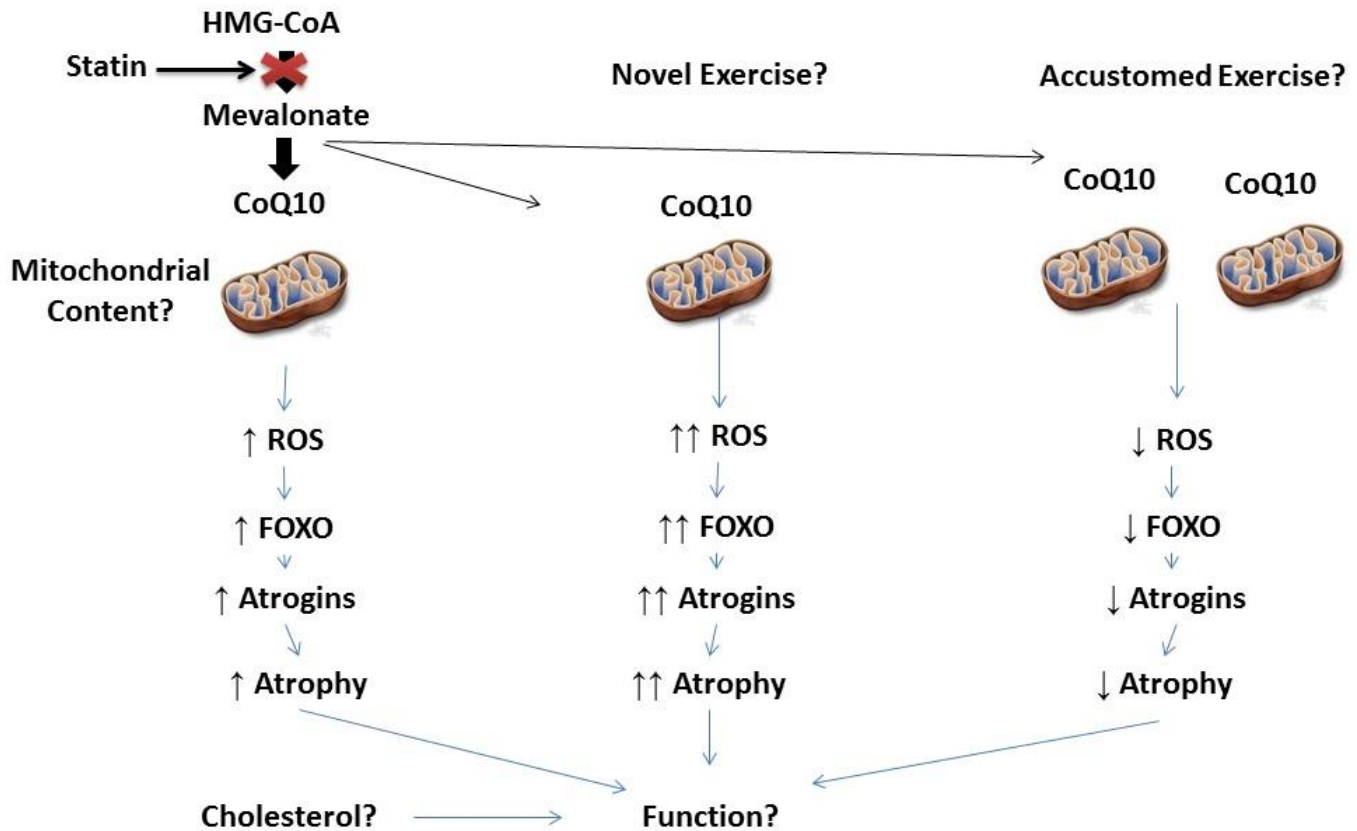
*Hypothesis 1:* We hypothesize that novel exercise will exacerbate statin-induced myopathy, while accustomed exercise will protect against statin-induced myopathy in ApoE<sup>-/-</sup> mice.

**Objective 2:** Determine the extent to which novel and accustomed exercise alter mitochondrial content, muscle oxidative stress, and protein degradation in response to statin administration in ApoE<sup>-/-</sup> mice.

*Hypothesis 2:* We hypothesize that statin administration will decrease mitochondrial content, muscle oxidative stress, and protein degradation and that novel exercise will exacerbate this effect in ApoE<sup>-/-</sup>



mice. Conversely, accustomed exercise will protect against declines in mitochondrial content and inclines in muscle oxidative stress and protein degradation with statin administration.



**Figure 1. Objectives.** Statins inhibit HMG-CoA reductase. This results in lower levels of CoQ10, an important electron carrier in the inner mitochondrial membrane. Depletion of CoQ10 by statins leads to generation of mitochondrial reactive oxygen species (ROS) which promote translocation of forkhead box (FOX) transcription factors to the nucleus. In muscle, FOXO can stimulate the transcription of ubiquitin protein ligases such as atrogen-1, which can induce myofibrillar proteolysis. Exercise is expected to influence statin-induced myopathy, but in a manner dependent on treatment sequence. With novel exercise, the exercise stress added to the statin-stressed mitochondria is expected to exacerbate statins' effects. However, with prior exercise training—accustomed exercise—the increased mitochondrial volume and efficiency allows the muscle to cope with the stress of statin treatment, alleviating the myopathic impact.

## CHAPTER 2

### BACKGROUND AND LITERATURE REVIEW

**2.1 Cardiovascular Disease and Statins.** According to the American Heart Association, cardiovascular disease (CVD) accounted for about 32.8% of all deaths in 2008. Moreover, 2,200 Americans die of CVD each day.<sup>22</sup> Hyperlipidemia is the leading cause for cardiovascular diseases, such as atherosclerosis, coronary heart disease, and peripheral vascular disease. Though physical activity and diet may help control lipid levels, lifestyle interventions may not be sufficient for everyone. Therefore, pharmacological means to reduce cardiovascular risk may be needed. Several forms of medication have been developed to reduce cardiovascular risk; however, statins have proven to be the most effective. Today, statins are the most widely used form of medication used to combat hyperlipidemia.<sup>23, 24</sup>

**2.2 Mechanism of Action.** 3-hydroxy-3-methyl-glutaryl-Coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are the most frequently prescribed medication to reduce cholesterol.<sup>24,</sup>  
<sup>25</sup> These drugs are very effective and have been extremely well-tolerated in controlled clinical trials.<sup>26</sup> According to a survey conducted in 2010, Zocor (simvastatin) is the third most prescribed medication (more than 76 million prescriptions) and \$1.1 billion in retail sales in the United States.<sup>27</sup>

Cholesterol is synthesized in the body from acetyl-CoA (Figure 2). This molecule is converted to mevalonate, which is ultimately transformed into cholesterol through a series of complex reactions. HMG-CoA reductase is an extremely important enzyme in this pathway as it serves as a catalyst in the conversion of acetyl-CoA to mevalonate. This is the rate-limiting step in cholesterol synthesis, and the site of action for statins.<sup>28</sup>

Statins reduce cholesterol level in the circulation by reducing hepatic cholesterol synthesis, thus decreasing intracellular cholesterol. This stimulates the up-regulation of LDL receptors and increases the uptake of non-HDL particles such as LDL, VLDL, and IDL from the systemic circulation.<sup>29</sup>

In addition to lowering cholesterol, statins have other positive effects such as: increasing endothelial function, stabilizing of atherosclerotic plaques, lowering inflammation and oxidative stress, and inhibition of the thrombogenic response in the vascular wall.<sup>2, 30-32</sup> Furthermore, statin therapy has been associated with a 30% decrease in cardiovascular events in both patients with coronary artery disease and in healthy persons.<sup>33</sup>

**2.3 Adverse Effects of Statins in Skeletal Muscle.** Despite benefits to the cardiovascular system, all statins are associated with skeletal myopathy, with symptoms ranging from mild complaints-such as myalgia, cramps, and weakness, to rhabdomyolysis with renal failure.<sup>24, 32</sup> Aside from pain, statin-related myopathy can also manifest as general fatigue that is not often measurable by physical examination.

Myalgia, myositis, myopathy, and rhabdomyolysis are forms of myotoxicity that are characterized by severity of muscle pain, inflammation, and elevation of creatine kinase (CK) levels. Myalgia is defined as muscle pain such as stiffness, muscle tenderness, heaviness, cramping, or weakness that may present with or without CK elevation.<sup>34</sup> Myositis is defined as inflammation of the muscles with CK elevation.<sup>35</sup> Myopathy is defined as muscle aches and CK level about a 10 fold higher than the upper limit of normal.<sup>36</sup> Rhabdomyolysis is defined as a condition in which damaged skeletal muscle tissue break down and characterized by CK level above 10,000 IU/L, or above 10-fold upper limit normal in combination with an elevation in serum creatinine, usually with compared renal function and increases in urinary myoglobin.<sup>37, 38</sup> Rhabdomyolysis is very rare and muscle fatigue, pain, or weakness (subclinical indicators of muscle damage) are more common.<sup>39</sup> In a meta-analysis, Kashani et al.,<sup>40</sup> showed that there is a non-significant trend toward a higher incidence of myalgia in a statin treated group compared with a placebo treated group. Other recent studies also have shown relatively low rates of myalgia in patients enrolled in clinical trials.<sup>41, 42</sup> The prevalence of statin associated myopathy is quite low and occurs in five patients per 100,000 people per years (~0.01 %).<sup>37</sup> Statin-associated rhabdomyolysis is rare and occurs in 1.6 patients per 100,000 people per years (0.002 %).<sup>37, 38</sup> Although the reported rate of myalgia and myopathy is low in the aforementioned studies, others have found a 22% prevalence of musculoskeletal discomforts amongst individual taking statins.<sup>43</sup> In the Prediction of Muscular Risk in Observational Conditions (PRIMO) study, approximately 8,000

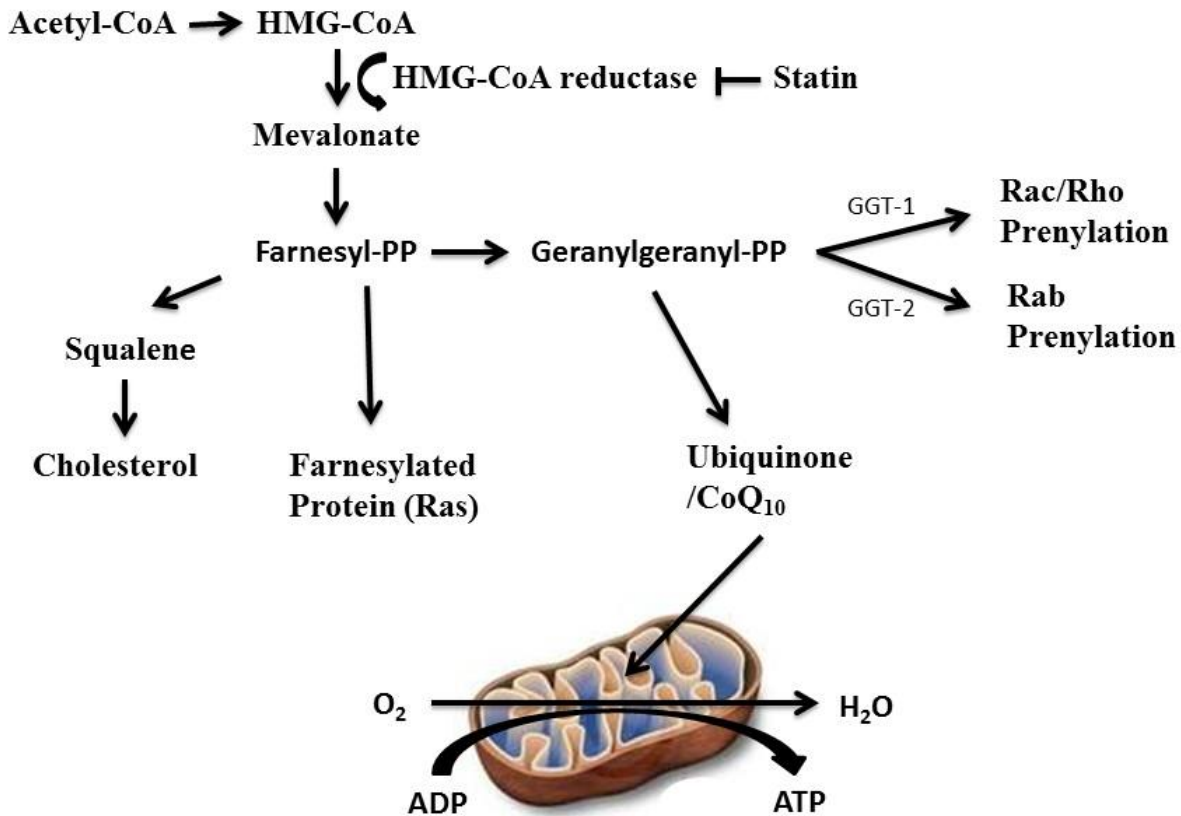
hyperlipidemic patients were treated with high dose statins for 12 months and muscle symptoms were reported by 11% of patients.<sup>4</sup>

**2.4 Lipophilic Statins.** Of the two types of statins available, researchers have shown that lipophilic statins (Cerivastatin, Simvastatin, Fluvastatin, and Atorvastatin) are more toxic than hydrophilic statins (Rosuvastatin and Pravastatin).<sup>13, 44</sup> For example, lovastatin (lipophilic), can induce the expression of atrogen-1, a gene which is responsible in promoting muscle damage.<sup>13</sup> In addition, Kaufmann et al., have shown that lipophilic statins (cerivastatin, atorvastatin, and simvastatin) decrease mitochondrial function (electron transport chain, coupling of oxidation and phosphorylation and/or mitochondrial  $\beta$ -oxidation) in skeletal muscle, while the hydrophilic statins (pravastatin) are significantly less toxic.<sup>44</sup> Both *in vitro*<sup>45</sup> and *in vivo*<sup>46</sup> studies have also produced similar results, in that lipophilic statins caused greater skeletal muscle damage than their hydrophilic counterpart. Cerivastatin, which was the most lipophilic of the statin medications, produced serious and adverse effects due to its ability to inhibit vascular smooth muscle proliferation resulting high incidences of myopathy. As a result, the drug was removed from the market.<sup>47</sup>

The mechanisms responsible for the relationship between statin lipophilicity and myopathy are uncertain. However, lipophilic statins permeate plasma membranes much more easily thereby producing greater pleiotropic effects.<sup>48</sup>

**2.5 Possible Mechanisms of Statin-Induced Myopathy.** The biological basis for statin-induced myopathy is not clear, but potential mechanisms include 1) decreased isoprenoid content and subsequent reduction of prenylated small GTP-binding regulatory proteins involved in cell function<sup>42, 49</sup>, and 2) decreased ubiquinone or coenzyme Q10, an important electron carrier necessary for ATP synthesis in mitochondria (Figure 2). Other mechanisms include: depressed fat metabolism (fat oxidation in the blood and respiratory exchange ratio was reduced)<sup>50, 51</sup>, increased skeletal muscle uptake of cholesterol by increased LDL receptor expression<sup>52</sup>, failure to catabolize damaged muscle proteins, and alterations in intracellular calcium (increase in cytoplasmic  $\text{Ca}^{2+}$ , SR- $\text{Ca}^{2+}$  overload, and  $\text{Ca}^{2+}$  waves)<sup>9, 53</sup>.

**2.6 Isoprenoids and small G protein prenylation.** In order to synthesize cholesterol in the body, HMG-CoA must be reduced to mevalonate through the action of HMG-CoA reductase (Figure 2). A main target of statins is the inhibition of mevalonate synthesis. It has been suggested that a decline or inhibition of other products in this pathway may be the cause of statin-induced muscle myopathy.<sup>54-56</sup> Mevalonate is converted to isopentenyl pyrophosphate, which is then condensed to form farnesyl pyrophosphate (F-PP).<sup>57</sup> Two molecules of F-PP are further condensed to form squalene, the precursor of cholesterol. In addition, F-PP is also the precursor for geranylgeranylpyrophosphate (GG-PP). Prenylation by GGT 1/2 is crucial for the activation of small GTPases (Rab, Ras, Rac, Raf, and Rho families).<sup>58</sup> (Figure 2)



**Figure 2. Effect of HMG-CoA reductase inhibitors on the mevalonate pathway.** The mevalonate pathway consists of biochemical reactions converting HMG-CoA to farnesyl-PP, which is converted to cholesterol, farnesylated protein (Ras), ubiquinone, etc. Statins inhibit HMG-CoA reductase, which is involved in the rate limiting step of HMG-CoA to mevalonate. This results in not only lower levels of cholesterol, but decreased Ras, Rac/Rho, Rab, and ubiquinone/CoQ<sub>10</sub> as well.

To better understand the mechanisms for statin-induced myopathy, Wagner et al<sup>59</sup> cultured C2C12 myotubes with statins in the presence of several intermediates in the cholesterol metabolic pathway—including cholesterol, CoQ, FPP, and GGPP—as well as pathway inhibitors to determine which of these intermediates may be contributing to statin-induced myopathy *in vivo*. Statin administration caused a drop in intracellular ATP, which was not reversed by the co-administration of cholesterol, CoQ<sub>10</sub>, or FPP to the medium. However, the addition of GGPP completely rescued the drop in ATP levels.<sup>60</sup>

Wagner et al further investigated using GGTI-2133 (an inhibitor of GGT-1, which is responsible for prenylation of Rac and Rho family proteins) and BMS3 (inhibitor of GGT-2, which is responsible for prenylation of Rab family proteins) to determine more specifically the pathways/compounds causing the drop in ATP levels with statin administration. Their findings indicate that the inhibition of GGT-2 by BMS3—the pathway leading to Rab prenylation—resulted in lower myotube ATP levels, even when GGPP was supplied in the media. ATP reductions in the presence of statins, GGPP, and BMS3 were similar to those seen with statins alone, which was also similar to BMS3 alone. These data indicate that GTPase prenylation—most specifically Rab prenylation—is involved in maintaining cellular ATP levels, and may be a pathway mediating statin-induced myopathy. Significant depletion of ATP may have detrimental effects on many critical cellular systems, including reducing the activity of plasma membrane ATP-dependent sodium pump, reduction of the activity of  $\text{Ca}^{2+}$  pumps leading to an influx of  $\text{Ca}^{2+}$ , and detachment of ribosomes from the rough ER, with a consequent reduction in protein synthesis.<sup>61-63</sup> Ultimately, these events may lead to fatigue, exercise intolerance, pain, and loss of muscle mass.

These mechanistic investigations into prenylation inhibition support the theory that inhibition of GTPase prenylation by statins is a potential contributor to muscle myopathy, possibly leading to reductions in ATP levels.<sup>59</sup> Collectively, these studies suggest that downstream factors in the mevalonate pathway may be responsible for this side effect of statin treatment.



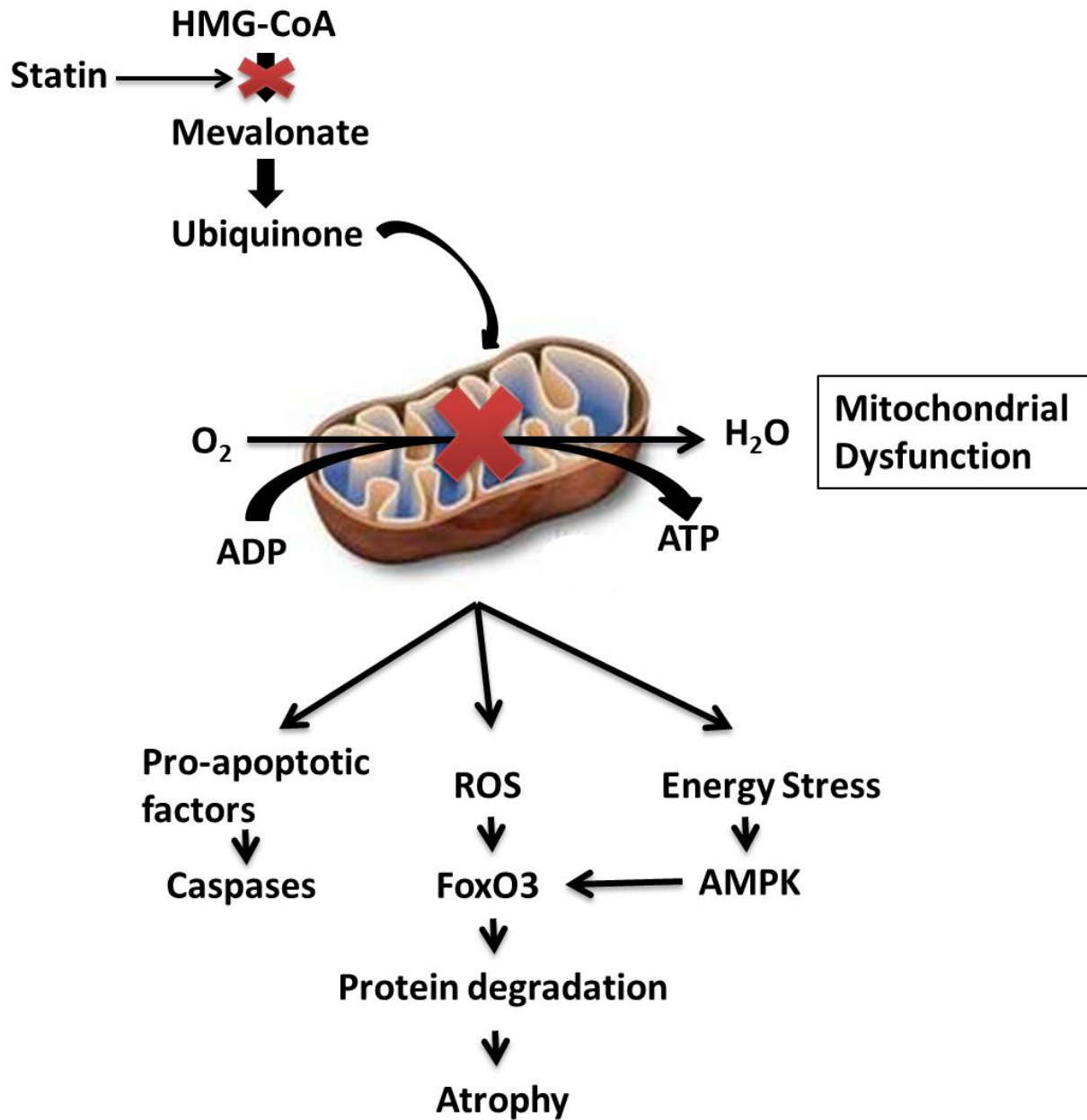
**2.7 Mitochondrial dysfunction.** Studies have proposed that statin-related myopathy may be associated with mitochondrial dysfunction and damage.<sup>64</sup> Statins block the formation of Co-enzyme Q10 (CoQ10), a protein that plays an important role in cellular respiration as it is one of the electron carriers in the electron transport chain.<sup>1</sup> CoQ10 is located within the inner mitochondrial membrane and acts as an electron carrier between complex 1 and 3 of the electron transport chain<sup>11</sup>. Reductions in this protein affect oxidative phosphorylation and mitochondrial adenosine triphosphate (ATP) production (Figure 3). CoQ10 supplementation in statin treated individuals has resulted in lower incidences of myopathy.<sup>65</sup> Animal studies have reported similar findings.<sup>66</sup> Muraki et al. demonstrated that statin treatment in rats affected running distance and induced mitochondrial dysfunction by reducing ubiquinone content. Supplementation of 10 mg/l of CoQ10 in water eliminated the reductions in both ubiquinone content and running distance.

Simvastatin (80 mg/day) for 8 weeks can decrease mitochondrial citrate synthase enzyme and respiratory chain activities, as well as decrease mitochondrial number and volume, in human skeletal muscle.<sup>67, 68</sup> Moreover, simvastatin has been reported to negatively affect mitochondrial function by disturbing membrane depolarization.<sup>9</sup> It has been suggested that lipophilic statins may affect the electron transport chain and decrease mitochondrial membrane potential, which can be regarded as a marker of mitochondrial function and integrity.<sup>44</sup> In patients receiving statin treatments, mitochondrial respiration (in skeletal muscle) is adversely affected through the alteration of the frequency and amplitude of calcium spikes.<sup>10</sup> In support of this, acute application of statins to rat skeletal muscle in

vivo and vitro cause large releases of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum and mitochondria. This can result in a malfunction in  $\text{Ca}^{2+}$ -induced opening of the permeability transition pore (PTP) and a loss of mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ).<sup>9, 69</sup> Human subjects administered simvastatin experienced greater mitochondrial dysfunction as reflected by lower ADP-stimulated oxygen consumption, and increases in mitochondrial superoxide/hydrogen peroxide generation, both signs of mitochondrial oxidative stress in primary human skeletal myotubes.<sup>70</sup>

Mitochondria are crucial for ATP synthesis, maintenance of cellular energy homeostasis. Any disturbance in mitochondrial function can result in the generation and release of reactive oxygen species (ROS) which are destructive to cellular proteins.<sup>71</sup> Thus, oxidative stress may play an important role in the cytotoxic activity of statins.<sup>72</sup>

**2.8 Muscle Atrophy.** A primary mechanism leading to the accumulation of muscle mass is the release of IGF-1, which occurs through continuous muscle contraction. Increased IGF-1 can increase mTOR activity through the PI3/AKT pathway, which promotes protein synthesis.<sup>73</sup> Increased AKT activation can phosphorylate and inhibit nuclear translocation of the Forkhead box gene family (FOXO)-1, -3, and -4, a family of transcription factors that can regulate gene expression of muscle specific ubiquitin (E3) ligases (muscle atrophy F-box, or MAFbx and muscle RING finger-1, or MuRF-1), or atrogenes important for myofibrillar protein degradation.<sup>74</sup>

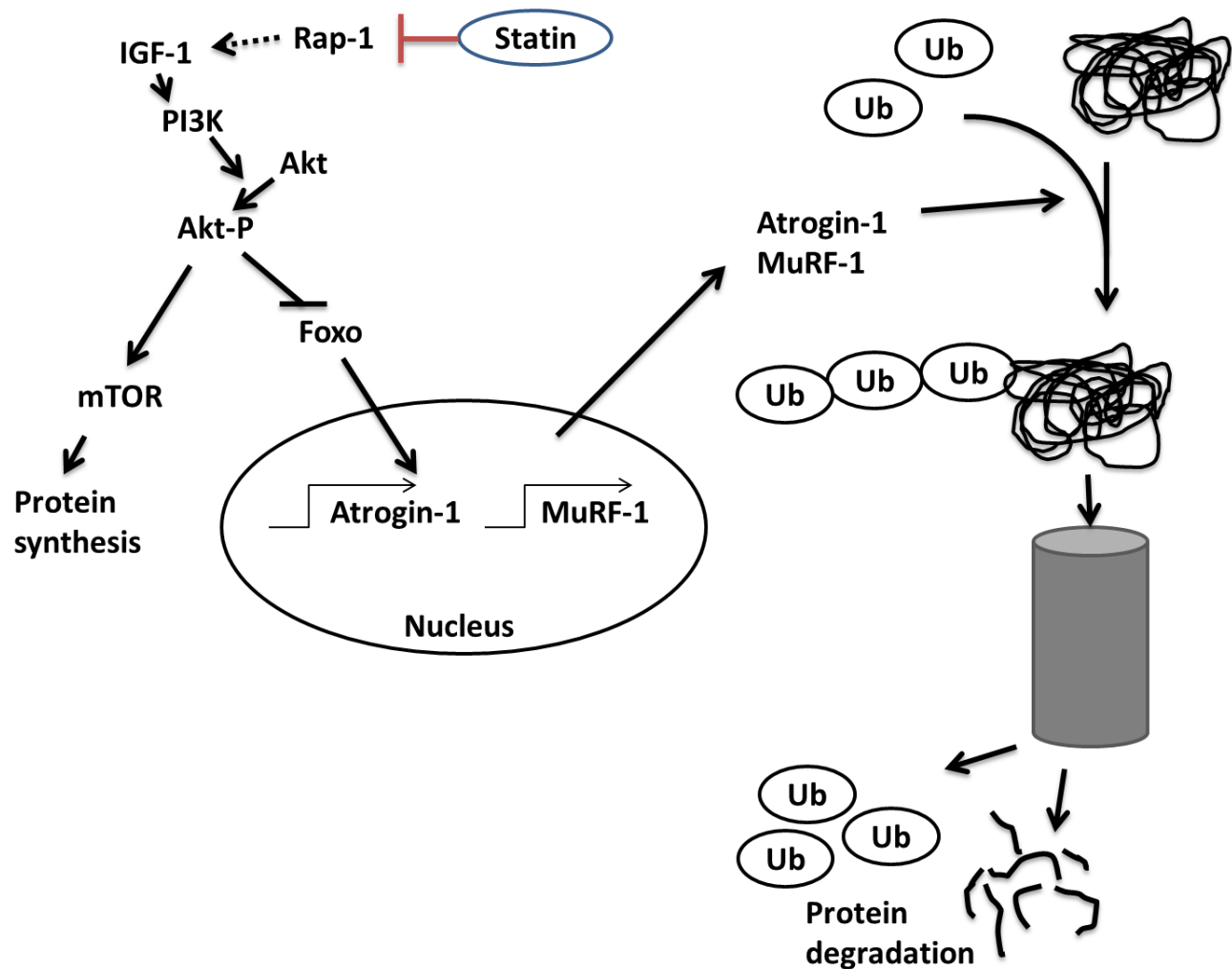


**Figure 3. Effect of HMG-CoA reductase inhibitors on pathways associated with mitochondrial function.** Inhibition of HMG-CoA reductase by statins leads to decreased ubiquinone or coenzyme Q10 (CoQ10) levels in the mitochondria. CoQ10 functions as an important antioxidant and electron carrier between complex I and complex III of the electron transport chain. Depletion of CoQ10 by statins leads to increased protein degradation by generation of mitochondrial reactive oxygen species (ROS).

In contrast, inhibition of AKT activation results in an increase in FOXO translocation, which amplifies atrogene levels and activates the ubiquitin proteasome pathway (UPP).<sup>75</sup> Studies suggest important involvement of this pathway in atrophy, as well as inflammation and apoptosis within the cell.<sup>14, 76, 77</sup>

Increased gene expression of UPP proteins have been observed in individuals that are treated with statins.<sup>14</sup> Evidence of this is also observed in zebrafish, whereby statin administration resulted in an increase in atrogin-1.<sup>13</sup> Studies have also indicated that statin-induced atrophy results from FOXO dephosphorylation.<sup>12</sup> Furthermore, it has been shown that ROS can induce activation of FOXO.<sup>78</sup> Taken together, it is possible that oxidative stress associated with mitochondrial dysfunction may play an important role of statin-induced myopathy through an ROS-FoxO-UPP mechanism. (Figure 3)

**2.9 Risk factors for statin-induced muscle myopathy.** The American College of Cardiology, American Heart Association, National Heart, lung and Blood institute suggest that statin-induced myopathy can be exacerbated when statins are taken by individuals with the following conditions: hyperthyroidism, alcoholism, previous bouts or a family history of myopathy, liver and kidney disease, and large fluctuations of CK levels.<sup>90</sup> Other situations that may worsen myopathy are advanced age (especially >80 years of age), the female gender, cramps, perioperative periods, the ingestion of grapefruit juice, multisystem disease (especially diabetes mellitus), elevated CK levels, small body size, and excessive physical activity.<sup>4, 17, 42, 79, 80</sup>



**Figure 4. Effect of HMG-CoA reductase inhibitors on skeletal muscle protein turnover.** IGF-1 pathway is involved in muscle atrophy and hypertrophy. IGF-1 signaling activates the PI3K/Akt pathway. Akt phosphorylates mTOR, which promotes protein synthesis. Akt also phosphorylates FOXO transcription factors, promoting their cytoplasmic retention and functional inactivation. FOXO proteins stimulate the transcription of the atrophy promoting factor such as atrogin-1 and MuRF-1, which can induce muscle atrophy. Statins inhibit Rap-1 which may inhibit IGF-1 signaling and induce muscle atrophy.

As mentioned, statins taken in combination with certain medications is not recommended. For example, some statins should not be taken in conjunction with protease inhibitors as statins are metabolized through P4503A4 (CYP3A4).<sup>81</sup> Protease inhibitors block CYP3A4, thus allowing statins

to accumulate.<sup>82</sup> Simvastatin taken with Amiodarone, an antiarrhythmic agent used for various types of cardiac dysrhythmias, can increase statin levels in the blood. This condition has been shown to increase the risk of myopathy by 10 fold.<sup>83, 84</sup>

**2.10 Statin-induced muscle myopathy with physical activity levels.** Another significant risk factor for statin-induced myopathy is excessive exercise. Studies have suggested that both previously trained athletes and individuals beginning an exercise program after being prescribed statin medication have a higher-risk of incurring myopathy.<sup>1, 16, 17</sup> For example, Thompson et al. measured changed in creatine kinase (CK) levels after an acute bout of exercise performed before and after four weeks of statin therapy. Following the statin therapy, the bout of exercise produced an 11% increase in CK as compared to the bout before the treatment<sup>85</sup> Though muscular problems affect only 10 % of the general population using statins, studies suggest that this phenomenon may be significantly increased in professional athletes. Utilizing 22 professional athletes being treated with statins (due to familial hypercholesterolaemia), Sinzinger and O'Grady administered several different types of statins in order to examine their possible effects. Out of the 22 athletes, only 6 were able to tolerate one form of the drug. This result suggests only 20% of athletes are able to tolerate treatment without side-effects.<sup>17</sup> Regrettably, this study did not utilize a control group. However, this work is still important as it is one of the few studies to investigate how exercise training affects statin-induced myopathy. This is also

significant as physicians often recommend exercise and physical activity in conjunction with statin medication.

Until recently, the interactions between exercise and statin therapy had not been directly investigated. In order to investigate the effect of exercise on statin treatment, Thompson et al. administered 20 mg/dl of statin to subjects prior to a maximum treadmill test. The vast majority (12 out of 14 subjects) incurred no significant changes in CK levels post-exercise. However, two (out of 14 total) of the participants had CK levels increase 183% and 242% respectively.<sup>86</sup> In a subsequent study, the same group randomized subjects into 2 groups: statin (40 mg lovastatin) and placebo. At 5 weeks, subjects completed a single exercise session consisting of 45 minutes of downhill treadmill walking and one set of arm curls (10 reps of 50% max). Results from blood samples revealed significantly higher levels of CK in statin users when compared to the placebo group.<sup>21</sup> This suggests that injury associated with a single bout of eccentric, or lengthening, contractions may increase susceptibility to statin-induced myopathy.

Whereas most studies have focused on understanding the acute response to exercise with statin treatment, very few have evaluated the prevalence of myopathy in response to exercise training. In order to investigate the effects of long term exercise on statin-induced muscle myopathy, Coen et al conducted a study using 3 groups: statin (rosuvastatin), statin + exercise training (Ex), and control. Statin was administered for 20 weeks, and exercise training was conducted 10 weeks after the drug was first administered. Results indicate that both statin and Ex + statin lowered the blood lipid profile, and

a significantly larger decrease was observed in the Ex + statin group. Interestingly, CK levels in blood and muscle myalgia were the same in both statin groups at the end of the study.<sup>87</sup> Further investigating the effect of statin treatment on humans accustomed to exercise, Parker et al. recruited 80 subjects competing in the Boston marathon (37 statin, 43 non-statin) and assessed CK levels. Blood was obtained immediately before the start of the race, within 1 hr of crossing the finish line, and 24 hrs after finishing the race. Results indicate that the exercise-induced increase in CK 24 hrs after the marathon was greater in statin users than controls. In addition, increases in CK levels in the statin group were correlated with age, whereas this association was absent in the non-statin group.<sup>88</sup> Overall, these studies suggest that the effect of exercise training on statin-induced myopathy is unclear.

Individuals who are typically instructed to exercise in conjunction with statin therapy typically fall into two categories: 1) Those that have not exercised for an extended period of time (novel exercise), and 2) Those that have a history of exercise and are consistently training (accustomed exercise). It is unknown whether statins affect these two groups differently. To address this important question, Meador et al randomized mice into 2 groups: statin (cerivastatin) and saline. These mice were further randomized into 3 subgroups each: sedentary, novel exercise, and accustomed exercise groups. The sedentary group remained in their cage for 2 weeks, after which they were injected with statin or saline daily for an additional 2 weeks. In the novel exercise group, mice also remained in their cage for 2 weeks, but then received either saline or statin injections concurrent with free access to a running wheel for the remaining 2 weeks. Finally, in the accustomed group, mice were provided access to a running



wheel during the first two weeks prior to initiation of saline or statin injections concurrent with continuation of exercise. At the end of the study, maximal isometric force of hind limb muscles was measured. Results indicate that statin administration alone resulted in lower force and higher fatigability than saline controls. Similarly, mice in the novel exercise group were weaker than saline treated controls. Interestingly, no differences in strength were observed in the accustomed exercise group compare to saline treated group. These data suggests that starting a training program prior to taking statins may preserve muscle function.<sup>18</sup>

Muscle damage induced by statins is also well established.<sup>16</sup> As observed in the aforementioned studies and others, CK levels are a widely utilized measure for muscle damage. However, it should be noted that the lack of correlation between muscle soreness ratings and CK levels in several studies. CK levels observed in response to exercise are variable and are not well correlated with measures of muscle damage.<sup>30, 31</sup> Furthermore, many of studies have shown that statin-induced myopathy can occur without elevations in blood CK levels.<sup>2, 32, 46, 89, 90</sup> In a case study utilizing seven subjects, Sinzinger et al. prescribed statins to patients whose exercise background ranged from elite athletes to recreational exercisers. After or during exercise, these patients complained of muscular aches, pain, and fatigue. After speculating that statins caused these symptoms, each drug was substituted for another form of statin (lovastatin, simvastatin, fluvastatin, and pravastatin). This resulted in some symptoms ceasing; however, other symptoms did not change. In some cases, the symptoms vanished only to recur later. Interestingly, CK levels in these patients did not differ from the norm.<sup>90</sup> In other study, Reust and

colleagues randomized 10 healthy subjects into 2 groups: statin (Lovastatin) and placebo. Both groups were administered their intervention for 1 month and all subjects participated in a single bout of eccentric exercise. This exercise intervention consisted of walking on a treadmill at 3 km/h on a -14 degree decline. No changes in CK levels in either the placebo or Lovastatin group were observed after exercise.<sup>91</sup> In addition, normal levels of CK have been found in patients with muscle damage due to statin.<sup>32</sup> Therefore, CK levels, as a measure of muscle damage, may not be as reliable as previously thought.

## **2.11 Potential mechanisms of the interaction between statin-induced myopathy and acute exercise.**

The biological basis for increased susceptibility to statin-induced myopathy with an acute bout of eccentric exercise is not known. However, mitochondrial dysfunction and oxidative stress-mediated activation of the UPP may contribute to this event. In a study conducted by Urso et al., human subjects were randomized into two groups, statin treatment or placebo. After 4 weeks of continuous treatment, subjects completed a single bout of eccentric exercise. Eight hours after performing the task, skeletal muscle biopsies were collected and mRNA expression was evaluated. Results from this study indicated that statins altered the genes associated with ubiquitin proteasome pathway (an indicator of protein catabolism), inflammation and apoptosis.<sup>14</sup> In a separate study, Boutibir and colleagues, randomized rats into 4 groups: Control, Statin, Control + Ex, and Statin + Ex. Control animals were received saline and statin groups were administered atorvastatin by oral gavage via a cannula. The

animals were subjected to a single bout of treadmill running, performed until failure. Data showed that the Statin + Ex group ran the shortest distance. Interestingly,  $V_{\max}$ , a measurement of mitochondrial respiration, was significantly lower in Statin and Statin + Ex compared to the other two groups and ROS was increased to the greatest extent in the Statin + Ex group compared to all other groups. Additionally, a correlation between distance to exhaustion and mitochondrial respiration was observed.<sup>92</sup> In a similar study performed in humans, Kwak et al. reported that simvastatin increased oxidative stress and atrophy.<sup>70</sup> There is evidence that indicates inflammation may play a role. A very recent clinical case study indicates that statins combined with vigorous exercise can increase inflammation. The only subject was a 34 year old male receiving statin treatment. The exercise intervention consisted of a single bout of running for duration of 2 hours. This was repeated 6 months after the initial bout of exercise. Blood parameters were taken after both interventions and the results show an increase in immune cells (75% lymphocytes and 1200% eosinophils).<sup>93</sup> As important as these finding are, this study, unfortunately, did not measure any markers of muscle damage. Further studies are necessary to determine the full extent to which exercise can increase susceptibility to statin-induced myopathy, and the mechanisms responsible, particularly given the fact that lifestyle intervention is traditionally prescribed in combination with statins.

**2.12 Hypercholesterolemia and muscle myopathy.** Statins are prescribed to patients with vastly elevated cholesterol levels. Though statins have been implicated in inducing muscle myopathy,

evidence exists indicating high cholesterol level (by itself) can potentially cause muscle dysfunction.

A recent study examined some of the interactions between these different factors, using 3 groups:

familial hypercholesterolemia (FH), FH + statin, and normal cholesterol. These three groups were

subjected to a single bout of exercise and myoglobin levels measured 1 and 8 hours post-exercise.

Results indicated no difference in muscle damage between the FH and FH + statin groups. However,

both of these groups did have significantly higher levels of myoglobin than the normal cholesterol

group. This is in stark contrast to other controlled studies performed on subjects with normal

cholesterol levels at the time of the exercise intervention. These studies tend to indicate that high

cholesterol levels do play a role in muscle myopathy.<sup>19, 20</sup>

Mice with genetic alteration of apolipoprotein E (ApoE<sup>-/-</sup>) are often used as a model for

hypercholesterolemia.<sup>94</sup> Maxwell et al. investigated whether hypercholesterolemia impairs exercise

ability in these mice. Using both ApoE<sup>-/-</sup> and wild-type mice, this group assessed whether high

cholesterol levels had an effect on both aerobic and anaerobic capacity. Both ApoE<sup>-/-</sup> and wild-type

mice were randomized into two groups and fed different diets: chow and high-fat. Their results

demonstrated that exercise capacity progressively decreased in all hypercholesterolemic mice at 12-20

weeks of age, while anaerobic capacity was unaffected. Interestingly, the degree of aerobic

impairment was related to serum cholesterol levels.<sup>95</sup> In order to detect differences in muscle

morphology and healing between hyper- and normocholesterolemic subjects, Kang et al. compared this

model (ApoE<sup>-/-</sup>) with wild-type mice. Injuries to skeletal muscle were caused by 1.5 hours of

unilateral hind limb ischemia, followed by reperfusion. Results from this study also indicated that the rate of muscle repair was delayed in ApoE<sup>-/-</sup> mice during the chronic phase of reperfusion.<sup>96</sup> Similar studies suggest that statins can reduce effectiveness of repair in muscle following injury in humans.<sup>97</sup> Thus, delayed healing may provide the basis for myopathy often witnessed in hypercholesteremic subjects.

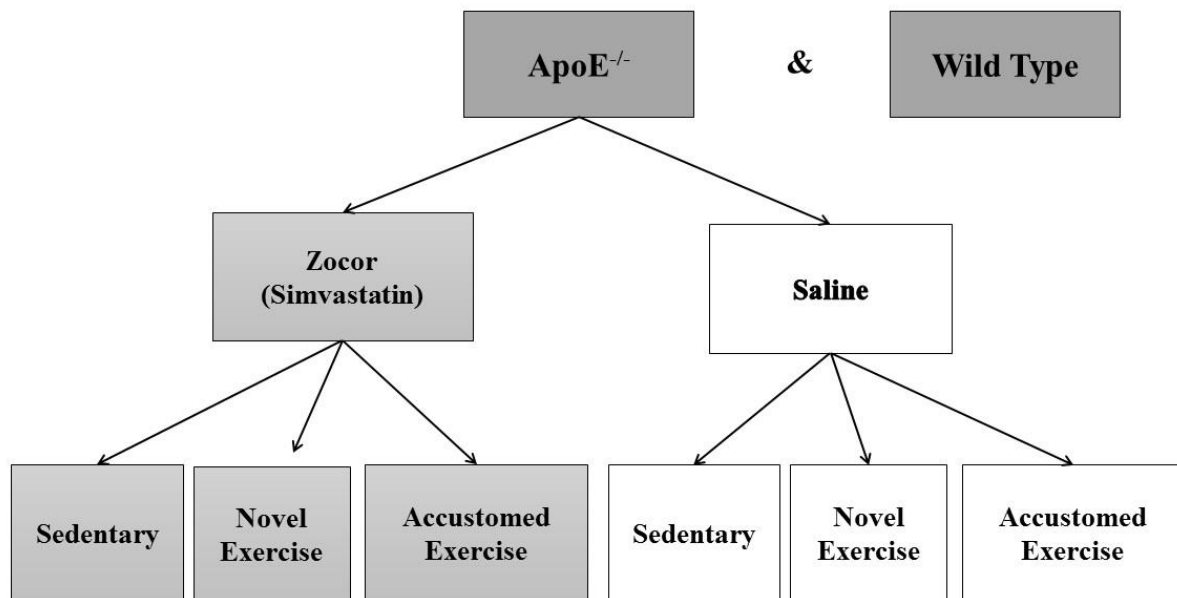
**2.13 Significance of proposed research.** The current literature addressing statins and myopathy is muddled by the lack of distinction between the effects of statins in normo- vs. hypercholesterolemic subjects. The proposed research will better define the poorly understood interactions between statin treatment, hypercholesterolemia, and exercise. Specifically, the results will indicate whether exercise training prior to the initiation of statin treatment might serve to counteract statins' myopathic effects in a hypercholesterolemic population as preliminary data indicates it can with normocholesterolemia. As a result, findings from this study may help guide future research to investigate how exercise impacts statin-induced myopathy, as well as attempt to provide insight on the biological basis for these observations.

## CHAPTER 3

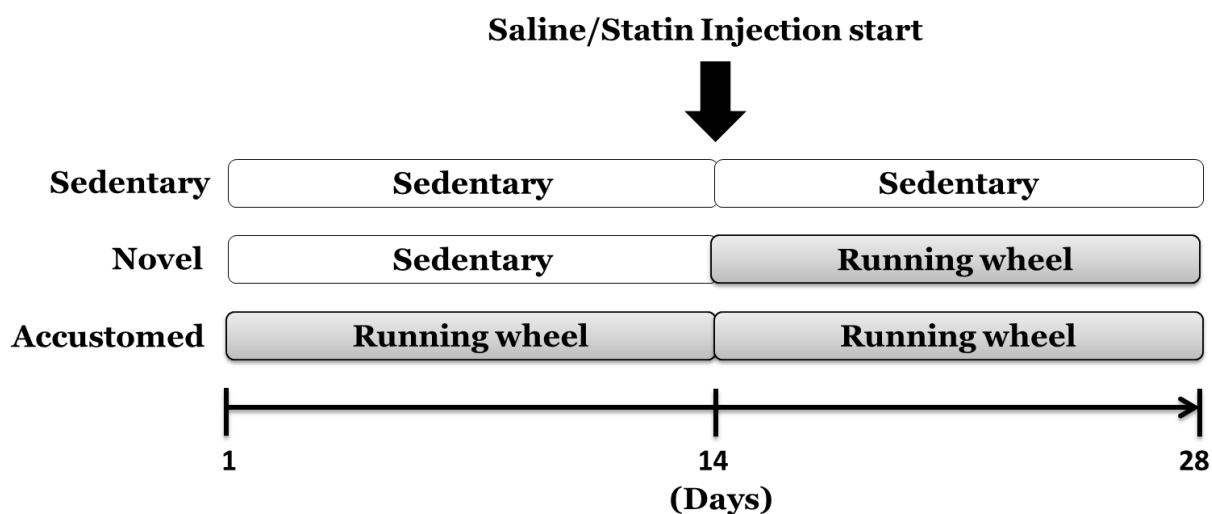
### RESEARCH DESIGN AND METHODS

**3.1 Study Overview:** To test the effects of simvastatin on muscle myopathy, 60 eight-week old male ApoE knockout mice (ApoE<sup>-/-</sup>) (Jackson Labs) were randomized to six groups. In addition, identical experiments were conducted in 60 eight-week old male C57BL/6J mice (Jackson Labs) for comparison purposes (Figure 5). Mice were first assigned to one of three groups: no exercise, voluntary novel exercise (initiation of exercise at 2 weeks, concomitant to initiation of statin or placebo), or voluntary accustomed exercise (exercise starting 2 weeks prior to statin or placebo administration). Exercise was administered through the use of a running wheel. At two weeks, mice were either injected with simvastatin (20 mg/kg/day) or an equivalent volume saline (Figure 6).

**3.2 Running Wheel Exercise:** Mice were monitored over a period of 28 days, housed in separate cages, fed a standard chow diet, provided water ad libitum, and kept on a 12/12 hour light/dark cycle. Mice assigned to the accustomed exercise group were provided access to an 11.5 cm running wheel (Mini-Mitter) in their cage on day one, while novel exercise mice were not given a running wheel until day 15. Activity levels were recorded until the 28<sup>th</sup> day. Distance was monitored using magnetic reed switches (Respironics, Mini Mitter) and a bicycle computer (Sigma). Activity was recorded every 24 hours at the time medication was administered.



**Figure 5. Study design.** Treatment groups based on combination of genotype, activity levels and statin usage.



**Figure 6. Study timeline.** Study timeline over 1-14 and 15-28 days.

**3.3 Statin Injections:** Starting on day 15, each mouse received an intraperitoneal injection of saline or statin every 24 hours. The majority of mice run during the dark cycle, so injections were given towards the start of the light cycle to minimize any direct effects of the injections.<sup>18</sup> Simvastatin/zocor (4893,

Medical Isotopes INC.) was given at a dose of 20 mg/kg/day from a solution of 0.25 mg/ml in sterile saline with a sterile insulin needle.<sup>98</sup> The metabolic rate of mice is 12.3 times higher than in humans, which means 20 mg/kg in a mouse is equivalent to 1.63 mg/kg in humans.<sup>136</sup> 1.63 mg/kg is slightly higher than the normal human dosage of 0.5 ~ 1 mg/kg/day depending on their body weight. This dose was also chosen due to pilot research conducted by this group, which demonstrated that 1 mg/kg and 10 mg/kg doses of simvastatin were too small to show any considerable change in only 2 weeks. Simvastatin and saline were administered to the mice via intraperitoneal injections starting on day 15, continuing until day 28. These injections were given every 24 hours at the beginning of the light cycle to minimize the impact on wheel running.

**3.4 Grip Strength Test:** Three days prior to the start of the running wheel exercise for the accustomed group (day -2) all mice had their all-limb strength tested using a force gauge (Columbus Instruments).<sup>99</sup> The mice were held up to the horizontal grip and then were pulled steadily backwards until they could not hold on any longer. This was repeated 3 times per mouse for each grip, and the highest force measurement was recorded. This procedure was repeated 2 days prior to the start of the exercise (day -1), and the highest number from both days was recorded as their maximum strength. This test was administered again on days 13, day 20 and day 27.



**3.5 Isometric Force Measurement:** Hindlimb plantarflexor isometric force testing was conducted on the 29<sup>th</sup> day of the study, approximately 24 hours following final injection. Mice were anesthetized with ketamine and xylazine, then the left sciatic nerve was dissected through the thigh. Electrodes were attached to the nerve and stimulated at 250 Hz for 1.5s to provoke a maximum force contraction. Strength was measured using a plate attached to a servomotor (305C-LR; Aurora Scientific). The limb was stimulated 10 times with a time interval of 5s between each shock, to test fatigability. As a measure of fatigue, the tenth contraction was compared with the maximum contraction and expressed as a percentage of maximal force.

**3.6 Blood Draws and Tissue Collection:** One day prior to the start of the running wheel exercise for the accustomed group (day 0) all the mice had approximately 0.3 mL of blood drawn via their jugular vein using an animal lancet (Bioseb) stored in heparin-treated tube (Bioseb). Blood was again collected using this method on day 14. On day 29 approximately 1 mL of blood was extracted from each mouse via their inferior vena cava after euthanasia using a heparin-treated syringes and collection tubes. The samples were centrifuged at 1200g for 10 minutes at 4°C, and plasma was collected and stored at -80°C. After the isometric force testing, gastrocnemius muscles were also collected and stored in a -80°C freezer.

**3.7 Blood Chemistry:** Plasma total cholesterol was measured by standard enzymatic methods using commercially available assay kits (Infinity Incorporated, Melbourne, Australia). 4-hydroxynonenal (4-HNE), a marker of oxidative stress, was measured in duplicate using commercially available ELISA kits (Cell Biolabs OxiSelect™ HNE Adduct ELISA kit, San Diego, CA) as per the manufacturer's instructions.

**3.8 RNA and DNA extraction:** RNA was extracted from 50-100 mg of gastrocnemius muscles tissue using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and then quantified using spectrophotometry. After the separation of RNA and protein from the tissue, DNA was extracted with phenol-chloroform then precipitated with ethanol, according to manufacturer's instructions. Spectrophotometry was used to quantify the amount of RNA and DNA extracted.

**3.9 Evaluation of gene expression:** Real-time RT-PCR for the genes of interest was conducted using the following procedure: one cycle at 48°C for 30 minutes, followed by 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. This procedure was conducted using an ABI PRISM 7700 sequence detector (Applied Biosystems, Roche, Branchburg, NJ) and a Taqman 100R × n PCR Core Reagent Kit (Applied Biosystems, Roche, Branchburg, NJ). Primers (atrogin-1; Cat #:4310893E and PGC1-α; Cat #:4331182, Applied Biosystems) were recreated using Primer Express Software version 2.0 (Applied Biosystems, Roche, Branchburg, NJ). The data was normalized by

dividing the target amount by the amount of Glyceraldehyde-3- phosphate dehydrogenase (GAPDH), which served as an internal control and was used as the housekeeping gene and all data are presented relative to its expression using the  $\Delta\Delta C_t$  method.

**3.10 Real-time PCR for mitochondrial DNA:** The ratio of the amount of mitochondrial DNA to nuclear DNA provides an assessment of the amount of mitochondria per cell in a given tissue. Nuclear DNA and mtDNA was amplified and quantified using real-time PCR with an ABI PRISM 7700 sequence detector (Applied Biosystems, Roche, Branchburg, NJ). A 120-bp long region of mtDNA was amplified for quantification by PCR and cloned into the plasmid pCDNAII, following the manufacture's procedure (Invitrogen, Carlsbad, CA), then sequenced to verify the identity of the DNA. The DNA concentration was estimated by spectrophotometry and calculated to give a stock of  $2.5 \times 10^{10}$  copy/ $\mu\text{L}$ . Amplification and quantification prior to cloning was completed using the following PCR procedure: one cycle at  $50^\circ\text{C}$  for 2 minutes, followed by  $95^\circ\text{C}$  for 10 minutes, then 40 cycles of  $95^\circ\text{C}$  for 15 seconds and  $60^\circ\text{C}$  for 1 minute. The amount of DNA was quantitated using the following assay during PCR: 50  $\mu\text{L}$  containing 10  $\mu\text{L}$  DNA template, 11  $\mu\text{L}$  of 25 mmol/L  $\text{MgCl}_2$ , 0.05  $\mu\text{L}$  AMPErase UNG (uracil-N-glycosylase), 15.25  $\mu\text{L}$  DI water, 0.25  $\mu\text{L}$  AmpliTaq Gold DNA Polymerase, 1  $\mu\text{L}$  of each dNTP, and 5  $\mu\text{L}$  of  $10\times$  buffer A. The copy numbers of the unknown was determined by creating a standard curve from a plasmid of known copy number. These results were normalized by also amplifying the 120-bp region, then cloning it (as described above).

**3.11 Statistical Analysis:** All outcome measures were analyzed and reported separately for ApoE<sup>-/-</sup> and WT mice. WT group results were used to determine the effect of hypercholesterolemia in ApoE<sup>-/-</sup> group on outcomes. Continuous variables are presented as means  $\pm$  standard deviation (SD). A 2-factor complete factorial model design with 2 levels of drug treatment (statin or saline) and 3 levels of exercise (sedentary, novel, or accustomed) was used to test differences in variables related to muscle myopathy. A two-way analysis of variance (ANOVA) was conducted to examine differences in handgrip strength, isometric maximal force, muscle fatigability, cholesterol levels, mtDNA, protein levels, and gene expression at the end of experiment. Additionally, repeated measures ANOVA was used to test differences in handgrip strength, daily running wheel activity and body weight during the course of the 4 week study. Strength was normalized to body weight only when differences in body weight were detected between groups. Turkey HSD post-hoc analysis was performed to examine group differences only if a significant interaction or main effect was detected. All statistical analysis were performed using SPSS version 19.0 and statistical significance was defined as  $p < 0.05$ .

## CHAPTER 4

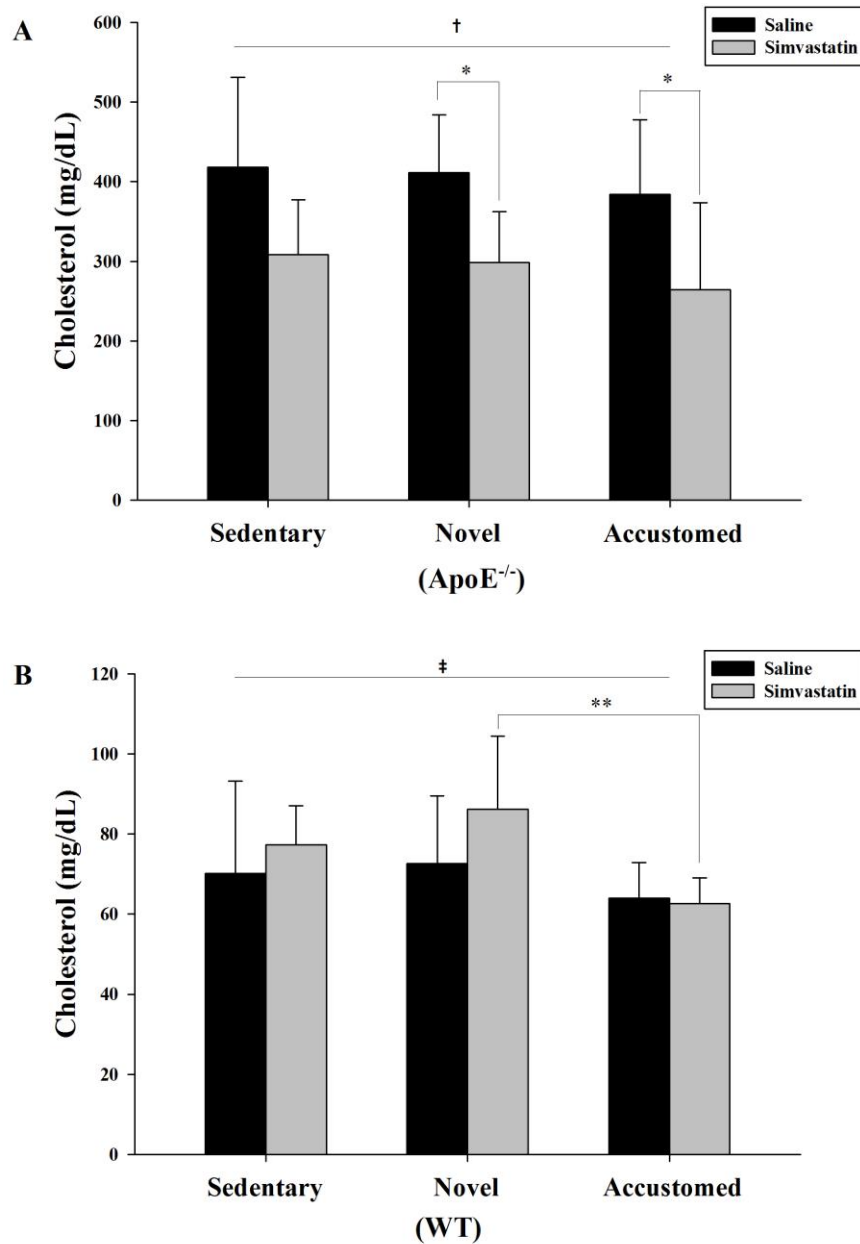
### RESULTS

**4.1 Body Weight.** No significant differences in body weight were detected between groups for either ApoE<sup>-/-</sup> or WT mice. However, final body weights were significantly increased in both ApoE<sup>-/-</sup> and WT mice compared to initial body weights (time main effect,  $F(1,47)=41.230$ ,  $p<0.001$  &  $F(1,48)=19.252$ ,  $p<0.001$  respectively) (Table 1).

<b>Table 1 : Body Weight</b>				
Wild Type			ApoE <sup>-/-</sup>	
Group	Initial BW (g)	Final BW (g)*	Initial BW (g)	Final BW (g)*
Sed/Saline	21.89 ± 1.62	23.42 ± 1.25	23.40 ± 1.58	25.17 ± 1.31
Sed/Statin	22.05 ± 1.35	22.70 ± 2.95	22.66 ± 3.10	24.58 ± 1.65
Novel/Saline	22.76 ± 2.41	23.59 ± 1.71	23.45 ± 3.62	25.29 ± 2.15
Novel/Statin	22.11 ± 1.79	23.96 ± 1.72	22.77 ± 3.15	24.97 ± 1.17
Accust/Saline	22.56 ± 1.36	23.38 ± 0.63	23.63 ± 2.47	24.99 ± 1.96
Accust/Statin	22.06 ± 1.40	23.19 ± 1.49	21.13 ± 1.26	23.77 ± 1.34

Data expressed as mean ± SD, \*  $p<0.001$  compared with initial body weight (BW), n = 8-10/group

**4.2 Cholesterol.** Statin treatment lowered cholesterol in ApoE<sup>-/-</sup> mice (drug main effect,  $F(1,43)=17.68$ ,  $p<0.001$ ) (Figure 7.A). Plasma cholesterol was reduced to the same extent in both novel and accustomed exercise groups receiving statin treatment compared to saline controls ( $p<0.001$ ). In WT mice, statin treatment had no effect, whereas exercise influenced cholesterol (exercise main effect,  $F(2,42)=4.688$ ,  $p=0.015$ ) (Figure 7.B).



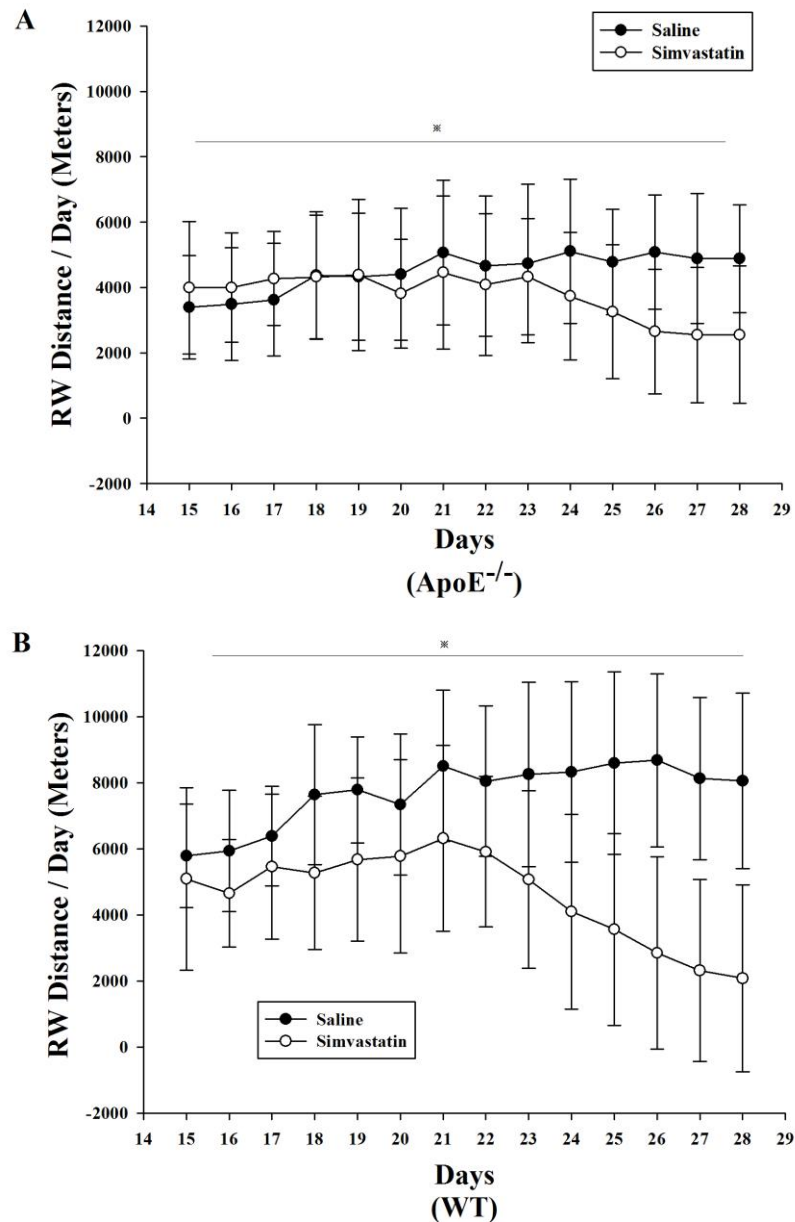
**Figure 7. Statin treatment decreases plasma cholesterol in ApoE<sup>-/-</sup> mice.** Plasma cholesterol after 4 weeks normal cage activity (Sedentary), two weeks normal cage activity and two weeks running wheel access (Novel), or 4 weeks running wheel access (Accustomed), with saline or simvastatin (20 mg/kg) during the final two weeks of treatment in ApoE<sup>-/-</sup> (A) and WT (B). Means  $\pm$  SD; n = 8-10/group. exercise  $\times$  drug interaction, p=NS; †=main effect of drug, p<0.05; ‡=main effect of exercise, p<0.05; \*=within group pairwise comparison, p<0.05; \*\*=difference between exercise p<0.05

**4.3 Running Wheel Activity.** Daily running wheel distances for 14 days (Day 15-28) in the novel and

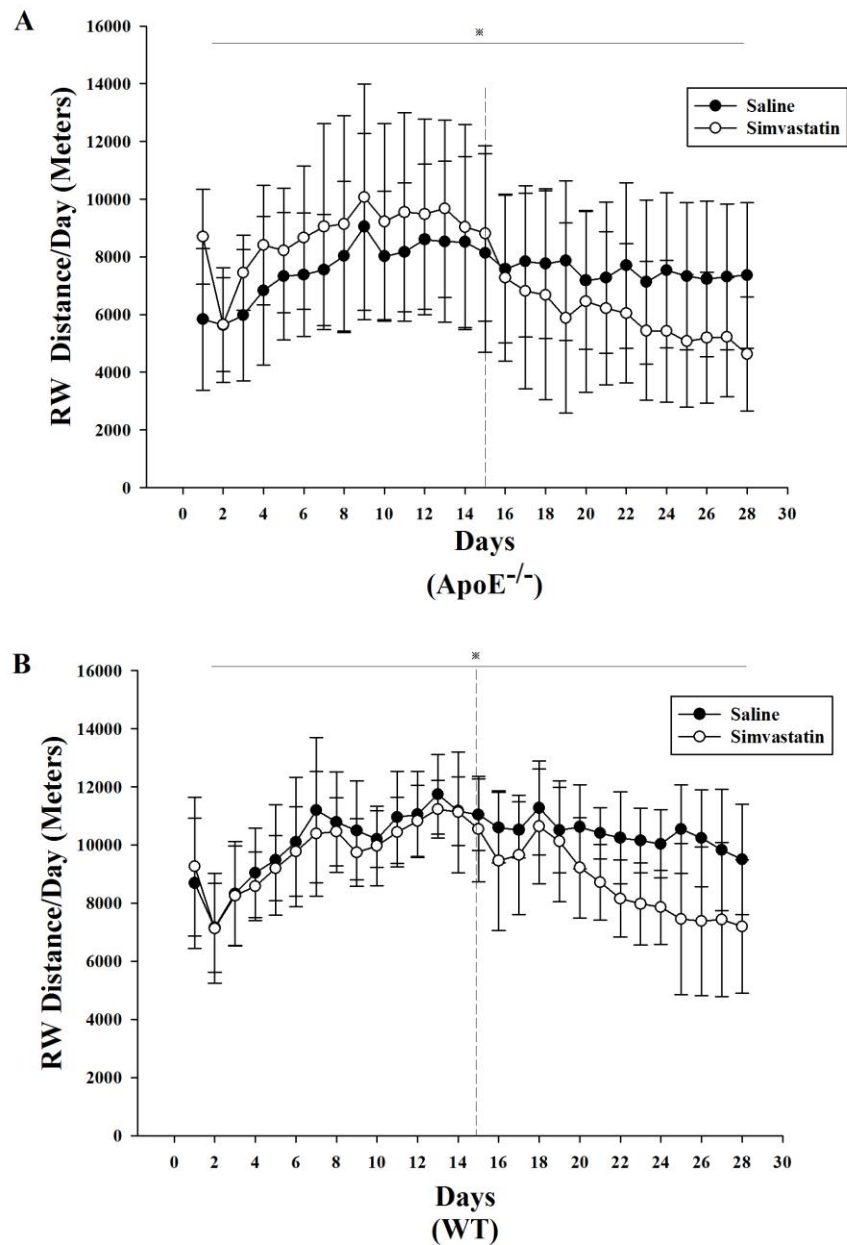
28 days in the accustomed exercise groups are shown in Figures 8 and 9, respectively. In both novel and

accustomed exercise groups, statin treatment lowered running distance over the course of the study

(drug x time interaction,  $p < 0.05$ ). This observation was true for both ApoE<sup>-/-</sup> (Figures 8.A and 9.A) and WT (Figures 8.B and 9.B) mice.



**Figure 8. Effect of statins on voluntary running wheel activity in novel exercise mice.** ApoE<sup>-/-</sup> (A) and WT (B) mice were provided access to a running wheel on the same day statin or saline treatment was initiated. Introduction of exercise concurrent with statin treatment decreases daily voluntary wheel running over the subsequent 14 days in both ApoE<sup>-/-</sup> (A) and WT (B) mice. Means  $\pm$  SD;  $n = 8-10/\text{group}$ . \* = drug x time interaction,  $p < 0.05$



**Figure 9. Effect of statins on running wheel activity in accustomed exercise mice.** ApoE<sup>-/-</sup> (A) and WT (B) mice were provided access to a running wheel on 2 weeks prior to statin or placebo administration. Statins caused a reduction in running wheel activity despite a 2 week period of training prior to statin administration in both ApoE<sup>-/-</sup> (A) and WT (B) mice. Means  $\pm$  SD; n = 8-10/group. \* = drug x time interaction, p < 0.05

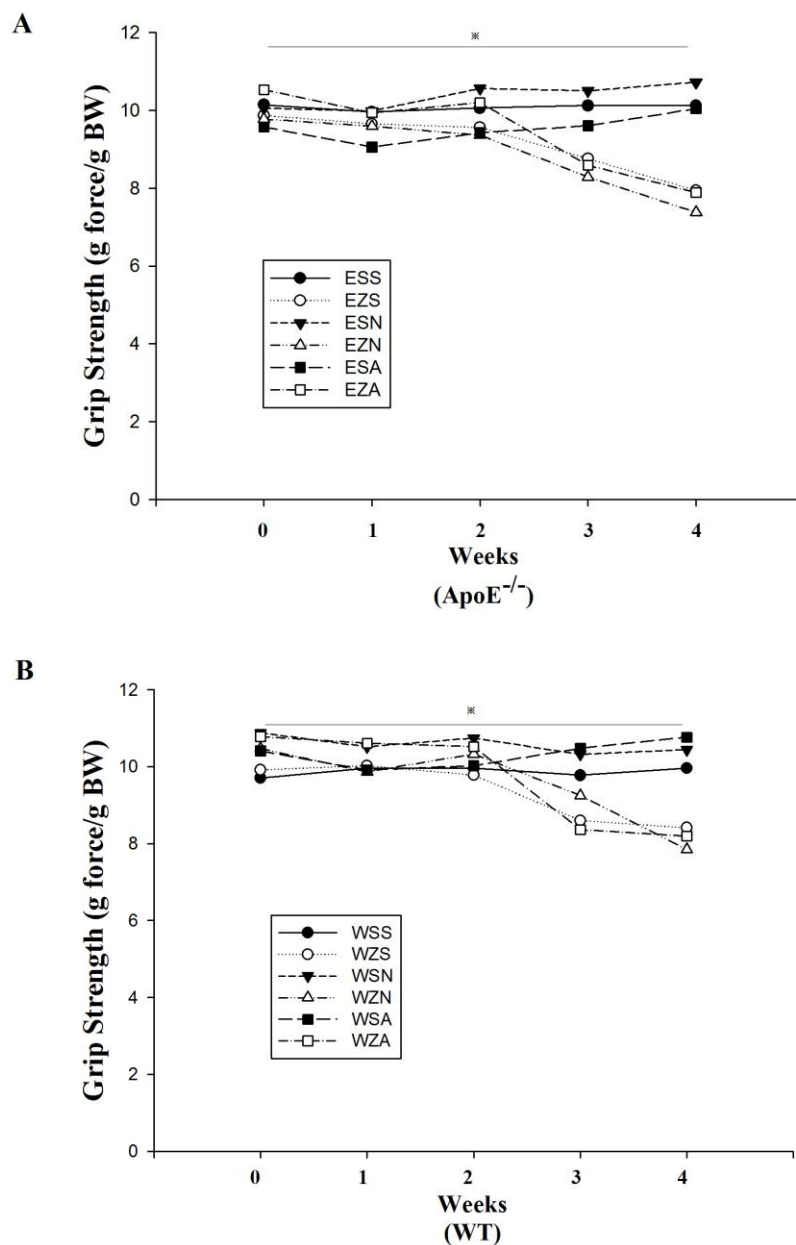
#### 4.4 Maximal Grip Strength. Statin treatment decreased maximal relative, all-limb grip strength in

ApoE<sup>-/-</sup> mice over the course of the study (drug x time interaction, p < 0.001) (Figure 10.A). Maximal

strength was significantly lower with statin treatment compared to saline at week 3 and week 4 (p < 0.001

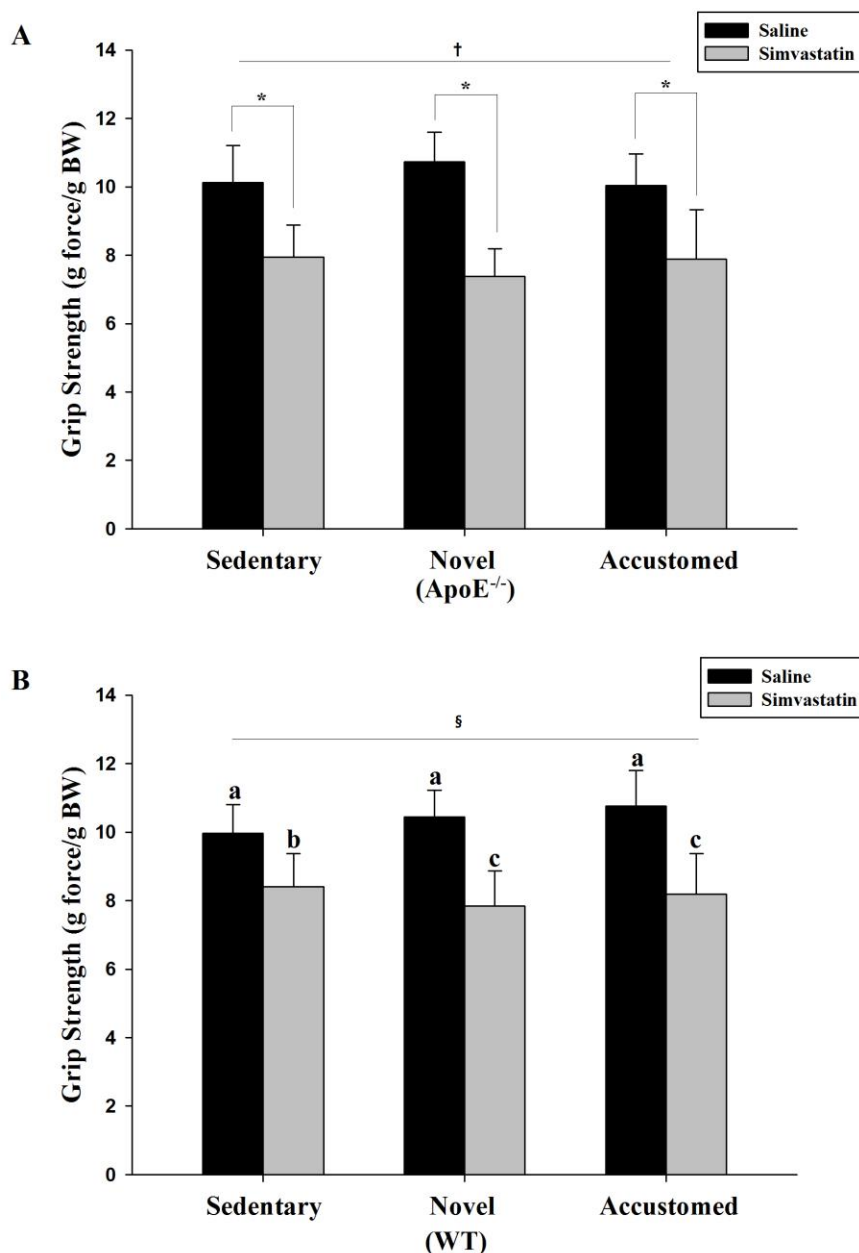


for both). In WT mice, statin treatment similarly decreased maximal strength compared to saline-treated controls over the course of the study (drug x time interaction,  $p < 0.003$ ) and significant differences were noted at week 3 and week 4 ( $p < 0.001$  for both) (Figure 10.B). No differences in strength were detected between statin-treated novel and accustomed exercise groups for ApoE<sup>-/-</sup> or WT mice.



**Figure 10. Statin treatment decreases maximal grip strength at week 3 and week 4 in both ApoE<sup>-/-</sup> (A) and WT (B) mice.** W: Wild Type, E: ApoE<sup>-/-</sup>, Z: Zocor (simvastatin), S: Saline, S: Sedentary, N: Novel, A: Accustomed. Means; n = 8-10/group. \* = drug x time interaction,  $p < 0.05$

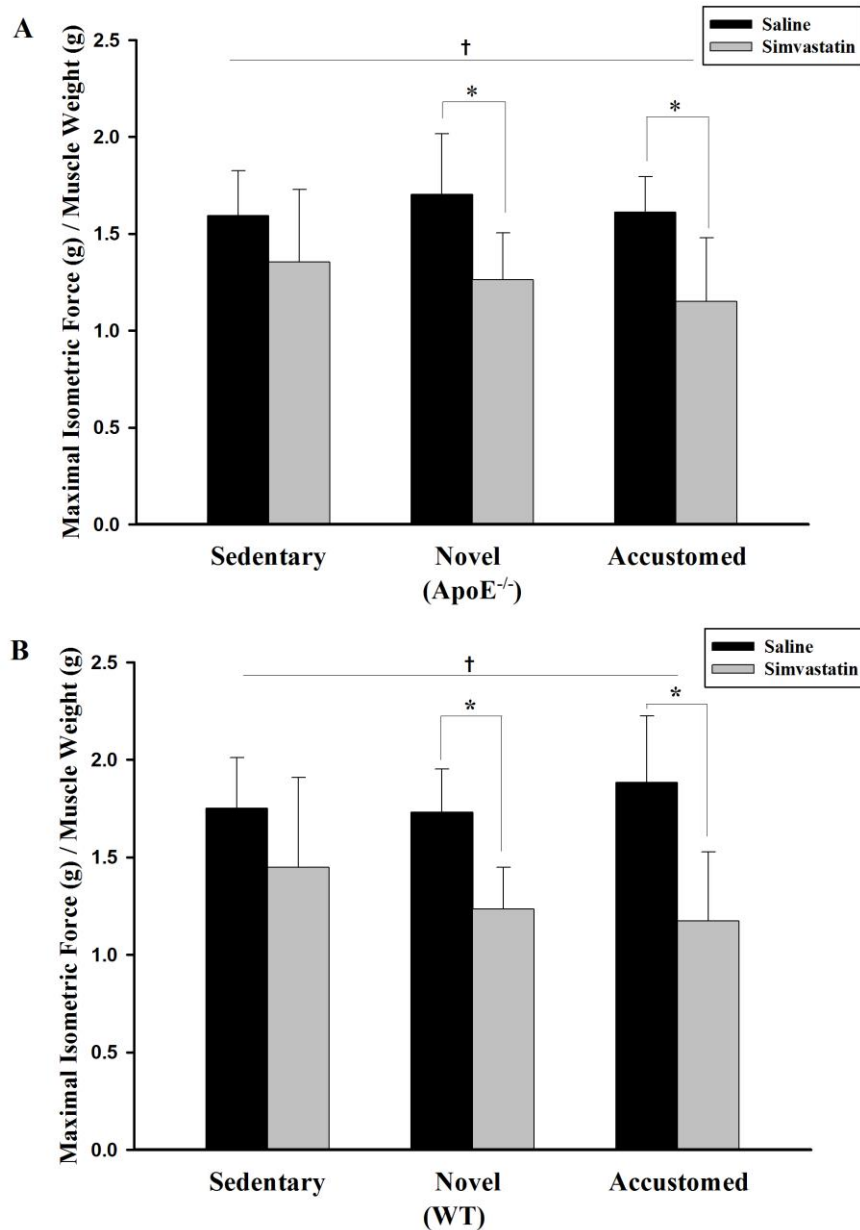
**4.5 Change in Grip Strength from Baseline to End of Study.** Maximal grip strength normalized to body weight was calculated for each animal and the difference from baseline to end of study was determined.



**Figure 11. Statin treatment decreases maximal grip strength.** Grip strength after 4 weeks normal cage activity (sedentary), two weeks normal cage activity and two weeks running wheel access (Novel), or 4 weeks running wheel access (Accustomed), with saline or simvastatin (20mg/kg) during the final two weeks of treatment in ApoE<sup>-/-</sup> (A) and WT (B) mice. Means  $\pm$  SD; n = 8-10/group. †=main effect of drug,  $p < 0.05$ ; §=exercise  $\times$  drug interaction,  $p < 0.05$ ; \*=within group pairwise comparison,  $p < 0.05$ ; a,b,c=different letters indicate statistically significant differences at 95% confidence.

Statin treatment decreased relative strength in ApoE<sup>-/-</sup> mice (drug main effect,  $F(1,53)=106.732$ ,  $p<0.001$ ). Statin treatment lowered strength in ApoE<sup>-/-</sup> mice to the same extent in all groups compared to saline-treated controls ( $p<0.001$  for all 3 groups) (Figure 11.A). In WT mice, relative strength was decreased by statin treatment and this was exacerbated in the exercise groups (drug x exercise interaction,  $F(2,54)=3.216$ ,  $p=0.048$ ). Based on 95% confidence interval comparisons, statin treatment significantly lowered grip strength compared to saline-treated controls ( $p<0.001$  for all 3 groups). Novel ( $p=0.007$ ) and accustomed ( $p=0.02$ ) exercise lowered strength to a greater degree than sedentary conditions following statin treatment (Figure 11.B).

**4.6 Isometric Muscle Force.** Statin treatment reduced maximal isometric force in ApoE<sup>-/-</sup> mice (drug main effect,  $F(1,48)=22.322$ ,  $p<0.001$ ) (Figure 12.A). Maximal isometric muscle force was significantly lower following statin treatment in novel and accustomed exercise groups compared to saline controls ( $P<0.001$ ). In WT mice, statin treatment similarly reduced maximal isometric force (drug main effect,  $F(1,51)=35.807$ ,  $p<0.001$ ) (Figure 12.B). Maximal isometric muscle force was significantly lower following statin treatment in novel and accustomed exercise groups compared to saline controls ( $P<0.001$ ). For both ApoE<sup>-/-</sup> and WT mice, no differences in maximal isometric force were detected between statin-treated novel and accustomed exercise groups.



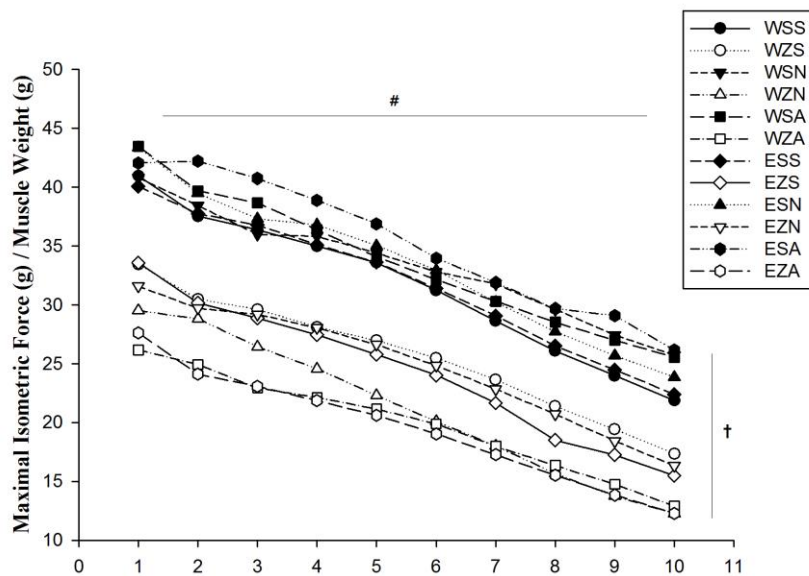
**Figure 12. Statin treatment reduces maximal isometric muscle force.** Hind-limb plantarflexor strength after 4 weeks normal cage activity (Sedentary), two weeks cage activity and two weeks running wheel access (Novel), or 4 weeks running wheel access (Accustomed), with saline or simvastatin (20mg/kg) during the final two weeks of treatment in ApoE<sup>-/-</sup> (A) and WT (B) mice. Means  $\pm$  SD; n = 8-10/group. exercise  $\times$  drug interaction, p=NS; †=main effect of drug, p<0.05; \*=within group pairwise comparison, p<0.05

#### 4.7 Muscle Fatigability (% of Maximum Isometric Force). Isometric force was reduced over time

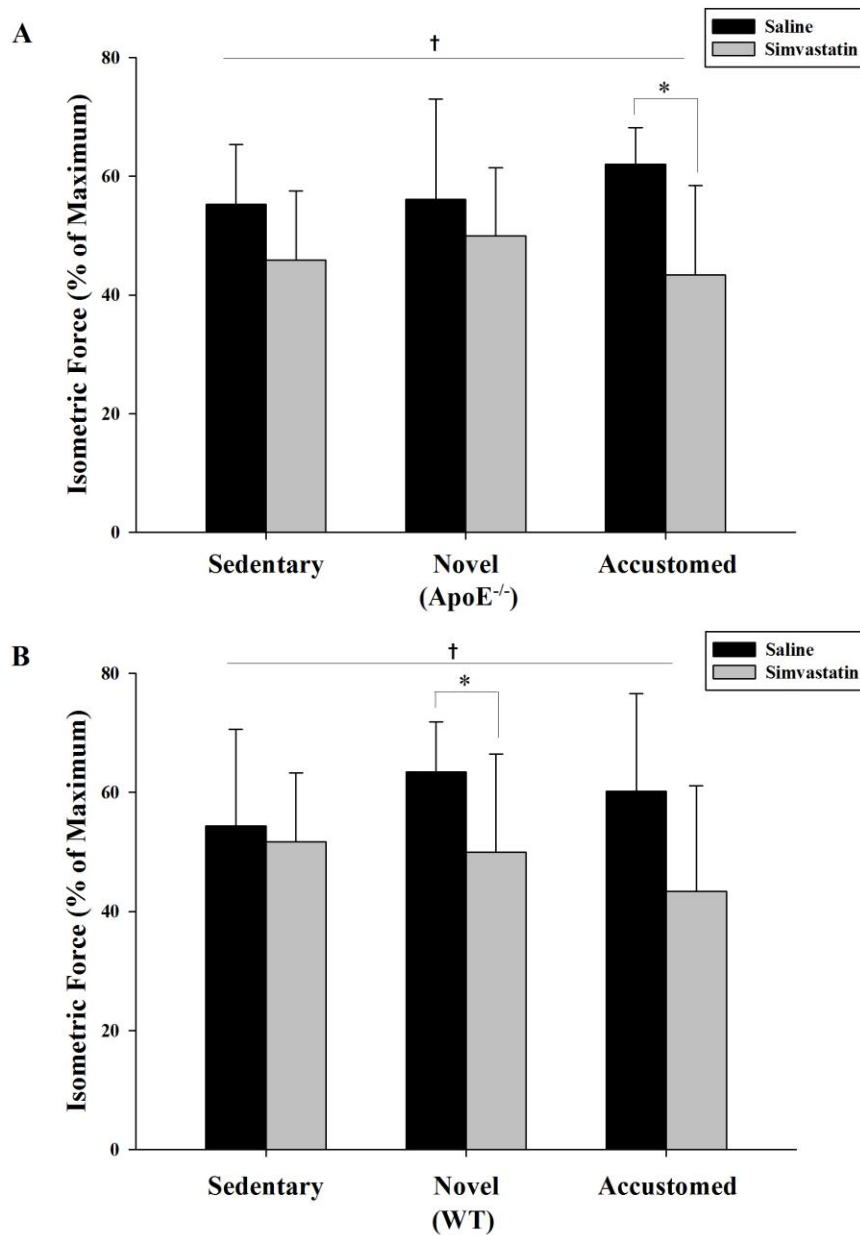
in all groups (time main effect by repeated measures ANOVA); however, there was no time  $\times$  drug

interaction indicating that the rate at which force declined did not differ between groups (Figure 13).

Statin treatment increased susceptibility to muscle fatigue in ApoE<sup>-/-</sup> mice (drug main effect,  $F(1,48)=10.654$ ,  $p<0.002$ ) (Figure 14.A). Muscle fatigability was significantly higher following statin treatment with accustomed exercise compared to saline controls ( $P<0.002$ ) (Figure 14.A). Statin treatment similarly increased muscle fatigue in WT mice compared to saline controls (drug main effect,  $F(1,51)=10.648$ ,  $p<0.002$ ) (Figure 14.B). Muscle fatigability was significantly higher following statin treatment with novel exercise compared to saline controls ( $P<0.002$ ) (Figure 14.B). For both ApoE<sup>-/-</sup> and WT mice, no differences were detected between statin-treated novel and accustomed exercise groups.

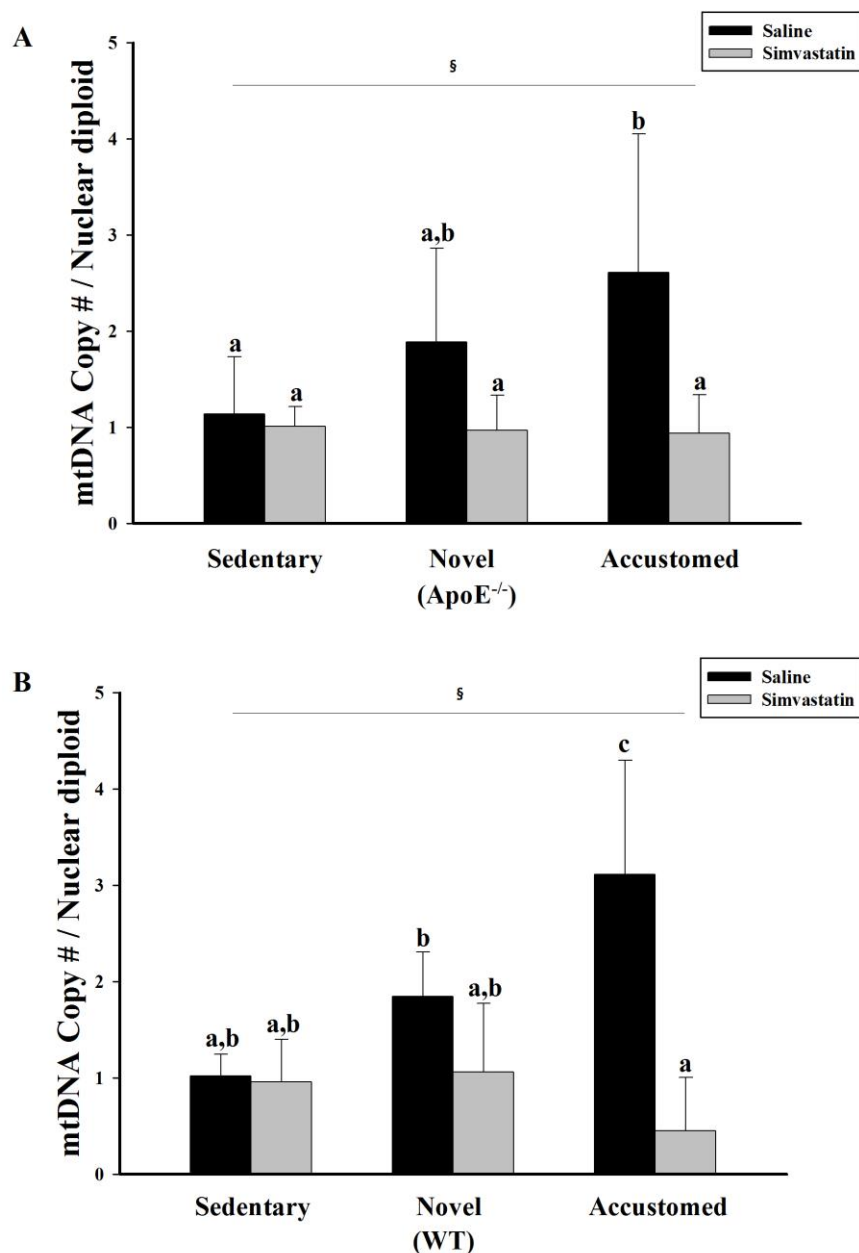


**Figure 13.** The contraction by stimulation of the sciatic nerve was repeated 10 times in order to examine muscle fatigability. W: Wild Type, E: ApoE<sup>-/-</sup> Z: Zocor (simvastatin) S: Saline S: Sedentary, N: Novel, A: Accustomed. n = 8-10/group. # = main effect of time,  $p<0.05$ ; † = main effect of drug,  $p<0.05$



**Figure 14. Statin treatment increases muscle fatigue.** Final isometric contraction force after 4 weeks of normal cage activity (Sedentary), 2 weeks of normal cage activity and 2 weeks of running wheel access (Novel), or 4 weeks of running wheel access (Accustomed), with saline or simvastatin (20 mg/kg) during the final 2 weeks of treatment in ApoE<sup>-/-</sup> (A) and WT (B) mice. Fatigue was calculated as percent maximal isometric force on the 10<sup>th</sup> repetition. Data expressed as %. Means  $\pm$  SD; n = 8-10/group. exercise  $\times$  drug interaction, p=NS; †=main effect of drug, p<0.05; \*=within group pairwise comparison, p<0.05

**4.8 Mitochondrial DNA Content (mtDNA).** Mitochondrial DNA content in skeletal muscle was altered by drug treatment and exercise in ApoE<sup>-/-</sup> mice (drug x exercise interaction,  $F(2, 42)=4.632$ ,  $p < 0.015$ ) (Figure 15.A).

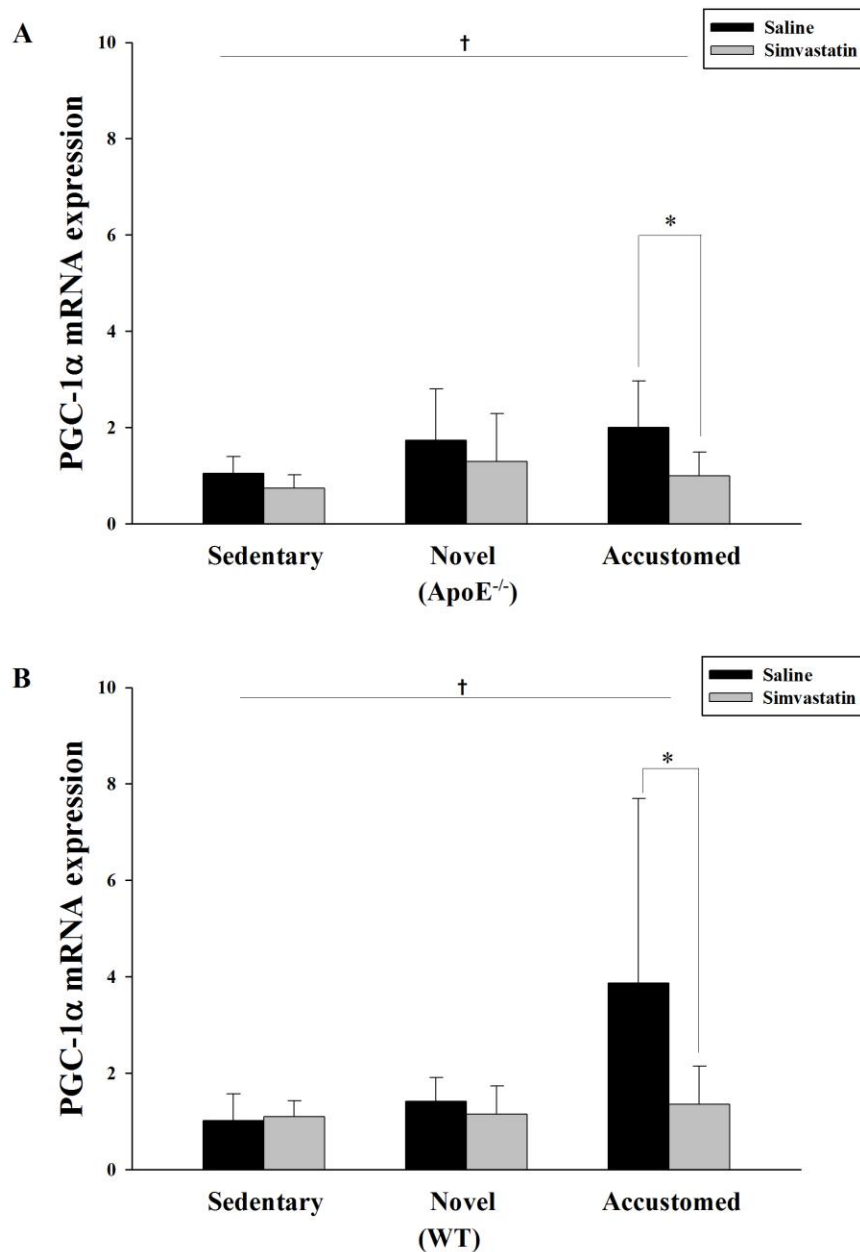


**Figure 15. Statin treatment prevents mitochondrial DNA content accumulation following accustomed exercise.** Real time PCR analysis of mitochondrial DNA content in gastrocnemius muscles after 4 weeks of normal cage activity (Sedentary), 2 weeks of normal cage activity and 2 weeks of running wheel access (Novel), or 4 weeks of running wheel access (Accustomed), with saline or simvastatin (20 mg/kg) during the final 2 weeks of treatment in ApoE<sup>-/-</sup> (A) and WT (B) mice. Means  $\pm$  SD; n = 8-10/group. <sup>§</sup>=exercise  $\times$  drug interaction,  $p < 0.05$ ; <sup>a,b,c</sup>=different letters indicate statistically significant differences at 95% confidence.

Accustomed exercise increased mitochondrial DNA content concurrent with saline injection compared to the sedentary control group and all mice receiving statin treatment ( $p < 0.001$ ). Similar results were obtained for WT mice (drug x exercise interaction,  $F(2, 42) = 16.159$ ,  $p < 0.001$ ) (Figure 15.B). In WT mice, accustomed exercise increased mitochondrial DNA content concurrent with saline injection compared to all other groups.

**4.9 Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1- $\alpha$ ).** Skeletal muscle PGC1- $\alpha$  mRNA expression was higher in saline-treated ApoE<sup>-/-</sup> mice compared to mice that received statin treatment (drug main effect,  $F(1,34) = 6.053$ ,  $p = 0.019$ ) (Figure 16.A). PGC1- $\alpha$  mRNA expression was significantly higher following saline treatment with accustomed exercise compared to similarly exercised mice that received statin treatment ( $P < 0.018$ ). In WT mice, PGC1- $\alpha$  mRNA expression was similarly higher with saline compared to statin treatment (drug main effect,  $F(1,36) = 4.186$ ,  $p = 0.048$ ) (Figure 16.B). PGC1- $\alpha$  mRNA expression was significantly higher following saline treatment with accustomed exercise compared to similarly exercised mice that received statin treatment ( $p < 0.009$ ), as well as mice that received saline concurrent with sedentary conditions ( $p = 0.005$ ) or novel exercise ( $p = 0.018$ ) (Figure 16.B).

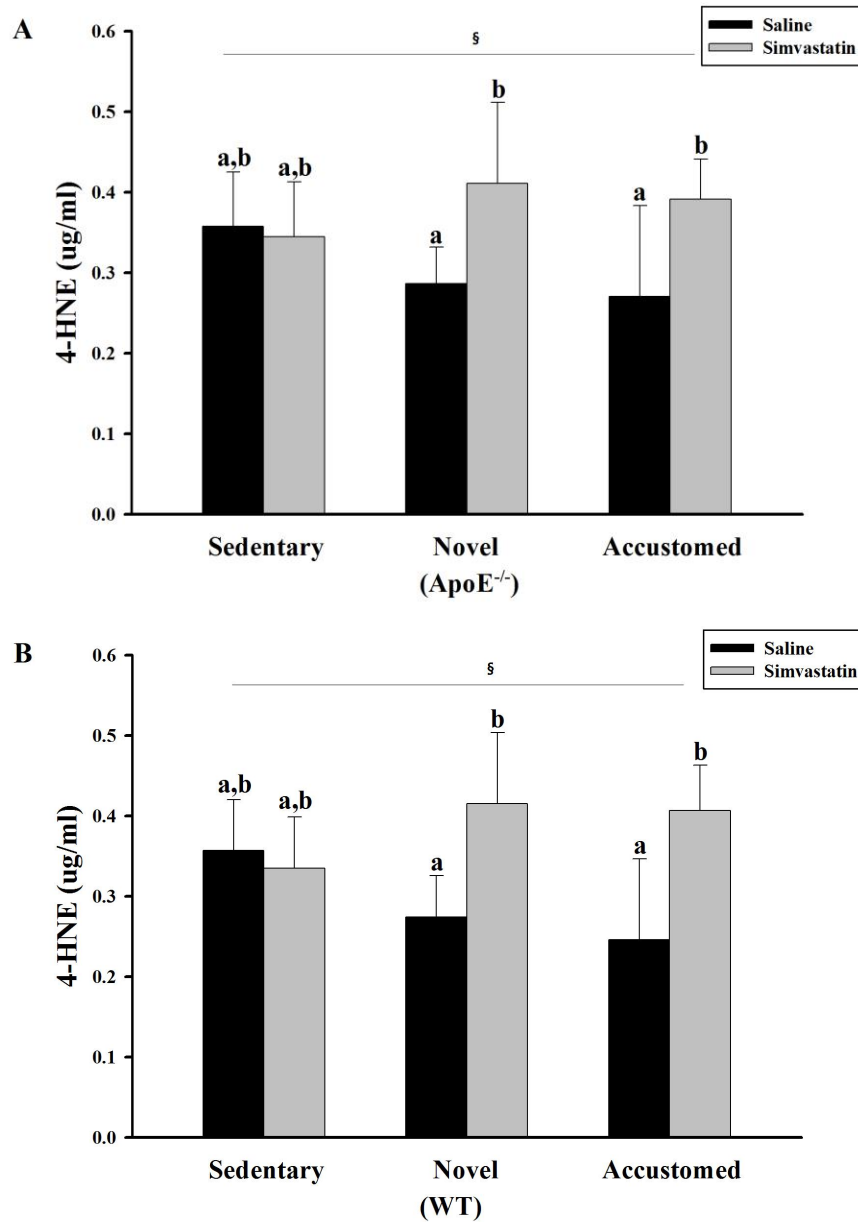




**Figure 16. Statin treatment inhibits an increase in skeletal muscle PGC1- $\alpha$  mRNA expression following accustomed exercise.** Real time RT-PCR analysis of PGC1- $\alpha$  mRNA expression in gastrocnemius muscles after 4 weeks of normal cage activity (Sedentary), 2 weeks of normal cage activity and 2 weeks of running wheel access (Novel), or 4 weeks of running wheel access (Accustomed), with saline or simvastatin (20 mg/kg) during the final 2 weeks of treatment in ApoE<sup>-/-</sup> (A) and WT (B) mice. Means  $\pm$  SD; n = 8-10/group. †=main effect of drug, p<0.05; \*=within group pairwise comparison, p<0.05

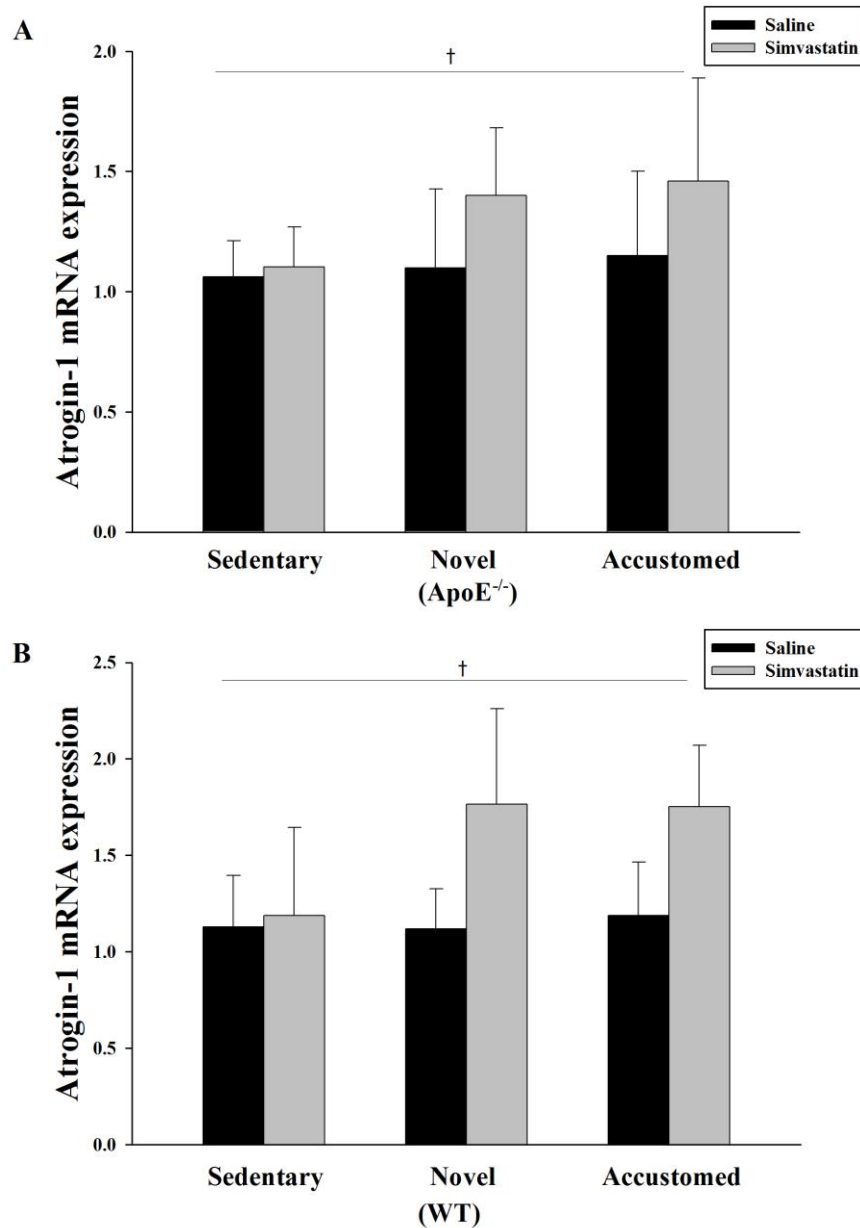
**4.10 4-hydroxynonenal (4-HNE).** 4-HNE was altered with statin and exercise intervention in ApoE<sup>-/-</sup> mice (drug x exercise interaction,  $F(2,35)=3.555$ ,  $p=0.039$ ) (Figure 17.A). Statin treatment increased

skeletal muscle 4-HNE compared to saline treatment only in combination with novel ( $p<0.006$ ) and accustomed ( $p<0.006$ ) exercise (Figure 17.A).



**Figure 17. Statin treatment prevents a decline in skeletal muscle oxidative stress with novel or accustomed exercise.** ELISA analysis of 4-HNE in gastrocnemius muscles after 4 weeks of normal cage activity (Sedentary), 2 weeks of normal cage activity and 2 weeks of running wheel access (Novel), or 4 weeks of running wheel access (Accustomed), with saline or simvastatin (20 mg/kg) during the final 2 weeks of treatment in ApoE<sup>-/-</sup> (A) and WT (B) mice. Means  $\pm$  SD;  $n = 8-10/\text{group}$ . §=exercise  $\times$  drug interaction,  $p<0.05$ ; <sup>a,b,c</sup>=different letters indicate statistically significant differences at 95% confidence.

In WT mice, 4-HNE was similarly altered with statin treatment and exercise (drug x exercise interaction  $F(2,42)=7.796$ ,  $p=0.001$ ). Statin treatment increased 4-HNE compared to saline treatment only in combination with novel ( $p<0.001$ ) and accustomed ( $p<0.001$ ) exercise (Figure 17.B).



**Figure 18: Statin treatment in combination with novel or accustomed exercise increases atrogin-1 gene expression in skeletal muscle.** Atrogin-1 mRNA in gastrocnemius muscles after 4 weeks of normal cage activity (Sedentary), 2 weeks of normal cage activity and 2 weeks of running wheel access (Novel), or 4 weeks of running wheel access (Accustomed), with saline or simvastatin (20 mg/kg) during the final 2 weeks of treatment in  $ApoE^{-/-}$  (A) and WT (B) mice. Means  $\pm$  SD;  $n = 8-10$ /group. exercise  $\times$  drug interaction,  $p=NS$ ; †=main effect of drug,  $p<0.05$

**4.11 Atrogin-1.** Atrogin-1 mRNA was higher in statin-treated ApoE<sup>-/-</sup> mice compared to saline controls (drug main effect,  $F(1,35)=4.381$ ,  $p=0.044$ ) (Figure 18.A) Atrogin-1 mRNA was similarly higher in statin-treated WT mice compared to saline controls (drug main effect,  $F(1,35)=12.865$ ,  $p=0.001$ ) (Figure 18.B). After combining novel and accustomed exercise groups in WT mice, there was a significant drug  $\times$  exercise interaction ( $p = 0.023$ ), indicating that statins only increased the atrogin-1 mRNA in comparison to controls when combined with exercise.

## **CHAPTER 5**

### **DISCUSSION**

Prior studies suggest that statins cause muscle damage, and this damage is exacerbated by exercise in human and animal models.<sup>4, 17, 100-102</sup> The purpose of this study was to compare the effects of novel exercise – starting exercise upon onset of statin treatment, and accustomed exercise – two weeks of exercise training prior to the onset of statin treatment, on statin-induced myopathy in hypercholesterolemic mice. We found that two weeks of statin treatment impaired muscle function as demonstrated by decreased running wheel activity, isometric force, and grip strength. However, the decline in muscle function was not dependent on exercise, as statin treatment reduced all parameters of strength to the same extent in all mice regardless of their activity group. Due to the short-term time course of this study, we also examined several indices of muscle health that might reflect cellular stress and/or increased susceptibility to myopathy, including mitochondrial content, oxidative stress, and atrogin-1 gene expression. In saline-injected animals, both novel and accustomed exercise increased mitochondrial content, but this effect was blunted by statins. Furthermore, there was a similar trend for PGC1- $\alpha$  levels, with exercise-induced increases in PGC1- $\alpha$  expression being blunted by statins. We also examined the effects of statins and exercise on muscle 4HNE as a marker of oxidative stress, and atrogin-1 gene expression as a marker of protein degradation. For both, there was an interaction effect between statins and exercise. Specifically, there was an overall trend for a decrease in these markers

when statins were provided to sedentary mice, though both novel and accustomed exercise increased their levels. Taken together, this suggests a pattern of increased oxidative stress and protein degradation when exercising concomitant to statin treatment. However, exacerbated declines in muscle function and activity caused by the combination of exercise and statin treatment may take more time to manifest than the 2 to 4 week intervention period examined here. Importantly, these detrimental responses were demonstrated to the same degree in both ApoE<sup>-/-</sup> and WT mice, suggesting that high cholesterol does not independently enhance susceptibility to statin-induced myopathy.

### **The effect of statin and/or exercise on muscle function**

The present study demonstrates that two weeks of statin treatment significantly impaired muscle function, as evidenced by decreased running wheel activity, isometric force, and grip strength, as well as increased fatigue. However, the timing of the introduction of statin administration, either after two weeks of exercise training or concurrent with the initiation of training, resulted in similar declines in physical function.

A decline in muscle strength has been previously reported with statin therapy.<sup>21</sup> Phillips et al., demonstrated that statin treatment can reduce hip abduction and flexion strength<sup>103</sup>, and Scott et al., reported a decline in leg strength in periodic statin users compared to control subjects<sup>104</sup>.

Based on the possible positive relationship between exercise training and statin-induced muscle damage, Meador et al. further examined whether exercise prior to statin prescription (accustomed

exercise) may result in a different response compared to the initiation of exercise coincident with statin therapy (novel exercise).<sup>18</sup> This study found that accustomed, but not novel exercise before statin administration preserved muscle function as measured by isometric force measurement. In the present study, a protective effect of prior exercise training was not shown in relation to statin-induced declines in muscle function. Both exercise groups demonstrated the same trend as observed in the sedentary group – lower strength, decreased activity levels, and increased muscle fatigability in statin-treated mice. Importantly, daily wheel running distances were gradually decreased after statin treatment started, a response not observed with saline treatment. The dosage of simvastatin used currently, 20 mg/kg, is higher than normally prescribed, which may cause tissue damage and evoke an inflammatory response, resulting in a central nervous system-mediated decline in activity, or sickness behavior. To distinguish between myopathy and sickness behavior, we measured serum amyloid A (SAA), a marker of systemic inflammation. Although no differences were detected between saline- and statin-treated mice in the sedentary group, significant increases (5-fold) in SAA were detected in both the novel and accustomed exercise groups with statin treatment (data not shown). This suggests that the interactive effects of statins and exercise on muscle strength or function may be mediated in part by inflammation. While further investigation is needed, SAA, especially when normalized to total HDL<sup>105, 106</sup>, may represent a diagnostic tool in the early detection of statin-associated myopathy in active individuals.

## **Statins blunted exercise-induced increases in mitochondrial DNA content**

Chronic aerobic exercise training has been widely shown to have mitochondrial and antioxidant benefits. Exercise training increases mitochondrial content and improves mitochondrial function<sup>107</sup>, and an incremental relationship has been shown between physical activity levels and mitochondrial content<sup>108, 109</sup>. The exercise-induced increase in mitochondrial content has been shown to effectively neutralize excessive oxidative stress.<sup>107</sup> Thus, it is plausible that exercise training would provide a protective environment against the statin-induced mitochondrial defects and increased oxidative stress levels which eventually result in diminished muscle function in statin users. However, very little is known about the impacts of exercise training on statin-induced mitochondrial abnormalities and antioxidant capacity. In fact, previous observational studies suggest the opposite effect, with exercise training disturbing muscle function in the presence of statin toxicity in muscles.<sup>17</sup>

In the present study, mitochondrial content was enhanced in saline-treated exercise groups and this response was greater with accustomed exercise compared to novel exercise in WT mice. However, this beneficial exercise adaptation was completely blunted in statin-treated mice. The observation that exercise in combination with statin treatment significantly decreased PGC1- $\alpha$  gene expression suggests that statins interfere with upstream cellular signaling pathways responsible for mitochondrial biosynthesis, such as p38.<sup>110, 111</sup> Indeed, other research has indicated that statin users have attenuated exercise training-induced improvements in cardiorespiratory exercise capacity compared to non-statin users.<sup>112</sup>



Although the mechanisms of statin-induced skeletal muscle myopathy are not well understood, reduced mitochondrial content and mitochondrial dysfunction are potential causes.<sup>10, 113, 114</sup> Inefficient mitochondrial function can be induced by a decrease in Coenzyme Q10 levels<sup>115</sup>. Coenzyme Q10 is an electron carrier in the electron transport chain, making it vital for mitochondrial respiration. Reductions in Coenzyme Q10 levels may reduce adenosine triphosphate (ATP) production.<sup>116, 117</sup> Normally coenzyme Q10 is not fully saturated in the mitochondria, and an increase in concentration results in an increase in mitochondrial function.<sup>118</sup> Statins decrease mevalonic acid, an important precursor for coenzyme Q10 (Figure 2). Several studies have demonstrated that statin treatment can cause a significant reduction in coenzyme Q10 in the blood<sup>119</sup>, and decreased coenzyme Q10 has been associated with mitochondrial dysfunction and muscle myopathy<sup>115</sup>.

According to a recent study, simvastatin suppressed mitochondrial biogenesis (via PGC1- $\alpha$ ), content (by flow cytometry and immunocytochemistry), function, and metabolism (by quantification of extracellular acidification rate and oxygen consumption rate) by lowering ubiquinol (a chemically reduced form of CoQ10). The reduction was rescued with ubiquinol prescription.<sup>120</sup> Hence, we postulate that statin-induced reductions in CoQ10 in combination with exercise negatively affect mitochondrial content and ROS, which is likely to lead to dysregulation of muscle protein synthesis/degradation homeostasis, and eventually contribute to impaired muscle functional capacity.

## **Effect of Statins and exercise on oxidative stress in skeletal muscle.**

One possible cause of increased protein degradation includes elevated oxidative stress. Statins play a role as an anti-oxidant in the circulation. Indeed, statins reduce extracellular LDL oxidation and intracellular oxidative stress by reducing NO, possibly contributing to their role in reducing cardiovascular disease<sup>121</sup>. In addition, fluvastatin has been shown to lower oxidative stress levels in blood and improve endothelial function.<sup>122</sup>

The mechanisms by which statins promote oxidative stress in skeletal muscle is poorly studied. Unlike in the circulation, reactive oxygen species in skeletal muscles are increased with 2 weeks of statin treatment in rats, and in patients with statin-induced myopathy.<sup>123</sup> These researchers hypothesized that mitochondrial dysfunction and consequent muscle pain may result from excessive oxidative stress in skeletal muscle induced by statins. However, moderate exercise could oppose this increase in oxidative stress by increasing mitochondrial biogenesis.<sup>124</sup> In this study, 4-HNE, a marker of oxidative stress in muscle, was not affected by statin treatment alone, but only by the combination of statins and exercise. The exercise-induced decrease in oxidative stress was blunted by statin treatment. While exercise training is capable of decreasing oxidative stress at rest through enhancing stress-defense mechanisms, acute exercise is known to generate acute increases in oxidative stress. Considering the effects of exercise training in relation to statins, we postulate that the favorable enhancement in mitochondrial content and function seen with two weeks of exercise training likely fail to overcome excessive oxidative stress resulting from statin treatment.

Bouitbir et al. observed that ROS levels were elevated in atorvastatin-prescribed rats compared to control rats. Exhaustive exercise caused a further increase in ROS levels in the statin treated rats. Furthermore, both maximal mitochondrial respiration capacity ( $V_{\max}$ ) and running capacity were reduced compared to the control group and exercise further exacerbated the reductions in  $V_{\max}$  in skeletal muscles in the statin treated mice.<sup>92</sup> This suggests that statins and exercise may combine to induce excessive oxidative stress and mitochondrial dysfunction that can in turn negatively impact muscle function and performance.

#### **Atrogin-1 mRNA expression increased by statin treatment.**

Atrogin-1, a marker for muscle degradation and activation of the ubiquitin proteasome pathway<sup>125, 126</sup>, was significantly increased after 2 weeks of statin treatment in both the novel and accustomed exercise groups. This deleterious effect of statins on muscle is consistent with previous findings in patients undergoing statin treatment.<sup>13, 127, 128</sup> There was a trend for exercise, both novel and accustomed, to potentiate the statin-induced increase in atrogin-1 expression. Though this effect was no significant when the exercise groups were assessed independently, when the two exercise groups were combined to increase the power, atrogin-1 expression was significantly increased compared to in the statin treated group that did not exercise. This suggests that the combination of exercise and statins may exacerbate muscle catabolism in working muscles more than statins alone.

Previous studies found that statins increase muscle catabolic rate through disruptions in skeletal muscle membrane integrity and increases in ubiquitin proteasome pathway (UPP) activity. Cholesterol lowering drugs, including statins, reduce membrane fluidity by reducing cholesterol availability.<sup>129, 130</sup> Furthermore, Hani et al showed that atrogin-1 expression was increased after lovastatin treatment.<sup>13</sup> Proposed mechanisms for statins inducing atrogin-1 include FoxO dephosphorylation, nuclear localization, and transcription of the atrogin-1 gene.<sup>12, 74</sup> Increases in protein degradation, as measured by atrogin-1 expression, may contribute to muscle myopathy.<sup>13</sup> This may explain the decreased muscle function measures in the statin-treated mice of the present study. That is, the statin-induced protein degradation may contribute to impaired muscle function.

We postulate that the daily exercise in the present study, though voluntary, might produce detrimental stressors to mitochondrial energy-generating processes, as well as mitochondrial antioxidant capacity. This consequently led to protein degradation in muscles and eventually caused muscle dysfunction in the statin-treated mice.

### **Moving towards a mechanism**

Aerobic exercise training increases the number of mitochondria, improving the ability to increase exercise volume.<sup>107</sup> We previously hypothesized that the increased mitochondrial content may provide a protective adaptation against statin-associated myopathy, likely via the enhanced handling of ROS. Contrary to this hypothesis, the accustomed exercise group in the current experiment showed an

immediate drop in the running wheel activity levels with the commencement of statin treatment that was statistically similar to the response seen in the novel exercise group. This indicates that the increased mitochondrial content that should have occurred with the two weeks of prior training on the running wheel did not protect against statin-associated myopathy. Indeed, it is instructive to note that with the initiation of statin treatment, the reductions in running wheel and hand grip performance in the accustomed exercise group happened immediately, as opposed to the delayed reduction that was seen in the novel exercise group. This suggests that a reduction in mitochondrial content beyond some physiological threshold is not itself a primary mechanism for statin-induced myopathy in exercise, and that the role of the mitochondria is more likely to promote myopathy through its own damage or dysfunction. This statin-induced mitochondrial dysfunction may have contributed to strength declines in our study. Although mitochondrial function, which is dependent on aerobic metabolism, and maximal strength performance, which depends on the ATP-PC system, rely primarily on different energy systems, previous research has shown that mitochondrial damage can lead to contractile dysfunction in skeletal muscles<sup>131</sup>. This supports the idea that statin-associated myopathy causes declines in physical function by damaging skeletal muscle contractile functions through mitochondrial damage, rather than by impairing mitochondrial energy pathways. This is further supported by our findings that statin treatment decreased physical performance but did not affect cellular measures in the sedentary mice. While mitochondrial DNA copy number, muscle 4HNE levels, and atrogin-1 expression were not affected by statins in the sedentary animals, the sedentary animals did show

similar declines in functional measures such as grip strength and isometric force. Taken together, these observations suggest that the measured cellular markers are not the primary mechanisms for statin-induced impairment in physical function. The current results would not be expected if one of the main mechanisms was either inhibiting mitochondrial proliferation, increasing oxidative stress, or upregulating atrogin-1. In the sedentary mice, these cellular markers were unaffected, yet functional measurements still showed declines, and the accustomed exercise group showed no relative delay in the declines of running wheel activity or grip strength. Therefore, while the measured markers do seem to play some role in the interaction with exercise, it would appear that they are not causal factors in statin-induced myopathy.

One possible alternative explanation might be that statins cause mitochondria to release toxins such as cytochrome c<sup>44, 132, 133</sup>, malonate<sup>134</sup>, superoxide<sup>135</sup>, H<sub>2</sub>O<sub>2</sub><sup>132</sup>, etc. In this scenario, an increase in mitochondrial volume may only lead to higher levels of toxicity which, in turn, negatively affects physical performance to a greater extent. An increase in the capacity to deal with oxidative stress—as brought about by exercise training would also not necessarily be protective under these circumstances. This theory is consistent with both the mechanistic and functional results of the current study. Namely that; 1) WZS (statin + sedentary) group is worse-off than WSS (saline + sedentary) because of this toxin release; 2) WZN (statin + novel) is not better off than WZS (statin + sedentary) because the combination of statins and these toxins prevent any exercise adaptation; 3) WZA (statin + accustomed) is not better off than WZN (statin + novel) or WZS (statin + sedentary) because there are more

mitochondria to release these toxins, thereby causing a more rapid decline. Our measured outcomes did not allow thorough examination of statin-induced mitochondrial toxicity; therefore, further investigation is warranted to examine this potential mechanism.

### **Role of hypercholesterolemia**

There were no differences in any measures between ApoE<sup>-/-</sup> and WT mice in the present study. Previous research has shown that increased cholesterol levels can cause muscle damage<sup>19, 20</sup> consequently harming muscle function<sup>95</sup>. Furthermore, elevated levels of cholesterol delays healing from muscle damage.<sup>96</sup> Statins drugs are also known to down regulate muscle repair and regeneration.<sup>97</sup> Because of this, we had hypothesized that hypercholesterolemia may itself be playing a role in the interactions between statins and exercise. However, in the present study, mice with high levels of cholesterol (ApoE<sup>-/-</sup>) showed the same trends in all functional and cellular measures as seen in WT mice. Therefore, it appears that the detrimental effects of statins on muscle damage are not confounded by high cholesterol levels, as we had previously hypothesized<sup>19</sup>. Additionally, this data refutes the supposition that reductions in cholesterol levels are contributing to statin-associated myopathy through mechanisms such as reduced membrane fluidity.

## **Strengths and limitations**

This is one of the first studies to determine whether novel or accustomed exercises produces a negative impact on statin-induced myopathy by examining parameters related to muscle function such as isometric force, grip strength, and running wheel activity, and is the first to associate them to a repeated measure of muscle strength—the grip strength test. Previous studies are mostly observational, examining the relationship between exercise and statins, and even experimental studies used a single functional metric in most cases. Furthermore, the present study confirms the hypothesis that mitochondrial abnormalities, muscular oxidative stress, and consequent increases in atrogen-1 are closely interrelated and likely cause detrimental exercise effects in muscles of exercising statin users. However, while these cellular markers do seem to be associated with the interactions of exercise and statin-associated myopathy, our findings indicate that they are likely not the primary cause of statin-induced myopathy itself. Further investigation into the primary mechanisms is still needed.

Our cellular data is in agreement with the previous observations supporting the detrimental effect of exercise on statin-induced myopathy, while results from our functional testing do not support the hypothesis that exercise potentiates these effects. These discrepant results between cellular and functional measures may be explained by the exercise mode and its relationship with the measures in the present study. Our exercise training protocol, voluntary running wheel exercise for 2 weeks (novel exercise) or 4 weeks (accustomed exercise), effectively increased mitochondrial content and mitochondrial biogenesis and decreased oxidative stress levels and protein degradation rates as a



successful adaptation to aerobic exercise in the saline treated mice. On the other hand, the functional measures—grip strength and maximal isometric force—are not expected to be changed to a great extent with chronic running, although the same exercise protocol led to a detectable change in isometric force in our preliminary study.<sup>18</sup> Additionally, cellular abnormalities precede muscular dysfunctional symptoms, with high variabilities in their types, occurrence rates, and onset time. Therefore, the low sensitivity of the functional measures and the potentially low relevance of some of the functional measures to the exercise mode in the present study may limit the ability to detect an added effect of exercise on statin-induced functional declines.

One important strength of the study is the dual-genotype design, which allowed examination of the role of hypercholesterolemia on statin induced myopathy. This may be important due to the fact that statins are generally prescribed to those with significantly elevated cholesterol levels.

### **Future study direction**

Mikus et al., demonstrated that simvastatin blunted or decreased improvements in cardiorespiratory fitness ( $\text{VO}_{2\text{ peak}}$ ) and mitochondrial content (skeletal muscle citrate synthase activity) after 12 weeks of exercise training.<sup>112</sup> Therefore, it appears that instead of providing a protective environment or compensational balance, concomitant exercise training exacerbates muscle myopathy when combined with statin treatment. In other words, due to the myotoxicity of the statins, exercise training may become a repeated, stressful stimulus similar to acute exercise, rather than being a mild stimulus to

continuously achieve an enhanced defense system. In the same context, one possible strategy to avoid the apparently synergistic harm from exercise and statins might be alternating between two treatment modes. For example, it may be desirable for a hypercholesterolemic patient to alternate periods of statin-free exercise training with periods of exercise-free statin treatment. This continually alternating strategy may be able to bring benefits from both exercise and statins with fewer negative side effects. Further studies are needed to investigate the effectiveness and feasibility of this strategy.

## CHAPTER 6

### CONCLUSION

Statins are effective in lowering cardiovascular disease risk, and an increasing number of patients consume statins worldwide. Despite their established safety, muscle myopathy is frequently reported in statin users, ranging from muscle discomfort to rhabdomyolysis. When statins are prescribed, exercise is often recommended as an adjunct cardiovascular disease risk lowering strategy. However, observational studies indicate that exercise training status may exacerbate statin-induced myopathy.

In the present study, we found that statin treatment had a negative impact on muscle function and cellular regulation in muscles, both in normocholesterolemic and hypercholesterolemic mice. The combination of exercise and statin treatment decreased mitochondrial content and the expression of PGC1- $\alpha$ , a marker of mitochondrial biogenesis. Additionally, muscle oxidative stress and the protein degradation marker atrogin-1 were increased by the combination of exercise and statins while exercise alone elicited mitochondrial and antioxidant benefits. Even though no effect of exercise training was seen on muscle strength, the reduction in daily wheel running activity may reflect an elevation in muscle discomfort in statin treated animals.

The information in the present study is clinically important to prevent possible muscle damage in statin patients. The two well-known cardiovascular disease-prevention strategies—statins and exercise—should be administered together only cautiously, because exercise may produce additive

stress on muscles and thereby worsen statin-induced myopathy. Additional information about the roles of mitochondrial dysfunction on statin-associated myopathy could have been obtained had potential mitochondrial toxins been explored.

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