DIETARY CHOLESTEROL AND RESISTANCE TRAINING AS COUNTERMEASURES TO ACCELERATED MUSCLE LOSS

A Dissertation

by

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ABSTRACT

Cholesterol plays an important role in physiology, serving as a membrane constituent and steroid hormone precursor. Recently, cholesterol has also been associated with skeletal muscle homeostasis, leading to the purpose of this research, which was to examine the role of cholesterol metabolism during perturbations of skeletal muscle homeostasis. We evaluated skeletal muscle responses and proteins involved in cholesterol metabolism [sterol regulatory element-binding protein-2 (SREBP-2) and low-density lipoprotein-receptor (LDL-R)] under conditions of unloading, exercise and dietary cholesterol (D-CL) administration. We hypothesized that skeletal muscle mass, fractional synthesis rates (FSR), SREBP-2, and LDL-R would be lower in unloaded muscle while exercise and D-CL would have a positive effect.

The first study examined the effect of hindlimb unloading (HU) on muscle mass, SREBP-2, and LDL-R. HU animals showed lower muscle mass and a trend towards lower gastrocnemius SREBP-2 than cage controls (CC).

The second study examined the effect of D-CL and resistance training (RT) on lean mass, FSR, SREBP-2, and LDL-R in ambulatory rats. Unexpectedly, rats performing exercise without added resistance [(RT-Control (RTC)] had greater lean mass responses than the RT groups. However, RT groups had higher plantaris to body mass ratio and FSR than RTC (plantaris FSR only) and CC (both variables) animals. RT plus high D-CL administration resulted in greater plantaris FSR than the RT group

consuming normal D-CL. Quadriceps SREBP-2 trended towards an increase in response to RT.

The third study investigated the effect of D-CL and RT on muscle mass, FSR, SREBP-2, and LDL-R in the context of HU. HU and HU rats performing RT had lower muscle mass than CC. HU rats showed higher liver mature LDL-R than CC but showed a trend towards lower gastrocnemius SREBP-2 than CC. There was no difference in FSR among activity or D-CL groups.

These studies show evidence of shifts in content of proteins related to cholesterol metabolism and muscle FSR when muscle activity is manipulated from RT to complete unloading. However, effects such as elevated FSR with RT and high D-CL were not consistent throughout the studies, leaving doubt of the effect of activity on cholesterol metabolism.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

The National Aeronautics and Space Administration (NASA) was established in 1958. Since then, tremendous progress has been made in space exploration and research. This includes manned missions to the Moon and more recently, the construction of the International Space Station. The future plans for NASA are to send astronauts into space for long duration missions. The first destination will be to the Moon to establish a lunar base and to conduct research on living in an environment outside of Earth. This knowledge will eventually be used to determine the feasibility of future manned missions to Mars.

Before these missions to the Moon and Mars can occur, many obstacles need to be overcome. Space radiation and the associated deleterious effects provide the greatest threat to space exploration. Additionally, the deterioration of the musculoskeletal system and deconditioning of the cardiovascular system may jeopardize the successful completion of a mission. As a result, countermeasures are being developed and tested to prevent or minimize these problems in space. Moreover, these interventions may also reduce the time needed for rehabilitation when crewmembers return to Earth.

The current intervention used in space to counteract skeletal muscle atrophy has been ineffective. The Advanced Resistive Exercise Device (ARED) is a type of exercise equipment that permits astronauts to lift weights while in space. Crew members using the ARED can perform a variety of exercises targeting a wide range of muscle groups

with a resistance of up to 600 lbs. Despite allocating sufficient time for exercise, astronauts returning from space still experience significant skeletal muscle loss, suggesting other countermeasures are necessary.

One promising countermeasure may be the use of dietary cholesterol, when consumed in conjunction with resistance training. Cholesterol has garnered much attention for its role in cardiovascular disease. However, cholesterol also plays a critical physiological role within cells through its functional role in membrane integrity and fluidity and biochemical precursor to steroid biogenesis. One previous study has reported that higher dietary cholesterol consumption was associated with greater lean mass gains following 12 weeks of resistance training (69). We hypothesized that dietary cholesterol is not only beneficial, but is also necessary to increase or preserve lean mass, when combined with resistance training.

This research was designed to provide support for the use of dietary cholesterol as a supplement to augment or maintain skeletal muscle mass. These results may provide evidence for the use of dietary cholesterol in other conditions that involve muscle atrophy, such as sarcopenia and bed rest.

CHAPTER II

BACKGROUND

Space Physiology and Skeletal Muscle

On Earth, the human body is exposed to gravitational forces equal to 1g. Once a person leaves the gravitation field of Earth and enters space, the body begins to experience microgravity (< 1g), which reduces the forces imposed on the body. As a result, muscles typically active on Earth, especially weight bearing muscles, are profoundly affected by the microgravity, resulting in muscle function being severely compromised (12). These muscles include the intrinsic back muscles, psoas, quadriceps femoris, hamstrings, gastrocnemius, soleus, and anterior leg muscles (34, 56).

In **Table 1,** muscle volume loss following various durations of spaceflight for the gastrocnemius, soleus, and quadriceps muscles are shown. Following short duration spaceflight (17 days), gastrocnemius and soleus muscle volume decreased by ~10% each while quadriceps muscle volume was reduced by 5-7% (56). During missions of longer duration (115-197 days; ~16-28 weeks), gastrocnemius and soleus muscle volume diminished by 16.9% and 16.8%, respectively, while quadriceps muscle volume decreased by 10.1% (56). It is important to note that although exercise countermeasures were employed during longer duration missions, muscle atrophy still persisted (56, 96). Additionally, the discrepancy observed in muscle volume loss between the LeBlanc (2000) study and the two other long duration studies could be attributed to the time when the data were collected (LeBlanc: landing day, Trappe: ~4 days after landing, and

Gopalakrishnan: ~5 days after landing). This would be problematic as volume measurements done days after landing have been reported to be higher than landing day (41).

Table 1. Muscle volume loss following various durations of spaceflight

Duration	Quadriceps	Gastrocnemius	Soleus	Subject Number, n
8 days	6% (57)	6.3%* (57)	6.3%* (57)	3-4
17 days	5-7% (56)	~10% (56)	~10% (56)	4
115-197 days	10.1% (56)	16.9% (56)	16.8% (56)	16
161-192 days		10% (96)	15% (96)	9
161-194 days	5.9% (41)	10.3%† (41)	18.6% (41)	4

*denotes gastrocnemius and soleus were measured together.
†denotes only medial gastrocnemius was measured.

To understand why muscle loss occurred, an examination of the muscle fiber composition would be insightful. Following spaceflight, type I muscle fibers atrophy as well as diminish in number, and many of the postural muscles contain a considerable proportion of type I muscle fibers (2, 8, 33, 103) (**Table 2**). Because type I muscle fibers possess abundant myoglobin, mitochondria, oxidative enzymes, and capillaries, they are capable of maintaining contractile activity for prolonged periods of time. Therefore, the atrophy of type I muscle fibers in response to microgravity appears reasonable since the stimulus (Earth's gravity) has been diminished and these muscle fibers no longer need to be constantly activated.

Table 2. Slow oxidative fiber percentage in normal human lower limb muscles

Muscle	Percentage of slow oxidative fibers
Soleus	70% (28)
Gastrocnemius	50% (28)
Quadriceps	
Vastus Intermedius	47% (28)
Vastus Lateralis	32% (28)

Due to limitations in conducting scientific studies in space, models simulating microgravity have been developed, including the bed rest paradigm that NASA prefers as its primary model of unloading. Bed rest involves the subject lying supinely in bed throughout a predetermined period of time (91). In one version, a participant's head is also tilted downward by 6 degrees, which mimics the cardiovascular fluid shift observed in space (91). Unfortunately the costs, as well as availability of facilities, make conducting bed rest studies difficult (10). Nevertheless, this paradigm has provided useful similarities in skeletal muscle atrophy for comparison with actual spaceflight (**Table 3**). Following 14 days of bed rest, gastrocnemius muscle volume decreased between 7.7 to 9.4% while soleus volume, which was analyzed with the flexor hallucis longus, diminished by 6.2% (9). Furthermore, rectus femoris and vasti (collectively the quadriceps) muscle volume was reduced by 4.1 and 6.7%, respectively (9). These muscle volume losses following 14 days of bed rest are similar to the losses observed after 17 days of spaceflight for gastrocnemius and quadriceps. However, prolonged bed rest may not be an ideal simulation of spaceflight, as atrophy appears more severe in the former. After 90 days of bed rest, the volume of the triceps surae significantly

diminished by 29% while the volume of the quadriceps was significantly reduced by 18% (5).

Table 3. Comparison of muscle loss between spaceflight and models simulating spaceflight

	Spaceflight	Bed Rest	ULLS
Short Duration	17 days	14 days	20-23 days
Quadriceps	5-7% (56)	4.1-6.7% (9)	6.9% (3)
Gastrocnemius	~10% (56)	7.7-9.4% (9)	8.8% (77)
Soleus	~10% (56)	6.2% (9)	7% (77)
Long Duration	115-197 days	90 days	35 days
Quadriceps	10.1% (56)	18% (5)	11% (18)
Gastrocnemius	16.9% (56)	29%* (5)	
Soleus	16.8% (56)		11% (18)

* denotes combined gastrocnemius and soleus muscle volume loss. Unilateral Lower Limb Suspension, ULLS.

In addition to the bed rest model, another human paradigm of disuse has been used. This model is known as unilateral lower limb suspension (ULLS). Participants subjected to ULLS wear an elevated shoe on one leg while the other leg remains mobile and non-weight bearing, permitting the suspended leg to be unloaded (2, 45). Subjects utilize crunches in order to walk around on the limb with the elevated shoe (2). From the results of short-term ULLS, it appears that the muscle losses exhibited for the quadriceps and gastrocnemius are comparable to that of short-duration spaceflight (**Table 3**). Quadriceps muscle volume significantly declined by 6.9% after 20 days of ULLS while soleus and gastrocnemius muscle volumes decreased by 7 and 8.8%, respectively, following 23 days of ULLS (3, 77). Moreover, prolonged ULLS has reported results similar to long-duration spaceflight, at least for the quadriceps muscle.

After a group of men and women completed 5 weeks of ULLS, significant reductions of 11% in both soleus and vastus lateralis muscle mass were observed (18). Thus, both human models can reasonably simulate spaceflight, which permits further investigation into the mechanisms responsible for muscle atrophy associated with unloading.

Hindlimb Unloading as a Model

In addition to humans, rodents have also been flown in space to advance research on muscle atrophy associated with unloading. When rats are in space, movement is achieved exclusively with their forelimbs (71). As a result of microgravity, skeletal muscle size diminishes, which can be attributed to a reduced (20 to 40%) muscle fiber cross sectional area (71). Additionally, fast twitch muscle fibers appear to become more prevalent in rats following spaceflight (71).

The number of rats that can be flown in space is limited. However, a ground-based rodent model simulating spaceflight does exist. This paradigm, known as hindlimb unloading (HU) or hindlimb suspension (HS), was developed in the mid-1970s (62). The current model involves the rodent lifted up by its tail, which causes its hindlimbs to be elevated off the floor and the head angled downward (54). Typically, the tail is suspended until a 30º angle is formed between the ground and the body (62). At this angle, the forelimbs are subjected to only half of its body mass (62). Furthermore, this paradigm requires the rat to ambulate with only its forelimbs, which mimics the movement of space-flown rats.

To validate this model, HU rats had been tested against space-flown rats. When HU rats were compared to space-flown rats, the decrease in muscle mass was similar (62). Moreover, HU rats were similar to space-flown rats in other aspects, such as alterations in bone, heart, and immune function (62). As a result, the HU paradigm has been regarded as the gold standard for ground-based rodent models simulating spaceflight.

Extensive research has been conducted using the HU model. Similar to humans in space, the slow twitch muscles of HU rodents decrease in size (12). After 4 days of unloading, HU rats had 15% less soleus muscle mass than cage control rats (36) (**Table 4**). When rats were suspended for 28 days, their soleus muscle mass atrophied to about 49% below control levels (106). Additionally, while this concept is unsubstantiated in humans, rodent type I muscle fibers have been reported to change into type II muscle fibers with unloading (11). To prevent muscle atrophy, allowing standing for 1h, 2h, or 4h daily was investigated as countermeasures, but was ineffective in completely ameliorating soleus muscle loss (62%, 72%, and 88%, respectively, of controls) in rats unloaded for 4 weeks (106). Thus, the pursuit of additional countermeasures is warranted to alleviate this muscle atrophy.

Table 4. Soleus muscle loss following various durations of hindlimb suspension

Days	Relative to Control Rats' Soleus	Relative to Own Body Mass (mg/g)
4	85% (36)	0.38 (36); 0.46 (72)
7	82% (50)	0.35 (50); 0.39 (72)
11		0.32 (72)
14	63% (50)	0.27 (50); 0.28 (72)
28	45% (54); 51% (106)	0.20 (54); 0.22 (106)

Cell Signaling Leading to Muscle Gain and Muscle Loss

Skeletal muscle is a dynamic tissue that results from the balance between muscle protein synthesis and protein degradation. In order to gain muscle mass, net muscle protein balance needs to be in favor of muscle protein synthesis. Conversely, to lose skeletal muscle, net muscle protein balance needs to be in favor of muscle protein degradation. Both muscle protein synthesis and protein degradation involve complex and integrative pathways.

Two independent pathways have been associated with protein synthesis. The first pathway involves the mammalian target of rapamycin (mTOR) (**Figure 1**). Several different signals have been reported to turn this pathway on (14). For example, when insulin or insulin-like growth factor 1 (IGF-1) associates with the insulin receptor, this leads to insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) phosphorylation, which go on to bind to and activate phosphatidylinositol 3-kinase (PI3K) (14, 58). Once PI3K is turned on, it leads to the phosphorylation of phosphatidylinositol-dependent protein kinase (PDK1), which then activates Akt (14). Three events result from Akt activation: glycogen synthase kinase (GSK) becomes inhibited, tuberous sclerosis complex 2 (TSC2) becomes inhibited, and mTOR becomes activated (14). When GSK becomes inactivated, its substrate, eukaryotic initiation factor 2B (eIF2B), is no longer phosphorylated and can participate in translation initiation (14). After Akt phosphorylates TSC2, this protein, which usually combines with tuberous sclerosis complex 1 (TSC1), loses its ability to inactivate ras homologue enriched in brain (Rheb) (14). Rheb can then activate mTOR through an unknown pathway (14). As for mTOR,

before it can carry out its function, GβL and the regulatory associated protein of target of rapamycin (raptor) must associate with it, forming the mTOR complex 1 (mTORC1) (14). Following mTORC1 formation, eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and ribosomal protein S6 protein kinase 1 (S6K1) get phosphorylated (14). 4E-BP1 becomes inactivated when it is phosphorylated, which causes 4E-BP1 to relinquish eIF4E and permit that factor to participate in messenger ribonucleic acid (mRNA) translation initiation (14). Conversely, S6K1 becomes activated when it is phosphorylated, leading to certain mRNA to be translated more (14). Collectively, these events increase protein synthesis.

The second pathway involved with protein synthesis is the mitogen-activated protein kinase (MAPK) pathway (**Figure 2**). While mTOR signaling affects mainly translation, both transcription and translation appear to be the targets of the MAPK pathway (61, 102). Similar to mTOR signaling, the insulin receptor can also initiate MAPK signaling (102). After the insulin receptor is turned on, the SHc-Growth factor receptor-bound protein 2 (Grb2) complex is assembled, which leads to the activation of Ras (102). When Ras associates with Raf, they become known as MAPK/extracellular signal-regulated kinase (ERK) kinase kinase (MEKK) that targets the protein MEK for phosphorylation (102). MEK then activates its substrate, the mitogen activated protein kinase ERK (102). After ERK is turned on, mitogen- and stress-activated protein kinase (MSK) and p90 ribosomal S6 kinase (p90rsk) become phosphorylated (102). When MSK is phosphorylated, this kinase is turned on, which can then activate the transcription factor cyclic adenosine monophosphate response element-binding protein

(CREB) (102). The activated p90rsk phosphorylates its substrate, eukaryotic elongation factor-2 kinase (eEF2K), causing it to be inhibited and leading to elevated mRNA translation (61). Together, these events increase gene expression.

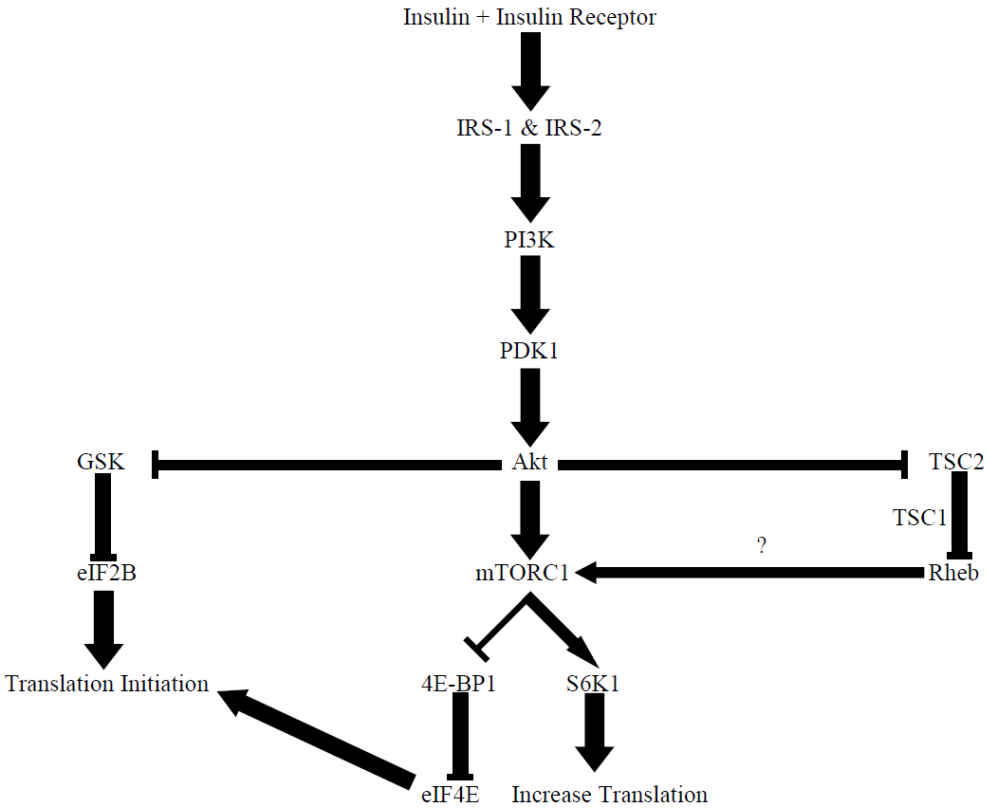

Figure 1. Mammalian target of rapamycin (mTOR) pathway. → denotes the target protein or process is stimulated while ┤ denotes the target protein is inhibited. The question mark signifies Rheb activating mTORC1 through an unknown mechanism. IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; PDK1, phosphatidylinositol-dependent protein kinase 1; GSK, glycogen synthase kinase; TSC, tuberous sclerosis complex; eIF2B, eukaryotic initiation factor 2B; mTORC1, mammalian target of rapamycin complex 1; Rheb, ras homologue enriched in brain; 4E-BP1, eukaryotic initiation factor 4E-binding protein; S6K1, ribosomal protein S6 protein kinase; eIF4E, eukaryotic initiation factor 4E.

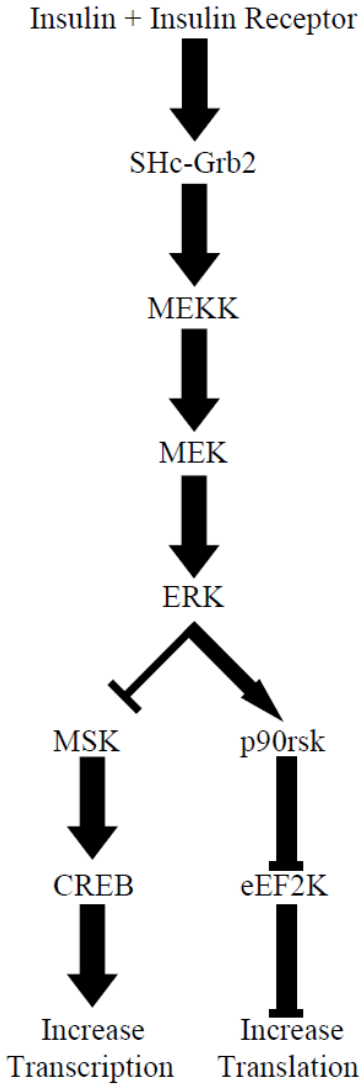

Figure 2. Mitogen activated protein kinase (MAPK) pathway. → denotes the target protein or process is stimulated while ┤ denotes the target protein or process is inhibited. Grb2, growth factor receptor-bound protein 2; MEKK, MAPK/extracellular signal-regulated kinase kinase; MEK, MAPK/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; MSK, mitogen-and stress-activated protein kinase; p90rsk, p90 ribosomal S6 kinase; CREB, cyclic adenosine monophosphate response element-binding protein; eEF2K, eukaryotic elongation factor-2 kinase.

Pathways leading to protein degradation have also been investigated. The primary route of protein breakdown is through the ubiquitin-proteasome degradation pathway. When proteins become dysfunctional, ubiquitin polymers are attached to them, targeting them for 26S proteasome catabolism (68). Three separate proteins are required to add ubiquitin to the target molecules. The initial protein, ubiquitin-activating enzyme or E1, activates the ubiquitin molecule, which is followed by the next protein, ubiquitin carrier protein or E2, taking ubiquitin to the final protein, ubiquitin ligase or E3 (68). E3 catalyzes the addition of ubiquitin from E2 to the target molecule (68). Although there are many different E3 enzymes, the two most important for muscle protein degradation is the muscle RING finger 1 (MuRF1) and the muscle atrophy F-box (MAFbx) (40). Regulatory and structural proteins at the myofibril have been reported to be broken down by MuRF1 while phosphorylated and other post-translationally altered compounds are typically acted on by MAFbx (40). Thus, the action of this pathway may lead to significant protein degradation and subsequently, muscle atrophy.

Resistance Training

Resistance training (RT) is a countermeasure known to promote muscle hypertrophy or prevent atrophy in compromised situations on Earth (1, 21, 32, 63, 98). Following a resistance exercise (RE) session, both muscle protein synthesis and degradation rise, with synthesis increasing relatively more than degradation and leading to increased net muscle protein balance (66, 93). This improvement in net muscle protein balance remains for up to 2 days after exercise (66). When RE is repeated

chronically (i.e. RT) and appropriate nutrition is provided, the proteins in skeletal muscle begin to accrue, which leads to increases in muscle mass (93).

Many human studies have investigated the effectiveness of RT in maintaining muscle mass during unloading. Following 6 months of spaceflight, thigh muscle volume of 4 exercising astronauts decreased between 4 to 7% while calf muscle volume diminished by 10 to 16% (41). When a group of men exercised with a gravity independent flywheel RE device during 29 days of bed rest, knee extensor muscle loss was prevented while plantarflexor deterioration was attenuated but remained significantly less (4). Furthermore, similar results were reported after these same subjects were assessed following 90 days of bed rest (5). In one study involving 5 weeks of ULLS, the group exercising with the flywheel significantly increased vastus lateralis muscle mass (9%), but similar to bed rest, there was atrophy of soleus muscle mass (18).

RT has also been examined with unloaded animals. Soleus muscle mass to body mass ratio and protein synthesis rates were reduced in rats undergoing 4 weeks of HU (37). When HU rats performed RT with the flywheel, that group of rats and the cage control group demonstrated comparable rates of protein synthesis (37). Although RT attenuated the decline in soleus muscle mass to body mass ratio due to unloading, the ratio in the HU rats performing RT remained significantly lower in comparison to the cage control rats (37). Consistent with these results, 2 weeks of RE reduced, but did not completely prevent, the decline in soleus muscle mass to body mass ratio (26). Thus, RT alone is not a sufficient countermeasure to preserve skeletal muscle mass.

Dietary Effects on Muscle

Nutrients such as amino acids and carbohydrates affect net muscle protein balance (93). Following consumption of amino acids, protein synthesis in skeletal muscle increases to the extent that net muscle protein balance is positive (93). Moreover, RE and amino acid ingestion produces a synergistic effect on protein synthesis and net muscle protein balance (93).

Carbohydrates also influence muscle protein balance. Following consumption of carbohydrates, protein synthesis in skeletal muscle is stimulated, but this effect requires an adequate supply of amino acids for protein synthesis (93). The addition of RE with carbohydrate consumption results in an attenuation of protein degradation, but has only a limited effect on protein synthesis (93). When carbohydrates are combined with amino acids and RE, the largest effect on net muscle protein balance is achieved (93).

Carbohydrates promote the release of insulin from pancreatic beta cells, which leads to the initiation of the mTOR pathway (93). When amino acids are present, mTOR and raptor become linked (14). Furthermore, S6K1 and 4E-BP1 are more likely phosphorylated in the presence of the amino acid leucine and activated mTOR (14).

Several studies have explored the use of amino acids as a dietary countermeasure to unloading. In one 60 day bed rest study, a group of women consumed either a normal diet or a high protein diet, which included high levels of leucine (95). The group consuming the high protein diet demonstrated knee extensor and plantarflexor muscle volume atrophies of 24% and 28%, respectively, which were comparable or even worse than bed rest only subjects (95). In another study, subjects underwent 28 days of bed

rest with and without a supplement consisting of carbohydrates and essential amino acids (65). The subjects receiving the supplement preserved leg lean mass while the bed rest only subjects lost mass (+0.2 vs. –0.4 kg, respectively) (65). However, those subjects receiving the supplement also showed elevated bone resorption (108). Finally, during 28 days of bed rest, a group of males were supplemented with essential amino acids and performed RT (16). The group supplemented with the essential amino acids and performing RT significantly attenuated leg lean mass loss (approximately –5%) when compared to a group that received amino acids only (–7%) (16). Thus, amino acids and/or RT may not be completely effective in preserving lean mass following unloading.

Dietary Cholesterol as a Countermeasure

One promising strategy may be to supplement cholesterol in conjunction with RT. Cholesterol plays multiple roles in the body, including serving as a precursor for Vitamin D and steroid hormones [androgens (testosterone), estrogens, progesterone, mineralocorticoids (aldosterone), and glucocorticoids (cortisol)]. The bile that is involved in lipid digestion and absorption contains cholesterol as well as bile acids, which are derived from cholesterol (109). Cellular membranes, including skeletal muscle sarcolemma, contain cholesterol, which provides fluidity to the membrane (84). Cholesterol, along with sphingolipids, can be found aggregated on the cell membrane to form lipid rafts that may be important structurally for proper cell signaling (83). After skeletal muscle is sufficiently overloaded following a RE session, lipid rafts are believed

to participate in the signaling pathways associated with muscle growth (70). This suggests that cholesterol may play an essential role in muscle hypertrophy not only as the building blocks for new sarcolemma but also to optimize cell signaling.

There is only a limited number of studies that examined blood cholesterol levels and RE. In one study, a decline in serum total cholesterol was observed right after a group of men completed a bout of eccentric RE (79). An earlier study reported that plasma total cholesterol decreased and remained low for 3 days after a group of young men performed a session of eccentric exercise (85). Similarly, a group of women performing eccentric contractions demonstrated reduced serum total cholesterol on days 2-4 following exercise (64). The decline in blood cholesterol may coincide with the use of cholesterol as a cellular membrane constituent for skeletal muscle repair.

Besides blood cholesterol, the transcription factor regulating the expression of the genes involved in cholesterol metabolism is also affected by RE. In one study, the mRNA for the transcription factor sterol regulatory element-binding protein-2 (SREBP-2) was elevated immediately after RE in skeletal muscle of men performing eccentric contractions to failure (59). Furthermore, at 48h post-RE, transcription of the genes targeted by SREBP-2 as well as the gene regulating SREBP-2 was augmented (59).

One training study assessed the effects of dietary cholesterol on muscle gain after 12 weeks of full body RT in elderly men and women and found that greater dietary cholesterol intake was associated with larger lean mass gains (69). However, a comparable training study did not find a relationship between dietary cholesterol and lean mass change (51). The earlier study adjusted dietary cholesterol for lean mass

while the second reported total dietary cholesterol intake. The approach taken by the first study may be more appropriate, because the adjustment accounted for muscle, the tissue that is hypothesized to utilize the cholesterol following RT (69). Thus, dietary cholesterol, when combined with RT, may prove to be an effective countermeasure against muscle atrophy.

Cholesterol Metabolism

Cholesterol in the body is derived from the diet and endogenous synthesis. Dietary cholesterol is consumed in the diet, absorbed in the digestive tract, and transported to the liver as chylomicron remnants. Endogenous synthesis, which occurs primarily in the liver, involves the cellular production of cholesterol from precursor molecules. These two sources are interdependent, ultimately determining the amount of cholesterol present in the body.

Dietary cholesterol enters the body through a sequence of steps beginning at the mouth. Following initial oral digestion, food particles enter the stomach and then the small intestinal lumen. The small intestinal lumen also receives bile, which contains cholesterol and bile acids (molecules derived from cholesterol) (109). Subsequently, mixed micelles are formed, consisting of a surface of hydrolyzed lipids and conjugated bile acids, and a hydrophobic core of non-esterified cholesterol (43). Before cholesterol can be absorbed, this mixed micelle must carry the lipids past the unstirred layer of water, which is located next to the intestinal cell surface (43). Since free cholesterol is hydrophobic, it can easily diffuse across the intestinal cell membrane (43). Once inside

the cell, cholesterol can combine with a fatty acid molecule to form cholesterol ester, which gets integrated into a chylomicron that is released into the lacteals and eventually reaches the circulation (43). Ultimately, the liver removes these chylomicrons from the blood (43).

In addition to chylomicrons, the liver also plays a role in the metabolism of other lipoproteins. The liver is responsible for synthesizing and releasing the very low-density lipoprotein (VLDL) into circulation (43). While VLDL is in circulation, cholesterol esters begin to fill the center of the lipoprotein (43). Additionally, triglycerides in VLDL are degraded and released, which decreases VLDL size and converts it into intermediate density lipoprotein (IDL) (43). As IDL travels in the blood, it eventually loses most of its triglycerides, some of its cholesterol esters, and specific apolipoproteins, transforming it into low-density lipoprotein (LDL) (43).

The LDL particle continues to travel in the blood and delivers cholesterol to cells either through desorption or receptor-mediated endocytosis (84, 94). Desorption consists of the cholesterol from the particle being transferred to the extracellular face of the cell membrane (84). Receptor-mediated endocytosis involves cells with surface LDL-receptors (LDL-R) binding to and internalizing the LDL (43). Within the cell, lysosomes merge with the internalized vesicles containing LDL (43). The proteins and lipids comprising LDL are then degraded (43). The cell can now take the liberated cholesterol and send it to the plasma membrane or store it for later use (43).

Besides exogenous sources, the body can also synthesize the 27 carbon cholesterol molecule. The process involves an acetyl-CoA molecule being converted

into cholesterol through a minimum of 21 enzyme-catalyzed steps (**Figure 3** and **Table 5**) (43). The initial 2 steps occur in the cytoplasm, while the remaining reactions take place in the endoplasmic reticulum (44, 83). Towards the later end of this pathway, there is a division on where lanosterol goes (Bloch vs. Kandutsch-Russell), with the key difference attributed to when the reduction of the double bond at carbon 24 occurs (52).

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme, which is heavily regulated (17). In the presence of sterols, cholesterol synthesis is unnecessary, leading to the catabolism of the HMG-CoA reductase protein as well a decline in the amount of its mRNA (17).

The interdependence between dietary cholesterol and synthesized cholesterol can be attributed to SREBP, specifically SREBP-2. Three isoforms of SREBP exist: SREBP-1a, SREBP-1c/adipocyte determination differentiation factor-1 (ADD-1), and SREBP-2 (75, 101). Distinct differences exist between the 3 proteins. The SREBF1 gene encodes SREBP-1 while the SREBF2 gene encodes SREBP-2 (15, 82). Each isoform targets different genes, with SREBP-1c affecting genes for fatty acid production and SREBP-1a targeting both fatty acid and cholesterol synthetic genes (80). SREBP-2 is responsible for upregulating cholesterol uptake and biosynthetic genes (17).

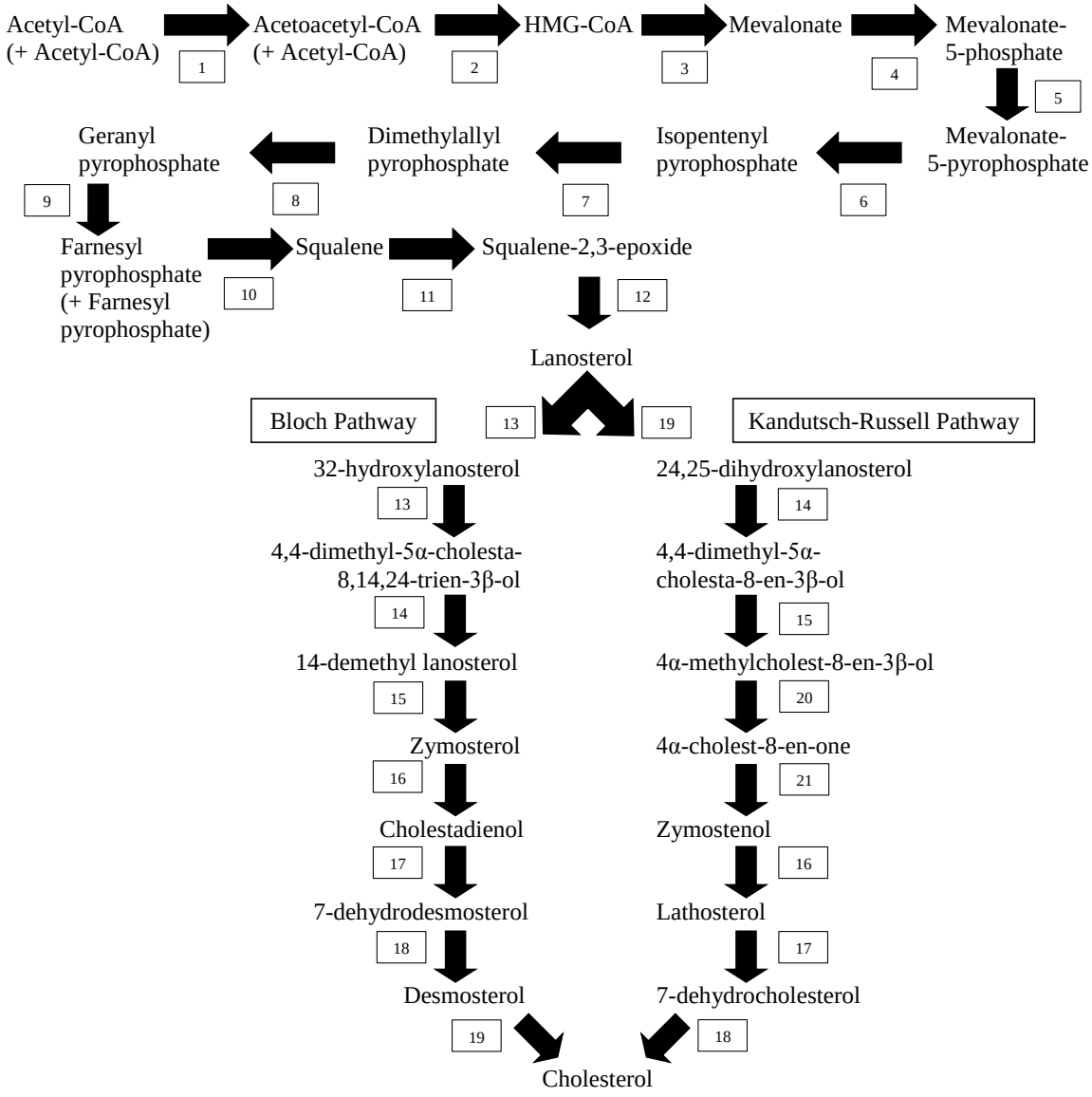

Figure 3. Cholesterol biosynthesis pathway. CoA, coenzyme A. The name of the enzymes catalyzing each respective numbered reaction is listed in Table 4. (Figure adapted from information found in 13, 44, 53, 81, and 104).

Table 5. Cholesterol biosynthesis enzymes

Reaction Number	Enzyme Name
1	Acetyl-CoA acetyltransferase
2	3-hydroxy-3-methylglutaryl-CoA synthase
3	3-hydroxy-3-methylglutaryl-CoA reductase
4	Mevalonate kinase
5	Phosphomevalonate kinase
6	Diphosphomevalonate decarboxylase
7	Isopentenyl-diphosphate Δ-isomerase 1/2
8	Farnesyl-pyrophosphate synthase
9	Geranylgeranyl-pyrophosphate synthase
10	Squalene synthase
11	Squalene monooxygenase
12	Lanosterol synthase
13	Lanosterol 14α-demethylase
14	Lanosterol 14α-demethylase
	Δ14-Sterol reductase
	Lamin B receptor
15	Methylsterol monooxygenase 1
	Sterol-4-α-carboxylate 3-dehydrogenase
	3-Keto-steroid reductase
16	3-β-Hydroxysteroid Δ8,Δ7-isomerase
17	Lathosterol oxidase
18	7-dehydrocholesterol reductase
19	Δ24-Sterol reductase
20	Methylsterol monooxygenase 1
	3-Keto-steroid reductase
21	Sterol-4-α-carboxylate 3-dehydrogenase

In order to affect cholesterol metabolism, SREBP-2 needs to be activated to its truncated mature form. The process begins at the ER membrane with immature SREBP (125 kDa) linked to SREBP cleavage activating protein (SCAP), which is a protein that senses sterols (**Figure 4**) (17, 27). During times of elevated sterol levels, the sterol-sensing region on SCAP is occupied by cholesterol, which causes SCAP to bind to another protein, insulin-induced gene (Insig), and prevent SREBP-SCAP from leaving the ER (27). Insig interacts with SCAP when oxysterols attach to Insig, providing an

additional method of sterol regulation (17). However, when sterol levels are low, Insig no longer associates with SCAP (30). As a result, SREBP-2 can now be transferred to the Golgi apparatus by SCAP to become activated (17). To accomplish this, a cytoplasmic protein called Sar1attaches to the ER, leading to Sar1 attracting a protein complex known as Sec23/24 (17). The SREBP-2/SCAP complex then associates with Sec24 (17). Next, CopII-coated vesicles that include all these proteins leave the ER for the Golgi (17).

At the Golgi apparatus, SREBP-2 is cleaved into two membrane-bound molecules by site-1 protease (17). The SREBP-2 molecule not associated with SCAP is then cut by site-2 protease, which liberates the mature SREBP-2 (68 kDa) (29). Next, the emancipated transcription factor attaches to the protein importin and together traverses the nuclear membrane (27). This process permits mature SREBP-2 to initiate transcription of specific genes by attaching to the sterol regulatory element (SRE) DNA sequence in the nucleus (29). For example, HMG CoA-reductase and LDL-R are genes that possess the SRE in their promoters (30). In fact, SREBP-2 upregulates the majority of the genes encoding enzymes involved in cholesterol biosynthesis as well as other genes participating in cholesterol homeostasis (**Table 6**) (23, 48, 81). Thus, SREBP-2 provides the link between dietary and synthesized cholesterol.

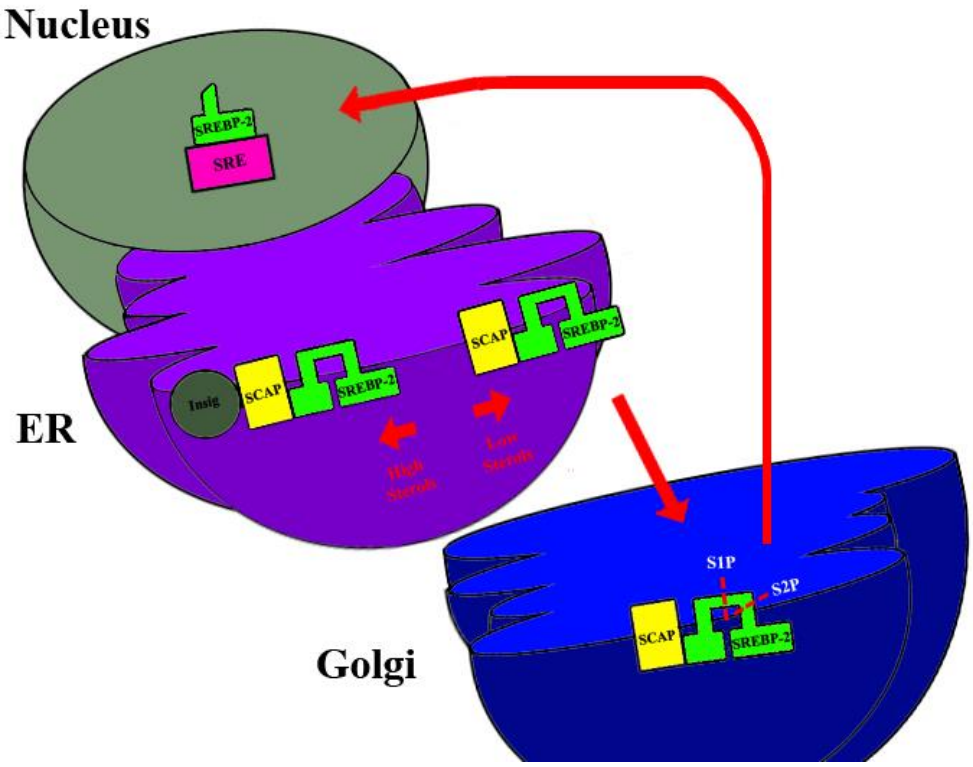

Figure 4. Sterol regulatory element-binding protein (SREBP)-2 pathway. When sterol levels are high, the SREBP-2-SCAP complex remains associated with Insig at the ER. As sterol levels become low, the SREBP-2-SCAP complex dissociates from Insig and gets transported to the Golgi. At the Golgi, two proteases, S1P and S2P, cleave the immature SREBP-2 to release a mature SREBP-2. This shortened fragment then enters the nucleus, binds to the SRE and activates the transcription of certain genes. SREBP-2, sterol regulatory element-binding protein-2; SRE, sterol regulatory element; ER, endoplasmic reticulum; Insig, insulin-induced gene; SCAP, SREBP-cleavage activating protein; S1P, site-1 protease; S2P, site-2 protease.

Several cell culture studies have provided evidence that the signaling involved in protein synthesis may also regulate SREBP-2. In Chinese Hamster Ovary cells, the SREBP-2 pathway has been reported to be activated by Akt (58). Akt is believed to activate a signal that promotes SREBP-SCAP delivery to the Golgi from the ER (24). Depending on the cell type examined, one of the downstream targets of mTORC1 may play a more prominent role in controlling cholesterol metabolism than another (100). For example, cholesterol metabolism in hepatocellular carcinoma cells is controlled by S6K1 while in NIH 3T3 cells, 4E-BP1 appears more significant (100). Besides this, MAPK signaling may regulate SREBP-2 activity in HepG2 cells (55).

Table 6. Cholesterol metabolism genes

Gene	Enzyme Coded	Function	SREBP-2 Target?
ACAT2	Acetyl-CoA acetyltransferase 2	Cholesterol Biosynthesis	Yes (59, 81)
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase	Cholesterol Biosynthesis	Yes (81)
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	Cholesterol Biosynthesis	Yes (81)
MVK	Mevalonate kinase	Cholesterol Biosynthesis	Yes (81)
PMVK	Phosphomevalonate kinase	Cholesterol Biosynthesis	Yes (81)
MVD	Diphosphomevalonate decarboxylase	Cholesterol Biosynthesis	Yes (81)
IDI1/2	Isopentenyl-diphosphate Δ-isomerase 1/2	Cholesterol Biosynthesis	Yes (81)
FDPS	Farnesyl-pyrophosphate synthase	Cholesterol Biosynthesis	Yes (81)
GGPS1	Geranylgeranyl-pyrophosphate synthase	Cholesterol Biosynthesis	Yes (81)
FDFT1	Squalene synthase	Cholesterol Biosynthesis	Yes (81)
SQLE	Squalene monooxygenase	Cholesterol Biosynthesis	Yes (81)
LSS	Lanosterol synthase	Cholesterol Biosynthesis	Yes (81)
CYP51A1	Lanosterol 14α-demethylase	Cholesterol Biosynthesis	Yes (81)
TM7SF2	Δ14-Sterol reductase	Cholesterol Biosynthesis	Yes (81)
LBR	Lamin B receptor	Cholesterol Biosynthesis	No (81)
SC4MOL	Methylsterol monooxygenase 1	Cholesterol Biosynthesis	Yes (81)
NSDHL	Sterol-4-α-carboxylate 3-dehydrogenase	Cholesterol Biosynthesis	Yes (81)
HSD17B7	3-Keto-steroid reductase	Cholesterol Biosynthesis	Yes (81)
EBP	3-β-Hydroxysteroid Δ8,Δ7-isomerase	Cholesterol Biosynthesis	Yes (81)
SC5D	Lathosterol oxidase	Cholesterol Biosynthesis	Yes (81)
DHCR7	7-dehydrocholesterol reductase	Cholesterol Biosynthesis	Yes (81)
DHCR24	Δ24-Sterol reductase	Cholesterol Biosynthesis	Yes (81)
SREBF2	SREBP-2	Transcription Factor	Yes (59)
LDLR	Low-density lipoprotein receptor	Cholesterol Uptake	Yes (48, 59)
INSIG1	Insulin induced gene 1	Regulates SREBP-2	Yes (17, 59)
INSIG2	Insulin induced gene 2	Regulates SREBP-2	No (17)

Another key regulator of cholesterol metabolism is adenosine monophosphate-activated protein kinase (AMPK). As AMP levels rise, this enzyme becomes active, which leads to the inhibition of pathways that use energy and stimulation of those that produce energy (105). HMG-CoA reductase is a target of AMPK and becomes inactive following AMPK phosphorylation (105). Collectively, this evidence suggests that cholesterol may play an important role in cellular growth and may serve as a potentially useful countermeasure for conditions associated with muscle loss, such as unloading.

CHAPTER III

THE EFFECTS OF HINDLIMB UNLOADING ON SREBP-2 AND LDL-R IN RAT SKELETAL MUSCLE

Introduction

Dietary cholesterol has garnered a negative reputation as a result of its role in cardiovascular disease (99). However, cholesterol plays an essential role in cellular homeostasis as a critical constituent of all plasma membranes, a precursor to steroid hormones such as testosterone, estrogen and vitamin D, and a component of bile acids (70, 84, 109). Furthermore, dietary and low-density lipoprotein (LDL) cholesterol have been associated with greater lean mass gains following 12 weeks of RT in older adults (69).

Cholesterol found in the body is derived from one of two sources, the diet or endogenous synthesis. Interestingly, these two seemingly distinct sources are connected to each other through a key transcription factor, SREBP-2 (17). When intracellular sterol (such as cholesterol) levels become low, inactive SREBP-2 is transported from the endoplasmic reticulum to the Golgi apparatus by SCAP (27, 30). At the Golgi apparatus, two proteases sequentially convert the 125 kDa precursor into a 68 kDa mature transcription factor (17, 29). This mature SREBP-2 then traverses the nuclear envelope and upregulates the expression of genes involved in cholesterol metabolism by attaching to their promoter (29). Target genes include cholesterol biosynthetic enzymes as well as the LDL-R, which serves a vital role in the cellular uptake of cholesterol from

the circulation (30). The increase in SREBP-2 and LDL-R can lead to higher intracellular cholesterol levels. Since cholesterol plays a critical role in cellular homeostasis and is positively associated with changes in lean mass, SREBP-2 and LDL-R may also play an important part in maintaining or altering muscle cell size in response to changing environments.

Hindlimb unloading is an established animal paradigm that has been used to mimic spaceflight and the associated muscle atrophy (, 36, 37, 50, 54, 62, 72, 106). Only one study to date has examined SREBP-2 in the context of HU (rat liver), but SREBP-2 has not been investigated in skeletal muscle (97). The purpose of this study was to examine the effects of 28 days of HU on SREBP-2 and LDL-R in rat skeletal muscle. We hypothesized that SREBP-2 and LDL-R protein expression in rat skeletal muscle would be reduced in response to HU since muscle cell size is smaller and subsequently cholesterol needs may be lower.

Methods

Animals

Thirteen 6-month-old male Sprague Dawley rats were obtained from Harlan Laboratories (Houston, TX). Following 14 days of acclimation, the rats were allocated to individual cages in a climate and light regulated (12h light/12h dark cycle) room. Rats were given free access to a standard rodent diet (Harlan Teklad 8604) and water. Rats were then assigned to one of two groups based on body mass: cage control (CC,

n=7) or hindlimb unloading (HU, n=6). All experimental procedures were approved by Texas A&M University's Institutional Animal Care and Use Committee.

Hindlimb Unloading

Rats in the HU group underwent 28 days of tail suspension (90). Following anesthesia with isoflurane, the rat's tail was cleaned with a brush and soap, rinsed, and dried with a towel. A gauze pad containing acetone was applied to the tail and a hair dryer was used to dry the tail. The tail was then covered with a quick drying adhesive (Cramer Q.D.A. tape adherent, Gardner, KS), which was allowed to dry before an additional adhesive was applied to the sides of the tail (Amazing Goop, Eclectic Products, Pineville, LA). A harness consisting of cloth tape, a bobby pin, a paper clip, and staples was placed over the second adhesive on the tail. After allowing approximately 30 min. for the second adhesive to dry, the animal was placed in an HU cage, and the harness was fixed to a pulley and cable apparatus that was situated across the top center region of the cage. This attachment was adjusted to create an approximately 30° head-down tilt in order to preclude the animal's hindlimbs from touching the walls and floor of the cage, but to permit it to move freely with its forelimbs everywhere else.

Tissue Harvest

After 28 days of HU, rats were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and medetomidine (0.5 mg/kg), and the gastrocnemius and plantaris muscles were excised, weighed and frozen immediately in liquid nitrogen for

subsequent analysis. Adrenal glands were also removed and wet weights were measured to determine the stress responses to HU.

Western Blotting

Following pulverization in liquid nitrogen, approximately 40 mg of gastrocnemius and plantaris muscle was weighed and placed in individual vials with 270 µl of a lysis buffer [25 mM HEPES, 5 mM ß-glycerophosphate, 200 µM ATP, 25 mM Benzamidine, 2 mM PMSF dissolved in DMSO, 4 mM EDTA, 10 mM $MgCl_2$, and 0.5% protease inhibitor (Sigma-Aldrich, St. Louis, MO)] and 30 µl of 10% Triton X-100. The samples were then homogenized, placed on ice for 1-1.5h and centrifuged at 14,000 rpm at 4°C for 30 min. The supernate, which contained the cytosolic and membrane-bound proteins, was saved for the bicinchoninic acid protein assay and electrophoresis (86).

Eighty micrograms of protein was obtained from each sample and mixed with Laemmli sample buffer. The samples were heated for 10 minutes and separated by SDS-PAGE for 101 min at 30 mA. The proteins from the gels were transferred to nitrocellulose membranes through a 3-step wet transfer protocol (1.3 mA/cm^2 for 1h, 3.8 mA/cm^2 for 14h, 7.5 mA/cm^2 for 1h). The membranes were blocked with non-fat milk in TBS (blocking buffer) for 1h at room temperature, washed with TBS for 5 min for 3 replicates and then incubated with either SREBP-2 (Santa Cruz Biotechnology, Dallas, TX) or LDL-R (Abcam, Cambridge, MA) primary antibodies overnight at 4ºC. The membranes were subjected to a set of 3 TBS washes and then incubated with secondary antibody for 1h at room temperature. After another series of 3 TBS washes, enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo

Scientific, Rockford, IL) was used to detect (FluorChem Alpha Innotech) the protein bands of interest. The integrated density values (IDV) of the bands were normalized to the standard for the respective membranes and to the Ponceau stain as a loading control (74). The values were reported in arbitrary units with the cage control values set at 1.

Statistical Analysis

All data were analyzed using Statistical Analysis System (SAS) Enterprise Guide (v.7.1). An independent samples t-test was used to analyze differences between HU and CC groups. For gastrocnemius mature SREBP-2, adrenal mass and adrenal mass to body mass ratio, since these variables did not pass the Shapiro-Wilk Test of Normality, a Wilcoxon rank-sum test was used instead. All data were expressed as mean ± standard error (SE) and a p-value of < 0.05 was considered significant.

Results

Effect of 28 Days of Hindlimb Unloading on Body, Muscle and Adrenal Mass

Following 28 days of unloading, there was no significant difference in body mass ($p = 0.09$) (**Table 7**), but there was a significant difference in the change in body mass between HU and CC groups (-23.8 ± 2.8 g and 12.4 ± 3.7 g, respectively, $p < 0.05$). Gastrocnemius and plantaris masses and the body mass ratios were also significantly lower ($p < 0.05$) in the HU rats than control rats. Adrenal mass to body mass ratio ($p < 0.05$), but not adrenal mass ($p = 0.08$), was significantly higher in the HU rats than the CC rats.

Table 7. Effect of 28 days of hindlimb unloading on body, skeletal muscle and adrenal mass

	Cage Control (*n = 7*)	Hindlimb Unloading (*n = 6*)
Baseline body mass (g)	481.6 ± 15.0	478.8 ± 12.8
Post-study body mass (g)	494.0 ± 16.2	455.0 ± 12.1
Gastrocnemius (mg)	2553.8 ± 77.8	1907.1 ± 53.5[a]
Gastrocnemius to body mass (mg/g)	5.18 ± 0.09	4.20 ± 0.13[a]
Plantaris (mg)	535.7 ± 19.9	420.7 ± 15.7[a]
Plantaris to body mass (mg/g)	1.08 ± 0.02	0.93 ± 0.04[a]
Adrenal mass (mg)	28.8 ± 2.3	51.7 ± 18.9
Adrenal to body mass (mg/g)	0.06 ± 0.01	0.11 ± 0.04[b]

Data are means ± standard error (SE).

[a]Significantly different ($P < 0.005$) from cage control animals

[b]Significantly different ($P < 0.05$) from cage control animals

Effect of 28 Days of Hindlimb Unloading on SREBP-2 and LDL-R in Skeletal Muscle

There were no significant differences between groups in mature SREBP-2 for the gastrocnemius ($p = 0.14$) or plantaris muscles ($p = 0.56$) (**Figure 5**). Similarly, there was no significant difference between groups in precursor LDL-R in the gastrocnemius muscle ($p = 0.48$) (**Figure 6**).

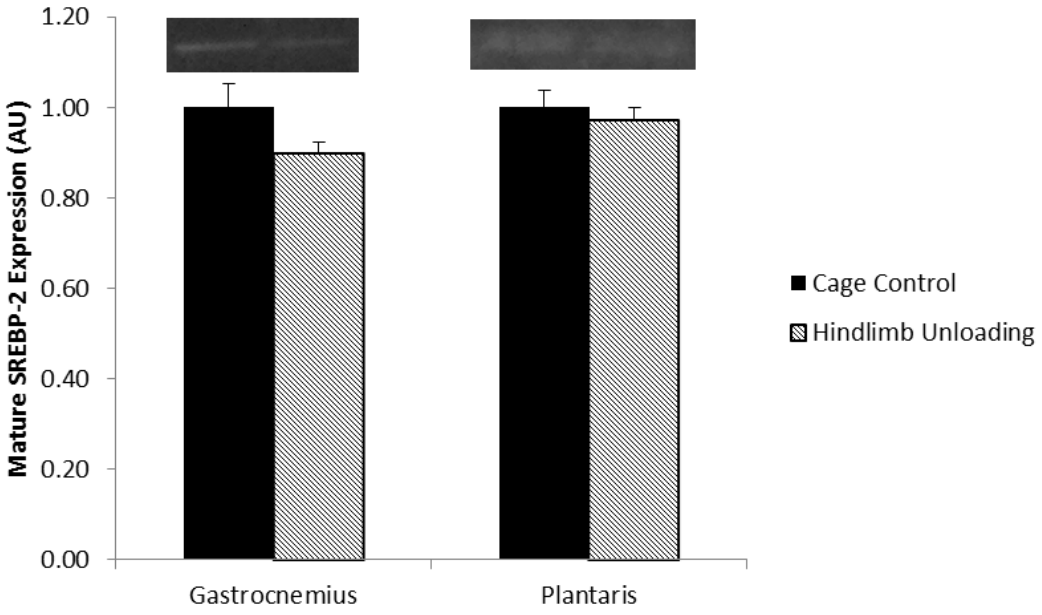

Figure 5. The effect of 28 days of hindlimb unloading on mature sterol regulatory element-binding protein-2 (SREBP-2) in gastrocnemius and plantaris muscles. Data are means ± standard error (SE). Cage Control (*n = 7*). Hindlimb Unloading (*n = 6*).

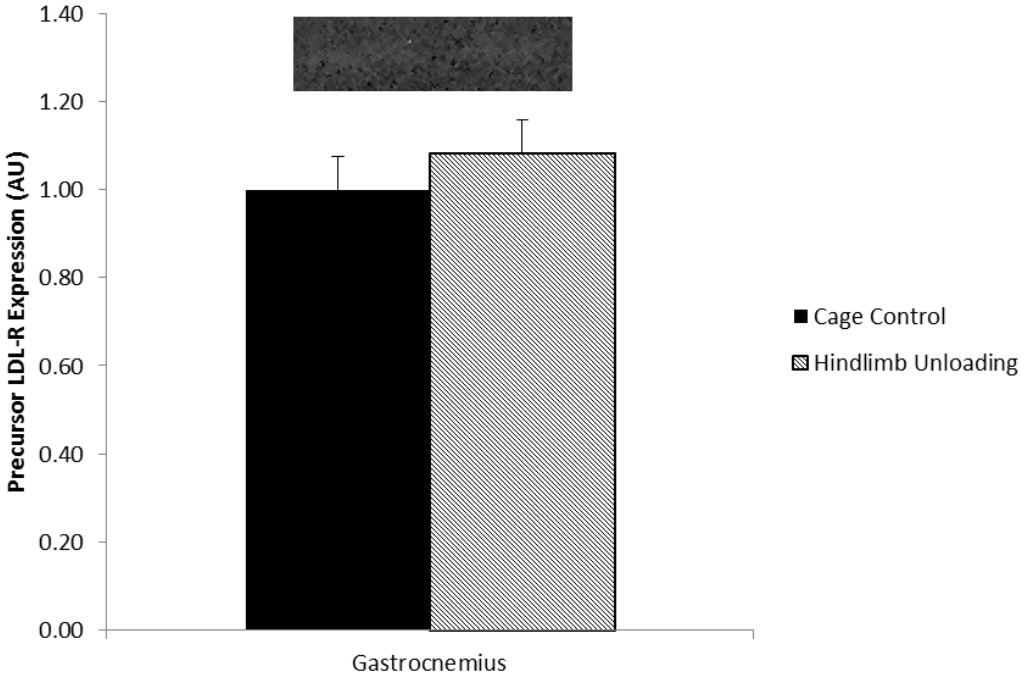

Figure 6. The effect of 28 days of hindlimb unloading on precursor low-density lipoprotein-receptor (LDL-R) in gastrocnemius muscle. Data are means ± standard error (SE). Cage Control (*n = 6).* Hindlimb Unloading (*n = 4*).

Discussion

This current study investigated the effect of 28 days of HU on muscle mass, SREBP-2, and LDL-R in rat skeletal muscle. Following HU, both gastrocnemius and plantaris muscle masses and their muscle mass to body mass ratios were significantly lower in the HU animals. Several studies previously reported significant atrophy of the gastrocnemius and plantaris muscles following various durations of HU (50, 54, 106). The gastrocnemius muscle mass to body mass ratios in the HU (4.20 mg/g) and CC (5.18 mg/g) animals in the current study were comparable to the ratios in a separate 28-day unloading study (HU: 4.05 mg/g; CC: 5.31 mg/g) (54). Furthermore, the plantaris

muscle mass to body mass ratios in the HU (0.93 mg/g) and CC (1.08 mg/g) rats also were similar (HU: 0.91 mg/g; CC: 1.08 mg/g) to that study's ratios, demonstrating the effectiveness of the HU methodology utilized in this investigation. However, to our knowledge, no studies have explored the effects of HU on SREBP-2 or LDL-R in skeletal muscle.

In the current study, mature SREBP-2 protein levels in the gastrocnemius and plantaris muscles were not significantly different between groups. However, there appeared to be a trend ($p = 0.14$) towards significance for mature SREBP-2 in the gastrocnemius muscle, with HU rats expressing lower levels. In a previous study investigating the liver of rats undergoing 14 days of HU, mature SREBP-2 expression was reported to be elevated in comparison to CC animals (97). Those results, in combination with our data, present contrasting effects of HU on cholesterol metabolism in the liver and muscle. In the liver, since HU rats have been reported to have higher hepatic cholesterol synthesis than control animals, the elevated mature SREBP-2 expression in unloaded rats may lead to higher cholesterol synthesis (97). In the gastrocnemius muscle, the lower levels of mature SREBP-2 in the HU animals compared to CC animals may reflect a diminished need for cholesterol in the atrophied muscle. Since skeletal muscle is a relatively low endogenous producer of cholesterol, SREBP-2 activation should target uptake more than biosynthesis (76, 87). Lower levels of mature SREBP-2 may lead to less cholesterol uptake, which may partially explain the elevated blood cholesterol levels observed in HU rats in a previous study (97). In one study investigating the effects of resistance exercise on mRNA levels in skeletal muscle,

SREBP-2 mRNA was reported to be elevated immediately postexercise in men (59). The greater amount of SREBP-2 may lead to higher levels of cholesterol in muscle to facilitate repair following exercise. These contrasting results demonstrate the importance of skeletal muscle activity on SREBP-2 and possibly cholesterol. Future studies should further explore the effects of RE and unloading on SREBP-2 and cholesterol.

After 28 days of HU, there was no difference in the protein expression of precursor (130 kDa) LDL-R in the gastrocnemius muscle between HU and CC groups. During western blot analysis, the LDL-R may separate into a mature (160 kDa) form and two precursor forms (110 kDa and 130 kDa). In this study, the 110 kDa precursor and 160 kDa mature LDL-R could not be detected with our technique. However, disuse has been compared to aging since both conditions are associated with decrements in muscle mass (49). In one aging study, the gastrocnemius muscle of older rats exhibited significantly lower mature LDL-R when compared with younger rats (76). Since muscle mass is lowered in both conditions, it is possible that the reduction in mature LDL-R protein content may occur in unloading as well, coinciding with a diminished need for cellular cholesterol and consequently a decrease in SREBP-2 protein levels. The reduced uptake of cholesterol by LDL-R could be problematic as this may lead to elevated LDL cholesterol and consequently, increased risk for cardiovascular disease (42).

One limitation of this study was the inability to measure total cholesterol content within the cell. Since only SREBP-2 and LDL-R protein expressions were measured,

the effects of these proteins on cholesterol homeostasis were not directly observed and can only be hypothesized. Future studies assessing cholesterol content within the cell in conjunction with measuring the expression of these essential proteins should be considered.

In summary, 28 days of HU resulted in significantly lower muscle mass in HU rats compared with CC animals. Furthermore, HU animals exhibited a trend towards lower mature levels of SREBP-2 in the predominantly fast glycolytic gastrocnemius muscle. However, unloading did not significantly affect protein levels of mature SREBP-2 in the predominantly fast oxidative plantaris or precursor LDL-R in the gastrocnemius muscles of HU animals when compared with CC rats. Thus, there may be a relationship between HU, decreased muscle mass and the proteins involved in cholesterol metabolism but the exact nature of that association remains unclear.

CHAPTER IV

THE EFFECTS OF DIETARY CHOLESTEROL AND RESISTANCE TRAINING ON PROTEIN SYNTHESIS, SREBP-2 AND LDL-R IN RAT SKELETAL MUSCLE

Introduction

The loss of muscle mass (sarcopenia) and concomitantly strength, poses a serious risk to the elderly population. Every decade starting around the age of 40, adults lose about 8% of their muscle mass, which may lead to greater body fat and higher risk for chronic diseases such as cardiovascular disease and diabetes (35, 49). Thus, there is a need to maintain and preferably increase muscle mass in this population to minimize this risk.

Resistance training with and without dietary supplementation are current interventions that have shown success in increasing or maintaining muscle. Following an acute session of RE, skeletal muscle protein synthesis rises above resting levels (66). The addition of a supplement (amino acids and carbohydrates) after a single bout of RE augments this increase in protein synthesis (67). Through repeated bouts of RT with and without supplementation, the elevated rates of protein synthesis may lead to muscle accretion, even in the elderly. For example, 10 weeks of RT increases muscle mass and strength in the elderly (32). Furthermore, postmenopausal women increased lean mass and muscular strength following 24 weeks of RT with post-exercise dietary supplementation (protein and carbohydrate) (47).

Besides dietary supplementation with protein and carbohydrate, another nutrient has also been associated with increasing lean mass. In particular, dietary cholesterol, in conjunction with RT, has been strongly associated with improvements in lean mass. We observed that older adults who performed 12 weeks of RT had significantly higher gains in lean mass with greater intakes of dietary cholesterol (69). Moreover, greater LDL cholesterol was also associated with larger gains in lean mass independent of dietary cholesterol (69).

Cholesterol is a critical constituent of all plasma membranes, including skeletal muscle sarcolemma, and provides membrane fluidity (84). Additionally, cholesterol is a component of lipid rafts, which plays a role in cellular signaling involved with muscle growth (70). One of the key proteins involved in cholesterol metabolism is SREBP-2. SREBP-2 is a transcription factor that increases the expression of cholesterol biosynthetic enzymes and cholesterol uptake proteins (LDL-R) when intracellular sterol levels are low (30). The purpose of this study was to examine the effects of dietary cholesterol on skeletal muscle responses following 5 weeks of RT in rats. We hypothesized that dietary cholesterol and RT would enhance skeletal muscle mass, elevate rates of protein synthesis and increase the protein expression of SREBP-2 and LDL-R when compared to the cage control animals and rats performing RT only.

Methods

Animals and Operant Conditioning

Forty-two male Sprague-Dawley rats (age = 5-6 months) were purchased from Harlan Laboratories (Houston, TX) and resided in individual cages within a temperature and light (12h light/12h dark cycle) regulated room at an animal housing facility. Following 7 days of acclimation, rats were randomized by body mass to one of the following groups: cage control (CC, n=7), cage control + cholesterol (CC+CL, n=4), resistance training control (RTC, n=7), resistance training control + cholesterol (RTC+CL, n=8), resistance training (RT, n=8) or resistance training + cholesterol (RT+CL, n=8). The Institutional Animal Care and Use Committee at Texas A&M University approved all experimental procedures.

Operant conditioning was used to train the RT and RTC groups to pull down on an illuminated bar situated high above the rat to turn it off. To achieve this movement, the rat needed to rise up on its hindlimbs to depress the switch. A foot shock of short duration (1 mA, 60 Hz) was utilized as negative reinforcement to promote this learning process. These animals performed this exercise for 4 sessions of 50 repetitions without any added resistance. Following these 4 sessions, an additional 2 bouts were completed with the rats performing the same protocol while wearing a non-weighted, leather and Velcro vest. Throughout the study, rats in the cage control groups did not perform any operant conditioning or RT.

Resistance Training

During the study, the RT groups engaged in 5 weeks of progressive resistance training. Each week, 3 resistance exercise sessions were completed, which were separated by at least 48h. For the 1st session, 80 g was added to the vest, and rats needed to perform 50 repetitions. Subsequently, weight was increased but repetitions were concomitantly reduced, resulting in total volume gradually decreasing by 5% per week such that the final session consisted of 16 repetitions with 410 g. The RTC groups performed similar training and experienced the same number of shocks as the RT groups; however, the former groups exercised without resistance.

Food

All rats were given free access to a standard rat chow diet (Purina Test Diet 5001). This diet consisted of 54% carbohydrates, 24% protein, 12% fat, 7% ash, 5% fiber and vitamins. However, at the start of the study, the CL groups were fed a similar chow that differed only in cholesterol levels (1800 ppm for CL vs. 180 ppm for standard chow).

Dual Energy X-ray Absorptiometry (DEXA)

At baseline and at the end of the study, DEXA (GE Lunar Prodigy) scans of the animals using a special program designed for rats were taken after administering anesthesia via Ketamine (40-55 mg/kg) and Medetomidine (0.4 mg/kg) in order to determine alterations in body composition.

Deuterium Oxide for Protein Synthesis

Twenty hours before the final resistance exercise session, 1.5 ml of deuterium oxide was injected intraperitoneally into each rat. Animals were also given free access to drinking water containing 4% deuterium oxide to maintain enrichment levels (25).

Tissue Harvest

After the rats were euthanized with Ketamine and Medetomidine, blood samples were obtained from the carotid artery, and then skeletal muscle (soleus, gastrocnemius, plantaris and quadriceps femoris) was harvested, weighed, and immediately placed in liquid nitrogen. To assess stress responses to the exercise protocol, adrenal glands were also removed and weighed.

Fractional Synthesis Rates (FSR)

Plasma samples and standards were prepared and analyzed as described previously (39). Briefly, 20 µl of plasma/standard, 4 µl of 5% acetone in acetonitrile (vol/vol), and 2 µl of 10N NaOH were combined and permitted to settle for 24h. Each sample/standard was thoroughly mixed with 600 µl of chloroform and 0.5 g of Na_2SO_4. The sample/standard was analyzed on a GC-MS [Agilent 5973N-MSD furnished with an Agilent 6890 GC System and DB17-MS capillary column].

Approximately 0.030 g of muscle (soleus and plantaris) from each sample was homogenized with 0.3 ml of 10% TCA and centrifuged for 15 min at 3,800 rpm at 4°C. The resultant pellet was then washed with 10% TCA, centrifuged, and disposed of the supernate for an additional 3 times. The pellet was then mixed with 6N HCl and incubated for 18h at 100°C. The hydrolysate was then freeze dried for 24h before 100 µl

of a 3:2:1 ratio of methyl-8, methanol and acetonitrile were added to each sample and analyzed on the GC-MS.

The following equation was used to determine the mixed muscle protein FSR:

$$E_A \cdot [E_{BW} \times 3.7 \times t]^{-1}$$

E_A signifies the quantity of ^{2}H-labeled alanine in protein (%), E_{BW} indicates the amount of ^2H_2O found in body water (%), and t represents time in h (39).

Western Blotting

Approximately 0.040 g of pulverized quadriceps femoris or red gastrocnemius muscle sample was homogenized with a lysis buffer [25 mM HEPES, 5 mM β-Glycerophosphate, 200 μM ATP, 25 mM Benzamidine, 2 mM PMSF in DMSO, 4 mM EDTA, 10 mM $MgCl_2$, 0.5% protease inhibitor (Sigma-Aldrich, St. Louis, MO)] and 10% Triton X-100. After at least 1h on ice, homogenates were centrifuged (30 min, 14,000 rpm, 4ºC) to isolate membrane-bound and cytosolic proteins (supernate) from myofibrillar proteins (pellet). The supernate was retained for SDS-PAGE, with an aliquot taken to determine protein concentration via the bicinchoninic acid method (86). Eighty micrograms of protein from each sample was combined with Laemmli buffer, heated and separated by electrophoresis on 8% gels for 101 minutes (30 mA). The proteins in the gels were transferred to nitrocellulose membranes using a 3-step wet transfer (1.3 mA/cm^2 for 1h, 3.8 mA/cm^2 for 14h, 7.5 mA/cm^2 for 1h) procedure. The membranes were blocked with 5% non-fat milk in TBS for 1h at room temperature and

washed with TBS for 3 replicates at 5 min each. The membrane was incubated with SREBP-2 (Santa Cruz Biotechnology, Dallas, TX) or LDL-R (Abcam, Cambridge, MA) antibody at 4ºC overnight. The membranes were washed again with TBS for 3 replicates at 5 min each and subsequently reacted with secondary antibody for 1h at room temperature. Following another 3 washes with TBS, the membranes were imaged (FluorChem Alpha Innotech) using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, IL). The integrated density value (IDV) for each sample was normalized to the IDV of the standard for that particular membrane and to the Ponceau stain (74). The cage control values were set at 1, with all values reported in arbitrary units.

Statistical Analysis

IBM (SPSS) Statistics 23 was used to analyze all data, which were presented as mean ± standard error (SE). Two-way analysis of variance (ANOVA) was performed for activity and cholesterol groups. A p-value of < 0.05 was accepted as significant. Post hoc analysis with the Tukey-Kramer test was used to determine significant differences between groups.

Results

Effect of Dietary Cholesterol and/or Resistance Training on Body Mass and Composition, Food Intake and Organ Masses

At baseline, there was no significant difference in body mass among groups (**Table 8**). After 5 weeks, body mass was significantly less in the RT animals compared

to the RTC groups, with no effect of dietary cholesterol. Furthermore, weight gain was significantly lower in the RT rats compared to the RTC and cage control animals. Food intake in the RT and RTC groups were significantly less than the cage control rats.

Table 8. Effect of dietary cholesterol and/or resistance training on body mass, food intake, and body composition

Variable		CC	RTC	RT
Baseline body mass (g)	No CL	408.0 ± 6.7	418.0 ± 10.3	403.9 ± 10.4
	CL	404.5 ± 10.0	412.5 ± 11.4	400.1 ± 12.0
Post-study body mass (g)	No CL	443.4 ± 14.4	463.1 ± 8.1	404.7 ± 16.7[a]
	CL	444.9 ± 12.5	444.2 ± 15.3	417.6 ± 15.1[a]
Average daily food intake (g/day)	No CL	24.1 ± 0.6	21.7 ± 0.9[b]	20.4 ± 0.8[b]
	CL	24.0 ± 0.3	22.8 ± 0.8[b]	21.4 ± 0.4[b]
Baseline lean mass (g)	No CL	338.6 ± 4.5	345.7 ± 9.8	338.8 ± 7.4
	CL	353.5 ± 15.7	344.8 ± 7.0	327.1 ± 9.4
Post-study lean mass (g)	No CL	350.9 ± 8.6	375.7 ± 10.7	336.3 ± 9.9[a]
	CL	356.5 ± 4.9	362.0 ± 11.5	343.1 ± 12.6[a]
Baseline fat mass (g)	No CL	43.6 ± 2.6	50.6 ± 6.2	46.0 ± 3.4
	CL	43.3 ± 4.4	45.4 ± 4.6	43.8 ± 2.4
Post-study fat mass (g)	No CL	72.3 ± 6.3	54.9 ± 5.9[b]	42.8 ± 6.1[b]
	CL	63.8 ± 4.5	51.8 ± 4.7[b]	43.9 ± 4.4[b]

Data are means ± standard error (group size n). CC = Cage Control. RTC = Resistance training control. RT = Resistance training. CL = Cholesterol. *n = 4-8* per group.
[a]Significantly different ($P < 0.05$) from RTC groups.
[b]Significantly different ($P < 0.05$) from CC groups.

Based on DEXA data, there were no significant differences in total lean or fat mass among groups at baseline. However, at the end of the study, the RT animals had significantly less total lean mass than the RTC groups while the RTC and RT rats had significantly lower body fat compared to the cage control groups.

Soleus, soleus to body mass ratio, gastrocnemius, gastrocnemius to body mass ratio, and plantaris were not significantly different among groups (**Table 9**). However,

plantaris to body mass ratio was significantly higher in the RT rats than the cage control animals. Adrenal mass and adrenal mass to body mass ratio were significantly higher in the RTC and RT groups compared to the cage control rats. It should be noted that gastrocnemius to body mass ratio and adrenal mass failed Levene's test for homogeneity of variances.

Table 9. Effect of dietary cholesterol and/or resistance training on muscle and adrenal mass

Variable		CC	RTC	RT
Gastrocnemius (mg)	No CL	2250.5 ± 56.3	2305.4 ± 60.4	2058.6 ± 120.0
	CL	2318.9 ± 65.5	2300.9 ± 80.8	2191.7 ± 107.7
Gastrocnemius to BM (mg/g)	No CL	5.12 ± 0.12	4.98 ± 0.07	5.18 ± 0.19
	CL	5.21 ± 0.06	5.19 ± 0.10	5.32 ± 0.09
Soleus (mg)	No CL	183.9 ± 13.3	185.6 ± 7.4	168.9 ± 9.7
	CL	183.8 ± 10.2	183.6 ± 10.6	174.3 ± 8.7
Soleus to BM (mg/g)	No CL	0.42 ± 0.05	0.40 ± 0.01	0.42 ± 0.01
	CL	0.41 ± 0.02	0.41 ± 0.02	0.42 ± 0.01
Plantaris (mg)	No CL	452.3 ± 20.6	487.4 ± 13.8	444.9 ± 19.6
	CL	462.3 ± 9.1	472.1 ± 17.4	460.8 ± 20.1
Plantaris to BM (mg/g)	No CL	1.03 ± 0.05	1.05 ± 0.02	1.12 ± 0.03[a]
	CL	1.04 ± 0.02	1.07 ± 0.03	1.12 ± 0.02[a]
Adrenal mass (mg)	No CL	39.3 ± 7.0	63.9 ± 10.5[a]	50.9 ± 4.5[a]
	CL	38.3 ± 2.7	65.1 ± 7.1[a]	64.4 ± 2.3[a]
Adrenal to BM (mg/g)	No CL	0.09 ± 0.01	0.14 ± 0.02[a]	0.13 ± 0.01[a]
	CL	0.09 ± 0.01	0.14 ± 0.01[a]	0.16 ± 0.01[a]

Data are means ± standard error (group size n). CC = Cage Control. RTC = Resistance training control. RT = Resistance training. CL = Cholesterol. BM = Body mass. *n = 4-8* per group.
[a]Significantly different ($P < 0.05$) from CC groups.

Effect of Dietary Cholesterol and/or Resistance Training on Fractional Synthesis Rates

After 5 weeks of exercise, the RT groups had significantly higher plantaris mixed muscle FSR than the RTC and CC animals (**Figure 7**). Furthermore, there was a

significant effect of cholesterol, with the CL groups showing greater plantaris mixed muscle FSR than the No CL rats. There was no significant difference among groups for soleus mixed muscle FSR (**Figure 8**). It should be noted that plantaris FSR failed both Levene's test for homogeneity of variances and the Shapiro-Wilk test of normality.

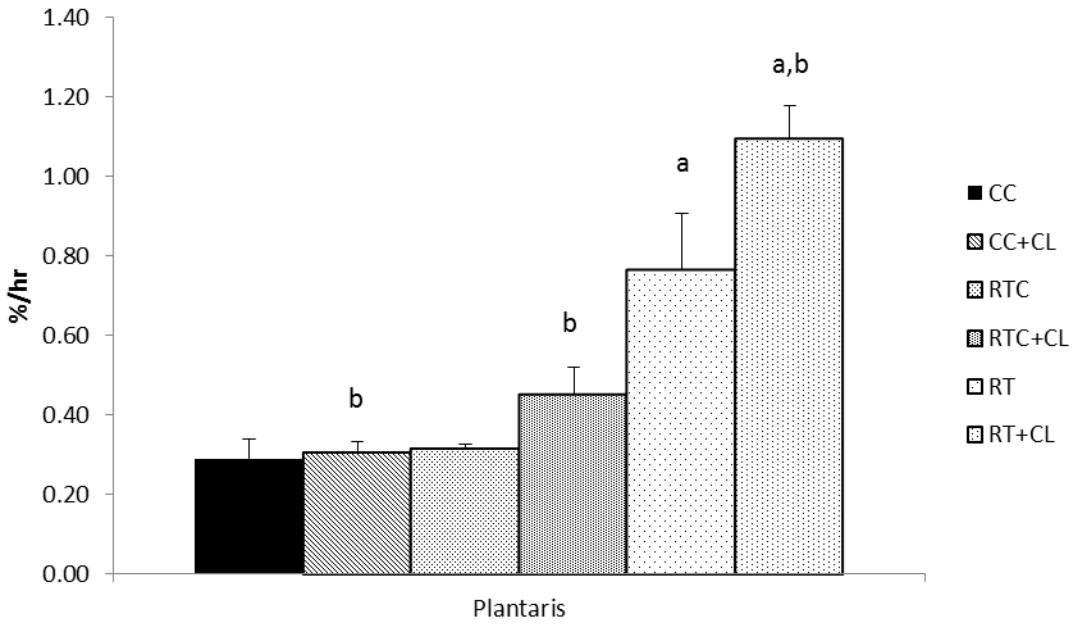

Figure 7. The effect of dietary cholesterol and/or resistance training on plantaris fractional synthesis rates. Data are means ± standard error (SE). *n = 3-5* per group.
[a]Significantly different (P < 0.05) from CC and RTC groups.
[b]Significantly different (P < 0.05) from No CL groups.

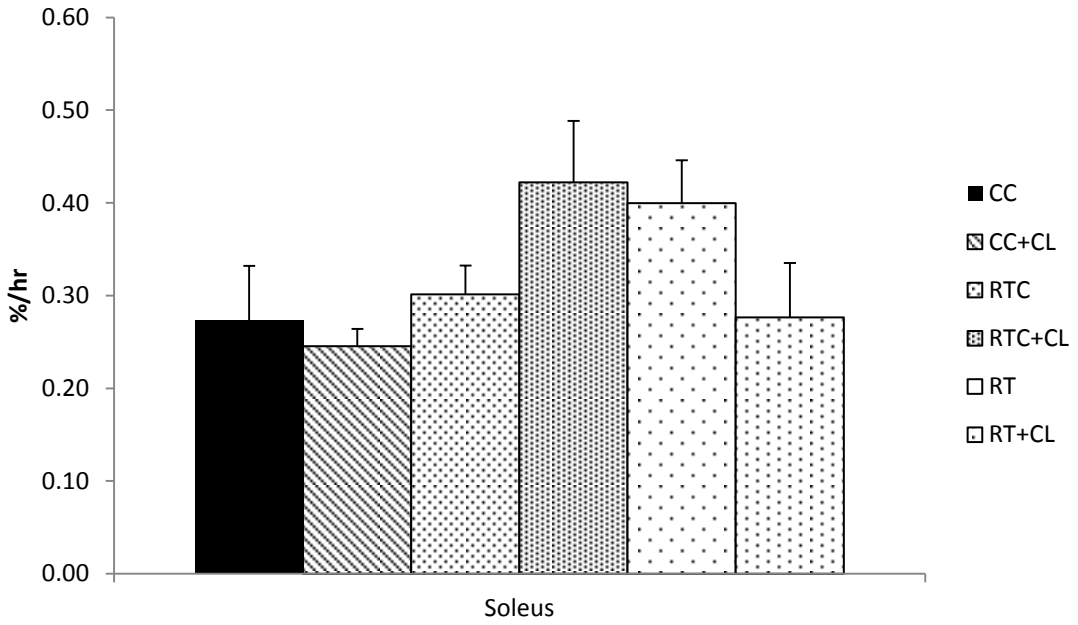

Figure 8. The effect of dietary cholesterol and/or resistance training on soleus fractional synthesis rates. Data are means ± standard error (SE). *n = 4-5* per group.

Effect of Dietary Cholesterol and/or Resistance Training on SREBP-2 and LDL-R in Skeletal Muscle

Following 5 weeks of exercise, there was no significant difference among groups for mature SREBP-2 in the red gastrocnemius (**Figure 9**) or quadriceps femoris (**Figure 10**) muscles. Similarly, there was no significant difference among groups for precursor (130 kDa) or mature LDL-R in the quadriceps femoris muscle (**Figure 11**). It should be noted that mature LDL-R for the quadriceps femoris failed the Shapiro-Wilk test of normality.

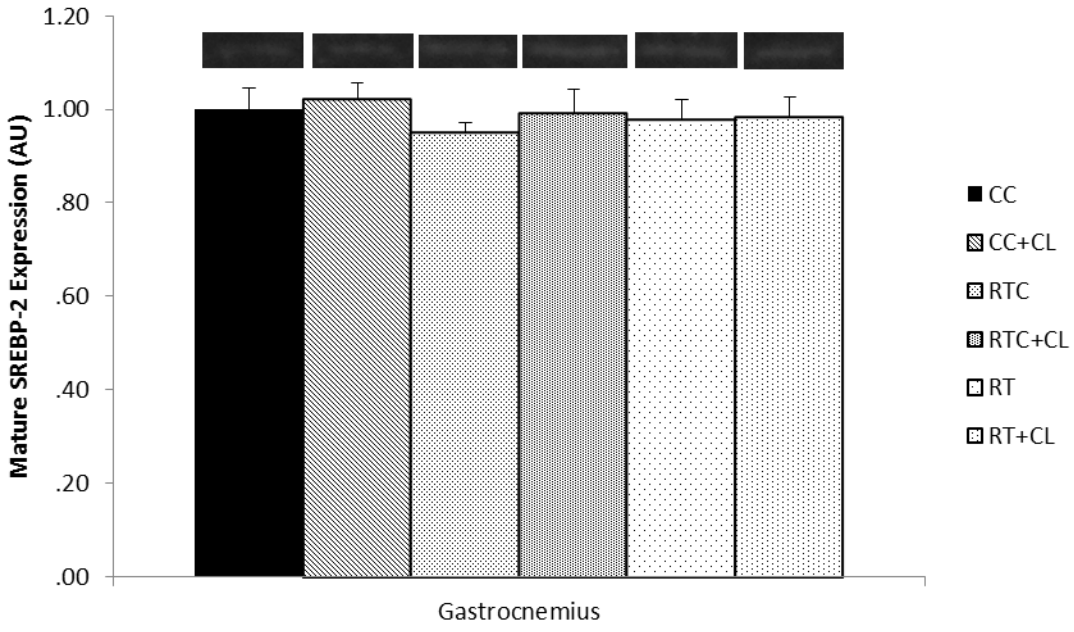

Figure 9. The effect of dietary cholesterol and/or resistance training on mature sterol regulatory element-binding protein-2 (SREBP-2) in gastrocnemius muscle. Data are means ± standard error (SE). *n = 3-8* per group.

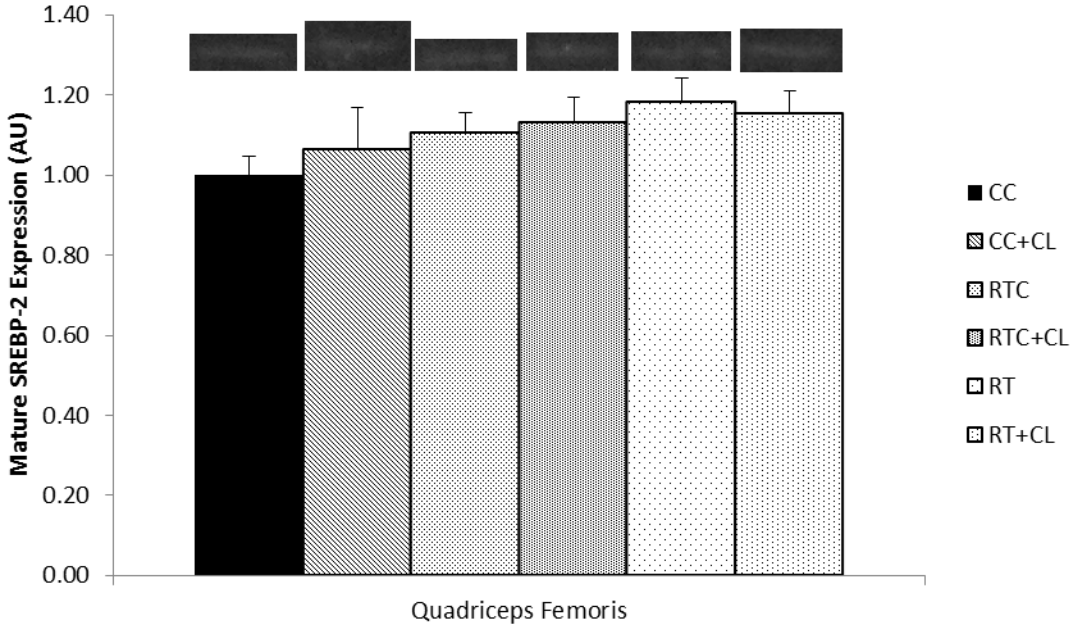

Figure 10. The effect of dietary cholesterol and/or resistance training on mature sterol regulatory element-binding protein-2 (SREBP-2) in quadriceps femoris muscle. Data are means ± standard error (SE). *n = 4-8* per group.

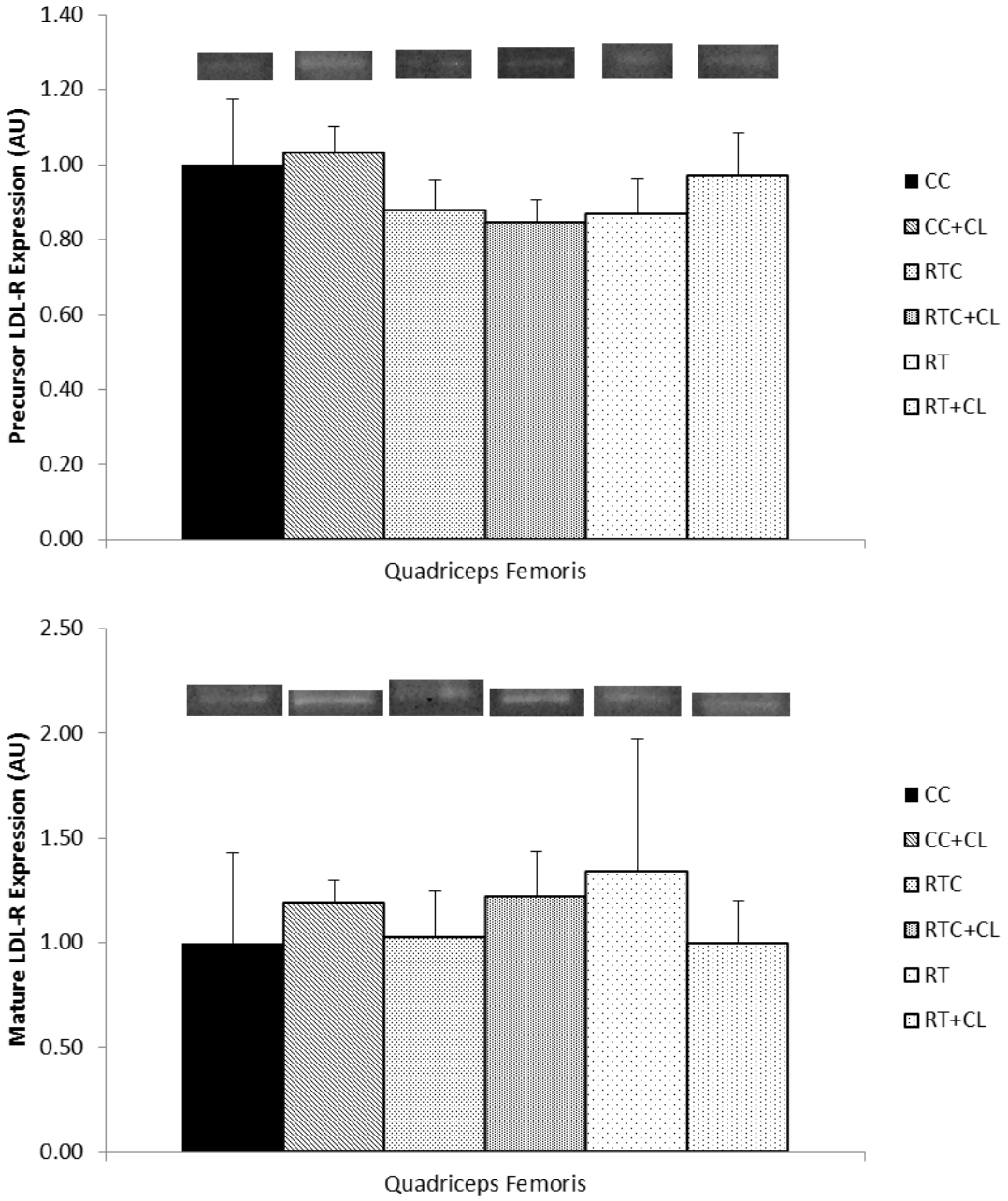

Figure 11. The effect of dietary cholesterol and/or resistance training on low-density lipoprotein-receptor (LDL-R) in quadriceps femoris muscle. (A) Precursor LDL-R. n = 3-6 per group. (B) Mature LDL-R. Data are means ± standard error (SE). *n = 3-7* per group.

Discussion

In the present study, the effects of dietary cholesterol and RT on muscle mass, protein synthesis, SREBP-2, and LDL-R were investigated in rat skeletal muscle. After 5 weeks of training, the RT animals gained significantly less body and lean mass than the RTC groups. This attenuated increase may be attributed to two related issues. The exercise performed by the RT and RTC rats was stressful, as demonstrated by the significantly higher adrenal mass and adrenal mass to body mass ratio than the cage control animals. Second, the RT and RTC groups consumed on average, significantly less food per day than the cage control rats, with the RT animals consuming the least amount. In fact, the exercise may have contributed to the lower food intake, with the stress deterring the rats from consuming more food. Thus, the combination of exercise and lower food consumption may explain why the RT groups gained less lean and body mass than the RTC animals.

The RTC groups gained the most lean mass (23.2 g) while the RT animals gained the least (6.8 g). Moreover, the cage control rats gained more lean tissue (8.9 g) than the RT groups. With the exception of the plantaris to body mass ratio, gastrocnemius, soleus and plantaris and their respective body mass ratios did not show any significant differences among groups. Additionally, post-study total fat mass was significantly lower in the RT & RTC groups than the CC animals. Taken together, this exercise protocol did not enhance muscle size but did promote overall leanness for the RTC groups. One study that utilized an exercise protocol similar to this study also reported no change in muscle mass for these particular muscles (31). However, that study did not

measure total lean mass. Future studies investigating RT in animals should include total body lean mass to provide an overall effect of the exercise protocol.

Dietary cholesterol did not appear to play a significant role in skeletal muscle gains with RT, but did with plantaris mixed muscle FSR. We observed significant gains in lean mass with greater dietary cholesterol intake following 12 weeks of RT in older adults (69). Other investigators have reported no significant effect of dietary cholesterol on lean mass gains with RT (51). Since the RT and RTC animals consumed less food than the cage control animals, the potential beneficial effects of dietary cholesterol following RT may not have been observed. Future studies should address this issue of food intake.

Following 5 weeks of exercise, the RT groups had significantly higher plantaris mixed muscle FSR and plantaris to body mass ratio than the CC animals. Since fast twitch fibers are more responsive to RT, predominantly fast twitch muscles such as the plantaris muscle are more likely to hypertrophy in response to training than slow twitch muscles (soleus) (6, 7). From one study on acute RE, both plantaris and soleus FSR were not significantly different between exercising rats and cage control animals (39). Those results, combined with our data, suggest that repeated resistance exercise is necessary to observe changes in FSR.

At the end of 5 weeks of training, SREBP-2 was not significantly different among groups in either the red gastrocnemius or quadriceps femoris muscles. However, there was a trend ($p = 0.17$) towards higher mature SREBP-2 with greater activity in the quadriceps femoris. Consistent with those results, SREBP-2 mRNA levels were higher

immediately after an acute resistance exercise session (59). We previously reported that HU animals demonstrate a trend towards lower protein levels of mature SREBP-2 than cage control rats (Chapter III). Collectively, these data suggest that cholesterol plays a role in skeletal muscle's response to various conditions. With a decrease in activity levels, mature SREBP-2 protein levels diminish, coinciding with a reduced need for cholesterol. Through activities that promote muscle growth (RT), SREBP-2 mRNA and protein levels rise, eventually leading to elevated amounts of cholesterol. This additional cholesterol could be utilized in building new cell membrane for skeletal muscle repair and growth.

LDL-R was not affected by dietary cholesterol or RT. Following 5 weeks of training, there were no significant differences among groups in either precursor (130 kDa) or mature LDL-R protein content in the quadriceps femoris. In one study, animals consuming normal or high cholesterol diets did not show significant difference in hepatic LDL-R activity or protein content (73). These data suggest that dietary cholesterol or RT may not play a significant role in promoting LDL-R protein content and that the amount of LDL-R available is sufficient to meet the needs of each environment.

In summary, 5 weeks of exercise led to greater gains in lean and body mass in the RTC rats than RT animals but higher plantaris to body mass ratio and mixed muscle FSR in the RT than the RTC (plantaris FSR) and CC (both) animals. Moreover, the groups consuming cholesterol had greater plantaris FSR than the rats not consuming cholesterol. Mature SREBP-2 in quadriceps femoris exhibited a trend where the RT groups were

greater than the CC animals. However, mature SREBP-2 in red gastrocnemius muscle and LDL-R protein levels in the quadriceps femoris were not significantly different among groups. Thus, this study provides evidence for cholesterol metabolism being associated with skeletal muscle homeostasis.

CHAPTER V

THE RESPONSE TO DIETARY CHOLESTEROL AND RESISTANCE TRAINING AS COUNTERMEASURES TO ACCELERATED MUSCLE LOSS IN RATS

Introduction

Accelerated muscle loss poses a serious problem for a variety of populations. Sarcopenia, or age-associated loss of muscle mass, is a concern for the elderly population as it may lead to higher risk for chronic diseases (49). Astronauts exposed to only 8 days of microgravity have also been reported to lose muscle (57). Countermeasures such as RT have been reported to be effective for the former, but less efficacious for the latter (32, 56). After a single session of RE, rates of protein synthesis rise above baseline levels (66). Through repeated bouts of RT, the elevated rates of protein synthesis may lead to skeletal muscle hypertrophy.

Hindlimb unloading is an established animal model of accelerated muscle loss (62, 107). After 4 weeks of unloading, soleus muscle mass and protein synthesis rates were reduced in HU rats (37). However, HU rats that performed RT demonstrated soleus protein synthesis rates comparable to that of cage control animals (37). Although RT attenuated the decline in soleus muscle mass due to unloading, the muscle mass in the HU rats performing RT remained significantly lower in comparison to cage control animals (37).

One potential solution may be to include dietary cholesterol in conjunction with RT. We reported that older adults consuming higher amounts of dietary cholesterol had

greater lean mass gains (69). Furthermore, greater LDL cholesterol was correlated with higher lean mass gains, which was independent of dietary cholesterol (69). Those effects may be attributed to cholesterol's important role as a plasma membrane constituent as well as its involvement in cellular signaling for muscle growth (70).

The cholesterol found in the body can be attributed to two sources, diet and endogenous synthesis. These two distinct sources are connected to each other through a transcription factor, SREBP-2. Normally, SREBP-2 is situated in the unit membrane of the endoplasmic reticulum in a precursor form (27). When sterol levels within the cell diminish, SREBP-2 is transported to the Golgi complex to be enzymatically cleaved (17). This mature form of SREBP-2 then enters the nucleus to upregulate genes for proteins involved with cholesterol uptake (LDL-R) and biosynthesis (17, 23).

We previously investigated the effects of HU as well as the combination of dietary cholesterol and RT on muscle mass, SREBP-2, and LDL-R. The purpose of this study was to examine the effects of those countermeasures on protein synthesis and proteins involved in cholesterol metabolism in the context of HU. We hypothesized that RT and cholesterol supplementation will maintain both protein synthesis and muscle mass at levels similar to the cage control animals. We also hypothesized that SREBP-2 and LDL-R protein levels will be down-regulated in HU rats when compared to control rat muscles but will be up-regulated with RT and dietary cholesterol.

Methods

Animals and Operant Conditioning

Thirty-six male Sprague Dawley rats (6-7 months old) were purchased from Harlan Laboratories (Houston, TX). Upon arriving, animals were housed in separate cages within a temperature controlled 12h light-12h dark cycle room. Following at least 7 days of acclimation, all rats were operant conditioned to depress an illuminated switch located above the animals to turn off the light. The movement necessary to achieve this resembles that of a jump squat. To facilitate this learning process, negative reinforcement through the use of a brief foot shock (1 mA, 60 Hz) was employed to encourage the rat to locate, jump and turn off the light switch. For the 1st 3 sessions, the rat was trained to depress the light switch or get close to it for 50 repetitions. On the 4th session, a weighted leather and Velcro vest was attached to the rat's trunk, and the rat then completed 50 repetitions.

After operant conditioning and a baseline DEXA scan, rats were randomized by body mass to one of the following groups: cage control (CC, n = 6), cage control + cholesterol (CC+CL, n = 6), hindlimb unloading (HU, n = 6), hindlimb unloading + cholesterol (HU+CL, n = 6), hindlimb unloading + resistance training (HURT, n = 6), or hindlimb unloading + resistance training + cholesterol (HURT+CL, n = 6). All experimental procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Hindlimb Unloading (HU)

Animals in the HU groups underwent 28 days of tail suspension (90). Following anesthesia with Ketamine (50 mg/kg) and Dexmedetomidine (0.5 mg/kg), the rat's tail was cleaned with soap and a brush and then dried with a paper towel. Acetone was applied to the tail with a gauze and dried with a hair dryer. Cramer Q.D.A. tape adherent (Gardner, KS) was sprayed on the tail and also dried with the hair dryer. A harness constructed of cloth tape, a paper clip, a bobby pin, and staples was trimmed to the width of the tail, and Marine Goop (Pineville, LA) was applied to the harness. The harness with glue was then attached to the sides of the rat's tail and Durapore tape was added along the length of the harness to provide additional support. The glue was allowed to dry for ~1h before the harness was attached to a pulley on a cable located along the top center region of a HU cage. The attachment height was adjusted (approximately 30° head-down tilt) to ensure that the hindlimbs did not reach the bottom or sides of the cage while permitting the animal to ambulate freely throughout the rest of the cage on its forelimbs.

Resistance Training (RT)

Rats in the RT groups participated in a progressive resistance training program (3 days per week for 4 weeks) consisting of the same exercise from operant conditioning. During the 1st week of training, the rats wore the same leather and Velcro vest from operant conditioning and performed 2 sets of 25 repetitions, with 1-2 min of rest between sets. The animals then performed the same number of sets and repetitions but the rest period was increased to 3 min and weight was added to the vest such that the

resistance was ~0.2 g, ~0.3 g, and ~0.4 g/g of body mass for weeks 2, 3, and 4, respectively.

Food

Both laboratory rodent diets were purchased from Lab Supply (Fort Worth, TX). All rats were fed ad libitum a diet (Laboratory Rodent Diet 5001) that consisted of 48.7% nitrogen-free extract, 23.9% protein, 10.7% fat, 7% ash, 5.1% fiber and vitamins. The CL groups consumed a similar diet but total cholesterol content was increased to 2000 ppm (normal diet = 200 ppm).

Dual Energy X-ray Absorptiometry (DEXA)

At baseline, day 14, and day 26 of the study, DEXA (GE Lunar Prodigy) scans using a special program designed for rats were taken after the rats were anesthetized as previously described. The same investigator completed all the body composition analyses to ensure experimental consistency.

Deuterium Oxide for Protein Synthesis

On the final day of exercise (day 27), 99.8% $^{2}H_2O$ (Cambridge Isotope Laboratories, Andover, MA) was injected intraperitoneally (20 μl/g body mass) 24h before euthanization and 8h before RE. Additionally, animals were allowed free access to drinking water containing 4% $^{2}H_2O$ up until tissue harvest.

Tissue Harvest

Sixteen hours after RE, rats were anesthetized as previously described, and blood was obtained through a cardiac puncture and separated by centrifugation. The resulting plasma was saved and immediately frozen for future analysis. Soleus and gastrocnemius

muscles as well as liver were then harvested, weighed, and immediately frozen in liquid nitrogen for later analyses (7). Adrenal glands were also excised and weighed to assess stress responses to the HU and RT protocols.

Fractional Synthesis Rates (FSR)

Plasma samples and standards were analyzed as described previously (39). Briefly, 20 µl of plasma sample/standard was mixed with 10N NaOH and 5% acetone in acetonitrile (vol/vol). After 24h, each sample/standard was mixed with Na_2SO_4 and chloroform. The sample/standard was then analyzed on a GC-MS [Agilent 5975 MSD furnished with an Agilent 7890 GC System]. All samples were run in triplicate.

For mixed muscle FSR, ~30 mg of soleus and gastrocnemius samples were mixed with 10% TCA and homogenized. For myofibrillar FSR, the myofibrillar pellets from the Western Blotting preparations were mixed with 10% TCA and homogenized. The homogenates for both analyses were centrifuged and the resulting supernate was discarded. A sequence of steps involving the addition of 10% TCA, centrifugation, and removal of supernate was repeated 2 additional times. The pellet was mixed with 6N HCl and incubated for 24h at 100ºC. Fifty µl of sample/standard was heated at 100ºC and mixed with 3:2:1 methyl-8, methanol and acetonitrile. The mixtures were heated for 1h at 70ºC before GC-MS analysis.

The following equation was utilized to assess FSR:

$$E_A \cdot [E_{BW} \times 3.7 \times t]^{-1} \times 100$$

Where E_A represents the amount of ^2H-labeled alanine in protein (mole % excess), E_{BW} signifies the quantity of ^2H_2O found in body water (mole % excess), and t indicates time in hours (39).

Western Blotting

Following pulverization, approximately 0.030 g of soleus, 0.060 g of gastrocnemius, and 0.040 g of liver from each animal was placed in individual vials and mixed with 10% Triton X100 and a kinase buffer [25 mM HEPES, 5 mM ß-Glycerophosphate, 200 µM ATP, 25 mM Benzamidine, 2 mM PMSF in DMSO, 4 mM EDTA, 10 mM $MgCl_2$, 0.5% (v/v) Protease Inhibitor (Sigma-Aldrich, St. Louis, MO)]. This mixture was homogenized and allowed to settle on ice for at least 1h. The samples were centrifuged (14,000 rpm, 30 min, 4°C) and the resulting supernates containing cytosolic and membrane-bound proteins were retained for analysis. Ten microliters of sample was removed from the supernates to determine protein concentration by the bicinchoninic acid method (86). Eighty µg of protein for each sample was separated by SDS-PAGE on an 8% gel for 101-120 minutes at 30 mA and transferred to nitrocellulose membranes through a 3 step wet transfer method (1.3 mA/cm^2 for 1h, 3.8 mA/cm^2 for 14h, 7.5 mA/cm^2 for 1h). The membranes were blocked in 5% non-fat milk in TBS (blocking buffer) at room temperature for 1h and washed 3 times with TBS for 5 min each time. The membranes were incubated overnight at 4ºC in primary antibodies [SREBP-2 (Santa Cruz Biotechnology, Dallas, TX) and LDL-R (Abcam, Cambridge, MA)]. The membranes were washed 3 times with TBS for 5 min each time and subsequently exposed to secondary antibodies for 1h at room temperature. Following

another 3 replicates of washes with TBS, the protein bands of interest were visualized (FluorChem Alpha Innotech) by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, IL). The protein bands were reported as integrated density values (IDV) that were normalized to the standard used in each membrane and to the Ponceau stain (74). The cage control values were set at 1 (arbitrary units).

Statistical Analysis

All data was analyzed on IBM (SPSS) Statistics 23. Data are presented as mean ± standard error (SE). Two-way analysis of variance (ANOVA) was conducted for cholesterol and activity groups. Significance was set at a p-value of < 0.05. Post hoc analysis using the Tukey-Kramer test was utilized to assess significant differences among groups.

Results

Effect of Hindlimb Unloading, Hindlimb Unloading plus Resistance Training, and/or Dietary Cholesterol on Body Mass and Composition, Food Intake, and Organ Mass

At the start of the study, there were no significant differences among groups in body mass (**Table 10**). After 28 days of hindlimb unloading, body mass in the HU and HURT animals were significantly lower than the CC rats, with no effect from cholesterol. Moreover, change in body mass was significantly different among groups with the HU and HURT groups losing mass (CC groups 10.9 ± 4.1 g, HU groups -47.2 ± 7.0 g, HURT groups -65.5 ± 5.4 g, $p < 0.01$). Food intake in the HU animals was

significantly higher than the CC rats. In contrast, the CL groups consumed significantly less per day than the No CL animals. It should be noted that post-study body mass and change in body mass failed the Levene's test of homogeneity of variances and the Shapiro-Wilk test of normality.

Table 10. Effect of hindlimb unloading, hindlimb unloading plus resistance training, and/or dietary cholesterol on body mass, food intake, and body composition

Variable		CC	HU	HURT
Baseline body mass (g)	No CL	496.1 ± 26.3	497.5 ± 18.9	504.3 ± 12.1
	CL	468.4 ± 25.2	477.6 ± 16.1	483.4 ± 13.9
Post-study body mass (g)	No CL	501.6 ± 22.3	444.0 ± 13.7[a]	437.6 ± 10.4[a]
	CL	484.9 ± 25.1	436.7 ± 15.3[a]	419.1 ± 8.7[a]
Average daily food intake (g/day)	No CL	23.4 ± 1.1	26.1 ± 0.9[a]	25.4 ± 1.1
	CL	22.1 ± 0.7[b]	25.5 ± 0.7[a,b]	22.2 ± 0.5[b]
Baseline lean mass (g)	No CL	427.5 ± 22.5	439.7 ± 16.8	438.5 ± 8.6
	CL	412.3 ± 18.3	433.3 ± 16.2	442.8 ± 6.2
Day 14 lean mass (g)	No CL	456.2 ± 25.0	414.7 ± 13.3	413.0 ± 6.1
	CL	436.5 ± 18.0	421.8 ± 17.9	408.8 ± 6.5
Day 26 lean mass (g)	No CL	450.8 ± 22.4	424.7 ± 15.8	412.5 ± 8.1
	CL	437.3 ± 20.1	428.2 ± 17.5	415.2 ± 8.4
Baseline fat mass (g)	No CL	50.8 ± 5.4	52.2 ± 7.5	55.0 ± 4.3
	CL	45.5 ± 7.1[b]	41.8 ± 5.4[b]	38.8 ± 5.0[b]
Day 14 fat mass (g)	No CL	50.5 ± 4.9	23.3 ± 5.6[a]	22.7 ± 6.5[a]
	CL	44.8 ± 7.6	19.0 ± 4.5[a]	16.4 ± 1.8[a]
Day 26 fat mass (g)	No CL	54.2 ± 6.1	20.5 ± 2.9[a]	19.3 ± 6.2[a]
	CL	48.5 ± 9.6	20.3 ± 1.7[a]	4.8 ± 1.2[a]

Data are means ± standard error. CC = Cage control. HU = Hindlimb unloading. RT = Resistance training. CL = Cholesterol. *n = 5-6* per group.
[a] = Significantly different ($P < 0.05$) from CC groups.
[b] = Significantly different ($P < 0.05$) from No CL groups.

At the beginning, middle and end of the study, there were no significant differences in total lean mass among groups as determined by DEXA (**Table 10**). However, there was a significant difference in total lean mass change, with all three

activity groups significantly different from each other (CC groups 24.2 ± 2.5 g, HU groups -10.1 ± 5.0 g, HURT groups -26.8 ± 5.1 g, $p < 0.01$). At baseline, the No CL groups had significantly more body fat than the CL groups. At day 14 and 26, fat mass was significantly lower in the HU and HURT groups than the CC animals, with no effect of cholesterol. Furthermore, there was a significant change in fat mass with the HU and HURT rats losing fat (CC groups 3.2 ± 2.6 g, HU groups -26.6 ± 3.9 g, HURT groups -36.5 ± 4.1 g, $p < 0.01$). It should be noted that post-study fat mass and post-study lean mass failed the Shapiro-Wilk test of normality while the day 14 lean mass failed the test of normality and Levene's test for homogeneity of variances.

Gastrocnemius, soleus, and their respective body mass ratios were all significantly lower in the HU and HURT animals when compared to the CC rats (**Table 11**). Adrenal mass was significantly lower in the HURT groups than the CC animals, but there were no significant differences among any groups for adrenal mass to body mass ratio. It should be noted that soleus, soleus to body mass ratio, gastrocnemius and adrenal mass failed the Shapiro-Wilk test of normality while the gastrocnemius to body mass ratio failed the Levene's test for homogeneity of variances.

Table 11. Effect of hindlimb unloading, hindlimb unloading plus resistance training, and/or dietary cholesterol on muscle and adrenal masses

Variable		CC	HU	HURT
Gastrocnemius (mg)	No CL	2677.1 ± 132.8	1859.2 ± 39.6[a]	1905.6 ± 58.0[a]
	CL	2609.4 ± 115.4	1764.3 ± 59.0[a]	1759.2 ± 72.3[a]
Gastrocnemius to BM (mg/g)	No CL	5.33 ± 0.08	4.20 ± 0.09[a]	4.35 ± 0.06[a]
	CL	5.40 ± 0.14	4.08 ± 0.23[a]	4.19 ± 0.12[a]
Soleus (mg)	No CL	205.7 ± 17.0	87. 3 ± 5.0[a]	94.4 ± 5.3[a]
	CL	200.0 ± 18.3	86.0 ± 4.5[a]	99.4 ± 2.3[a]
Soleus to BM (mg/g)	No CL	0.41 ± 0.02	0.20 ± 0.01[a]	0.22 ± 0.01[a]
	CL	0.41 ± 0.02	0.20 ± 0.01[a]	0.24 ± 0.01[a]
Adrenal mass (mg)	No CL	54.5 ± 4.9	45.4 ± 3.3	44.3 ± 2.3[a]
	CL	48.8 ± 5.1	47.7 ± 2.8	40.2 ± 2.5[a]
Adrenal to BM (mg/g)	No CL	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
	CL	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.01

Data are means ± standard error. CC = Cage control. HU = Hindlimb unloading. RT = Resistance training. CL = Cholesterol. BM = Body mass. *n = 6* per group.
[a] = Significantly different ($P < 0.05$) from CC groups.

Effect of Hindlimb Unloading, Hindlimb Unloading plus Resistance Training, and/or Dietary Cholesterol on Fractional Synthesis Rates

Gastrocnemius mixed muscle and myofibrillar FSR were not significantly different among activity or cholesterol groups (**Figure 12**). Similarly, there were no significant differences among activity or cholesterol groups in soleus mixed muscle or myofibrillar FSR (**Figure 13**). It should be noted that the gastrocnemius mixed muscle FSR and soleus myofibrillar FSR did not pass the Shapiro-Wilk test of normality.

Effect of Hindlimb Unloading, Hindlimb Unloading plus Resistance Training, and/or Dietary Cholesterol on SREBP-2 and LDL-R in Skeletal Muscle and Liver

Following 28 days of hindlimb unloading, there was no significant difference in mature SREBP-2 among groups in gastrocnemius, soleus, or liver (**Figures 14**, **15**, and

16, respectively). Gastrocnemius mature LDL-R (**Figure 17**) and liver precursor LDL-R (**Figure 18**) was also not significantly different among groups. Liver mature LDL-R was significantly different, with the HU groups higher than the CC animals (**Figure 18**). It should be noted that gastrocnemius mature LDL-R failed the Shapiro-Wilk test of normality.

Discussion

HU has consistently been reported to induce body mass loss as well as muscle loss in hindlimb musculature (37, 38, 46, 54). One previous study has demonstrated a partial mitigation of muscle loss with the use of RT (37). In this study, we did not observe a partial restoration of muscle mass with RT. The discrepancies between the two studies could be attributed to the intensity of the RT. Unlike that study, which utilized flywheel technology to provide variable resistance, we used a progressive RT protocol, starting with 0.1 g/g of body mass and increasing the resistance by 0.1 g/g of body mass each week. This amount of resistance may not have been sufficient to elicit an anabolic response, which is reflected by the non-significant differences among groups in FSR.

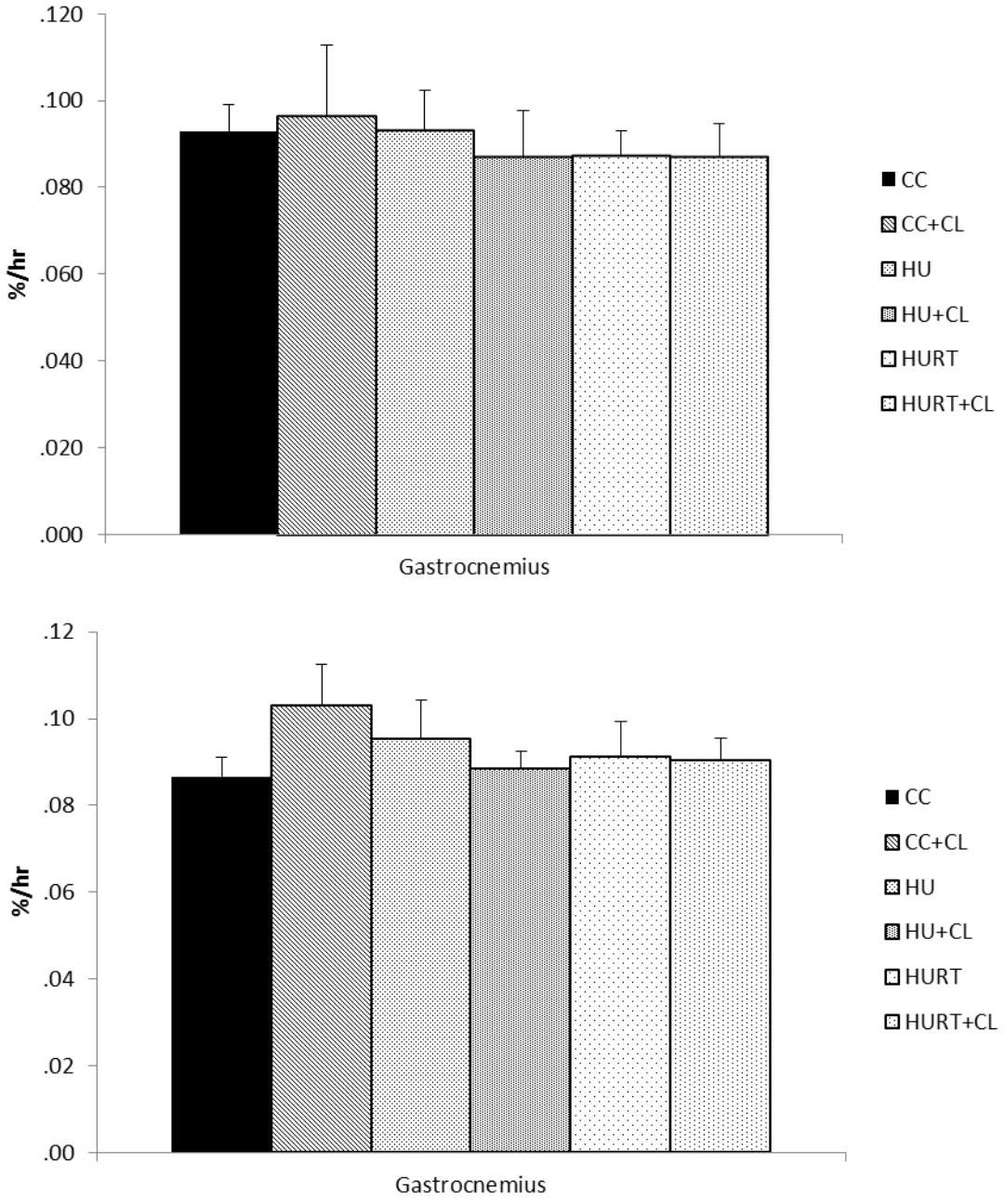

Figure 12. The effect of hindlimb unloading, hindlimb unloading plus resistance training, and/or dietary cholesterol on gastrocnemius fractional synthesis rates (FSR). (a) Mixed muscle FSR. (b) Myofibrillar FSR. Data are means ± standard error (SE). *n = 6* per group.

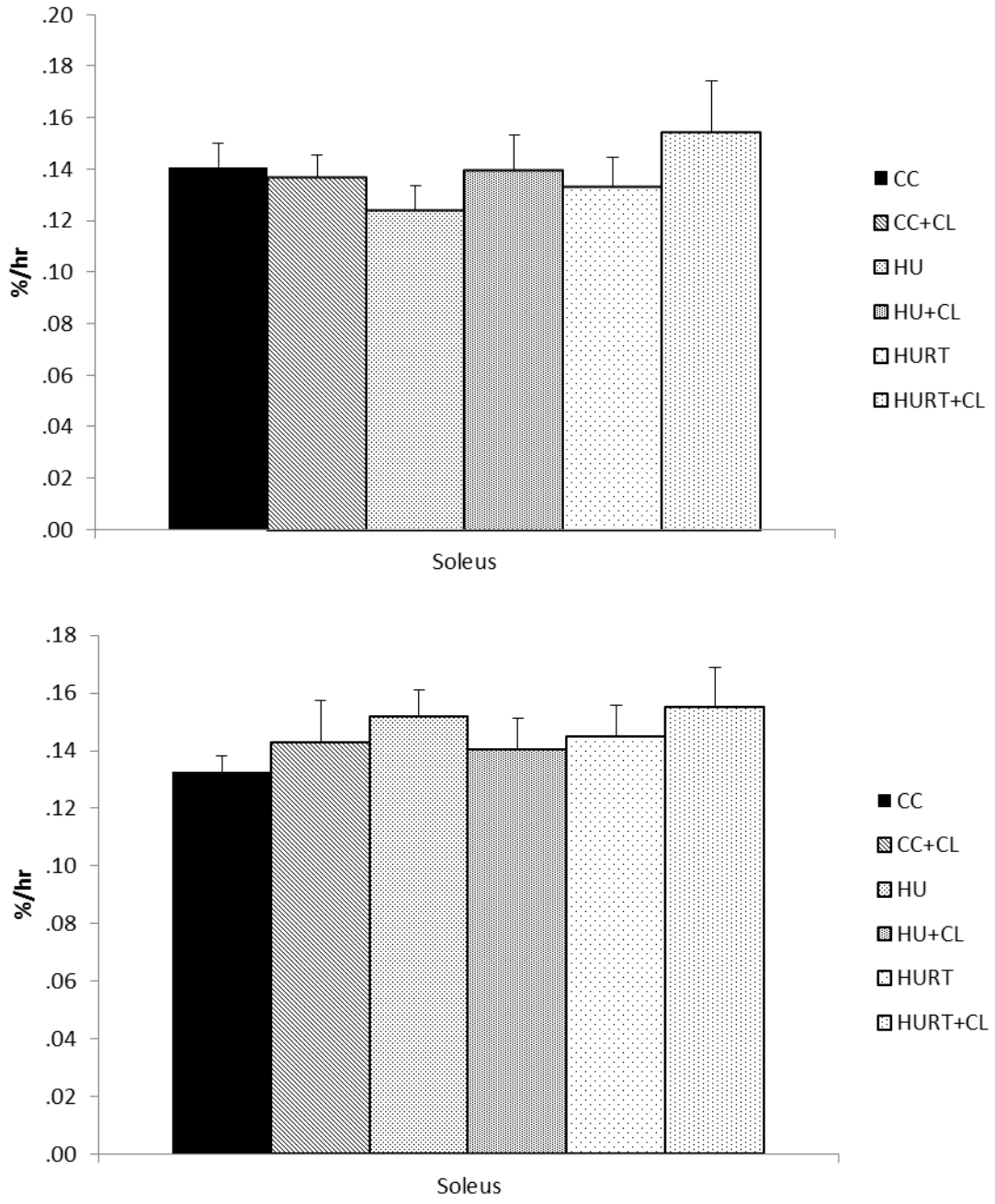

Figure 13. The effect of hindlimb unloading, hindlimb unloading plus resistance training, and/or dietary cholesterol on soleus fractional synthesis rates (FSR). (a) Mixed muscle FSR. (b) Myofibrillar FSR. Data are means ± standard error (SE). *n = 6* per group.

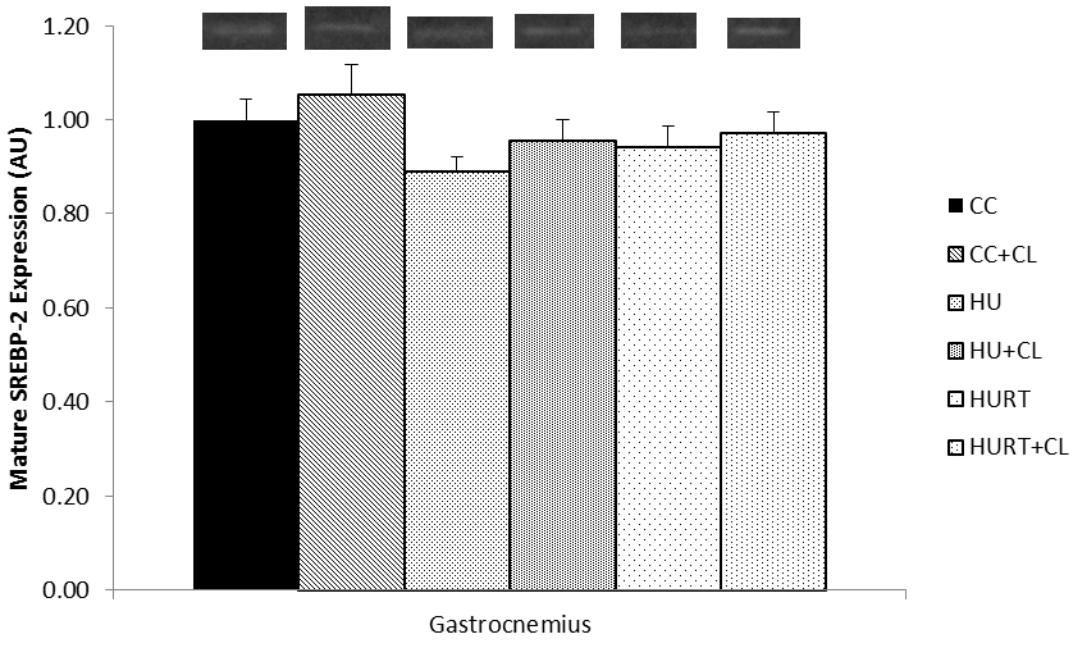

Figure 14. The effect of hindlimb unloading, hindlimb unloading plus resistance training, and/or dietary cholesterol on mature sterol regulatory element-binding protein-2 (SREBP-2) in gastrocnemius muscle. Data are means ± standard error (SE). *n = 6* per group.

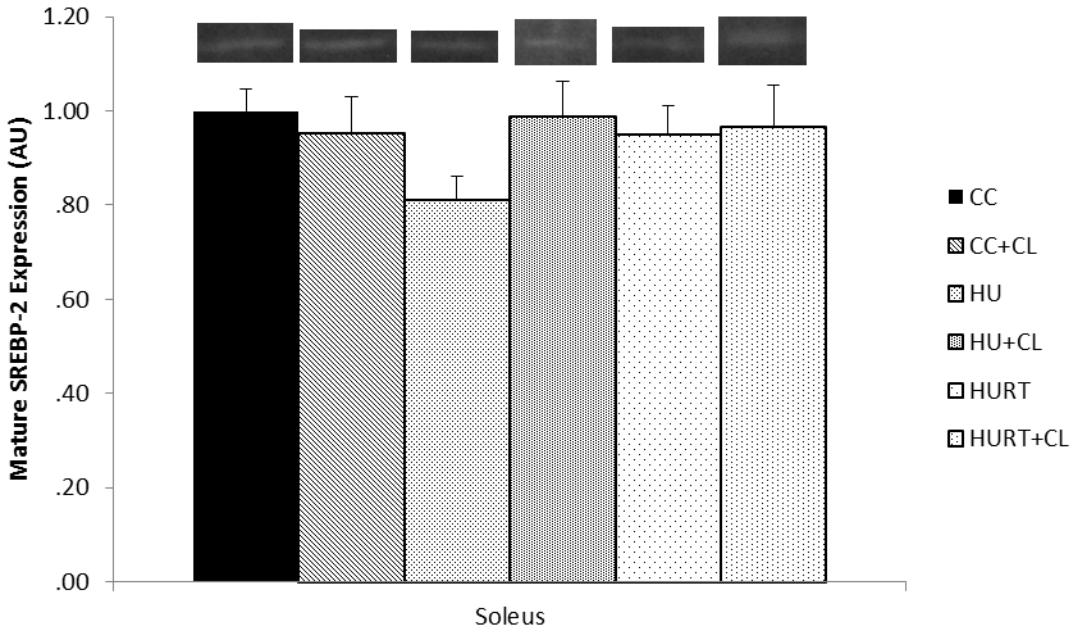

Figure 15. The effect of hindlimb unloading, hindlimb unloading plus resistance training, and/or dietary cholesterol on mature sterol regulatory element-binding protein-2 (SREBP-2) in soleus muscle. Data are means ± standard error (SE). *n = 6* per group.

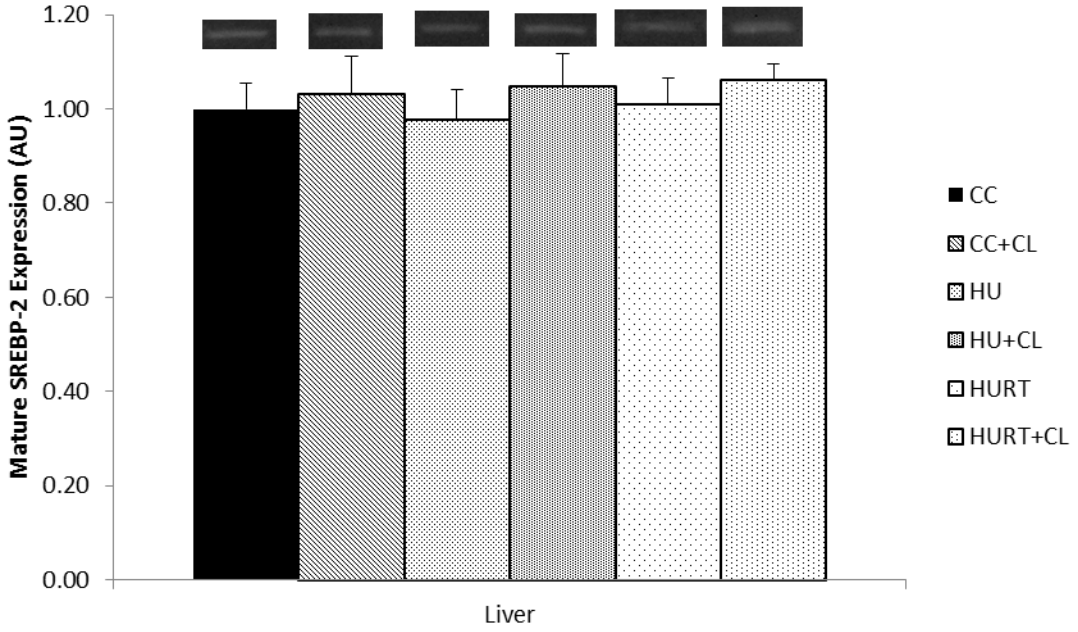

Figure 16. The effect of hindlimb unloading, hindlimb unloading plus resistance training, and/or dietary cholesterol on mature sterol regulatory element-binding protein-2 (SREBP-2) in liver. Data are means ± standard error (SE). *n = 6* per group.

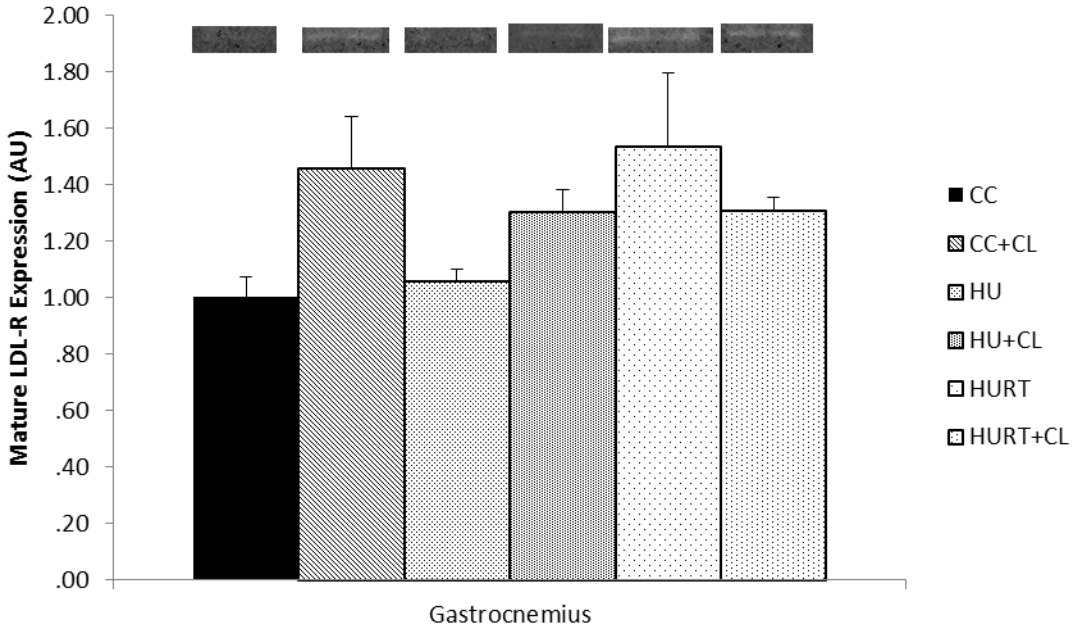

Figure 17. The effect of hindlimb unloading, hindlimb unloading plus resistance training, and/or dietary cholesterol on low-density lipoprotein-receptor (LDL-R) in gastrocnemius muscle. Data are means ± standard error (SE). *n = 2-4* per group.

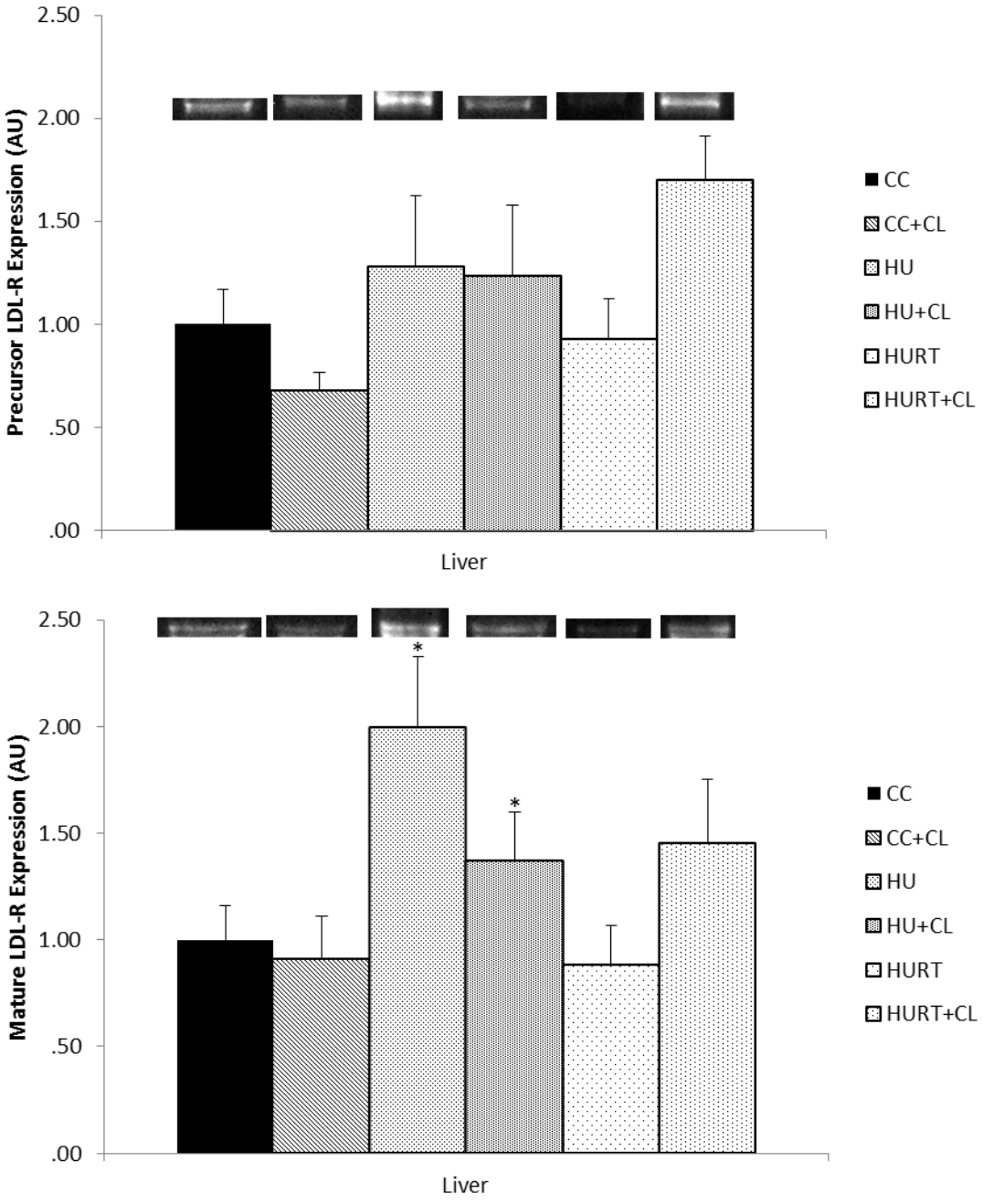

Figure 18. The effect of hindlimb unloading, hindlimb unloading plus resistance training, and/or dietary cholesterol on low-density lipoprotein-receptor (LDL-R) in liver. (a) Precursor LDL-R. (b) Mature LDL-R. Data are means ± standard error (SE). *n = 3-6* per group.
* = Significantly different ($P < 0.05$) from CC groups.

Soleus and gastrocnemius mixed muscle and myofibrillar FSR were not significantly different among groups. In a previous study, soleus muscle rates of protein synthesis were lower in HU rats while rats performing RT had rates comparable to cage control rats (37). The difference between the studies may be due to the type of RT. Fluckey (2002) used flywheel technology, which permits the animals to exert maximal effort during each repetition (5). This maximal effort from the flywheel may be necessary to increase FSR and consequently muscle mass.

Dietary cholesterol did not play a role in mitigating muscle loss or enhance rates of protein synthesis as the HU + CL and HURT + CL groups had similar muscle masses to the HU and HURT groups that did not consume additional cholesterol. This result may be attributed to the CL groups consuming significantly less food per day than the normal chow animals. Without sufficient food intake, these rats would not be provided with sufficient calories to maintain body mass as well as enough cholesterol to repair the muscle following RT. Another related concern may be a diminished uptake of cholesterol, as well as other molecules, by unloaded muscle. However, cholesterol uptake in unweighted muscle may not be impaired as one study reported muscle glucose uptake, as measured through hindlimb perfusion, is actually enhanced with unloading (89).

After 28 days of unloading, there were no significant differences among groups for mature SREBP-2 in the gastrocnemius and soleus or mature LDL-R in the gastrocnemius. Although the p-value ($p = 0.09$) did not reach significance, there appears to be a trend in the gastrocnemius with the HU animals showing lower mature SREBP-2.

This result for HU rats was similar to the results that we observed in a previous study with animals suspended for 28 days (Chapter III). Since unweighted skeletal muscle is reduced in size, there might not be a need for additional cholesterol and consequently more SREBP-2. However, RT creates an environment where extra cholesterol may be required for the repair of muscle plasma membrane and consequently growth (70). This effect may be more important in predominantly fast twitch muscles (gastrocnemius muscle) than in predominantly slow twitch muscles (soleus) that are less responsive to RT (6). Since sterol synthesis is relatively low in rat skeletal muscle, the higher SREBP-2 may lead to the greater cholesterol through uptake from the circulation (87). Our data indicate that mature LDL-R in the gastrocnemius was not significantly different among groups.

Another paradigm that has demonstrated phenotypically similar muscle atrophy is aging (49). In one study, older rats demonstrated significantly less mature LDL-R in the gastrocnemius muscle than younger animals (76). The discrepancy between the two studies may be due to this study being limited by the small sample for the 6 groups. Collectively, these results suggest a role for cholesterol in skeletal muscle homeostasis that requires further elucidation.

In the liver, mature SREBP-2 and precursor LDL-R was not significantly different among groups but mature LDL-R was different, with HU animals showing higher LDL-R protein expression than the control and HU rats performing RT. Our results on liver mature SREBP-2 differ in contrast to a previous study (97). That study reported a significant difference between groups, with the HU group expressing a higher

amount of mature SREBP-2 than the control group (97). The discrepancy between the two studies may be attributed to those authors examining mature SREBP-2 in a nuclear fraction while this study explored mature SREBP-2 in a lysate. The lysate includes mature SREBP-2 from both the nucleus and the cytoplasm, which might have diluted the higher amount of mature SREBP-2 in the HU rats than the control and HU rats performing RT. Vecchini's (2003) result for greater hepatic mature SREBP-2 suggests that unloading promotes an upregulation in cholesterol metabolism in the liver. Since that study showed greater hepatic cholesterol synthesis in unloaded rats than control animals and hepatic cholesterol concentration did not change significantly, cholesterol uptake may be decreased (97). In contrast, we observed higher mature LDL-R in the unloaded animals than the cage controls. Since Vecchini (2003) did not measure LDL-R but only SREBP-2 protein expression, future studies should further examine these proteins concurrently with hepatic and blood cholesterol content.

In summary, following 28 days of unloading, HU and HURT rats had significantly lower body and muscle masses than control animals. Furthermore, mature SREBP-2 in the gastrocnemius and mature LDL-R in the liver had contrasting effects with HU. However, gastrocnemius and soleus mixed muscle and myofibrillar FSR were not different among groups. Collectively, these results provide some evidence for the role of cholesterol metabolism in determining skeletal muscle homeostasis.

CHAPTER VI

CONCLUSIONS

Overall Conclusions

Cholesterol's role in cardiovascular disease is undisputed but what is largely forgotten is this sterol's importance in normal physiological homeostasis. For example, all cells with a unit membrane contain cholesterol, steroid hormones that are needed for normal daily functions are synthesized from this sterol and the digestion of fats requires bile acids, which are derived from cholesterol. These experiments were conducted to examine the role of cholesterol metabolism on skeletal muscle homeostasis. Based on our results, we can conclude that cholesterol metabolism is linked to skeletal muscle homeostasis in a variety of conditions.

Muscle and Lean Mass

Skeletal muscle loss poses a serious problem for older adults as well as individuals experiencing disuse (astronauts) (60, 78, 88, 92). Resistance training has been reported to be effective for the former, but not completely for the latter (19, 20, 22, 41). The effect of dietary cholesterol with resistance training on lean muscle mass gains has been equivocal (51, 69). In Chapters IV and V, we observed that the addition of dietary cholesterol with resistance training was not able to restore muscle mass that was lost due to unloading or promote muscle gains in normal ambulatory rats. However, animals performing exercise (Chapter IV) or fed the high cholesterol diet (Chapter V) consumed less food, which may prevent a benefit from the cholesterol from being

observed. Future studies should consider developing a method that ensures adequate food intake for the unloaded animals.

Muscle Protein Synthesis

Resistance exercise has been reported to increase muscle protein synthesis (66). In Chapter IV, we observed a significantly greater fractional synthesis rate in the plantaris of rats performing resistance training than cage controls and rats performing exercise without additional resistance. Moreover, groups that consumed the high cholesterol diet had higher plantaris fractional synthesis rate than normal cholesterol consuming rats. These results suggest that cholesterol does influence protein synthesis rates following resistance training, especially in the predominantly fast twitch plantaris muscle. In Chapter V, this result was not observed as gastrocnemius and soleus mixed muscle and myofibrillar fractional synthesis rates were not different among groups. This discrepancy in fractional synthesis rates could be due to the unloaded animals that performed resistance training not consuming enough rodent chow, preventing those animals from receiving the benefits of the additional cholesterol.

Proteins Involved in Cholesterol Metabolism

SREBP-2 is the master regulator of cellular cholesterol homeostasis. The production of mature SREBP-2 leads to the upregulation of cholesterol biosynthetic enzymes and LDL-R (17). In Chapters III-V, we observed trends toward significance in mature SREBP-2 in the gastrocnemius and quadriceps femoris muscles. Gastrocnemius mature SREBP-2 was lower in the hindlimb unloaded animals than cage control rats while quadriceps femoris mature SREBP-2 was higher in groups performing resistance

training than in cage control animals. These trends support our initial hypothesis involving SREBP-2 and its response to the extreme conditions tested in these studies. The greater or lesser mature SREBP-2 in each condition should consequentially upregulate or downregulate, respectively, the LDL-R. We did not observe any significant difference among groups in precursor and mature LDL-R in select muscles. We did notice that liver mature LDL-R was significantly higher in the hindlimb unloaded animals than the cage control rats. Despite greater LDL-R protein content, plasma cholesterol has been reported to be significantly higher in unloaded rats (97). The higher LDL-R reported here might not necessarily be fully functional, contributing to the higher plasma cholesterol. Future studies should assess the LDL-R protein content as well as LDL-R activity in the liver to elucidate the effects of unloading.

Final Thoughts

Through the work completed in this dissertation, the important physiological role of cholesterol was being studied in the context of activity levels and cholesterol consumption. The consumption of dietary cholesterol, once thought to contradict a healthy lifestyle, is complicated by important considerations such as if individuals are engaging in exercise, specifically resistance training. Future studies that attempt to resolve these complex roles of cholesterol on health and disease should provide a more lucid understanding of the importance of this sterol, particularly in the context of skeletal muscle in a variety of activity levels.

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