

Oxidative Stress: Aging and Disuse

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Abstract

Sarcopenia, the age-related decline of muscle mass and strength, is one major risk factor for frailty and mobility disability of the elderly. Muscle disuse due to bed rest or surgery (such as joint replacements) exacerbates the ongoing decline of muscle function in the elderly. The decline of muscle function with disuse is greater in aging muscles. However, the cellular mechanism responsible for the greater functional decline of aging muscles with disuse is unknown. Oxidative stress, a condition where the balance between oxidant production and removal is disrupted, is a shared mechanism of age and disuse related muscle dysfunction. Thus, the overall aim of my dissertation is to understand the role of oxidative stress in the age-related muscle dysfunction with disuse.

Using an animal model of muscle disuse (hindlimb unloading), I tested the hypothesis that the ability of aging muscles to cope with the increased oxidative stress associated with muscle disuse is compromised. There are three major findings: (1) the regulation of glutathione (GSH), an essential endogenous antioxidant, is impaired in aging muscles with disuse; (2) the decline of GSH levels in aging muscles with disuse is associated with the decrease of glutamate cysteine ligase (GCL) activity and the reduction of the catalytic subunit of GCL content; (3) using proteomic techniques, I identified two proteins (carbonic anhydrase III and four-and-a-half LIM protein1, FHL1), which show changes in the oxidation levels with disuse and aging. The changes in the oxidation levels of these two proteins with disuse occur in adult rats but not old rats. However, old rats have greater baseline levels of oxidized FHL1.

In summary, the series of studies demonstrate that the response of muscles with disuse is age-dependent. The ability to maintain GSH levels with disuse is compromised in aging muscles. In addition, the changes of protein oxidation

with muscle disuse occur in specific proteins and that the changes are age-related.

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Introduction

Skeletal muscles adapt with aging as well as with altered physical activity. The adaptations involve changes in muscle morphology, contractile properties and metabolic parameters. One proposed mechanism underlying the adaptations of skeletal muscles with aging and disuse is oxidative stress, a condition where the rate of the free radical production is greater than its elimination. In clinics, older individuals are observed to experience more severe muscle weakness after a period of muscle disuse due to cast immobilization or bed rest. Thus, in overall, my interest is to understand whether the disturbance of redox balance with muscle disuse is more severe in aging muscles, where the oxidative stress is already ongoing. Specifically, my thesis focuses on understanding whether the responses of muscles with disuse are age-dependent in terms of antioxidants and the accumulation of oxidized proteins.

In order to provide the background information for my thesis studies, I will first review the structure, classifications, and adaptations of skeletal muscles. Next, oxidative stress and the link between oxidative stress and muscle dysfunction are discussed. Last, the unanswered questions and thesis goals are identified (Chapter 1). In Chapters 2, 3, and 4, three related studies (my thesis studies) are described and discussed. Finally, in Chapter 5, I will summarize the main findings of my studies and demonstrate how these results contribute to the field of muscle aging and disuse.

Chapter 1

I. Skeletal Muscle

In this section, I will give an overview of skeletal muscles regarding the structures, classifications, size regulations and adaptations. The review will provide the necessary background, support and rationales of the study design used in my thesis studies.

1. Structure of skeletal muscle

Skeletal muscle is an organ consisting of muscle tissue, connective tissue, nerve tissue and blood tissue. Different from cardiac muscles and smooth muscles that contract

involuntarily, the contraction of skeletal muscles (such as force and velocity) can be controlled through the nervous system. A whole muscle (e.g., soleus muscle) is composed of many fascicles and is wrapped in a layer of connective tissue, epimysium. With the connective tissues and tendons, muscles are attached to the skeleton (bones) (**Figure 1**).

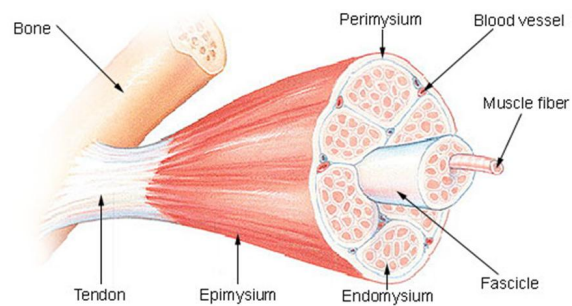


Figure 1. The structure of skeletal muscle. A whole muscle (wrapped in epimysium) is composed of many fascicles (wrapped in perimysium), which is composed of a bundle of muscle fibers (wrapped in endomysium). Source: http://training.seer.cancer.gov/module_anatomy/unit_4_2_muscle_structure.html

Fascicle. A fascicle is composed of a bundle of muscle fibers and is surrounded by a layer of connective tissue called perimysium. Small blood vessels and motor axons transverse the perimysium to connect with muscle fibers. In addition, muscle spindles (sensory receptors that detect the changes of muscle length) are found in the perimysium. In a fascicle, fibroblasts are located

between the muscle fibers and secrete collagen fibers to form the connective tissue matrix, the endomysium.

Muscle fiber. A muscle fiber is a muscle cell. Different from cardiac muscle cells, smooth muscle cells and other types of cells, skeletal muscle cells are multinucleated and have satellite cells (muscle stem cells) adjacent to them. The cell membrane of a muscle fiber is also called the sarcolemma. The basement membrane, located on the outer side of the sarcolemma, is composed of a loose collagen network with glycoproteins. Satellite cells are located quiescently in the space between the sarcolemma and the basement membrane until activated.

Myofibril. Same as other types of cells, skeletal muscle cells have organelles (nucleus, mitochondria, proteasomes, et al.) and proteins (ubiquitin, ion channels, enzymes, et al.) that ensure the multiple cellular functions. Different from other cell types, in skeletal muscles, contractile proteins and

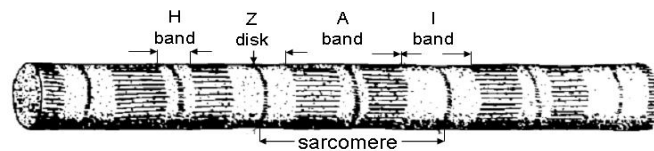


Figure 2. The structure of a myofibril. A myofibril is a serial assembly of sarcomeres and appears striated under the light microscope, due to alternative dark (A) and light (I) bands. Modified from Bloom, W. & Fawcett, D.W. 1994. *A Textbook of Histology*, ed. 12, Chapman & Hall, New York.

structural proteins are arranged into highly ordered arrays called myofibrils (**Figure 2**). Each myofibril is a serial assembly of sarcomeres, the contractile units of the muscle cells. Myofibrils appear striated under the light microscope, and the dark areas are termed A bands and the light areas are termed I bands. More detailed definitions of the terms used in describing myofibrils will be discussed later.

Sarcomere. A sarcomere is defined from Z disk to Z disk in a myofibril (**Figure 2**). Sarcomeres are the contractile units of the muscles and are composed of contractile proteins and structural proteins. Contractile proteins (myosin and actin) function to generate muscle contraction. Structural proteins (such as titin

and α -actinin) integrate the contractile proteins in the sarcomeres and maintain the integrity of sarcomeres when the lengths of the muscles are changed (**Figure 3**).

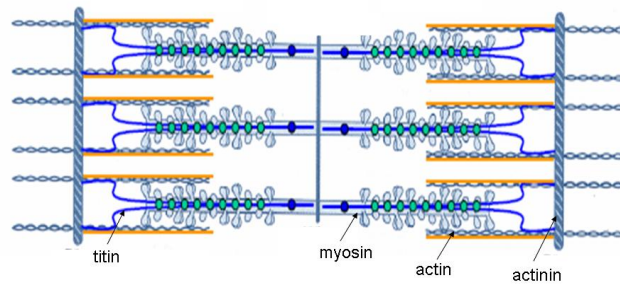


Figure 3. Structure of sarcomere

Proteins in skeletal muscles can be categorized into 3 types: (1) proteins that perform contractile functions (contractile proteins), (2) proteins that integrate the contractile proteins and link them to the sarcolemma (structural proteins), and (3) proteins that maintain cellular functions (house keeping proteins). The three types of proteins are discussed in detail below.

1.1 Contractile proteins

Contractile proteins comprise about 80% of total proteins and are considered the major proteins in skeletal muscle cells (74). The main contractile proteins are myosins and actins. A myosin molecule is composed of two identical myosin heavy chains with a molecular weight of approximately 200 kDa each. The two myosin heavy chains bind with four light chains (2 regulatory light chains and 2 essential light chains) to form thick filaments. The thick filaments contribute to the dark area (A band) observed under the microscope in myofibrils (**Figure 2**). Actins (globular proteins, G-actins) are molecules with a molecular weight of approximately 42 kDa. The G-actin polymerizes into double helical strands called F-actins or actin filaments. Actin filaments together with

regulatory proteins (tropomyosin, troponin I, troponin C and troponin T) form the thin filaments. The areas where thin filaments do not overlap with thick filaments are called I bands in the myofibrils (**Figure 2**).

The interactions between thick and thin filaments result in the length changes of the sarcomeres that ultimately give rise to muscle contraction. Thus, any damage in the proteins that comprise the thick and thin filaments may influence the normal function of muscles. In addition, since contractile proteins are the major proteins in the muscle cells, the degradation of these proteins will significantly reduce the fiber (muscle) size.

1.2 Structural proteins

There are two main groups of structural proteins in muscle cells. One group of structural proteins is involved in anchoring the contractile proteins in the sarcomere and links sarcomeres in series. This group of structural proteins is primarily the proteins in the Z disc. The other group of structural proteins is involved in anchoring the contractile apparatus to the sarcolemma.

Proteins in the Z disc function as a scaffold that link the contractile apparatus in series. These proteins include titin, α -actinin, muscle LIM proteins, four and a half LIM protein 1 (FHL1), telethonin, et al. Titin is the protein that runs from Z disc to the middle of A band (**Figure 3**) and functions as an elastic element that connects myosin with the Z disc. α -actinin is the main protein in the Z disc and links actin filaments and titins to the Z disc. Muscle LIM proteins are located in the Z disc and can translocate to the nucleus. There are two main functions of muscle LIM proteins: (1) stabilizing the structure of the Z disc when they are in the Z disc and (2) being the signals for myogenesis when they are translocated to the nucleus. FHL1, one member of LIM-only protein family, is found predominantly at the I band. FHL1 plays a role in the assembly of the sarcomere by interacting with myosin-binding protein C (113). In addition, FHL1

also functions as a regulator of myogenesis and muscle growth (113, 114, 116, 135). Telethonin is located in the Z disc and functions as anchors of titin (139).

Proteins that anchor the contractile apparatus to the sarcolemma include dystrophin, talin, desmin, et al. This group of proteins not only translates the force generated from myofibrils but also stabilizes the cell membrane of muscles (sarcolemma). For example, dystrophin increases the stiffness of the sarcolemma, thus preventing the disruption of the sarcolemma during muscle contraction (124). Muscles lacking dystrophin, seen in individuals with Duchenne Muscular Dystrophy, undergo the contraction-induced muscle degeneration.

Collectively, structural proteins are crucial regarding the stability and the function of muscle cells. The damage or degradation of structural proteins may disrupt the integrity of sarcomeres that results in sarcomere disassembly. The consequence of the sarcomere disassembly is the release of myofilaments, which will result in protein degradation and muscle atrophy.

1.3 House keeping proteins

In addition to contractile proteins and structural proteins, many other proteins are in the skeletal muscle cells to maintain the survival of the cells. These proteins include proteins in the electron transport chain (ETC), enzymes, antioxidants, chaperones, proteasomes (proteins that degrade proteins), et al. House keeping proteins are critical for muscles to have normal contractile functions, and the damage of these proteins can affect muscle functions enormously. For example, ETC proteins function as the provider of energy for the muscle contraction. Damage of ETC proteins not only influences the supply of energy but also generates excessive free radicals that attack proteins, phospholipids and DNA. Another example of the link between muscle dysfunction and damaged house keeping proteins is the sarcoplasmic-reticulum

(SR) Ca^{2+} -ATPase (SERCA). SERCA is the protein responsible for the uptake of cytosolic Ca^{2+} into the sarcoplasmic reticulum, thus playing an essential role in muscle relaxation. Damage of SERCA reduces the efficiency of Ca^{2+} uptake, which not only retards the muscle relaxation but also disrupts the homeostasis of intracellular calcium. Last, since antioxidants, chaperones and proteasomes prevent, repair and remove damaged proteins, their dysfunctions lead to accumulation of damaged proteins in the cells that affect negatively the cell functions.

Collectively, this section demonstrates that muscle, the organ that allows movement and performance of daily activities, is composed of various types of cells and one of them is muscle cells. The normal function of muscle cells is essential for muscle contraction and force generation. Since there are many different proteins in a muscle cell, the specific functions of these proteins determine the overall function of the cell.

Mammals have various types of skeletal muscles depending on the isoforms of proteins in the cells. While all types of skeletal muscles have contractile functions, they are different in the contractile force, contractile velocity, fatigability, et al. The differences among various types of skeletal muscles are addressed next.

2. Types of skeletal muscle

Muscle fibers are classified genetically based on myosin heavy chain (MHC) isoforms: MHC I, MHC IIa, MHC IIx and MHC IIb. A muscle can be composed of more than one isoform and the percentage of each isoform within a muscle is different among muscles. In rats, soleus muscles are composed of 100% of MHC I, and gastrocnemius muscles are composed of 5% of MHC I, 7% of MHC IIa, 31% of MHC IIx and 57% of MHC IIb (127). Different isoforms of MHC

possess different biochemical properties that regulate contractile function of the muscle fibers. Muscle fibers containing primarily MHCI contract most slowly while fibers having primarily MHCIIb contract fastest (54).

Another classification of muscle fibers are based on their properties: myosin ATPase (type I, IIa and IIb), contractile speed (slow and fast), metabolic enzymes (oxidative and glycolytic) and fatigability (fatigue resistant and rapidly fatigued). Properties of different muscle fiber types are shown in

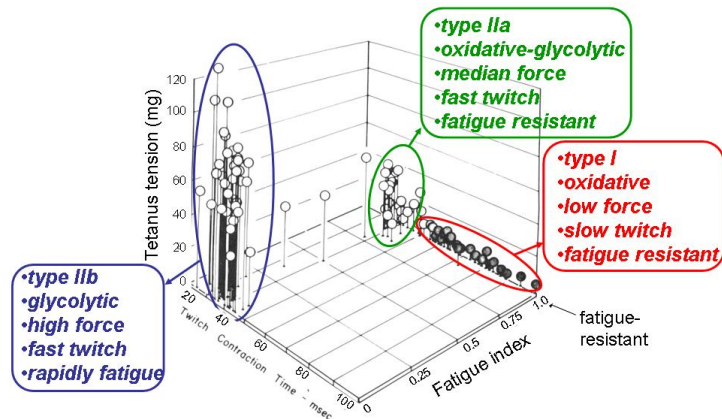


Figure 4. Fiber types based on contractile properties. Muscle fibers are classified into 3 types, type I, IIa and IIb, based on myosin ATPase, which affects the contractile speed of muscle fibers. Modified from Burke, *Handbook of Physiology, Section 1, Vol. II; p325, 1981.*

Figure 4. In brief, type I muscle fibers are more sensitive to recruitment (are activated at low load demand), are smaller in fiber size, have lower maximal contractile velocity as well as force, have more mitochondria and oxidative enzymes, and are fatigue resistant. Type I muscle fibers are activated in low intensity activities such as standing and walking. Postural muscles such as soleus muscles usually contain high percentage of type I muscle fibers. Type II muscle fibers, on the contrary, are less sensitive to recruitment (are activated at high load demand), are larger in fiber size, have higher maximal contractile velocity as well as force, have more glycolytic enzymes, and are rapidly fatigued. Type II muscle fibers can produce large power and are activated in activities that require higher force and velocity such as jumping. An example of muscles that contain high percentage of type II muscle fibers is the gastrocnemius.

Collectively, skeletal muscles are heterogeneous in terms of their morphological properties, contractile/physiological properties, and metabolic properties. Because of these differences, muscles are affected differently with specific stimuli. For example, the stimulus of muscle unloading has the most significant effect on muscles that are usually weight bearing (e.g. soleus muscle), and has less effect on muscles that do not bear weight such as the extensor digitorum longus. The muscle-dependent effect of stimuli provides the rationale of the selection of muscles that I investigated in my thesis studies.

3. Regulation of muscle size

The size of a whole muscle is primarily determined by the *number* and the *size* of muscle cells. The *number* of muscle cells that reflects the balance of cellular turnover remains relatively constant after adulthood and only changes significantly with aging. In contrast, the *size* of muscle cells that reflects the balance of protein turnover (protein synthesis and degradation) adapts with various conditions. Aging, physical activity, muscle length, hormones, growth factors, nutrition, mechanical stress and diseases are factors that influence muscle size via the regulation of protein synthesis and degradation pathways (120, 138).

3.1 Protein synthesis and degradation pathways

The main protein synthesis pathway in skeletal muscles is the serine/threonine kinase Akt-mammalian target of rapamycin (AKT-mTOR) signaling pathway. Muscle contractile activity, passive stretch, nutrients, hormones (such as glucocorticoids) and growth factors such as (insulin-like growth factor 1) are shown to activate AKT-mTOR pathway and result in increased protein synthesis.

Protein degradation is mainly regulated by nuclear factor-kappa B (NF- κ B) pathway and Forkhead box O (FoxO) pathway. NF- κ B pathway is activated by inflammatory cytokines, particularly TNF- α , which is elevated in inflammatory conditions and cachexia (a systemic muscle wasting that is usually accompanied with a chronic disease). FoxO pathway is triggered by free radicals and AMP-activated protein kinase (AMPK) that is activated with increases of intracellular AMP due to increased ATP consumption. Activation of NF- κ B and FoxO pathways leads to the up-regulation of genes *atrogenin-1/MAFbx* and *MuRF1*. Both genes encode ubiquitin ligases E3, thus up-regulation of these genes increases protein degradation through the ubiquitin-proteasome system (will be discussed in 3.2 *Protein degradation mechanism*). In addition to the up-regulation of the ubiquitin-proteasome system, FoxO pathway also activates the autophagy-lysosome system (will be discussed in 3.2 *Protein degradation mechanism*) by activating autophagy-related genes. Importantly, while protein synthesis and degradation pathways are distinct, they are inter-related. For example, protein synthesis AKT-mTOR pathway and protein degradation FoxO pathway were found to down-regulate each other (115, 138).

3.2 Protein degradation mechanism

Protein degradation in muscles is a coordinated process where more than one degradative process is involved. There are 3 main protein degradation pathways in muscles: (1) calcium dependent calpain proteolysis (**Figure 5A**), (2) proteasome proteolysis (**Figure 5B**), and (3) lysosomal proteolysis (**Figure 5C**). In the calcium dependent

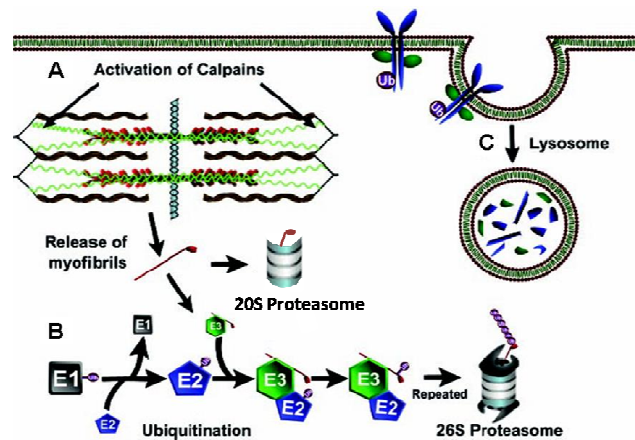


Figure 5. Three major proteolytic systems in skeletal muscles: (A) calcium dependent calpain proteolysis, (B) proteasome proteolysis, and (C) lysosomal proteolysis. Modified from Jackman et al., 2004.

calpain proteolysis, calpain, a protein that is activated by calcium, cleaves the structural proteins of sarcomeres including titin, vinculin, C-protein and nebulin (66). The cleavage of structural proteins disrupts the integrity of sarcomeres and results in the release of myofibrillar proteins. The release of myofibrillar proteins from the sarcomere allows the proteins to be degraded by the proteasomes, the primary degradation system for contractile proteins (62, 110).

There are various types of proteasomes depending on the different combinations of the catalytic core (the 20S proteasome) and the regulatory subunits (the 19S and the 11S). While 20S proteasomes (the proteasome containing no regulatory subunits) can degrade proteins in an ATP independent manner, the 26S proteasomes (composed of a 20S proteasome with a regulatory 19S complex connected to each end) degrade proteins that are poly-ubiquitinated. As shown in **Figure 5B**, proteins degraded by 26S proteasomes need to be ubiquitinated first in order to be recognized by the 26S proteasomes. The process of the protein ubiquitination requires 3 enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3). E1 utilizes ATP to activate ubiquitin. The activated ubiquitin is then transferred to E2. Next, E3 bound with the target protein attaches to the E2 carrying the activated ubiquitin where the activated ubiquitin is transferred to the target protein. This process of protein ubiquitination is repeated until at least four ubiquitin molecules are attached to the target protein. The poly-ubiquitinated protein is recognized and unfolded by the 19S complex of 26S proteasome, which is then fed into the 20S core proteasome for degradation (66, 78, 79, 129).

Lysosomal proteolysis plays a major role in degrading membrane proteins. Lysosomes contain several acid-optimal proteases such as cathepsin and degrade proteins by endocytosis. Studies show that the lysosomal proteolysis pathway does not play a critical role in the degradation of myofibrillar proteins

because inhibition of the lysosomal system does not attenuate myosin heavy chain degradation in muscles with increased oxidative stress (62, 66, 78, 79, 129).

In summary, the size of muscles is dynamic and responds to various stimuli. Protein degradation that leads to decreased muscle size is regulated by proteolytic systems in the cells. Thus, factors that up-regulate the proteolytic systems may cause the decrease of muscle size. Since muscle size is one determinant of muscle force, the changes of muscle size are usually accompanied with the changes of muscle force, the critical physiological parameter in terms of muscle function. The physiological adaptations of skeletal muscles with stimuli are discussed next focusing on the adaptations with aging and disuse.

4. Adaptation of skeletal muscle

Skeletal muscles change with aging and various stimuli such as altered physical activity, mechanical stress, nutrition and hormonal levels. Here I focus on reviewing the morphological and physiological adaptations of skeletal muscles with disuse and aging. The review of the functional adaptations of muscles with aging and disuse will provide the background, support and the rationales of my research questions.

4.1 Adaptation of skeletal muscle with disuse

The term “disuse” in this dissertation is used to describe muscles that are unloaded (non-weight-bearing) or immobilized. The disuse of skeletal muscles occurs with astronauts during the period of spaceflight and individuals who are bed rest or limb immobilized due to illness or surgeries. The primary adaptation of muscles to disuse is loss of muscle mass (atrophy). Studies have shown that

two weeks of muscle unloading and immobilization in young men cause the decline of muscle mass and strength (75, 144).

In order to better understand the mechanisms underlying the disuse-related muscle dysfunction, animal models are developed to mimic various conditions of muscle disuse in human populations. Examples of animal models of muscle disuse are limb immobilization, hindlimb unloading, and mechanical ventilation. The limb immobilization of animals is used to mimic cast immobilization in humans. Hindlimb unloading of animals (**Figure 6**) is used to mimic non-weight bearing conditions of human muscles that occur during post surgery (e.g., the non-weight bearing ambulation after joint replacement), bed rest or spaceflight. Last, mechanical ventilation of animals is used to mimic the disuse of diaphragms seen in patients using ventilators.

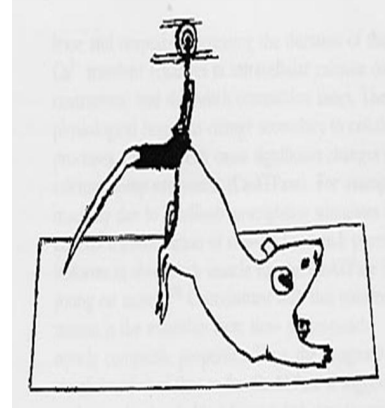


Figure 6. The animal model of hindlimb unloading. Source: Thompson, 2002.

The disuse-related muscle atrophy is contributed mainly by the decrease of fiber size but not the loss of muscle fibers. Decreased fiber size is mainly due to the loss of intracellular proteins with preferential loss of myofibrillar proteins (157). Type I muscle fibers are found to be more susceptible to the disuse-related muscle atrophy compared to type II muscle fibers. An explanation is that type I motor neurons have lower threshold of activation, and type I muscle fibers of lower limbs are activated even when a person is just standing still. In contrast, Type II muscle fibers are activated when the load requirement is increased such as jumping, thus they are affected less by the disuse induced by muscle unloading (129).

The disuse-induced muscle atrophy develops in the first week of disuse (2, 9). Decreased protein synthesis and increased protein degradation both contribute to the muscle atrophy with disuse. Recent studies show that muscle inactivity not only suppresses AKT-mTOR pathway (the pathway that activates protein synthesis) (8, 75) but also activates NF- κ B and FoxO pathways (pathways that activate protein degradation) (75, 120, 154). As shown in **Figure 7**, the initial loss of muscle mass caused by muscle unloading is primarily due to decreased protein synthesis. After that, the muscle loss is mainly attributed by increased protein degradation. Specifically, the synthesis of myofibrillar proteins of soleus muscles begins to decrease within the first 5 hours of hindlimb unloading and reaches the maximal reduction by 3 days of unloading. In contrast, the degradation of myofibrillar proteins starts at 2 days of hindlimb unloading and reaches the peak at 15 days of unloading (**Figure 7**) (157).

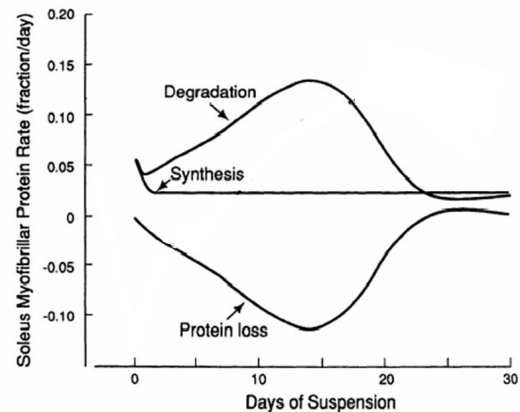


Figure 7. Time courses of protein synthesis, degradation and loss in soleus muscles from rats with hindlimb unloading. Modified from Thomason, 1990.

In addition to the changes in muscle size, contractile properties as well as metabolic properties of muscles adapt with disuse. Unloaded soleus muscles have lower percentage of type I fibers, greater contractile velocity and reduced absolute and normalized force production. Metabolically, disused muscles have greater intramuscular glycogen stores, lipid stores and glycolytic enzyme activity, and lower oxidative enzyme activity.

In summary, muscle disuse caused by limb immobilization and muscle unloading has a negative impact on muscle function. It decreases muscle size

and strength in both whole muscles and single muscle fibers. Specifically, type I muscle fibers are more susceptible to the influence of muscle disuse. Thus, soleus muscle, a posture muscle that contains high percentage of type I muscle fibers, becomes a good target muscle to investigate the influence of disuse on skeletal muscles.

4.2 Adaptation of skeletal muscle with aging

One age-related change in muscle is a loss in mass (109, 136). In 1989, Irwin Rosenberg used “sarcopenia” to describe the age-related decline of muscle mass in the elderly. In order to understand the prevalence of sarcopenia in populations as well as the effect of sarcopenia on individuals, an operational definition of sarcopenia was developed. The current operational definition of sarcopenia is determined by skeletal muscle mass index (SMI). SMI is derived from the equation: sum of muscle mass from four limbs by dual energy x-ray absorptiometry (DEXA) scan (kg) / height² (m²). Individuals with SMI below 2 standard deviation of the mean SMI of the middle-age reference male and female population are defined as sarcopenic (136). It has been reported that the loss of muscle mass becomes significant when people reach 50 years old and the average rate of muscle loss after that is 1-2% annually. Around 10% of people under 70 years old are sarcopenic, and 23% of people aged older than 80 years old are sarcopenic (109, 136). The main effect of the loss of muscle mass is reduced muscle strength. Studies have shown that the age-related loss of muscle mass and strength is a major risk factor for frailty, mobility disability and hospitalization of old individuals (109).

The age-related muscle loss is attributed by the decline of both fiber (cell) size and number (97). In addition to the changes in the muscle size, contractile properties and metabolic properties of muscles also adapt with aging. Maximal force and power production, maximal shortening velocity as well as fatigue resistance of muscles decrease with aging. Metabolically, muscles from aged

animals have lower capillary density, mitochondrial density, activities of oxidative enzymes as well as phosphocreatine repletion rate. While aging affects both type I and type II fibers, type II fibers are more susceptible to the age-related changes. An explanation for the fiber type-specific changes with aging is that type II muscle fibers are activated less because the intensity of physical activity an individual may perform is gradually decreased with aging.

Interestingly, the decrease of the contractile force with aging is more than the loss of muscle mass. As shown in **Figure 8**, specific force (force/muscle size), an indicator of muscle quality, decreases with aging (158).

The decreased specific force indicates that aging not only

decreases the quantity of muscle tissue but also reduces the quality of muscles.

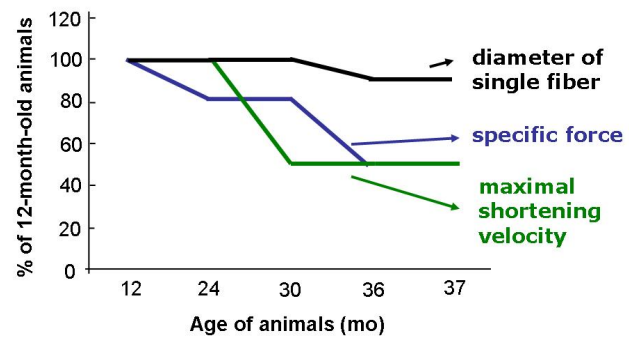


Figure 8. Contractile properties of single type I fibers from soleus muscle of different aged rats. The fiber size, specific force and maximal shortening velocity decrease with aging. Modified from Thompson, 2002.

The etiology and pathogenesis of the age-related muscle dysfunction are multifactorial including lack of physical activity, progressive denervation, age-related hormone adaptations, oxidative stress and apoptosis (136). The amount of physical activity that organisms perform decreases with aging. The decreased physical activity has a negative effect on muscle size and strength. Exercise, particularly resistance exercise, has been reported to significantly increase muscle mass, muscle strength and muscle quality in the elderly (155). Another etiology of age-related muscle dysfunction is progressive loss of type II α motor neurons (136). In response to this, the remaining α motor neurons (type I mainly) innervate the denervated muscle fibers resulting in a reduction of the number of motor units, and an increase in the size of the motor units. The preferential loss

of type II α motor neurons decreases muscle strength and power, and the increase in the size of motor units hinders the coordination of the fine motor movements.

Other etiologies of age-related muscle dysfunction are age-related hormone changes, oxidative stress and apoptosis. The levels of anabolic hormones such as testosterone, estrogen, growth hormone, insulin-like growth factor decline with age. Because these anabolic hormones increase protein synthesis, the decline in the hormone levels and/or decreased sensitivity potentiate the occurrence of sarcopenia. Aging is associated with increased oxidative stress where the rate of free radical generation is greater than the rate of their removal. The excess free radicals not only damage proteins, lipids and DNAs resulting in cellular dysfunction but also initiate signal cascades that lead to increased protein degradation. Apoptosis, a strictly regulated programmed cell death, increases with aging. It represents a common final mechanism for the loss of muscle mass in the elderly.

Importantly, the potential contributors discussed above (lack of physical activity, progressive denervation, age-related hormone change, oxidative stress and apoptosis) of sarcopenia are interrelated. For example, the age-related lack of physical activity is related to the hormone adaptations with aging. The loss of motor neuron axons is likely due to the decreased physical activity, increased oxidative stress and hormone adaptations. Physical inactivity increases oxidative stress and apoptosis, and oxidative stress per se plays a role in the apoptosis pathway. Overall, skeletal muscles become smaller (atrophy) and weaker with aging, and the changes have a detrimental effect on the physical function of the elderly. Multiple, interrelated factors contribute to the age-related modifications of skeletal muscles and oxidative stress is one of them.

4.3 Age-related remodeling of skeletal muscles in response to stimuli

The remodeling of skeletal muscles with stimuli is dependent on age. For example, older individuals were found to gain less from resistance exercise training, the type of exercise that specifically increases muscle mass and strength, compared to younger individuals. Slivka et al. reported that, while 12 weeks of resistance training increases 7% of the quadriceps muscle size in healthy men aged between 70-80 years old, the same training protocol only increases 2.5% of the quadriceps muscle size in healthy men aged 80-86 years old (149). Even worse than the reduced training effect that older organisms achieve, aging muscles may be more susceptible to the damage caused by exercise training. Cutlip et al. found while the chronic exposure of stretch shortening cycles (reciprocal concentric and eccentric muscle contractions) increases muscle size and force of muscles from young rats, it decreases the muscle force and has no effect on muscle size of the muscles from old rats (26).

The age-related modifications of skeletal muscles are also seen in the conditions of muscle disuse. Deschenes et al. found that aged men (average 68.5 years old) experience greater declines in muscle function after 7 days of muscle unloading compared to young men (average 21.7 years old). The declines of peak force and power of knee extensor at higher contractile velocity only happen in aged men, but not young men. Whereas, at lower contractile velocity, the declines of peak force and power of knee extensor were similar between aged and young men after 7 days of muscle unloading (29). Importantly, in addition to the decreased muscle strength, the decline of muscle quality with disuse is also more significant in aging muscles. The decline of muscle quality (tension/muscle size) was shown to be greater in soleus muscles of old rats with 7 and 14 days of hindlimb unloading compared to young rats with same periods of hindlimb unloading (59, 160).

In summary, muscle cells are composed of many proteins that perform contractile functions (contractile proteins), maintain the integrity of sarcomere and sarcolemma (structural proteins), provide energy, and maintain the homeostasis of cellular environment (house keeping proteins). The “team work” of these proteins makes muscles contract normally. Importantly, muscle cells are highly dynamic and can adapt with different stimuli. While the size of muscles increases with exercise training, especially resistance exercise, the size decreases with aging and muscle disuse. In addition to the morphological changes of muscle cells, the contractile functions also change with stimuli. For example, muscle strength and muscle quality (force generated per cross section area of the muscle fiber) decrease with aging and muscle disuse. Interestingly, although muscles can adapt with stimuli, their adaptability is influenced by the age of the organisms. The positive effects of exercise training on muscles decrease in the elderly, and the negative effects of muscle disuse are more significant in the elderly.

In the next section, oxidative stress, a common mechanism behind aging- and disuse-related muscle dysfunction, is introduced. This introduction will further provide the rationale of my research questions.

II. Oxidative Stress

Oxidative stress is defined as the disruption of the balance between free radicals (oxidants) and antioxidants, and the balance shifts toward free radicals. Skeletal muscles continuously generate free radicals at a low rate under basal conditions. The low level of free radicals (superoxide or hydrogen peroxide) is essential for the normal function of muscle cells. The low level of free radicals can reversibly oxidize amino acids, especially cysteines, resulting in posttranslational protein modifications that are involved in cellular signal transduction. In fact, depletion of free radicals by addition of antioxidants has been shown to decrease contractility of muscles (68, 150). Although free radicals are essential for normal muscle function, their regulatory function is disrupted when excess free radicals are generated in the cells. The excess free radicals cause irreversible modifications of lipids, proteins and DNAs that disrupt their normal functions and ultimately lead to cell dysfunction. Evidence for the links between oxidative stress and muscle function is that contractile function of muscles decreases with the exogenous addition of oxidants or deficiencies in nutritional antioxidants (131, 150).

Because of the role of oxidative stress in muscle dysfunction, in this section, I will introduce factors that affect the redox balance (free radicals and antioxidants) and the consequences of the increased oxidative stress (oxidative modification of proteins, and repair and removal of damaged proteins).

1. *Free radicals*

Free radicals are molecules that have unpaired electron in their outer valence shell. Because of this conformation, free radicals are unstable and tend to react with other molecules, which ultimately results in oxidative damage. Free radicals generated in skeletal muscles that can initiate oxidative damage cascades are superoxide anion ($O_2^{\bullet -}$) and nitric oxide (NO^{\bullet}). $O_2^{\bullet -}$ reacts with NO^{\bullet} and forms a highly cytotoxic compound peroxynitrite ($ONOO^-$). In addition to the

reaction with NO^\bullet , $\text{O}_2^{\bullet-}$ can be converted to hydrogen peroxide, which subsequently gives rise to a highly reactive compound, hydroxyl radicals (OH^\bullet), in the presence of catalytic transition metals (**Figure 9**).

Free radicals are generated exogenously as well as endogenously. Exogenous sources of free radicals include ionizing radiation (X-rays and γ -rays), ultraviolet light and environmental

toxins. Endogenous sites of free radicals formation are mitochondria, plasma membrane, sarcoplasmic reticulum and phospholipase A2 (**Figure 9**).

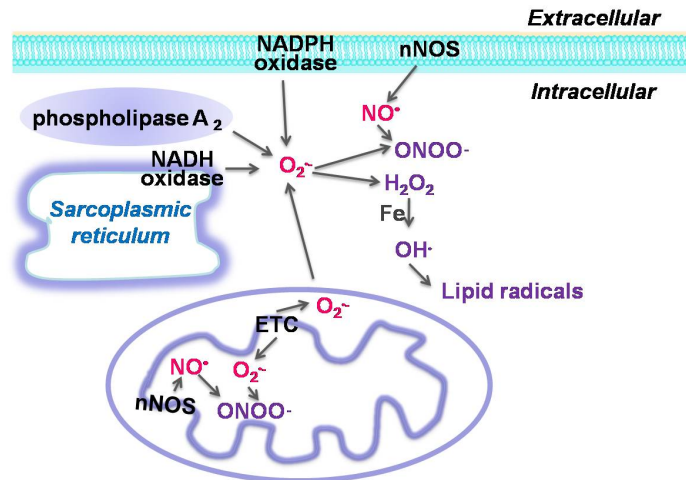


Figure 9. Intracellular sources of free radicals. Free radicals are generated intracellularly from mitochondria, plasma membrane, sarcoplasmic reticulum and phospholipase A2.

1.1 Endogenous sources of free radicals

Mitochondria, being the cellular power plants, are the sites where Krebs cycle and

oxidative phosphorylation occur. Oxidative phosphorylation in the mitochondria is achieved by the electron transport chain that establishes a proton gradient across the inner membrane by oxidizing NADH and FADH_2 produced from the Krebs cycle. Superoxide is generated when there is electron leak during the electron transfer in the electron transport chain. The leaked electron can react with oxygen and form superoxide. Studies indicate that iron-sulphur clusters and ubiquinone (coenzyme Q) are the main sites of electron leakage in the electron transport chain (68). The generated superoxides flow into both mitochondria matrix and intermembrane space of mitochondria. The superoxides in the intermembrane space (outside of inner membrane of mitochondria) can be released from the mitochondria into the cytosol (68).

Another superoxide generation site in skeletal muscles is the plasma membrane (sarcolemma). Under basal conditions, free radicals are continuously generated at low rates from membrane proteins, NADPH oxidase and NO[•] synthase. NADPH oxidase generates superoxide by transferring the electron from NADPH to oxygen when it is activated via cell membrane depolarization (34, 68). NO[•] synthase (NOS) is an enzyme that synthesizes NO[•] in the presence of L-arginine, NADPH and oxygen. NOS has three isoforms: neuronal NOS (type I, nNOS), inducible NOS (type II, iNOS) and endothelial NOS (type III, eNOS). nNOS is localized on the sarcolemma and is strongly expressed in type II muscle fibers. Inducible NOS is expressed in muscles in some inflammatory conditions, thus does not play a significant role in normal muscles. eNOS is localized on the muscle mitochondria. Studies show that nNOS is the primary isoform that produces NO in muscles (68).

The third endogenous site of free radical formation is the sarcoplasmic reticulum (SR). SR is a subcellular organelle that controls the contractile state of muscles by regulating the Ca²⁺ levels in the cytosol. SR increases the intracellular Ca²⁺ level by Ca²⁺ release channel (ryanodine-receptor, RyR), which releases Ca²⁺ from SR once it is activated by the voltage-sensor (Dihydropyridine receptors, DHPR). DHPR senses the depolarization of membrane caused by the stimulation of motor nerve. Studies show that SR contains NADH-dependent oxidases that produce superoxide, which in turn increases the activity of Ca²⁺ release channel. Taken together, superoxide is generated in the SR during normal physiological conditions where it helps regulate Ca²⁺ levels in the cytosol (68, 169).

Phospholipase A₂ (PLA₂), located in the cytosol of skeletal muscles, is another source of superoxide generation. PLA₂ catalyzes the hydrolysis of phospholipids into fatty acid and arachidonic acid. There are two types of PLA₂

in skeletal muscles: calcium-independent PLA₂ (iPLA₂) and calcium-dependent PLA₂ (cPLA₂). Under basal conditions, iPLA₂ produces superoxide that helps regulate the contractile function of muscles. However, elevated cytosolic calcium concentrations activate cPLA₂ that produces superoxide at supernormal rates (51, 68).

In summary, free radicals (superoxide and nitric oxide) are generated in normal cells at low levels from various sources. The low levels of free radicals play a role in regulating muscle function. The generation of free radicals, however, increases when muscles are under stress such as exercise and disuse.

1.2 Oxidants that are derived from free radicals

Peroxynitrite (ONOO⁻), generated from the reaction of NO[•] with O₂^{•-}, is a powerful oxidizing and nitrating agent (126). Although O₂^{•-} can be removed by superoxide dismutase (SOD), peroxynitrite still forms because the rate of the reaction of NO[•] and O₂^{•-} is approximately 3 times faster than the reaction between O₂^{•-} and SOD (15). Peroxynitrite attacks tyrosine residues of proteins and causes tyrosine nitration. Targets of peroxynitrite include manganese SOD, α-actinin, SERCA, aconitase, beta-enolase, triosephosphate isomerase, carbonic anhydrase III, myosin, actin, et al. (47, 126, 159). In addition, the activity of SERCA has been shown to be reduced with nitration (164).

Hydroxyl radicals (OH[•]), generated from hydrogen peroxides in the presence of catalytic transition metals, are highly unstable and can react with carbohydrates, DNA, lipids and amino acids, and impair their functions. For instance, oxidized DNA is related to gene mutations, replication errors, persistent DNA damage and genomic instability. These detrimental changes are associated with cell immortality (cancer) or early apoptosis (aging) (28). In addition, peroxidized membrane (phospholipids) alters the characteristics of fluidity, selective permeability, and integrity. The disrupted cell membranes permit cytotoxic

molecules such as calcium to flow into the cells. In addition, the disruption of cell membranes also adversely affects the function of transmembrane proteins (28).

Besides the direct disruption of normal function of membrane (phospholipids), the attack of OH^\bullet on lipids amplifies the detrimental effects of the free radical (35). The attack of OH^\bullet on lipids, preferentially polyunsaturated fatty acids of phospholipids (PUFA), generates lipid hydroperoxide (LOOH), which is easily degraded into reactive aldehydes such as 4-hydroxy-2-nonenal (HNE). HNE, compared to primary free radicals such as OH^\bullet , has a longer half life, thus can affect targets that are not close to the origin of generation. Studies have shown that HNE can diffuse across the membrane and covalently modify target proteins such as cytochrome c oxidase, heat shock proteins, myosin, actin, et al. (36, 53, 159). Last but not least, because reactive species generated from lipid peroxidation have relatively long half life, the amount of the reactive species and the amount of proteins modified by them are often used as biomarkers of oxidative stress.

Overall, the discussion in this section provides the rationales of selecting HNE and nitrotyrosine modified proteins as two markers of oxidized proteins in my thesis study.

2. Antioxidants

Antioxidants are molecules that buffer the burden of free radicals, thus protecting cells from oxidative damage. Some antioxidants are enzymatic such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX). Others are non-enzymatic such as the membrane bound antioxidant, vitamin E (α -tocopherol), and water-soluble antioxidants, vitamin C (ascorbic acid) and glutathione (GSH). Enzymatic antioxidants catalyze the re-dox reactions that reduce and stabilize the oxidants. Non-enzymatic antioxidants protect cells from

oxidative damage by reacting with the oxidants, which unavoidably causes a reduction in the antioxidants.

2.1 Endogenous enzymatic antioxidants

The key enzymatic antioxidants in muscles are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) (**Figure 10**). SOD has two isoforms: copper-zinc SOD (Cu-Zn SOD, SOD1) and manganese SOD (MnSOD, SOD2). Cu-Zn SOD is mainly located in the cytosol with a small portion in the mitochondria intermembrane space. MnSOD is the predominant isoform of SOD in the mitochondria. The function of SOD is to catalyze the conversion of superoxide to hydrogen peroxide ($O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$).

Hydrogen peroxide, produced from SOD, is toxic and needs to be removed quickly. Catalase and GPX are antioxidants that convert hydrogen peroxide to water.

While both enzymes (catalase and GPX) can catalyze the reduction of hydrogen peroxides, they function differently and have different compartmentalization. Catalase is distributed in the cytosol and catalyzes the decomposition of hydrogen peroxide to water and oxygen ($H_2O_2 \rightarrow 2 H_2O + O_2$) (5). GPX exists in both the cytosol and mitochondria. GPX catalyzes the reduction of hydrogen peroxide to water and the reduction of lipid hydroperoxides to their corresponding alcohols (64). In the reaction catalyzed by GPX, glutathione (GSH) is used as a cofactor where GSH is oxidized to glutathione disulfide (GSSG) (e.g., $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$).

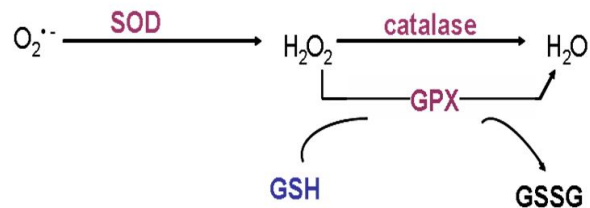


Figure 10. Functions of enzymatic antioxidants in skeletal muscles

2.2 Endogenous non-enzymatic antioxidants

Glutathione (GSH), a tripeptide composed of glutamate, cysteine and glycine, is the most important non-enzymatic antioxidant formed in cells. 80~90% of GSH is in the cytosol and it can be compartmentalized into other organelles including mitochondria, nucleus and sarcoplasmic reticulum after synthesized in the cytosol (45). GSH protects cells by being a cofactor in redox reactions as well as direct conjugation with oxidants. In redox reactions, GSH donates electrons to hydrogen peroxides that are reduced to water by enzyme GPX. In the direct conjugation reaction, GSH conjugates with a variety of oxidants via glutathione S transferase (GST) (**Figure 11**). The reaction between GSH and HNE is one major route of HNE detoxification (23). Although the GSH-dependent detoxifying reactions protect cells from oxidative damage, they reduce intracellular GSH levels.

The replenishment of GSH is achieved by redox recycling and biosynthesis. The redox recycling of GSH is catalyzed by glutathione reductase (GR) with NADPH as the cofactor. The biosynthesis of GSH is regulated by the substrate availability and the synthesis rate. Substrate availability is mainly determined by γ -glutamyl transferase (GGT) that initiates the extracellular degradation of GSH and GSH-conjugates. The intracellular synthesis of GSH is by two consecutive ATP-dependent enzymatic reactions. In the first reaction, glutamate is coupled with cysteine to form γ -glutamylcysteine catalyzed by glutamate cysteine ligase (GCL), the rate limiting enzyme of GSH biosynthesis. In the second reaction, γ -glutamylcysteine is coupled with glycine to form GSH catalyzed by GSH synthetase (GS) (**Figure 11**).

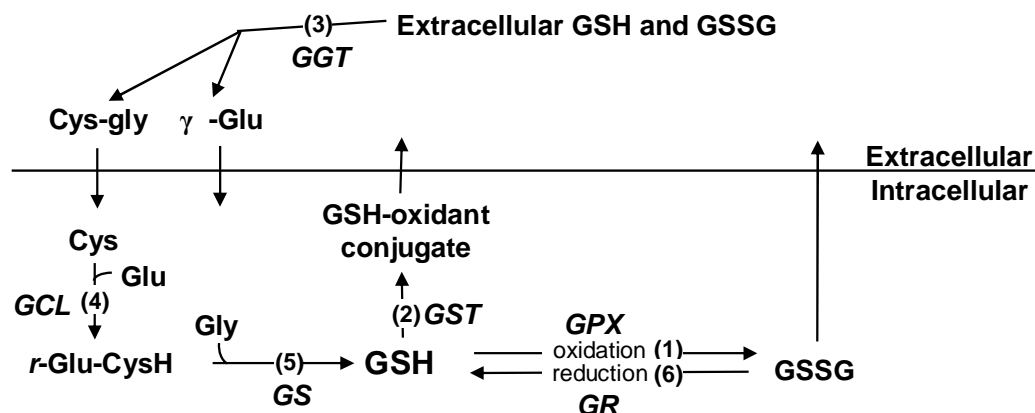


Figure 11. Metabolism of glutathione (GSH). GSH, a tripeptide composed of glutamate (Glu), cysteine (Cys) and glycine (Gly), is a crucial antioxidant in the cells. (1) GSH is used as a cofactor in the redox reaction catalyzed by glutathione peroxidase (GPX). In this redox reaction, hydrogen peroxide and other peroxides are reduced while GSH is oxidized to glutathione disulfide (GSSG). (2) The detoxifying function of GSH is also achieved by direct conjugation with oxidants. This direct conjugation reaction is catalyzed by glutathione S transferase (GST). (3) GSSG and GSH-oxidant conjugates are transported out of cells where they are degraded by γ -glutamyl transferase (GGT). This degradation ensures substrate available for GSH biosynthesis intracellularly. The intracellular synthesis of GSH is by two consecutive enzymatic reactions catalyzed by glutamate cysteine ligase (GCL) (4) and GSH synthetase (GS) (5). (6) GSH is also replenished by the reduction of GSSG catalyzed by glutathione reductase (GR).

GCL activity is regulated by its two subunits, the catalytic subunit (GCLC) and the modifier subunit (GCLM). GCLC (73 kDa) contains the active site for the ATP-dependent bond formation between glutamate and cysteine, and possesses all the catalytic activity of GCL (10). Studies have shown that GCLC alone is necessary and sufficient for γ -GC formation (27, 173). GCLM (31 kDa), although having no catalytic activity, enhances enzyme activity by lowering the K_m (increasing the affinity) of GCLC to glutamate and ATP, and increasing the concentration of GSH required to inhibit GCL activity (K_i). Yang et al. demonstrated that *Gclm* homozygous knockout mice had lower GSH levels, increased K_m (reduced affinity) of GCLC to glutamate and were more sensitive

to oxidative stress compared to wild type littermates (173). These results demonstrate the important contribution GCLM makes in regulating GSH levels.

In summary, the key antioxidants in muscles are SOD, catalase, GPX and GSH. These antioxidants work cooperatively to detoxify reactive species in the cells. Different from enzymatic antioxidant (SOD, catalase and GPX), GSH, the non-enzymatic antioxidant, reduces its intracellular level in the detoxifying reactions. Thus, enzymes that are involved in the GSH replenishment play an important role in the maintenance of redox balance of the cells. The key enzymes involved in GSH-replenishment include GR, GGT and GCL.

2.3 Regulation of antioxidants

The activation of antioxidant genes is influenced by both acute and chronic oxidative stress. Some antioxidant genes are up-regulated rapidly in response to acute oxidative stress resulting from infection, toxin or metabolic disturbance such as ischemia-reperfusion. Other antioxidant genes are up-regulated in response to chronic oxidative stress such as aging, exercise training and environmental changes (chronic smoking) (72). The up-regulation of antioxidants in response to oxidative stress helps maintain the redox balance of cells. The adaptability of antioxidants in response to reactive species makes the status of antioxidants an indicator of oxidative stress.

Many oxidants such as H_2O_2 , NO^* and oxidized proteins signal the activation of antioxidant genes (72). Transcription factors that play a role in the signaling pathways include nuclear factor- κB (NF κB), activator protein-1 (AP-1) and NF-E2-related factor 2 (Nrf2).

NF κB exists as a homodimer or heterodimer of two (out of five) NF κB family proteins: RelA (p65), RelB, c-Rel, p50/p105 (NF κB 1), and p52/p100 (NF κB 2). In mammals, NF κB remains in an inactive state in the cytosol with inhibitor of κB

(I κ B). NF κ B is activated when I κ B is degraded by the 26S proteasome following increased levels of H₂O₂ and proinflammatory cytokines, such as tumor necrosis factor α (TNF α), interleukin 1 (IL-1) and interleukin 6 (IL-6) (71). Once NF κ B is activated, it translocates into nucleus where it binds to DNA and activates the transcription of target genes (38). Antioxidants that have promoter regions for the binding of NF κ B are MnSOD, GPX and GCL (71, 103, 104).

Activator protein-1 (AP-1) is another transcription factor involved in the regulation of antioxidant genes. AP-1 is a heterodimer composed of activating subunit (c-Fos and c-Jun) and inhibitory subunits (Fra-1 and Fra-2). The composition of AP-1 is dependent on the cellular redox status, which subsequently determines the activation or inhibition of the transcription of antioxidant genes (72). Antioxidants that have promoter regions for the binding of AP-1 are GPX and GCL (71, 103).

Nrf2 is another transcription factor involved in the regulation of antioxidant genes. Nrf2 exists in an inactive state in the cytosol associated with its inhibitor Keap1 under non-stressful conditions. When Keap1 is alkylated by HNE and other electrophiles, the Keap1-Nrf2 complex is dissociated. Once free from Keap1, Nrf2 translocates to the nucleus and then activates the genes that contain antioxidant-response elements (ARE) in their promoter regions (53). The promoter regions of GCL and GGT contain the ARE (103). Importantly, there are complex cross talks among the different families of transcription factors. For example, Nrf2 can up-regulates the GCLC expression not only via ARE, but also by modulating the expression of AP-1 and NF κ B (171).

In summary, antioxidants are up-regulated in response to oxidative stress. The changes in antioxidants lessens the damage caused by reactive species and can be viewed as a compensatory change. Various transcription factors are involved in the signaling pathways of antioxidant regulation, thus the impaired

functions of transcription factors could influence the regulation of antioxidant expressions.

3. *Oxidative modification of proteins*

Oxidative modification of proteins by reactive species can lead to (1) oxidative modifications of amino acid side chains, (2) peptide bonds cleavage, and (3) cross linkages among proteins (152). Oxidative modifications of proteins can disrupt the normal structure of proteins and the relationships among protein subunits, which can cause protein dysfunction.

Evidence for the linkage between protein oxidative modification and function is found in studies where protein functions are evaluated in conditions with different levels of oxidative stress. For example, the affinity of adipocyte fatty acid binding protein (FABP) for fatty acids is found to reduce with HNE modification (53). The function of myosin, the key contractile protein in muscle cells, also shows a dose-dependent decrease with increased concentrations of hydrogen peroxide and peroxynitrite (25, 131, 151). Regarding enzymes, several enzymes such as MnSOD, Ca²⁺-ATPase, sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), cytochrome c oxidase (COX), Na/K ATPase, isocitrate dehydrogenase (ICDH), and 20S proteasome are shown to have reduced activities with increased oxidative stress in a dose- and time- dependent manner (22, 42, 48, 106, 146, 164, 172). Similarly, kinases, enzymes related to cellular signaling, change activities after oxidative modification. Oxidative modifications of extracellular signal-regulated kinase (ERK) and AMPK kinase inhibit kinase activities and attenuate downstream signal pathways (137).

In summary, protein oxidation is a result of oxidant insult. Thus, the level of oxidized proteins is used as an indicator of oxidative stress. In addition, because oxidative modifications of proteins may impair the functional status of

proteins, oxidized proteins are identified in my study to unveil the potential mechanisms underlying the disuse-related muscle dysfunction.

4. *Repair and removal of damaged proteins*

The intracellular levels of oxidized proteins are determined by the balance between the formation and the repair/removal of oxidized proteins.

Damaged proteins can be repaired by heat shock proteins (HSPs) that stabilize denatured or unfolded proteins. In addition to the repair of damaged proteins, HSPs facilitate the degradation of proteins that are irreversibly damaged. Thus, HSPs help protect cells from oxidative stress. Studies show that HSPs (HSP27, HSP60 and HSP70) have a compensatory up-regulation in aging muscles (24) and in muscles under a variety of stresses including exercise, hyperthermia and oxidative stress (80). Up-regulation of HSPs attenuates muscle atrophy induced by hindlimb unloading (121).

The removal of damaged proteins minimizes the intracellular accumulation of oxidized proteins. Oxidative-modified proteins are degraded primarily through two systems, the lysosomal system and proteasome system. Please refer to 3.2 *Protein degradation mechanism* for detailed discussion of these two systems. Importantly, although protein degradation machinery is up-regulated with mild oxidative stress, the machinery itself is affected by increased oxidants and becomes less efficient (42, 108).

In summary, free radicals in the cells are generated endogenously and exogenously. The low levels of free radicals generated under basal conditions are critical for cell functions. However, if the generation of free radicals exceeds the scavenging capacity of antioxidants, oxidative stress develops. The excessive reactive species attack proteins, lipids and DNA of the cells. Focusing on proteins, this section shows that oxidative modifications may

impair protein function. The detrimental effects of oxidative stress on proteins are minimized by repair/removal systems in the cells. However, the oxidative-modified proteins accumulate when the intracellular oxidative stress is too high (free radical generation >> scavenging capacity) or the efficiency of the repair/removal systems is reduced. In the next section, I will link oxidative stress with skeletal muscle dysfunction, focusing on aging and disuse.

III. Oxidative Stress and Skeletal Muscle Dysfunction

1. Oxidative stress and disuse-related muscle dysfunction

1.1 Oxidative stress is increased in disused muscles

Originally it was proposed that the production of ROS would be low in disused muscles because of the lower respiratory rates of mitochondria. Interestingly, oxidative stress is greater in disused muscles compared to muscles with normal weight bearing (37, 63, 86, 89, 140). The increased oxidative stress in disused muscles is characterized by increased generation of reactive species, altered antioxidant capacity, and increased oxidative-modified proteins.

Regarding the generation of reactive species, **Table 1** summarizes the published literature investigating the levels of reactive species in disused muscles. Overall, these studies, investigating different muscles from several animal models with various durations of muscle disuse, show that the level of reactive species is greater in disused muscles. Specifically, higher levels of hydrogen peroxide and ROS are found in diaphragm muscles with 12 hours of mechanical ventilation (37), in soleus muscles with 8 days of cast-immobilization (86) and in soleus muscles with HU for 1, 3, 6, 12 and 28 days (3, 89). Increased NO[•] level is detected in skeletal muscles of young mice with 7 and 14 days of HU (154).

Lipid peroxidation is also found to be increased in muscles with HU for 2, 5, 10, 14 and 21 days (63, 143, 147), and in muscles with cast immobilization for 8 and 12 days (85). Interestingly, while studies demonstrate that the level of reactive species increases with 8 and 12 days of cast-immobilization (85, 86), muscles with 21 days of cast-immobilization do not show an increase in lipid peroxidation (99). These results suggest the system that scavenges the breakdown of lipid peroxidation may be up-regulated and removes peroxidized lipids efficiently.

Altered antioxidant system is another indicator of oxidative stress. **Table 2** summarizes the published literature investigating the alteration of antioxidants in the disused muscles. Overall, results from these studies suggest that (1) the responses of antioxidants with muscle disuse are uncoordinated and (2) the responses of antioxidants with disuse are dependent on the duration of the disuse. In disused muscles, antioxidants change uncoordinatedly where some antioxidants are up-regulated, some are unchanged and some are down-regulated (86, 89, 141, 143, 147). Regarding the duration-dependent responses, most antioxidants are up-regulated with a short term period of muscle disuse (less than 21 days) (86, 141, 143, 147) but are down-regulated with longer periods of muscle disuse (more than 21 days) (89, 99).

Regarding the oxidative-modified proteins, studies to date indicate that the overall oxidative-modified proteins in muscles accumulate with disuse. Muscles with 14 days of HU are found to have more oxidized proteins characterized by protein carbonyls and nitration (83, 147). Similarly, muscles with 7 and 8 days of cast-immobilization are shown to have greater amounts of HNE and NT modified proteins (140, 141).

In summary, oxidative stress is increased in disused muscles. The generation of reactive species increases in disused muscle as early as 12 hours of mechanical ventilation, and 1 day of cast-immobilization and HU. Antioxidant systems in muscles up-regulate during the early phase of muscle disuse but down-regulate with a longer period of disuse. In addition, oxidized proteins are accumulating in the disused muscles. The accumulation of oxidized proteins may further impair muscle function. The review in this section provides the rationales for the selection of the durations of disuse (0, 3, 7 and 14 days) in my studies.

Table 1. The levels of reactive species in muscles with and without disuse

Reference	Species	Age	Disuse	Muscle	Outcome
Falk et al., 2006 ³⁷	Rats (Sprague-Dawley)	6 mo	MV for 12 h	diaphragm	ROS (DCF assay): ↑ in muscles with MV
Kondo et al., 1992 ⁸⁵	Rats (male Wistar)	3.5 mo	cast for 4, 8, and 12 d	sol	Lipid peroxidation (TBARS assay): ↑ in muscles casted for 8 and 12 d
Kondo et al., 1993 ⁸⁶	Rats (male Wistar)	3.75 mo	cast for 8 d	sol	Level of H ₂ O ₂ : ↑ in muscles casted for 8 d
Ikemoto et al., 2002 ⁶³	Rats (Wistar)	1.5 mo	HU for 2, 5, 10, 14 and 21 d	gas	Lipid peroxidation (TBARS assay): ↑ in muscles HU for 2, 5, 10, 14 and 21 d
Lawler et al., 2003 ⁸⁹	Rats (Sprague Dawley)	6 mo	HU for 28 d	sol	Level of H ₂ O ₂ : ↑ 21% Level of oxidants (DCF): ↑ 162%
Liu et al., 2005 ⁹⁹	Rabbits (New Zealand White)	3 mo	cast for 21 d	sol	Lipid peroxidation (TBARS assay): no difference
Servais et al., 2007 ¹⁴³	Rats (Wistar)	NA	HU for 14 d	sol	Lipid peroxidation (TBARS assay): ↑ 35%
Arbogast et al., 2007 ³	Mice (ICR)	NA	HU for 1, 3, 6, and 12 d	sol	ROS (DCF assay): ↑ in muscles with HU for 1, 3, 6 and 12 d (reaches peak at 3 d of HU and stays high)
Suzuki et al., 2007 ¹⁵⁴	Mice (C57BL/6)	3 mo	HU for 7 and 14 d	skeletal muscle	NO [•] level: ↑ in muscles with HU for 7 and 14 d
Siu et al., 2008 ¹⁴⁷	Rats (Fischer 344 X Brown Norway)	6 mo 30 mo	HU for 14 d HU for 14 d	medial gas medial gas	Level of H ₂ O ₂ : no change Lipid peroxidation (MDA + 4-HAE): ↑ 29% Level of H ₂ O ₂ : ↑ 43% Lipid peroxidation (MDA/4-HAE): ↑ 58%

4-HAE: 4-hydroxyalkenal; d: day; DCF: dichlorofluorescein; gas: gastrocnemius; h: hour; HU: hindlimb unloading; MDA: malondialdehyde; mo: month old; MV: mechanical ventilation; NO[•]: nitric oxide; sol: soleus; TBARS: thiobarbituric acid-reactive substance

Table 2. The activity/content of antioxidants in muscles with and without disuse

Reference	Species	Age	Disuse	Muscle	MnSOD	Cu-Zn SOD	GPX	catalase	GSH	GST
Kondo et al., 1992 ⁸⁵	Rats (male Wistar)	3.5 mo	cast for 4, 8, and 12 d	sol	NA	NA	NA	NA	↓ with 8 and 12 d of cast	NA
Kondo et al., 1993 ⁸⁶	Rats (male Wistar)	3.75 mo	cast for 4, 8, and 12 d	sol	↓ with 12 d of cast	↑ with 8 and 12 d of cast	no change	↑ with 12 d of cast	NA	↑ with 8 and 12 d of cast
Ikemoto et al., 2002 ⁶³	Rats (Wistar)	1.5 mo	HU for 2, 5, 10, 14 and 21 d	gas	NA	NA	NA	NA	↓ with HU for 5, 10, 14, and 21 d	NA
Lawler et al., 2003 ⁸⁹	Rats (Sprague Dawley)	6 mo	HU for 28 d	sol	↓	↑	↓	↓	NA	NA
Liu et al., 2005 ⁹⁹	Rabbits (New Zealand White)	3 mo	cast for 21 d	sol	NA	NA	NA	NA	↓	↓
Selsby et al., 2007 ¹⁴¹	Rats (Sprague Dawley)	NA	cast for 7 d	sol	No change	↑	No change	↑	NA	NA
Servais et al., 2007 ¹⁴³	Rats (Wistar)	NA	HU for 14 d	sol	No change	↑	↑	↑	NA	NA

Table 2. The activity/content of antioxidants in muscles with and without disuse (Cont.)

Reference	Species	Age	Disuse	Muscle	MnSOD	Cu-Zn SOD	GPX	catalase	GSH	GST
Siu et al., 2008 ¹⁴⁷	Rats (Fischer 344 X	6 mo	HU for 14 d	medial gas	No change	No change in protein content	No change	↑	NA	NA
	Brown Norway)	30 mo	HU for 14 d	medial gas	No change	No change in protein content	No change	↑	NA	NA

Cu-Zn SOD: copper-zinc superoxide dismutase; d: day; gas: gastrocnemius; GPX: glutathione peroxidase; GSH: glutathione; GST: glutathione S transferase; HU: hindlimb unloading; mo: month old; MnSOD: manganese superoxide dismutase ; NA: non-assessment; sol: soleus

1.2 Protein degradation is increased in disused muscles

It has been shown in human studies that the proteasome expression and the degradation of the overall proteins increase in the disused muscles (75, 156). Focusing on the degradation of myosin heavy chain (MHC), Ikemoto et al. reported an increased degradation of MHC in gastrocnemius muscles of young rats with 10, 14 and 21 days of HU (62, 63).

The increased protein degradation in the disused muscles is associated with the up-regulations of proteasome proteolysis pathway and lysosomal proteolysis pathway. The activation of proteasome proteolysis pathway is characterized by increased *ubiquitin expressions*, increased *expressions of ubiquitin enzymes*, increased *ubiquitinated proteins*, increased *proteasome expressions* and increased *proteasome activity*. Evidence of each is described below. The increased *ubiquitin expressions* (mRNA) were observed in soleus and gastrocnemius muscles of young rats with 14 and 21 days of HU (32, 62). The increased *expressions of ubiquitin enzyme*, E2, were found in gastrocnemius muscles of young rats with 10, 14 and 21 days of HU (62). Regarding the level of *ubiquitinated proteins*, two studies show an increase of ubiquitinated proteins with muscle disuse. Vermaelen et al. found that the level of ubiquitinated proteins increased in the myofibrillar fraction of soleus muscles from young rats with 4 and 8 days of HU (163). Similarly, Ikemoto et al. demonstrated an increased protein ubiquitination in gastrocnemius muscles of young rats with 14 days of spaceflight, and with 5, 10, 14 and 21 days of HU (62). The increased proteasome expressions were observed in soleus and gastrocnemius muscles of young rats with 14 and 21 days of HU (32, 62). The increased *proteasome activity* was found in the gastrocnemius muscles of young rats with 21 days of HU (62). Taken together, the results show that there is a tight co-activation of ubiquitin, ubiquitin enzymes and proteasome.

In addition to the proteasome pathway, activation of lysosomal proteolysis pathway is also found in the disused muscles. Gastrocnemius muscles of young rats show an increase of lysosomal cysteine protease activity with 5, 10, 14 and 21 days of HU (62). Collectively, these studies indicate that the protein degradation pathways are up-regulated in the disused muscles as early as 4 days of HU, which provide the rationales for the selection of the durations of disuse in my studies.

In summary, oxidative stress increases in the disused muscles, which may result in protein oxidation. Although the degradation of oxidized proteins minimizes the accumulation of damaged proteins, it is possible the damaged proteins interfere with cellular processes (e.g., contractility and signaling).

2. Oxidative stress and age-related muscle dysfunction

2.1 Free radical theory of aging

Multiple, interrelated factors orchestrate the aging process of an organism. Free radical theory of aging, proposed by Denham Harman in the mid-1950s, describes that aging is the result of accumulation of free radical-induced damage. Correlative studies and experimental studies are designed to test this theory. **Correlative studies** investigate whether older organisms have greater oxidative stress characterized by the amount of ROS in the cells, and the levels of damaged lipids, proteins and DNA. To further test the hypothesis that oxidative stress determines the lifespan and health span of organisms, experimental studies are designed. In **experimental studies**, transgenic and knockout animals are genetically manipulated to express more or less antioxidants, and antioxidant supplementations and calorie restriction (CR) are non-genetic interventions that lessen the oxidative stress of animals. The hypothesis is rejected if animals with lower oxidative stress (transgenic animals, antioxidant supplementation and CR) do not have longer lifespan or health span.

The hypothesis is also rejected if animals with increased oxidative stress (knockout animals) exhibit no difference in the lifespan or health span from wild type animals.

*Oxidative stress is increased in muscle: **correlative studies***

Table 3 summarizes the correlative studies that investigate whether aging muscles have greater oxidative stress. Overall, these studies indicate that muscles from older animals have greater oxidative stress characterized by more ROS generation (7, 67, 147) and lipid peroxidation (39, 55, 73, 87, 93, 123, 147, 175). Interestingly, although numerous studies show that muscles from aged animals exhibit enhanced oxidative stress, results about the accumulation of oxidized proteins are inconclusive. The inconsistent results of the levels of the oxidized proteins with aging are likely due to the ages of animals and the fiber type composition of muscles. For example, carbonyl content is found increased with aging in muscles composed of primarily type II muscle fibers such as vastus lateralis and gluteus maximus (18, 39, 123, 175). In muscles that are composed of mainly type I muscle fibers such as deep vastus lateralis and soleus, carbonyl content is found unchanged and even decreased with aging (7, 117). Using more specific markers of protein oxidation, Zainal et al. reported a positive correlation between HNE-modified proteins and age in vastus lateralis muscles of monkeys (175). However, while nitrated proteins is found increased in gastrocnemius muscles from older animals (147), it is not found to accumulate with aging in vastus lateralis muscles (94, 95, 175). In short, the accumulation of oxidized proteins is muscle specific and also depends on the type of modifications.

In the presence of oxidative stress, specific proteins are the targets of oxidative-modification and only some modified proteins show accumulation (47, 69). For example, although myosin and actin are modified by NT and 4-hydroxy-2-nonenal (HNE), the extent of modification does not increase with aging (159). In

contrast, the contents of specific oxidized proteins accumulate with aging. Examples of the age-related accumulation of oxidized proteins are sarcoplasmic reticulum Ca^{2+} -ATPase, aconitase, β -enolase, carbonic anhydrase III and triphosphate isomerase (41, 47, 164).

In summary, correlative studies demonstrate that oxidative stress is increased in aging muscles characterized by increased ROS generation and accumulation of oxidized proteins. The accumulation of the overall oxidized proteins is muscle specific and also depends on the types of oxidative modification. Furthermore, some proteins are targets for oxidation and part of them are more susceptible for accumulation. The proteins that accumulate their oxidized forms in muscles with aging may play a role in the age-related muscle dysfunction.

Table 3. The influence of aging on the levels of reactive species in muscles

Reference	Species	Age comparison	Muscle	ROS content	Lipid peroxidation	Level of oxidized proteins
Ji et al., 1990 ⁷³	Rats (Wistar-Furth)	Y: 4 mo O: 26 mo VO: 31 mo	VL	NA	O, VO > Y	NA
Leeuwenburgh et al., 1994 ⁹³	Rats (Fischer 344)	Y: 4.5 mo A: 14.5 mo O: 26.5 mo	VL	NA	MDA: A, O > Y	NA
Leeuwenburgh et al., 1997 ⁹⁵	Mice (C57BL/6Nia)	Y: 4 mo A: 14 mo O: 28~30 mo	mixed hindlimb muscle	NA	NA	cross-link tyrosine residues: O > Y
Leeuwenburgh et al., 1998 ⁹⁴	Rats (Long-Evans/Wistar hybrid)	Y: 9 mo O: 24 mo	VL	NA	NA	NT-proteins: no change
Bejma et al., 1999 ⁷	Rats (female Fischer 344)	Y: 8 mo O: 24 mo	deep VL	↑77% (DCF assay)	MDA: no change	carbonyls: no change
Zainal et al., 2000 ¹⁷⁵	Rhesus monkey	Y: 4 yr A: 15 yr O: 26 yr	VL	NA	MDA + 4-HAE: A, O > Y	carbonyls: O > A > Y HNE-protein: ↑ with aging; NT-proteins: no change
Cakatay et al., 2003 ¹⁸	Rats (Wistar)	Y: 5 mo A: 13 mo O: 24~30 mo	gluteus maximus	NA	NA	carbonyls: O > A, Y NT-proteins: O > A, Y

Table 3. The influence of aging on the levels of reactive species in muscles (Cont.)

Reference	Species	Age comparison	Muscle	ROS content	Lipid peroxidation	Level of oxidized proteins
Mosoni et al., 2004 ¹¹⁷	Rats (Wistar)	6, 9, 12, 15, 19, 22, 25, 28 mo	gas, EDL, sol	NA	NA	In gastrocnemius and EDL: no change (carbonyls) In soleus: decreased carbonyls at 28 mo rats
Gunduz et al., 2004 ⁵⁶	Rats (Wistar)	Y: 9 mo O: 21 mo	gas, sol	NA	TBARS assay: O > Y	NA
Lambertucci et al., 2007 ⁸⁷	Rats (Wistar-Kyoto)	Y: 2 mo O: 21 mo	sol	NA	TBARS assay: O > Y	NA
Kim et al., 2008 ⁸²	Rats (Fischer 344)	Y: 6 mo O: 24 mo	plantaris	H ₂ O ₂ : ↑ 22%	NA	NA
Siu et al., 2008 ¹⁴⁷	Rats (Fischer 344 X Brown Norway)	Y: 6 mo O: 30 mo	medial gas	H ₂ O ₂ : ↑	MDA + 4-HAE: O > Y	NT-proteins: O > Y
Pansarasa et al., 2000 ¹²³	Human	A: 17~40 yr M: 41~65 yr O: 66~91yr	VL+ rectus abdominis + gluteus maximus	NA	O > A, M (only in men)	carbonyls: O > A, M
Fano et al., 2001 ³⁹	Human	Y: ≤ 35 yr O: ≥ 70 yr	VL	NA	MDA: O > Y	carbonyls: O > Y

4-HAE: 4-hydroxyalkenal ; DCF: dichlorofluorescein; EDL: extensor digitorum longus; gas: gastrocnemius; HNE: 4-hydroxynonenal; MDA: malondialdehyde; mo: month old; NA: non-assessment; sol: soleus; TBARS: thiobarbituric acid-reactive substance; VL: vastus lateralis; yr: years old

*Oxidative stress is increased in muscle: **experimental studies***

In contrast to correlative studies, experimental studies alter the status of oxidative stress of animals. Using transgenic/knockout animals, antioxidant supplementations and calorie restriction (CR), experimental studies test the hypothesis that oxidative stress determines the *lifespan* and *health span* of organisms. Regarding the *lifespan*, overexpression of antioxidants decreases the oxidative stress and increases the lifespan of invertebrate transgenic animals (50). However, in mammals, it remains inconclusive on whether oxidative stress determines the lifespan. In support, increased oxidative stress decreases the lifespan of Cu-Zn SOD knockout mice (33). On the other hand, decreased oxidative stress does not extend the lifespan of transgenic mice with Cu-Zn SOD overexpression (58).

Although the data that support the association between oxidative stress and longevity is inconclusive in mammals, studies provide evidence of a link between oxidative stress and *health span* in mammals. Regarding the age-related pathologies, Cu-Zn SOD knockout mice show severe oxidative stress and is accompanied by an acceleration of sarcopenia, hearing loss, macular degeneration, etc (65, 112, 118). On the contrary, rats with antioxidant supplementations show reduced oxidative stress and reversal of age-related cognitive decline, although the lifespan of rats does not extend (76, 77).

In contrast to the transgenic animals and antioxidant supplementations, calorie restriction (CR) is shown to extend the lifespan in mammals. Importantly, CR also extends the health span of mammals (16, 30, 125). Focusing on muscles, calorie restricted mice and monkeys show lower levels of oxidized proteins (using dityrosine, HNE, carbonyls and nitrotyrosine as markers) in skeletal muscles (95, 175). In addition, life-long calorie restriction attenuates the age-related decline in the muscle size and strength of rats (125).

Collectively, experimental studies support the hypothesis that oxidative stress is one mechanism of age-related muscle dysfunctions. Increased oxidative stress (Cu-Zn SOD knockout mice) causes acceleration of sarcopenia in mice. On the contrary, decreased oxidative stress (by calorie restriction) reduces the accumulation of oxidized proteins in muscles and attenuates the age-related muscle dysfunctions.

2.2 Adaptation of antioxidants in muscles with aging

Generally, muscles from older animals have greater levels of antioxidant activity compared to muscles from younger animals (39, 55, 57, 73, 82, 87, 93, 123, 147) (**Table 4**). The up-regulation of antioxidants with aging helps buffer the increased ROS generation and lessens the possible negative effects caused by the ROS. Interestingly, the changes of antioxidants with aging are antioxidant specific. While most antioxidants are up-regulated with aging, some are down-regulated (39, 55, 57, 87). Moreover, studies found that the changes of enzyme activity, protein content and mRNA level with aging are not always coordinated (57, 87, 147). Taken together, these studies suggest that different antioxidants may be regulated differently and the regulations can occur at transcriptional, translational and post-translational levels.

In addition to the different regulations among various antioxidants, the responses of antioxidants with aging are muscle specific. For example, Hollander et al. investigated the changes of antioxidants in muscles with aging in both soleus (type I) and superficial vastus lateralis (type II) muscles (57). They found the activities of Cu-Zn SOD, GPX and catalase increased with aging in type IIb muscles but were unchanged in type I muscles with aging. Most likely, the increased oxidative stress with aging is better managed in type I fibers, which have greater antioxidant capacity than type II fibers (128).

In summary, the antioxidant system is up-regulated in aging muscles. The increased antioxidant capacity minimizes the damage that may be caused by the increased oxidative stress of aging muscles.

Table 4. The influence of aging on the activity/content of antioxidants in muscles

Reference	Species	Age comparison	Muscle	MnSOD	Cu-Zn SOD	GPX	catalase	GSH	GST
Leeuwenburgh et al., 1994 ⁹³	Rats (Fischer 344)	Y: 4.5 mo A: 14.5 mo O: 26.5 mo	VL	NA	NA	activity: A, O > Y	activity: O > A > Y	protein: O = A = Y	activity: A, O > Y
			sol	NA	NA	activity: A, O > Y	activity: O > A > Y	protein: O, A > Y	NA
Hollander et al., 2000 ⁵⁷	Rats (female Fischer 344)	Y: 8 mo O: 25 mo	sol	activity: O > Y protein: O = Y mRNA: O < Y	activity: O = Y protein: O > Y mRNA: O = Y	activity: O = Y	activity: O = Y	NA	NA
			gas (type II mix)	activity: O > Y protein: O = Y mRNA: O < Y	activity: O > Y protein: O > Y mRNA: O = Y	activity: O = Y	activity: O = Y	NA	NA
			superficial VL (type IIb)	activity: O < Y protein: O = Y mRNA: O < Y	activity: O > Y protein: O = Y mRNA: O = Y	activity: O > Y	activity: O > Y	NA	NA

Table 4. The influence of aging on the activity/content of antioxidants in muscles (Cont.)

Reference	Species	Age comparison	Muscle	MnSOD	Cu-Zn SOD	GPX	catalase	GSH	GST
Gunduz et al., 2004 ⁵⁵	Rats (Wistar)	Y: 9 mo O: 19 mo	gas	NA	activity: O > Y	activity: O = Y	activity: O > Y	NA	NA
			sol	NA	activity: O = Y	activity: O = Y	activity: O > Y	NA	NA
Ji et al., 1990 ⁷³	Rats (Wistar-Furth)	Y: 4 mo O: 26 mo VO: 31 mo	VL	activity: O, VO > Y	activity: VO > Y	activity: O, VO > Y	activity: VO > O, Y	NA	activity: VO > O, Y
Lambertucci et al., 2007 ⁸⁷	Rats (Wistar-Kyoto)	Y: 2 mo O: 21 mo	sol	activity: O > Y mRNA: O > Y	activity: O = Y mRNA: O > Y	activity: O = Y mRNA: O > Y	activity: O > Y mRNA: O > Y	NA	NA
Kim et al., 2008 ⁸²	Rats (Fischer 344)	Y: 6 mo O: 24 mo	plantaris	protein: O = Y	protein: O = Y	NA	NA	NA	NA
Siu et al., 2008 ¹⁴⁷	Rats (Fischer 344 X Brown Norway)	Y: 6 mo O: 30 mo	medial gas	activity: O = Y protein: O < Y mRNA: O < Y	protein: O = Y mRNA: O = Y	activity: O = Y	activity: O > Y mRNA: O < Y	NA	NA

Table 4. The influence of aging on the activity/content of antioxidants in muscles (Cont.)

Reference	Species	Age comparison	Muscle	Mn SOD	Cu-Zn SOD	GPX	catalase	GSH	GST
Pansaras a et al., 2000 ¹²³	Human	A: 17~40 yr M: 41~65 yr O: 66~91 yr	VL+ rectus abdominis+ gluteus maximus	NA	NA	activity: A = M = O	activity: O > A, A < Y	protein: A = M = O	NA
Fano et al., 2001 ³⁹	Human	Y: <= 35 yr O: >= 70 yr	VL	NA	activity: O = Y	activity: O = Y	activity: O < Y	NA	activity: O < Y

Cu-Zn SOD: copper-zinc superoxide dismutase; gas: gastrocnemius; GPX: glutathione peroxidase; GSH: glutathione; GST: glutathione S transferase; MnSOD: manganese superoxide dismutase; mo: month old; NA: non-assessment; sol: soleus; VL: vastus lateralis; yr: years old

3. Age-related changes in oxidative status with stress

The ability of the cellular protective system to adapt to stress is compromised in aging muscles. As shown in **Table 5**, the up-regulation of antioxidants with exercise training is attenuated in aging muscles (87, 93, 119). With an acute stimulus, Vasilaki and colleagues found that antioxidant activities and the content of heat shock proteins are increased in muscles from adult animals, but are unchanged or decreased in muscles from old animals (162). The compromised response of antioxidants in muscles to stress is also observed in muscles with disuse. The content of MnSOD decreases in muscles from old rats with 14 days of HU but remains unchanged in muscles from young rats with same period of HU (147). Collectively, these studies indicate that the ability of the antioxidant system to respond to stress is compromised with aging.

In addition to the compromised ability of aged organisms to modulate their antioxidant levels, ROS content in muscles of older animals accumulates more after the stress. Siu and colleagues reported that while the content of hydrogen peroxide remained unchanged in muscles of young rats with 14 days of HU, the content in muscles of old rats was increased after the same period of HU. In support of the increased ROS levels, the same study shows that the increase of lipid peroxidation after 14 days of HU is greater in muscles from old animals compared to that in the muscles from young animals (147).

Overall, the ability of aged organisms to modulate the antioxidant levels of muscles to stimuli is compromised. This finding provides the foundation of my thesis questions.

Table 5. Age-related adaptation of oxidative status with stimuli

Reference	Species	Age comparison	Stimulus	Muscle	Antioxidant/ chaperone
Leeuwenburgh et al., 1994 ⁹³	Rats (Fischer 344)	Y: 4.5 mo A: 14.5 mo O: 26.5 mo	Training: 10 wks of treadmill	VL sol	GPX activity: Y↑, A&O — Catalase activity: Y,A,O — GPX activity: Y,A&O: — Catalase activity: Y—, A& O ↓
Lambertucci et al., 2007 ⁸⁷	Rats (Wistar-Kyoto)	Y: 2 mo O: 21 mo	Training: 13 wks of treadmill	sol	mRNA of Mn SOD: Y↑, O↓ mRNA of Cu-Zn SOD & GPX: — in Y&O Mn SOD activity: Y↑, O ↓ Cu-Zn SOD activity: — in Y&O GPX activity: Y↑, O — catalase activity: Y↑, O —
Murlasits et al., 2006 ¹¹⁹	Rats (Fischer 344 X Brown Norway)	Y: 3 mo O: 30 mo	Training: resistance exercise (14 sessions)	AD	Cu-Zn SOD activity: Y↑, O↓ mRNA of HSP72,HSP70 & HSP25: Y&O — content of HSP72: Y&O↑ content of HSP70: Y&O — content of HSP25: ↑ more in Y
Vasilaki et al., 2006 ¹⁶²	Mice (B8 x SJL)	A: 12 mo O: 30 mo	One time stimulus: isometric contraction	TA, gas	Cu-Zn SOD activity: A↑, O↓ Catalase activity: A↑, O — HSP70: A↑, O↓ HSP25: A↑, O —
Siu et al., 2008 ¹⁴⁷	Rats (Fischer 344 X Brown Norway)	Y: 6 mo O: 30 mo	Stimulus: HU for 14 days	medial gas	Mn SOD content: Y—, O↓ Mn SOD&GPX activity: Y&O — Catalase activity: Y&O↑

AD: ankle dorsiflexor; Cu-Zn SOD: copper-zinc superoxide dismutase; gas: gastrocnemius; GPX: glutathione peroxidase; GSH: glutathione; HSP: heat shock protein; Mn SOD: manganese superoxide dismutase; mo: month old; sol: soleus; TA: tibialis anterior; VL: vastus lateralis

IV. Thesis Goal

The decline of muscle functions with disuse is greater in aging muscles. However, the cellular mechanisms underlying the greater functional decline of aging muscles with disuse are unknown. Research to date proposes that oxidative stress mediates both disuse and aging related muscle dysfunction. Thus, the *overall* goal of my thesis is to investigate the age-related changes of oxidative stress with muscle disuse (**Figure 12**).

Specifically, the *first goal* of my research is to investigate the age-related alteration of antioxidants in response to disuse. The hypothesis is that the alteration of antioxidants in response to muscle disuse is compromised in aging muscles. Enzyme activity and/or content in muscles from adult/old rats with/without muscle disuse were measured to test the hypothesis.

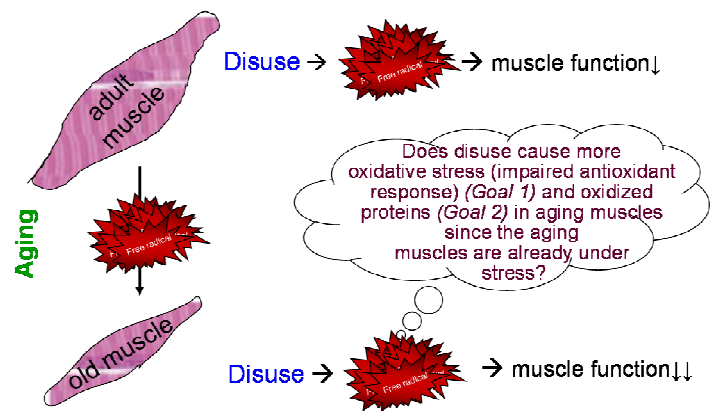


Figure 12. Illustration of research background and questions

The *second goal* of my research is to investigate the age-related differences in protein oxidative modifications with disuse. To date, research regarding the disuse of limb muscles is focused on young animals and only investigated the global oxidized proteins. Thus, it remains unknown whether the oxidized proteins accumulate more with disuse in aging muscles, and whether the accumulation of oxidized proteins is protein specific. The hypotheses are (1) the global accumulation of oxidized proteins with muscle disuse is greater in the aging muscles, and (2) the accumulation of oxidized proteins with disuse is protein specific, and (3) the alteration in the levels of the oxidation state of these

target proteins is age dependent. In this study, proteomic techniques were used to test hypotheses.

A two-factor experimental design (age x disuse) was used throughout my studies to test the hypotheses stated above. As shown in **Table 6**, there were 2 levels in the age factor and 4 levels in the disuse factor.

Table 6. Summary of study design

Animals (Fischer 344 rats) \ Disuse conditions	control	3d	7d	14d
Adult (13 months old)	n=10	n=10	n=10	n=10
Old (26 months old)	n=10	n=10	n=10	n=10

The 2 levels in the age factor were adult (100% strain survival) and old animals (25% strain survival). The 4 levels in the disuse factor were weight bearing (control) and hindlimb unloading (HU) for 3, 7, and 14 days (3d, 7d, and 14d, respectively). Hindlimb unloading, a technique that prevents the hindlimb muscles from weight bearing, was employed to mimic the bed rest condition in humans.

The durations of HU (3, 7 and 14 days) were chosen because studies have shown that the oxidative stress in muscles is increased with these durations of HU (3, 143, 147) and the degradation of myofibrillar proteins reaches the peak at 15 days of unloading (157). Since oxidized proteins are more susceptible to proteolysis, I predicted that the protein oxidation occurs during the first two weeks of HU. The soleus muscle was selected to be investigated in my studies because it is composed of predominantly type I fibers that are affected significantly by unloading and show age-related changes (59, 87). Collectively, the study design was used to test the stated hypotheses, and the results with discussions are presented in Chapters 2~4.

Chapter 2

Muscle Disuse: Adaptation of Antioxidant Systems Is Age-Dependent

In this chapter, the hypothesis “the adaptation of antioxidant systems to muscle disuse is age-dependent” is tested. The activities/contents of key antioxidants in muscles including Cu-Zn superoxide dismutase (SOD), catalase, glutathione (GSH) and glutathione peroxidase were measured. The main finding of this study is that the alterations of GSH level with muscle disuse are age-dependent. In adult animals, the homeostasis of GSH level in muscles is maintained during the 14 days of HU. However, the GSH level in aging muscles is dramatically decreased with 14 days of HU.

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I was responsible for the experimental design, data collection and analysis, and the writing of the manuscript of this study with the assistance of LaDora Thompson. Holly Brown-Borg and Sharlene Rakoczy were involved in the data collection, technical support and the revision of the manuscript.

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* “GSH peroxidase” is changed to “GPX” and “hind-limb” is changed to “hindlimb”. In figures and figure legends, “control” is replaced with “C”, “3d HU”

is replaced with “3d”, “7d HU” is replaced with “7d” and “14d HU” is replaced with “14d”.

I. Abstract

This study investigated the age effect on antioxidant adaptation to muscle disuse. Adult and old rats were randomized into 4 groups: weight bearing (control), 3 days of hindlimb unloading (HU), 7 days of HU, and 14 days of HU. Activities of Cu-Zn superoxide dismutase (SOD), catalase, and glutathione (GSH), as well as glutathione peroxidase (GPX) levels were measured in the soleus. Neither disuse nor aging changed the activity of Cu-Zn SOD. The old rats had greater GPX activity, whereas the activity of catalase had a compensatory increase with disuse, independent of age. Reduced GSH level and total glutathione (tGSH) level had age-related change with disuse. In old rats, the GSH and tGSH levels were lower with disuse, whereas the levels remained stable with disuse in adult rats. The depletion of intracellular GSH and tGSH levels of muscles from aged animals with disuse may make aged muscles more susceptible to oxidative damage.

II. Introduction

Individuals who are restricted to bed rest due to severe trauma (e.g., brain injury or complicated fracture) or serious disease (e.g., renal disease) are prone to experience muscle atrophy and weakness (170). This disuse-induced muscle dysfunction is thought to be related to the intracellular oxidative stress of the muscles (66, 129, 130). Oxidative stress develops when the production of oxidants exceeds the scavenger capacity of the antioxidant system. Support for the disuse-induced oxidative stress is found in investigations using young animals. These studies report increased oxidant production (3, 86, 89, 99), antioxidant adaptations (86, 140, 141), and the accumulation of oxidatively modified proteins (140) with muscle disuse.

The aging process is associated with increased oxidative stress. Numerous studies have shown that muscles from aged animals exhibit enhanced oxidative stress, characterized by lipid peroxidation, protein oxidative modification, and DNA damage (7, 49, 55). Muscle cells are able to adapt and respond to this increased oxidative stress by altering the expression of antioxidant enzymes. Several studies have demonstrated that the activity of antioxidant enzymes are increased in the aged muscles (49, 55, 57, 93, 105).

Although the antioxidant systems have the ability to adapt in the presence of increased oxidative stress, the adaptation may be influenced by the age of the animals. For example, the study of Leeuwenburgh and colleagues (93) found that exercise training increased the activity of antioxidant enzymes in muscles from young rats, but not in the muscles from aged rats. Other age-dependent responses are also observed in muscles following periods of disuse. Siu and colleagues (148) found that the level of heat shock protein 27, an antiapoptotic protein, increased in muscles from young animals following disuse but not in aged animals. Studies by Thompson and colleagues (160) found that hindlimb unloading deteriorated the muscle function in both adult and old animals.

However, this muscle function deterioration was more pronounced in old animals. In addition, they also showed that the ability of aged muscles to maintain the ratio of force to fiber size during hindlimb unloading was compromised (59). It is unknown whether adaptations of the antioxidant systems to muscle disuse are age-dependent and contribute to the different responses observed in muscle function.

The aim of this study was to investigate the influence of age on the ability of skeletal muscle tissue antioxidants to adapt to disuse. Based on the age-related responses in muscle function to disuse and to the already enhanced antioxidant capacity in aged muscles, we hypothesized that the adaptation of antioxidant systems to muscle disuse is age-dependent. The soleus muscle was chosen to be investigated in this study because it (a) is composed predominately of type I fibers, (b) is an antigravity, weight-bearing muscle, and (c) shows physiological and biochemical adaptations with unloading (86, 160, 163). The novel finding of this study was that, unlike the muscles from adult rats (13 months), which can maintain the intracellular glutathione (GSH) level at the control level after 14 days of muscle disuse, the GSH level in the muscles from aged rats (26 months) is reduced dramatically with disuse. This compromised antioxidant capacity of the aged muscles may be associated with the age-related responses of muscle functions with muscle disuse.

III. Methods

Animals and Hindlimb Unloading

Fifty-six male Fischer 344 rats aged 13 months (100% strain survival) ($n = 28$) and 26 months (25% strain survival) ($n = 28$) were purchased from the Minneapolis Veterans Administration Aged Rodent Colony. These rats were randomized into four groups: normal weight bearing (control), hindlimb unloading (HU) for 3 days (3d), 7 days (7d), and 14 days (14d). The hindlimb unloading intervention was achieved by attaching the tail of the rat to a swivel mounted at the top of the cage. The height of the suspension was adjusted to prevent the hind limbs from contacting the floor. This arrangement permits animals to walk around with their forelimbs while hind limbs are unloaded. All the animals were housed in a research animal facility and were checked daily for any abnormal response to suspension. The protocol of this study was approved by the University of Minnesota Institutional Animal Care and Use Committee.

Tissue Preparation

The rats were anesthetized with pentobarbital sodium (35 mg/kg body weight) after the intervention. Soleus muscles were harvested, weighed, and immediately frozen in liquid nitrogen. The frozen soleus muscles were stored in a -80°C freezer until homogenization. To measure enzyme activities of catalase and glutathione peroxidase (GPX), soleus muscles were homogenized in buffer containing 20 mM 3-(4-morpholino)propane sulfonic acid (MOPS), 62 mM sucrose, and 0.1 mM EDTA (pH 7.2). The supernatant that contained the extracted protein was collected after centrifuging at 12,000g for 25 minutes. Protein concentration was then measured by the Bradford method. For measurement of GSH content, soleus muscles were homogenized in 1% picric acid. The supernatant was collected after centrifuging at 12,941g for 30 minutes (12, 14).

Enzyme Activity Measurement:

Catalase activity. —Catalase activity was determined spectrophotometrically (1) by measuring the breakdown of hydrogen peroxide at a wavelength of 240 nm for 5 minutes at 30°C. Briefly, assay medium containing 0.34% hydrogen peroxide, 50 mM potassium phosphate monobasic, and 50 mM sodium phosphate dibasic (pH 7.0) was added to the samples to initiate the reaction as previously described (12, 14).

Glutathione peroxidase (GPX) activity.—GPX activity was determined spectrophotometrically (43) by measuring the oxidation of NADPH at a wavelength of 340 nm, 30°C for 5 minutes. Briefly, samples were incubated in an assay medium containing 50 mM Tris–EDTA buffer, 1 mM reduced GSH, GSH reductase at 5 U/mL, and 0.15 mM NADPH for 15 minutes. The reaction was then initiated by adding *t*-butyl hydroperoxide as previously described (12, 14).

Cu-Zn superoxide dismutase activity.—Cu-Zn superoxide dismutase (SOD) activity was determined using spectrophotometric assay (Bioxytech; Portland, OR).

Level of total glutathione, reduced GSH, and reduced to oxidized GSH ratio.—The GSH level was determined based on the standard curve, which was generated from the known concentrations of GSH and the formation of 2-nitro-5-thiobenzoic acid measured spectrophotometrically at a wavelength of 412 nm (52) using previously described procedures (12). Briefly, the reaction was initiated by adding GSH reductase to the medium containing 125 mM phosphate–EDTA buffer, 0.3 mM NADPH, 6 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and different amounts of reduced GSH. To measure oxidized glutathione (GSSG), 4-vinyl pyridine was added to the sample and incubated for 1 hour. This incubation conjugated the reduced GSH in the sample; therefore,

only oxidized GSH could reduce to GSH. The GSH level was determined by standard curve, and the GSSG level was calculated. The intracellular total glutathione level (tGSH) was indicated by GSH+GSSH. The ratio of reduced to oxidized glutathione (GSH/GSSG), which represented total tissue oxidative stress, was then determined.

Statistics

Data were presented as mean \pm standard error of the mean. Two-way analysis of variance (ANOVA) was used to determine the effect of aging and hindlimb unloading on the antioxidant system in the soleus muscle from rats. Tukey's honest significant difference test was used as a post hoc test when the main effect of aging or hindlimb unloading reached significance. A significant difference was considered achieved when $p < .05$.

IV. Results

Activity of Cu-Zn SOD

Cu-Zn SOD is an isoform of SOD that exists in the cytosol and converts superoxide anions to hydrogen peroxide. Neither hindlimb unloading nor aging changed the activity of Cu-Zn SOD (range: 18.06–29.27 U/mg protein). The activity of Cu-Zn SOD in the soleus muscle from both adult and old rats remained stable with hindlimb unloading.

Activity of glutathione peroxidase (GPX)

Hindlimb unloading did not change the activity of the antioxidative enzyme, GPX. However, the activity of GPX was significantly affected by age. The soleus muscle from old rats had greater GPX activity than did the muscle from adult rats (**Figure 1**). The soleus muscle from old rats had 28% greater GPX activity compared to the value seen in the adult muscle.

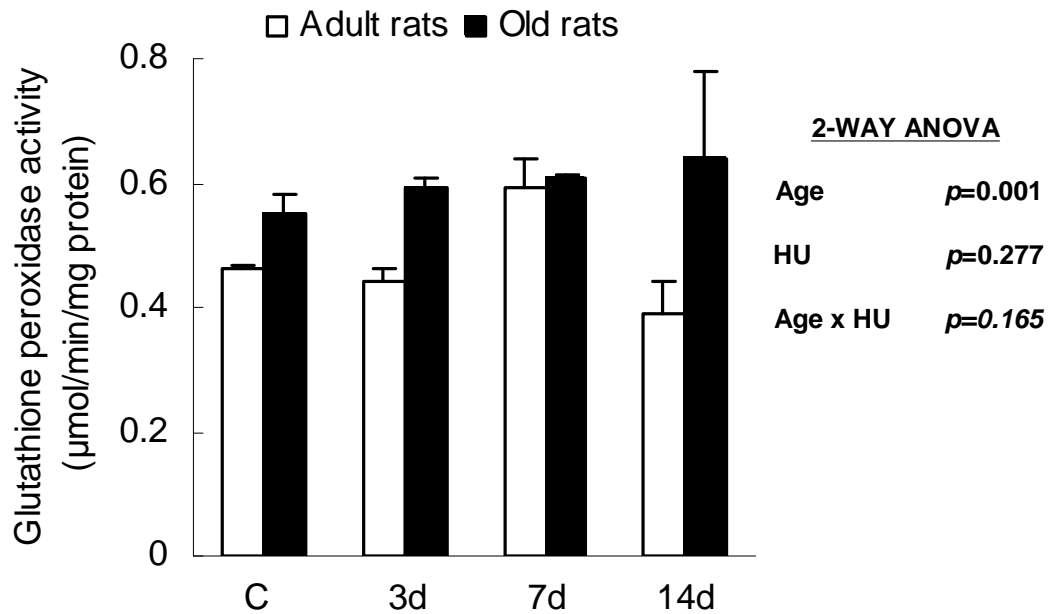


Figure 1. Activity of glutathione peroxidase from rats with weight bearing (C), hindlimb unloading (HU) for 3 days (3d), 7 days (7d), and 14 days (14d). Values are mean \pm standard error of the mean. The effect of age was independent of HU. ANOVA, analysis of variance.

Activity of Catalase

Catalase, an antioxidant enzyme that degrades hydrogen peroxide to water, reduces the H_2O_2 concentration of the cells. Hindlimb unloading altered the activity of catalase in the soleus muscles from rats, independent of age (Age \times HU, $p = .170$). Catalase activities of the 7d and 14d rats were 22% and 30% greater, respectively, than the rats with weight bearing (control group) (**Figure 2**).

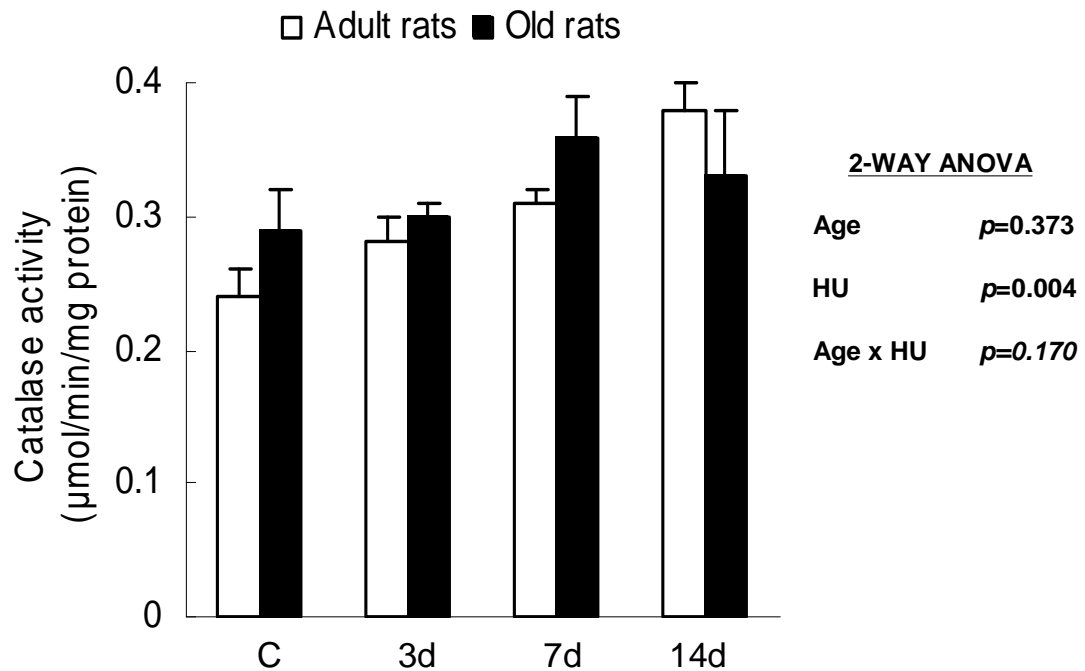


Figure 2. Catalase activity from rats with weight bearing (C), hindlimb unloading (HU) for 3 days (3d), 7 days (7d), and 14 days (14d). Values are mean \pm standard error of the mean. The effect of HU was independent of age. Catalase activities of the rats with 7d HU and 14d HU were higher than those of the rats with weight bearing (control). ANOVA, analysis of variance.

GSH Level

Hindlimb unloading affected the GSH level in the soleus muscle; however, the effect depended on the age of the rats (Age \times HU, $p = .004$) (**Figure 3**). In adult rats, the GSH level of soleus muscles remained stable with hindlimb unloading.

In old rats, the GSH level of muscle with 14 days of hindlimb unloading was 1.21 ± 0.15 mmol/g protein, which was significantly lower than the level of muscle with weight bearing and both 3 days and 7 days of hindlimb unloading (3.00 ± 0.24 , 2.39 ± 1.67 , and 3.14 ± 0.24 mmol/g protein, respectively).

In addition to disuse, aging also affected the GSH level. The GSH levels of soleus muscles were 130% greater in the old control rats compared to the level in the adult control rats ($p < .001$).

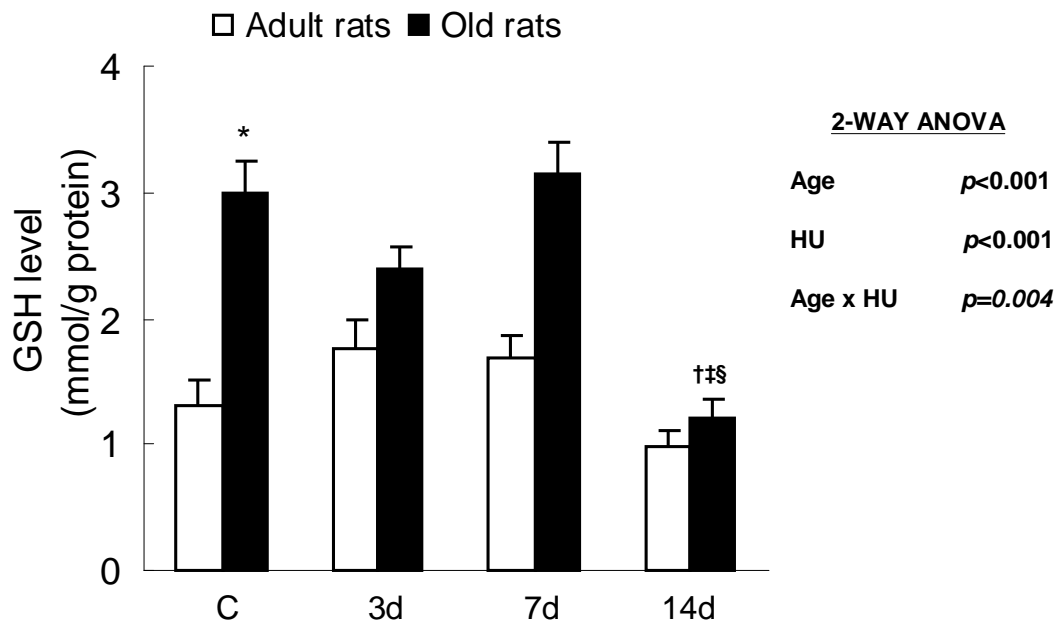


Figure 3. Glutathione (GSH) level from rats with weight bearing (C), hindlimb unloading (HU) for 3 days (3d), 7 days (7d), and 14 days (14d). Values are mean \pm standard error of the mean. The effect of HU was dependent on age. * Significantly different from adult control. † Significantly different from old control. ‡ Significantly different from old 3d rats. § Significantly different from old 7d rats. ANOVA, analysis of variance.

tGSH Level

tGSH level was the sum of GSH and oxidized glutathione in the muscle tissue. Hindlimb unloading affected the tGSH level in the soleus muscle; however, the effect depended on the age of the rats (Age \times HU, $p = .001$) (**Figure 4**). In adult rats, the tGSH level of soleus muscles remained stable with hindlimb unloading.

In old rats, the tGSH level of muscle with 14 days of hindlimb unloading was 1.37 ± 0.27 mmol/g protein, which was significantly lower than the tGSH level of muscle with weight bearing and both 3 days and 7 days of hindlimb unloading (3.29 ± 0.20 , 2.62 ± 0.19 , and 3.24 ± 0.20 mmol/g protein, respectively).

In addition to disuse, aging also affected the tGSH level. The tGSH levels of soleus muscles were 147% greater in the old control rats compared to the adult control rats ($p < .001$).

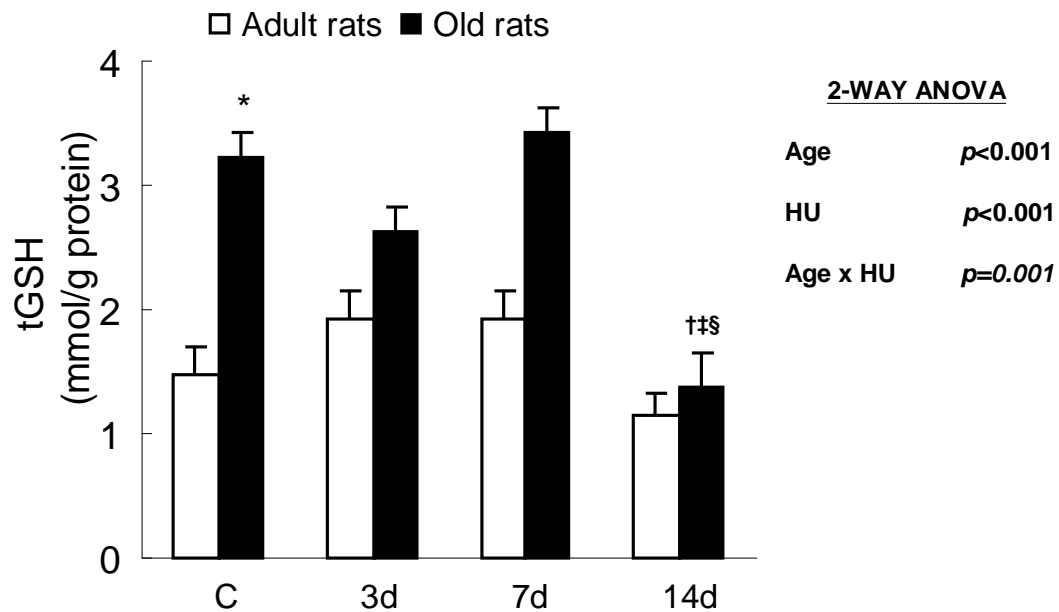


Figure 4. Total glutathione level (tGSH) from rats with weight bearing (C), hindlimb unloading (HU) for 3 days (3d), 7 days (7d), and 14 days (14d). Values are mean \pm standard error of the mean. The effect of HU was dependent on age. * Significantly different from adult control. † Significantly different from old control. ‡ Significantly different from old 3d rats. § Significantly different from old 7d rats. ANOVA, analysis of variance.

GSH/GSSG

GSH/GSSG tended to decrease with hindlimb unloading, although this change did not reach significance ($p = .066$). However, this ratio was significantly affected by age. The soleus muscle from old rats had a 38% greater GSH/GSSG (**Figure 5**).

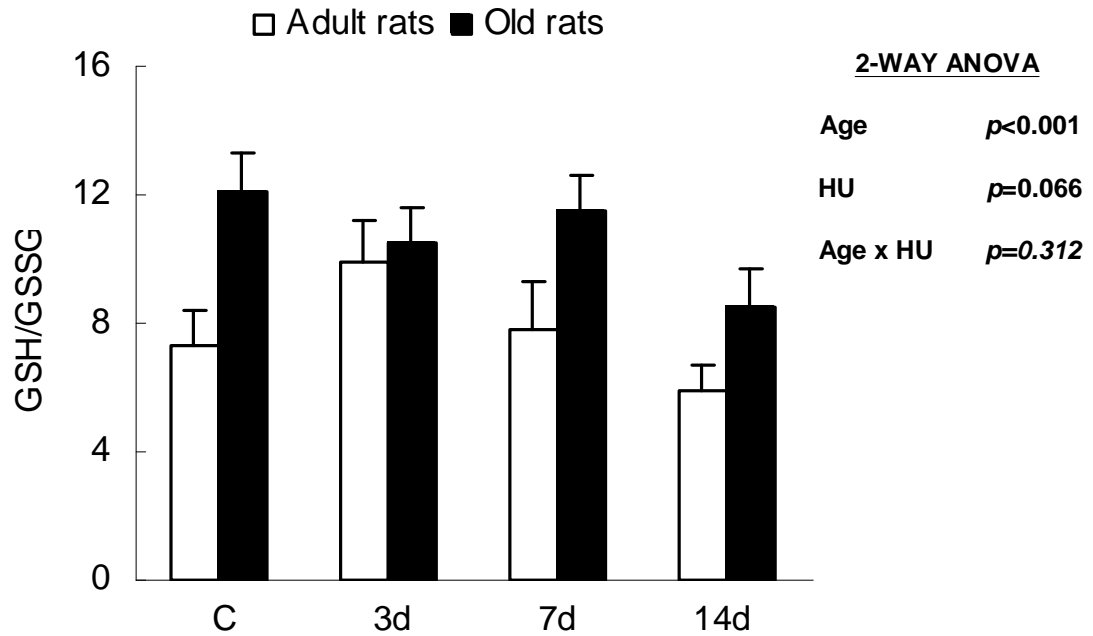


Figure 5. The reduced to oxidized glutathione ratio (GSH/GSSG) from rats with weight bearing (C), hindlimb unloading (HU) for 3 days (3d), 7 days (7d), and 14 days (14d). Values are mean \pm standard error of the mean. The effect of age was independent of HU. ANOVA, analysis of variance.

V. Discussion

The aim of this study was to investigate the influence of age on the adaptive ability of the antioxidant systems in the soleus muscle, a muscle composed of predominantly type I fibers, to disuse. We hypothesized that the adaptation of antioxidant systems to muscle disuse is age-dependent. In general, our results show that the antioxidant systems of the soleus muscles from both adult and aged rats did adapt to disuse (hindlimb unloading). Specifically, we found that the age of the rats influences the response of GSH and tGSH levels in the soleus muscle to hindlimb unloading. In adult rats, the levels of GSH and tGSH of muscles under 14 days of hindlimb unloading were not different from the levels seen in the muscles with normal weight bearing. However, the levels of GSH and tGSH in muscles from aged rats under 14 days of hindlimb unloading were approximately 60% lower than the levels seen in the muscles with normal weight bearing.

Age-Related Adaptation of Antioxidant Systems

Aging is associated with increased oxidative stress. Many studies show the age-related increase of lipid peroxidation, protein oxidative modification, and DNA damage (7, 49, 55). Similarly, the results of the current study suggest that the soleus muscles from aged rats have greater oxidative stress than muscles from adult rats. We find the tGSH level, the GSH level, and GPX activity of the aged muscles were 72%, 70%, and 28% higher, respectively, than the values seen in the adult muscles. Although the changes in the antioxidants reveal an enhanced oxidative environment of the cells, the changes demonstrate a positive adaptation of the antioxidant system with aging. This adaptation is positive to the cells because by increasing the content and/or activity of antioxidants, the impact (damage) of the increased intracellular oxidative stress with aging is minimized. Indeed, this positive adaptation is believed to be an important factor to cell survival. The age-related positive adaptation in skeletal

muscle is consistent with the work of Leeuwenburgh and colleagues (93) that demonstrated an age-related increase of the GSH level in the soleus muscles.

Activities of antioxidant enzymes, Cu-Zn SOD and catalase, were not different between the soleus muscles of adult and aged rats. The unchanged activity of Cu-Zn SOD and catalase in the soleus muscles with aging in our study is consistent with the findings of several studies that investigated the age related adaptation of antioxidant systems in this same tissue (57, 87). In contrast, other studies have shown increased activity of Cu-Zn SOD and catalase (57, 73). The different results of the enzyme adaptation with aging are likely due to the fiber type composition of the muscles that were investigated. For example, Hollander and colleagues (57) measured the age-related adaptation of the antioxidant systems in both type IIb and type I muscles. They found the activities of Cu-Zn SOD and catalase increased in type IIb muscles but remained unchanged in type I muscles with aging. Most likely, chronic oxidative stress can be better managed in type I fibers, which possess greater antioxidant capacity than do type II fibers (128).

Potential Mechanisms to Explain the Antioxidant Adaptation with Aging

The mechanisms to explain the age-related positive adaptation of antioxidant systems are unknown; however, the adaptation is likely associated with the chronic enhanced oxidative environment (stress). For instance, previous studies show that chronic moderate oxidative stress, such as exercise training and chronic smoking, induces a compensatory increase in GSH levels (133, 142). The finding of the age-related positive adaptation of antioxidant systems in the current study suggests that muscle cells are under chronic oxidative stress during the aging process.

In the current study, we investigated the response of GSH, tGSH, activity of GPX, and GSH/GSSG to muscle disuse in the soleus muscles from both adult

and old rats because this antioxidant system is a key pathway to prevent protein damage with oxidative stress. GSH, a tripeptide (γ -glutamyl-cysteinyl-glycine) containing a sulfhydryl ($-SH$) group, is a crucial peptide that protects cells from oxidants. This protective function of GSH is achieved by direct conjugation with radicals as well as electron donation in redox reactions. GSH is the substrate in redox reactions, and these reactions are catalyzed by GPX. This redox reaction oxidizes GSH to GSSG while hydrogen peroxide and other peroxides are reduced. The generated GSSG is recycled to GSH catalyzed by the NADPH-dependent GSH reductase. Previous studies clearly demonstrate that the GSH to GSSG cycling is crucial in regulating the redox status in the cell, and the intracellular GSH level is related to the redox status of the cells (107, 167). We found that muscles from old rats have a greater tGSH level, a greater GSH level, and a greater GPX activity. In addition, the GSSG level in muscles of old rats is no different from that in muscles of adult rats. The results suggest that the GSH-mediated redox system is up-regulated with aging to maintain the redox balance of the muscle cells.

It is thought that chronic oxidative stress induces an increase of intracellular GSH by increasing the expression of the rate-limiting enzyme involved in the synthesis of GSH, γ -glutamylcysteine synthetase (GCS). The transcription factors, activator protein-1 (AP-1) and nuclear factor- κ B (NF- κ B), have been shown to be redox sensitive and modulate gene expression of GCS (31, 101, 142) leading to increased GSH levels. Thus, the increased GSH levels in muscles from aged rats in the current study could result from the increased activity of GCS.

Adaptation of Antioxidant Systems to Muscle Disuse

There are many studies investigating the effects of muscle disuse on the intracellular adaptation of skeletal muscles; however, the investigations are mainly done on muscles from young or adult rats (63, 86, 89, 90, 99, 121, 163).

Few studies investigate the adaptative ability of the antioxidant systems in skeletal muscles to muscle disuse in aged animals (176). In general, muscle disuse induces greater oxidant production (63, 86, 89, 90, 99), accumulation of oxidative modified proteins (140), and antioxidant adaptations (63, 86, 89, 90, 99) in muscles from young and adult rats. Consistent with previous research, the results of this study show that hindlimb unloading increased oxidative stress of the muscle as indicated by the increased catalase activity. The increased catalase activity with muscle disuse is reported in other studies that have a muscle disuse duration similar to that of our study (86, 140). The finding of the unchanged activity of GPX with muscle disuse is also consistent with the studies that have a muscle disuse duration similar to that of our study (86, 140). In contrast, two studies report an increase of Cu-Zn SOD activity (86, 140), whereas our present data showed no change of Cu-Zn SOD activity with muscle disuse. The different results of the enzyme adaptation with disuse are likely due to different models of muscle disuse as well as different ages of the rats that were studied. For example, both Kondo and coworkers (86) and Selsby and Dodd (140) used limb immobilization by cast as the model whereas we used hindlimb unloading as the model of muscle disuse. In addition, different from the study by Kondo and coworkers, which investigated the antioxidant responses to disuse of young rats (15 weeks old), this current study investigated the responses of the adult and aged rats (13 months and 26 months, respectively).

The novel finding of the current study was the age-dependent response to muscle disuse. In general, the GSH level is maintained in soleus muscles from adult rats but is depleted in the muscles from aged rats with disuse. The GSH level in the muscles from aged rats decreased to 40% of the control value with 14 days of hindlimb unloading. The age-dependent response to muscle disuse is also observed in intracellular tGSH levels. The tGSH level of aged muscles decreased to 42% of the control value with 14 days of hindlimb unloading.

These decreases in GSH and tGSH levels compromise the overall antioxidant defense capacity of the aged muscle. Thus, this response of aged muscles to disuse implies a negative adaptation to cell survival.

Potential Mechanisms to Explain the Age-Dependent GSH Adaptation with Disuse

The differential intracellular GSH and tGSH response of soleus muscle to disuse between adult and old rats implies that the positive increase in GSH levels during the aging process leaves the aged muscles with fewer reserves and less adaptation ability.

The finding of the dramatic decrease of GSH and tGSH levels in the aged muscles with 14 days of disuse suggests that muscles with 14 days of disuse have (a) greater GSH consumption or/and (b) lower GSH production compared to the muscles in control weight-bearing animals.

Greater GSH Consumption-The greater GSH consumption is likely due to increased GSH utilization and efflux. GSH is utilized (a) to detoxify oxidants by direct conjugation (catalyzed by glutathione S-transferase [GST]) and (b) as an electron donor in a redox reaction (catalyzed by GPX). In the redox reaction, GSH is recycled by GSH reductase; however, GSH is consumed in direct conjugation with oxidants. In addition, GSH conjugates, GSH, and GSSG can be transported out of cells by γ -glutamyl transferase (GGT), a membrane-bound enzyme that breaks the γ -peptide bond of the GSH and degrades it. When the redox balance of cells is disrupted, intracellular GSSG and GSH-protein adducts accumulate and the GSH level decreases. The increased GSSG and GSH-protein adducts could be transported out of the cell, thus decreasing the intracellular tGSH level (107). As a vicious cycle, the decreased GSH level reduces the activity of enzymes that catalyze the reducing reaction of GSSG and GSH-protein adducts (40).

Thus, it is possible that reactive oxygen species (ROS) production significantly increases in the aged muscles with 14 days of disuse and the increased GST and GGT activities catalyze the utilization and efflux of the GSH in the cells. This hypothesis needs to be further tested.

Lower GSH Production- GSH production is affected by substrate availability and the synthesis rate. Cysteine is the limiting amino acid for GSH synthesis because the concentrations of glutamate and glycine are relatively high intracellularly. Factors that influence cysteine metabolism (such as insulin and growth factors) affect the intracellular GSH level. The synthesis of GSH is catalyzed consecutively by GCS and GSH synthetase. GCS expression and its activity are modulated by factors such as inflammation and oxidative stress at transcriptional, translational, and posttranslational levels. As discussed earlier, the greater GSH level of muscles with aging is a compensatory adaptation to the oxidative stress.

Thus, the dramatic decrease of GSH and tGSH levels in aged muscles with 14 days of disuse could be due to (a) the limitation of cysteine availability of the cells or (b) the fact that GCS cannot further adapt to the increased oxidative stress in the aged muscles with 14 days of disuse. Further investigation is needed to test this hypothesis.

Summary

Hindlimb unloading induced adaptations of antioxidant systems in the soleus muscles from both adult and old rats. This adaptation, however, was different between adult and aged muscles. In adult muscles, the levels of GSH and tGSH were maintained at the control level. In contrast, the levels of GSH and tGSH in the aged muscle were significantly reduced by 14 days of hindlimb unloading. This dramatic decrease in the GSH levels in aged muscles may

compromise the overall antioxidant capacity of the aged muscles. The compromised antioxidant capacity of the aged muscles with muscle disuse may predispose the proteins in the soleus muscle to damage. Future studies are needed to investigate the possible mechanisms of the impaired adaptation of GSH to disuse in the muscles of old animals.

Chapter 3

Aging Impairs the Expression of Glutamate Cysteine Ligase Catalytic Subunit in Muscle Under Stress

In the previous study, we found that the ability of aged organisms to adapt to additional stressors is compromised. In adult animals, the homeostasis of glutathione (GSH) level in muscles is maintained during the 14 days of HU. However, the GSH level in aging muscles is dramatically decreased with 14 days of HU (**Figure 1**). The finding gave rise to the study described in this chapter.

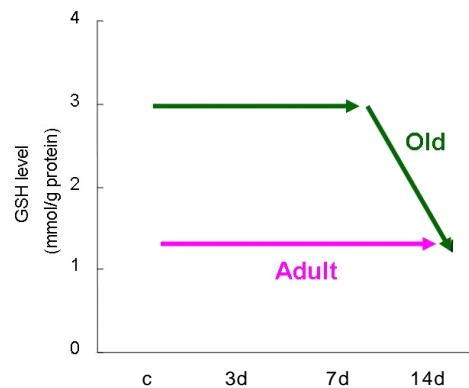


Figure 1. The simplified diagram shows that muscles from old animals could not maintain GSH homeostasis with 14 days of hindlimb unloading (HU). c: weight bearing; 3d: 3 days of HU; 7d: 7 days of HU; 14d: 14 days HU

The aim of this study was to investigate the mechanism behind the dramatic drop of GSH levels in disused muscles of old animals. Thus, enzymes that are associated with GSH metabolism were measured in this study. The main finding of this study is that the decrease of GSH synthesis explains the decline of GSH levels in aging muscles with disuse.

The content in this chapter will be submitted with the title “Aging Impairs the Expression of Glutamate Cysteine Ligase Catalytic Subunit in Muscle Under Stress”, and the authors Chen C-N, Brown-Borg HM, Rakoczy SG, Ferrington DA, Thompson LV.

I was responsible for the experimental design, data collection and analysis, and the writing of the manuscript of this study with the assistance of LaDora

Thompson. Holly Brown-Borg and Sharlene Rakoczy were involved in the data collection, technical support of enzyme assays (glutathione-S-transferase, glutathione reductase, and γ -glutamyl transferase) and the revision of the manuscript. Deborah Ferrington assisted in the development of enzyme assay of glutamate cysteine ligase and revised the manuscript.

I would like to acknowledge Sheng Zhong and Janice Shoeman for the assistance of animal care.

I. Abstract

Our previous study found that the ability of aging muscles to maintain the glutathione (GSH) level with stress (14 days of hindlimb unloading) is impaired. The purpose of this study is to investigate the mechanisms of the disrupted homeostasis of the GSH level in aging muscles with stress. Adult and old rats were randomized into 4 groups: weight bearing and 3, 7 and 14 days of hindlimb unloading (HU). Soleus muscles were harvested to investigate the activity/content of enzymes involved in GSH metabolism. Spectrophotometric assays were used to measure the activities of glutathione S transferase, γ -glutamyl transpeptidase, and glutathione reductase. The activity of glutamate cysteine ligase (GCL) was determined using a fluorescence assay. One-dimensional electrophoresis and Western Blot were used to determine the contents of the two subunits of GCL: catalytic subunit (GCLC) and modifier subunit (GCLM). The major finding of this study is that the decreased activity of GCL (the rate limiting enzyme of GSH synthesis) in aging muscles with 14 days of HU explains the observed GSH depletion in aging muscles with 14 days of HU. Furthermore, the failure to maintain the accelerated production of GCLC with stress contributes to the decline of GCL activity in aging muscle with 14 days of HU. Interestingly, although the content of GCLM had a 2 fold increase in aging muscles with 14 days of HU, it did not increase GCL activity. The results suggest that the regulation of GCL, especially the catalytic subunit, with stress may be compromised in aging muscles.

II. Introduction

Glutathione (GSH), a tripeptide composed of glutamate, cysteine and glycine, is a crucial antioxidant that protects cells by being a cofactor in redox reactions as well as direct conjugation with oxidants (**Figure 2**). In redox reactions, GSH donates electrons to hydrogen peroxides that are reduced to water by the enzyme glutathione peroxidase (GPX). In the reaction of direct conjugations, GSH conjugates with a variety of oxidants as well as protein cysteine residues (S-glutathionylation). The reaction of GSH conjugation with oxidants is catalyzed by the enzyme glutathione S transferase (GST). These GSH-dependent detoxifying reactions protect cells from oxidative damage, but consequently, they reduce intracellular GSH levels.

The replenishment of GSH is achieved by recycling and biosynthesis (**Figure 2**). The recycling of GSH is catalyzed by glutathione reductase (GR) with NADPH as the cofactor. The biosynthesis of GSH is regulated by the substrate availability and the synthesis rate. Substrate availability is mainly determined by γ -glutamyl transferase (GGT), which initiates the extracellular degradation of GSH-conjugates and GSH into γ -glutamyl amino acids and cysteine-glycine peptide. This reaction ensures substrate available for GSH biosynthesis intracellularly. The intracellular synthesis of GSH is by two consecutive ATP-dependent enzymatic reactions. In the first reaction, glutamate is coupled with cysteine to form γ -glutamylcysteine catalyzed by glutamate cysteine ligase (GCL), the rate limiting enzyme of GSH biosynthesis. In the second reaction, γ -glutamylcysteine is coupled with glycine to form GSH catalyzed by GSH synthetase (GS).

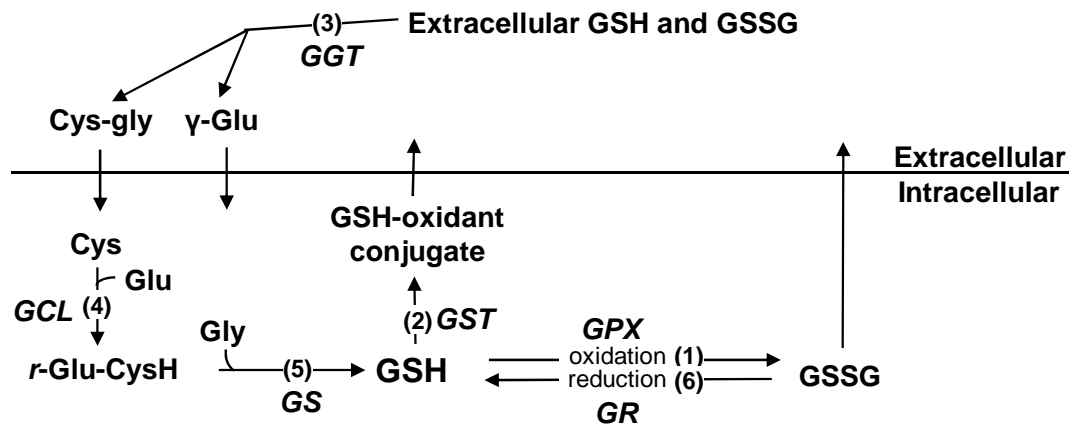


Figure 2. Metabolism of glutathione (GSH). GSH, a tripeptide composed of glutamate (Glu), cysteine (Cys) and glycine (Gly), is a crucial antioxidant in the cells. (1) GSH is used as a cofactor in the redox reaction catalyzed by glutathione peroxidase (GPX). In this redox reaction, hydrogen peroxide and other peroxides are reduced while GSH is oxidized to glutathione disulfide (GSSG). (2) The detoxifying function of GSH is also achieved by direct conjugation with oxidants. This direct conjugation reaction is catalyzed by glutathione S transferase (GST). (3) GSSG and GSH-oxidant conjugates are transported out of cells where they are degraded by γ -glutamyl transferase (GGT). This degradation ensures substrate available for GSH biosynthesis intracellularly. The intracellular synthesis of GSH is by two consecutive enzymatic reactions catalyzed by glutamate cysteine ligase (GCL) (4) and GSH synthetase (GS) (5). (6) GSH is also replenished by the reduction of GSSG catalyzed by glutathione reductase (GR).

The homeostasis of GSH is altered with various cellular stresses, including oxidative stress. Aging, a process of chronic oxidative stress, has been shown to affect GSH levels in a tissue-specific manner (107). In skeletal muscles, GSH levels are increased with aging in type I (oxidative) muscles (20, 93). The increased GSH level in aged muscles is likely a compensatory response for the chronic oxidative stress that helps maintain the redox balance of muscle cells.

Importantly, the ability of aged organisms to adapt to additional stressors appears to be compromised. Our previous study found that in adult animals, the GSH levels in muscles are stable with the additional stress of muscle disuse, which has been shown to induce oxidative stress of the affected muscles (20, 130). In contrast, GSH levels in aging muscles with the same stress dropped

dramatically (20). These findings prompted the focused examination of GSH metabolism in the current study. Thus, the aim of this study was to determine the enzymes that are associated with the impaired GSH homeostasis in aging muscles with additional stress.

III. Methods

Animals and Stress intervention

Eighty male Fischer 344 rats, ages 13 months (the age at which more than 90% of this rat strain has survived; n = 40) and 26 months (the age at which 25% has survived; n = 40), were purchased from the Minneapolis Veterans Administration Aged Rodent Colony that was maintained by the University of Minnesota. Rats were randomized into four groups: normal weight bearing (c) and hindlimb unloading (HU) for 3, 7, and 14 days. Muscle disuse by HU was used as a form of stress for skeletal muscles because previous studies have shown that HU altered antioxidant capacities (20, 63, 89), and increased protein oxidation and ubiquitination (21, 83, 163).

The HU intervention was achieved as described previously (20, 21). Briefly, the tail of the rat was attached to a swivel mounted at the top of the cage. The height of the suspension was adjusted to prevent the hind limbs from touching the floor. All the animals were housed in a research animal facility and were checked daily for any abnormal response to suspension. The rats were anesthetized with pentobarbital sodium (35 mg/kg body weight) after the intervention. Soleus muscles were harvested, weighed, and immediately frozen in liquid nitrogen. The frozen soleus muscles were stored in a –80°C freezer until later analysis. The protocol of this study was approved by the University of Minnesota Institutional Animal Care and Use Committee.

Enzyme activity assays

GR, GGT and GST activities— Frozen soleus muscles were homogenized in buffer containing 20 mM 3-(4-morpholino)propane sulfonic acid (MOPS), 62 mM sucrose, and 0.1 mM EDTA (pH 7.2). The supernatant collected after centrifuging at 12,000g for 25 minutes was used for the analysis of enzyme activities. Protein concentration in the supernatant was measured by the Bradford method (11).

GR activity was determined as previously described (12). Briefly, tissue homogenates were added to assay medium containing 100 mM thiourea, 10mM NADPH, 30mM GSSG, 200mM MOPS, 620mM sucrose and 1mM EDTA (pH 7.2) to initiate the reaction. GR activity was determined spectrophotometrically by measuring the disappearance of NADPH at a wavelength of 340 nm at 30°C for 5 minutes. Values were expressed in $\mu\text{mol}/\text{min}/\text{mg}$ protein.

GGT activity was measured as previously described (13). Briefly, homogenates were incubated in an assay medium containing 100 mM Tris-HCL (pH 8.0), 5mM γ -glutamyl-p-nitroanilide, and 100mM glycylglycine for 30 minutes at 37°C. The enzymatic reaction was stopped by precipitating proteins with 12N acetic acid followed by centrifugation at 8,160g for 3 minutes. GGT activity was then determined spectrophotometrically by measuring the absorbance of the supernatant fraction at 410 nm at 37°C (13). Values were expressed in nmol/mg protein.

GST activity was determined as previously described (13). Briefly, homogenates were mixed in a medium containing 100 mM KPO_4 (pH 6.5) and 20 mM GSH, and the reaction was initiated by adding 1-chloro-2,4-dinitrobenzene (CDNB). GST activity was then determined spectrophotometrically by measuring the formation of CDNB-GSH conjugates at 340 nm at 30°C for 5 minutes. Values were expressed in $\mu\text{mol}/\text{min}/\text{mg}$ protein.

GCL activity assay – GCL activity was determined by a fluorescence assay developed by White et al. and Wu et al. with our optimization of the reaction time specifically for muscle tissues (165, 168). In detail, frozen soleus muscles were homogenized in TES/SB buffer containing 20 mM Tris, 1 mM EDTA, 250 mM sucrose, 20mM sodium borate and 2mM serine. Homogenates were centrifuged at 10,000g, 4°C for 10 minutes. The supernatants were collected

and then centrifuged again at 15,000g, 4°C for 20 minutes. Protein concentrations in the supernatants were determined using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.

In the GCL activity assay, 30 µl of homogenate was added to 30 µl of GCL reaction cocktail (400 mM Tris, 40 mM ATP, 40 mM L-glutamic acid, 2 mM EDTA, 20 mM sodium borate, 2 mM serine and 40 mM MgCl₂) and incubated at 37°C for 5 minutes. After the 5 minutes of incubation, 30 µl of 30mM cysteine (dissolved in TES/SB buffer) was added and the mixture was incubated for 13 minutes at 37°C. The enzymatic reaction in the mixture was stopped by precipitating proteins with 200 mM 5-sulfosalicylic acid (SSA). After putting on ice for 20 minutes, the mixture was centrifuged at 2,000g, 4°C for 10 minutes.

Following centrifugation, 20 µl of supernatant that contained γ-glutamylcysteine (γ-GC) product was added to a 96 well plate designed for fluorescence detection. For each assay, 20 µl of γ-GC standards containing 30 µl of GCL reaction cocktail, 30 µl of 200 mM SSA, 30 µl of H₂O and 30 µl of γ-GC standard solution (0, 20, 40, 60, 80, 100, 120, 140 µM of γ-GC in TES/SB buffer) were added to the same 96 well plate to generate the standard curve. Next, 180 µl of 2,3-Naphthalenedicarboxyaldehyde (NDA) was added into each well. The plate was incubated in the dark at room temperature for 30 minutes. After the incubation, the formation of NDA-γ-GC was measured (472 excitation/528 emission) using a fluorescent plate reader (Applied Biosystems, Foster City, CA). The production of γ-GC in each sample was calculated with standard curve. Values were expressed in nmol/min/mg protein.

GCLC and GCLM protein content

Protein content of catalytic subunit of glutamate cysteine ligase (GCLC) and modifier subunit of glutamate cysteine ligase (GCLM) were determined by

Western blot. Briefly, soleus muscles were homogenized in DNase buffer containing 20 mM Tris (pH 6.8), 1 mM CaCl₂, 5 mM MgCl₂, and 150 units/mL DNase I (Roche Diagnostic, Indianapolis, IN). The homogenate was then put on ice for 40 minutes. Next, urea buffer containing 6M Urea, 2% SDS and 20mM Tris (pH 6.8) was added into the homogenate followed by homogenization. The homogenate was centrifuged at 600g, 4°C for 15 minutes and the supernatant was collected. Protein concentrations in the supernatants were determined using the BCA protein assay kit.

Equal amounts (20 µg for GCLC and 50 µg for GCLM, determined by linear responses of respective antibody) of protein were loaded onto 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and separated by electrophoresis using mini-vertical gel electrophoresis units (Amersham Biosciences, Cleaveland, OH, USA). 10 µl of GCLC positive control (Thermo Fisher Scientific, Fremont, CA, USA) was loaded in each gel for Western blot probed with GCLC antibody. Proteins resolved on the gels were transferred to polyvinylidene difluoride (PVDF) membranes using a Mini Trans-Blot Electrophoretic Transfer Cells (Bio-Rad, Hercules, CA) at 110V for 3 hours. An internal control (a muscle sample) was loaded and transferred on each blot. The band intensity of all samples was normalized to the intensity of this internal control, thus, permitting the comparison of samples across multiple blots.

The protein bound PVDF membranes were incubated overnight at 4°C with the polyclonal GCLC antibody (Thermo Fisher Scientific, Fremont, CA, 1:1000) or monoclonal GCLM antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000). Blots probed with GCLC antibody were incubated with goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Bio-Rad, 1:3000) at room temperature for 1 hour. Substrate BCIP-NBT (5-bromo-4-chlor-3'-iodolyl phosphate p-toluidine/nitroblue tetrazolium chloride) was used for colorimetric visualization of the immunoreactions on membranes. The immunoblots were

imaged using a GS-800 calibrated densitometer (Bio-Rad). Blots probed with GCLM antibody were incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, CA, 1:3000) at room temperature for 1 hour. Chemiluminescent substrate kits (SuperSignal West Pico, Fisher Scientific, Pittsburgh, PA) were used to detect HRP-labeled probes on the blots. The immunoblots were imaged using a ChemiDoc imaging system (Bio-Rad). The intensity of immunoreactions on the blots was quantified using Quantity One software (Bio-Rad) (21).

Statistics

Data are presented as mean \pm SEM. Two-way analysis of variance (ANOVA) and Tukey-Kramer Multiple Comparison post-hoc test were used to determine whether the age of the animals influences the responses of GSH metabolism-related enzymes in muscles under stress. If a *significant interaction* was detected between age and stress, the effects of age and stress were examined separately by one-way ANOVA with Tukey-Kramer Multiple Comparison Test as a post-hoc test. Differences were considered significant when $p < 0.05$.

IV. Results

Enzyme that is involved in GSH utilization

Activities of glutathione-S-transferase (GST)

Glutathione-S-transferase catalyzes the reaction of glutathione conjugation with endogenous compounds and xenobiotics. GST activities changed with hindlimb unloading and the changes were age-dependent (Age X HU, $p=0.046$) (**Figure 3**). In adult rats, GST activities in muscles with 14 days of HU were 0.109 ± 0.009 $\mu\text{mol}/\text{min}/\text{mg}$ protein, which were significantly higher than the values seen in the muscles of weight bearing control rats. In old rats, GST activities in muscles with 7 days of HU were higher than values seen in the muscles of weight bearing control rats (0.139 ± 0.009 and 0.101 ± 0.008 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively). However, GST activities in muscles of old rats with 14 days of HU (0.110 ± 0.009 $\mu\text{mol}/\text{min}/\text{mg}$ protein) were not different from the values seen in the muscles of old control rats.

Aging also affected GST activities. GST activities in normal weight bearing muscles of old rats were 38% higher than the values seen in the muscles of adult rats.

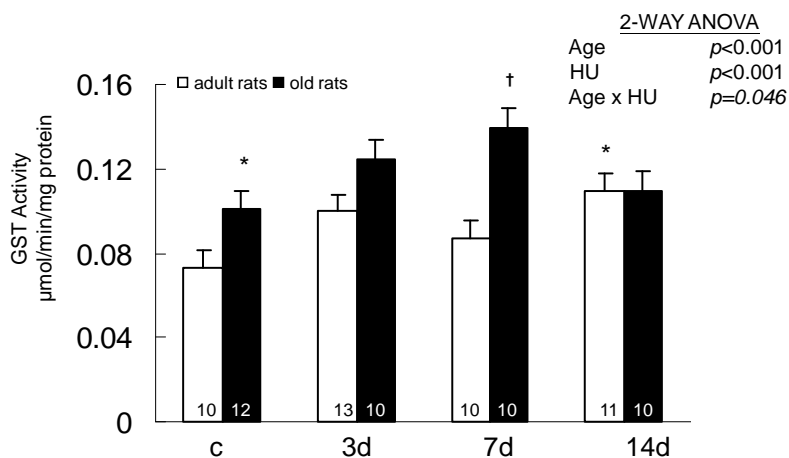


Figure 3. Activity of glutathione-S-transferase (GST) from rats with weight bearing (c) and 3, 7, and 14 days (d) of hindlimb unloading (HU). The number of animals in each group is noted. Values are mean \pm SEM. The effect of HU was dependent on age ($p=0.046$). *Significantly different from adult weight bearing rats. †Significantly different from old weight bearing rats. ANOVA = analysis of variance.

Enzymes that are involved in GSH replenishment

Activities of glutathione reductase (GR)

Glutathione reductase catalyzes the reduction reaction of glutathione disulfide (the oxidized form of glutathione) to glutathione (107). GR activities changed with hindlimb unloading age-specifically (Age X HU, $p=0.032$) (**Figure 4**). In adult rats, GR activity in muscles with 3 days of HU was 0.026 ± 0.002 $\mu\text{mol}/\text{min}/\text{mg}$ protein, which was 44% and 53% higher than the values seen in the muscles with 7 days and 14 days of HU (0.018 ± 0.001 and 0.017 ± 0.001 $\mu\text{mol}/\text{min}/\text{mg}$ protein, respectively). In old rat, GR activities did not change significantly with hindlimb unloading.

GR activities also changed with aging. GR activities in normal weight bearing soleus muscles of old rats were 37% lower compared to the values in adult rats.

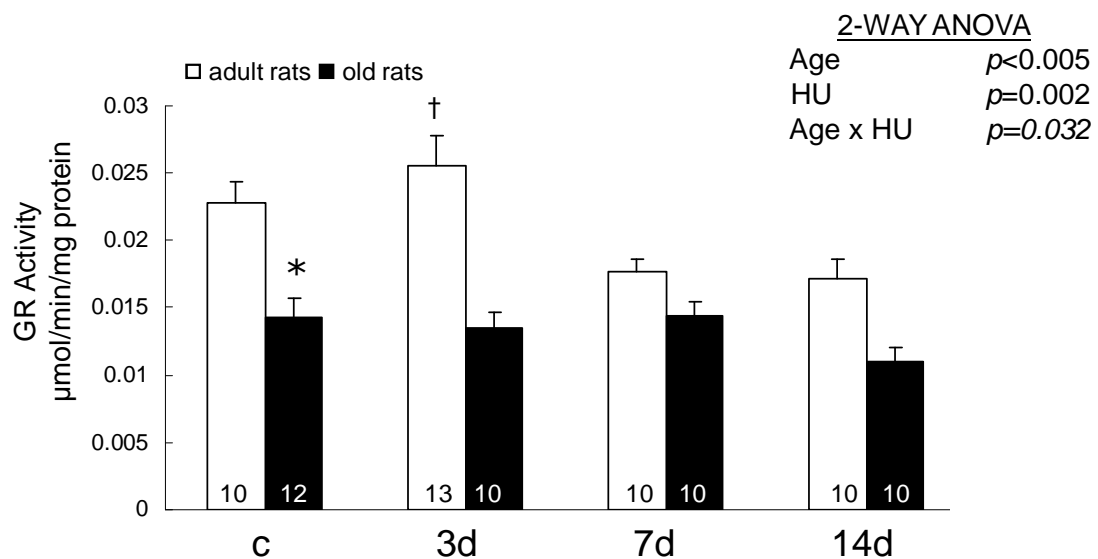


Figure 4. Activity of glutathione reductase (GR) from rats with weight bearing (c) and 3, 7, and 14 days (d) of hindlimb unloading (HU). The number of animals in each group is noted. Values are mean \pm SEM. The effect of HU was dependent on age ($p=0.032$). *Significantly different from adult weight bearing rats. †Significantly different from adult 7d and 14d rats. ANOVA = analysis of variance.

Activities of γ -glutamyltransferase (GGT)

γ -glutamyltransferase, a membrane bound enzyme, initiates the extracellular catabolism of GSH and GSH-conjugates, which makes amino acid substrates available for GSH synthesis intracellularly (103). GGT activities in muscles did not change with hindlimb unloading; however, they changed with aging (2-way ANOVA, $p=0.014$) (**Figure 5**). The activities of GGT were 18% lower in soleus muscles of old rats with normal weight bearing compared to the values seen in the adult rats.

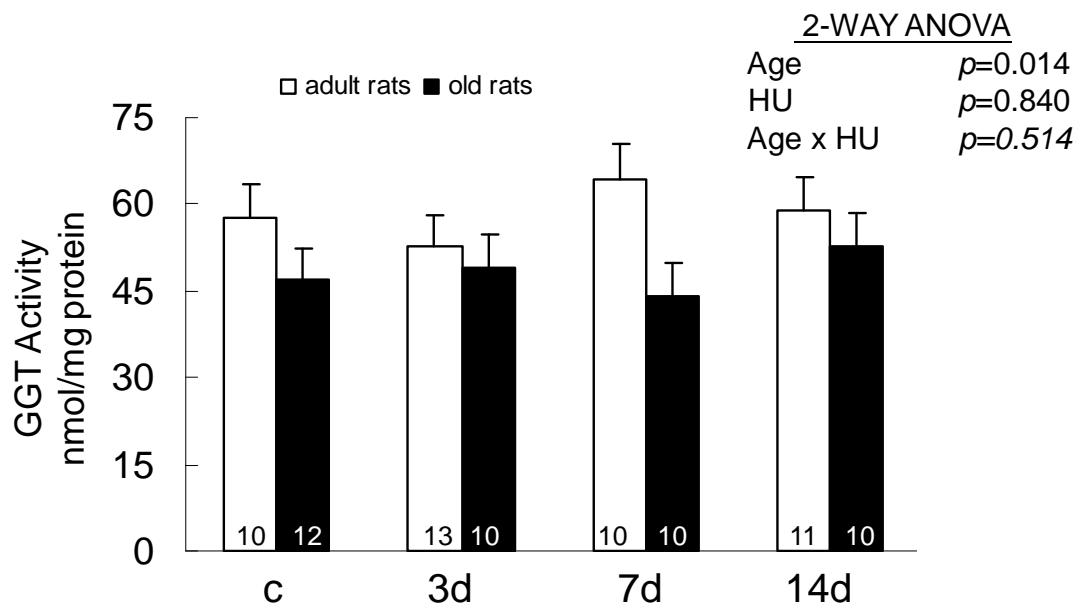


Figure 5. Activity of γ -glutamyltransferase (GGT) from rats with weight bearing (c) and 3, 7, and 14 days (d) of hindlimb unloading (HU). GGT activity is determined by assaying after 30 minutes of incubation. The number of animals in each group is noted. Values are mean \pm SEM. The effect of HU was independent of age. Adult rats had higher GGT activities than those of old rats. ANOVA = analysis of variance.

Activities of glutamate cysteine ligase (GCL)

Glutamate cysteine ligase is the rate-limiting enzyme of GSH synthesis (103). GCL activities change age-specifically with hindlimb unloading (Age X HU, $p=0.048$) (**Figure 6**). In adult rats, GCL activities did not change significantly with hindlimb unloading. In old rats, GCL activities in muscles with 14 days of HU were 0.13 ± 0.02 nmol/min/mg protein, which were significantly lower than the activities of muscles with 3 days and 7 days of HU (0.28 ± 0.03 and 0.31 ± 0.04 nmol/min/mg protein, respectively).

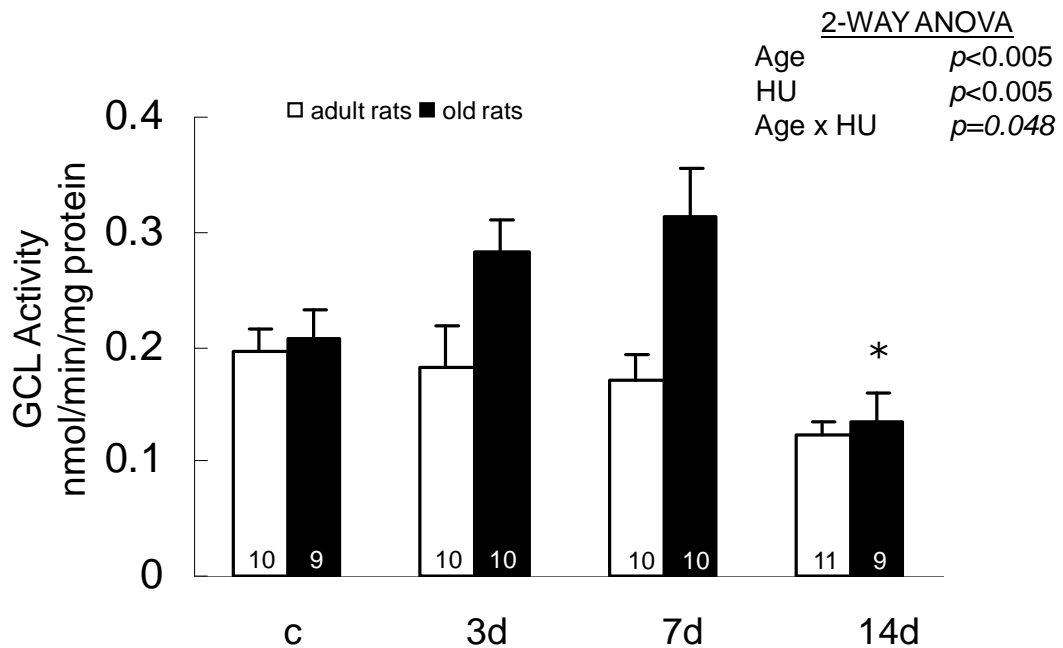


Figure 6. Activity of glutamate cysteine ligase (GCL) from rats with weight bearing (c) and 3, 7, and 14 days (d) of hindlimb unloading (HU). The number of animals in each group is noted. Values are mean \pm SEM. The effect of HU was dependent on age ($p=0.048$). *Significantly different from old 3d and 7d rats. ANOVA = analysis of variance.

GCL subunit protein contents

In order to understand why GCL activity in soleus muscles of old rats decreased from 7 days of HU to 14 days of HU, the protein content of the two subunits of GCL were determined.

The content of catalytic subunit of glutamate cysteine ligase (GCLC)

The catalytic subunit of GCL produces the catalytic function of GCL (46).

Figure 7 shows representative immunoblots (A) and densitometric analysis (B) of GCLC from soleus muscles of rats. The relative GCLC protein expressions changed with hindlimb unloading and the changes were age-related (Age X HU, $p=0.005$). In adult rats, the relative GCLC contents tended to increase with hindlimb unloading, although this change did not reach significance (one way ANOVA, $p<0.1$). In old rats, the relative GCLC contents in muscles with 3 days and 7 days of HU were 2.2 and 2.7 times higher, respectively, than the values seen in muscles with weight bearing. However, the GCLC contents in muscles of old rats with 14 days of HU returned to control levels.

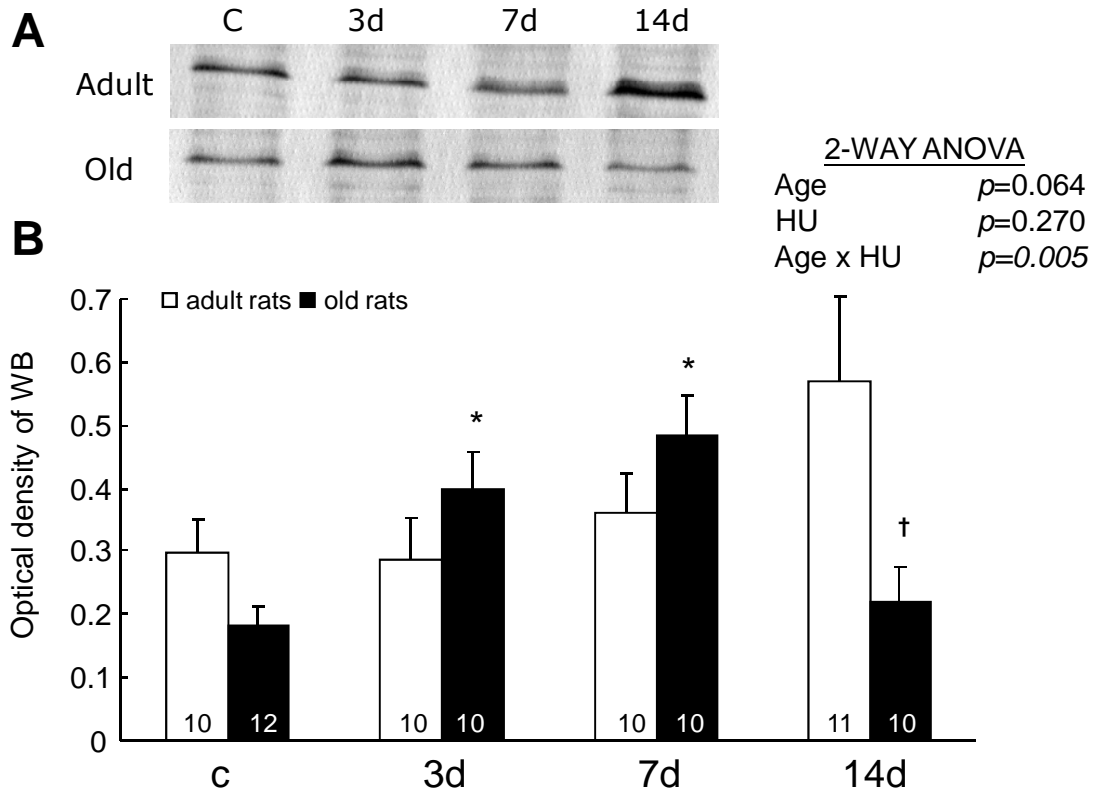


Figure 7. The relative content of the catalytic subunit of glutamate cysteine ligase (GCLC). Representative immunoblots (A) and densitometric analysis (B) are shown from rats with weight bearing (c) and 3, 7, and 14 days (d) of hindlimb unloading (HU). The number of animals in each group is noted. Values are mean \pm SEM. The effect of HU was dependent on age ($p=0.005$). *Significantly different from old weight bearing rats. †Significantly different from old 7d rats. ANOVA = analysis of variance.

The content of modifier subunit of glutamate cysteine ligase (GCLM)

The modifier subunit of GCL lowers the K_m (increases the affinity) for glutamate and ATP, and increases the concentration of GSH required for GCL inhibition (K_i) (46). **Figure 8** shows representative immunoblots (A) and summary of the densitometric analysis (B) of GCLM from soleus muscles of rats. The changes of the relative contents of GCLM with hindlimb unloading were age-specific (Age X HU, $p=0.022$). In adult rats, the relative GCLM contents in muscles did not change with hindlimb unloading. In old rats, the relative GCLM contents in muscles with 14 days of HU were greater than the values seen in the muscles with weight bearing, 3 days and 7 days of hindlimb unloading (3.48, 2.42 and 2.03 folds higher, respectively).

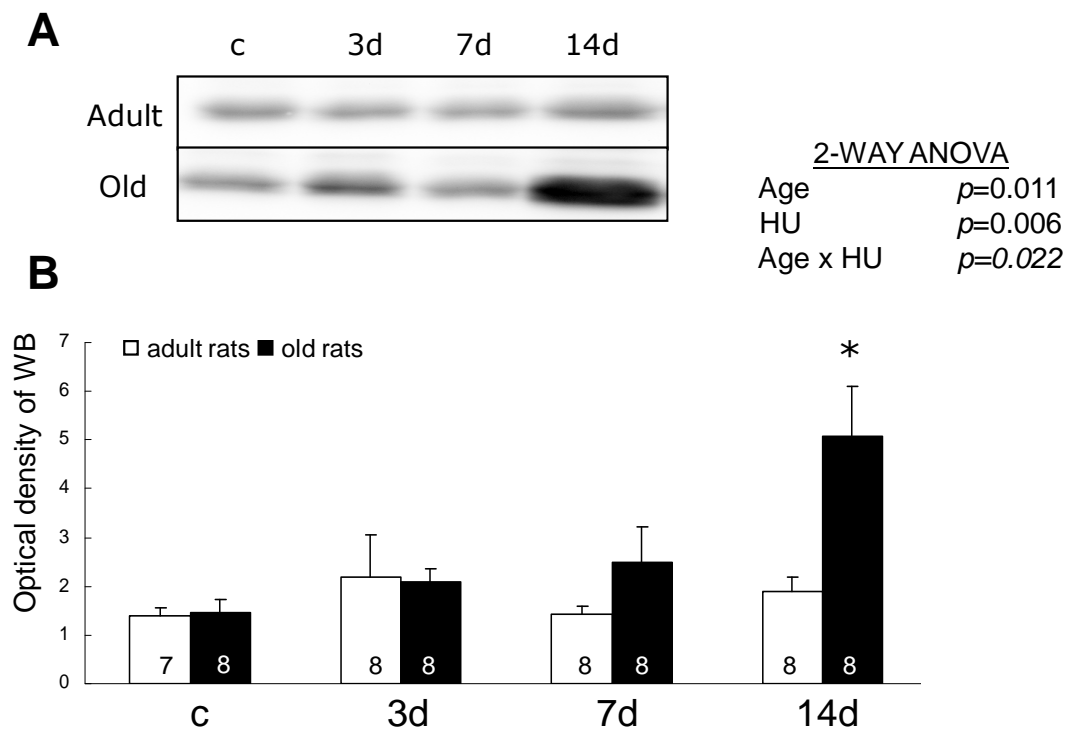


Figure 8. The relative content of the modifier subunit of glutamate cysteine ligase (GCLM). Representative immunoblots (A) and densitometric analysis (B) are shown from rats with weight bearing (c) and 3, 7, and 14 days (d) of hindlimb unloading (HU). The number of animals in each group is noted. Values are mean \pm SEM. The effect of HU was dependent on age ($p=0.022$). *Significantly different from old weight bearing, 3d and 7d rats. ANOVA = analysis of variance.

V. Discussion

With aging, organisms display a reduction in their ability to adapt to stress (80, 87, 148). Our previous study showed that aged muscles have an impaired ability to maintain the GSH level with extended stress (14 days of HU) (20). The aim of this study is to determine the mechanism behind the age-related differences in muscle GSH levels in response to stress. The major finding of this study is that the decreased activity of GCL, the rate limiting enzyme of GSH biosynthesis, explains at least part of the GSH depletion in muscles of old rats with stress (**Figure 9**). GCL activity in aging muscles declines under prolonged stress, and this decline is closely related to a reduction in the expression of the catalytic subunit of GCL.

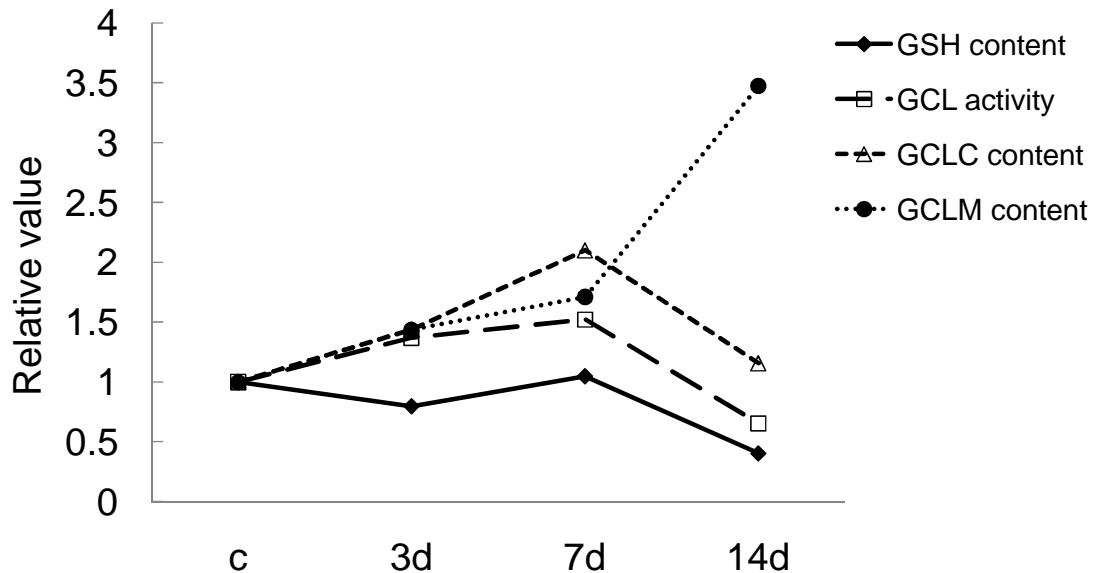


Figure 9. The changes of GSH protein content (Chen et al.,2008), GCL activity, GCLC protein content and GCLM protein content in old rats with weight bearing (c) and 3, 7, and 14 days (d) of hindlimb unloading (HU). Values are relative to control.

GSH metabolism is altered with aging

The alterations of GSH metabolism with aging (a condition of chronic oxidative stress) are tissue specific (57, 73, 81, 93, 100, 117, 122, 179). While brain and liver are the most studied tissues, few studies investigate the age-related

changes of GSH metabolism in skeletal muscles. Identifying the age-related changes of GSH homeostasis is important because GSH regulates the redox balance of the cells. Previously, we have shown that GSH content and glutathione peroxidase (GPX, an enzyme that uses GSH as a substrate in redox reactions) are up-regulated with aging (20). In the current study, we investigate GST, another key enzyme that facilitates GSH usage in the detoxification reaction, and found that GST activity increased in soleus muscles (composed of type I fibers) with aging (**Figure 2**). This age-related increase in GST activity was also observed in muscles composed of type II fibers (73, 93). Taken together, these results suggest that the GSH-related detoxifying machinery of skeletal muscles, both type I and type II muscles, is up-regulated with aging.

On the other hand, the enzymes that replenish GSH (GGT, GR and GCL) are not up-regulated in soleus muscles with aging. In fact, the current study shows that aging is accompanied by lower GR and GGT activities (**Figure 4 and 5, respectively**) and has no effect on GCL activity (**Figure 6**) in soleus muscles. The results are consistent with the finding of Leeuwenburgh et al. (93). The reduced GR and GGT activities suggest that the efficiency of these enzymes may be lower with aging.

In summary, the up-regulation of the GSH level and enzymes that utilize GSH suggest increased oxidative stress in aging muscle. The mechanisms underlying the age-related up-regulation of the GSH and the GSH-dependent detoxifying enzymes are unknown; however, the up-regulation is likely a compensatory adaptation responding to the chronic oxidative stress developed during the aging process (44, 87).

GSH metabolism is impaired in aging muscles under stress

Although the antioxidant system shows a compensatory adaptation with aging, the ability of aged organisms to positively respond to an additional stress appears to be compromised. Our previous study, focusing on the influence of age for skeletal muscle antioxidants to adapt to stress, revealed that GSH levels in muscles under stress (muscle disuse) are maintained in adult rats but not in old rats. Specifically, the GSH level in muscles of old rats did not change initially with stress (muscle disuse for 3 and 7 days); however, it dropped to 40% of the control values with prolonged stress (muscle disuse for 14 days). The main focus of the current study was to identify the mechanism underlying the dramatic decrease of GSH levels in the muscles of old rats with 14 days of HU.

GSH replenishment – One possible mechanism for the decreased GSH levels in the aging muscles with stress (20) is a decrease in GSH replenishment. The results of the present study suggest that the decline of GCL activity contributes to the decrease of GSH levels (**Figure 9**). GCL activity in muscles of old rats increased initially with stress but the elevated activity was not maintained with prolonged stress (i.e., activity level dropped with muscle disuse at 14 days). Moreover, the pattern of GCL activities (change in activity levels over a 14 day period of stress) is very similar to the kinetics of GSH content in muscles of old rats with additional stress (20).

GCL activity is regulated by its two subunits, the catalytic subunit (GCLC) and the modifier subunit (GCLM). GCLC (73 kDa) contains the active site for the ATP-dependent bond formation between glutamate and cysteine, and possesses all the catalytic activity of GCL (10). Studies have shown that GCLC alone is necessary and sufficient for γ -GC formation (27, 173). GCLM (31 kDa), although having no catalytic activity, enhances enzyme activity by lowering the K_m (increasing the affinity) of GCLC to glutamate and ATP, and increasing the concentration of GSH required to inhibit GCL activity (K_i). Yang et al.

demonstrated that *Gclm* homozygous knockout mice had lower GSH levels, increased K_m (reduced affinity) of GCLC to glutamate and were more sensitive to oxidative stress compared to wild type littermates (173). These results demonstrate the important contribution GCLM makes in regulating GSH levels.

In order to understand why GCL activity decreased in the muscles of old rats under prolonged stress, we investigated the protein content of the two subunits of GCL. We found that in adult animals, GCLC protein content did not change significantly during the 14 days of HU (**Figure 7**). In contrast, GCLC protein expression in old animals increased initially with stress (3 days and 7 days of HU) and then dramatically decreased with prolonged stress (14 days of HU). Since GCLC possesses catalytic function of GCL, the failure to maintain the accelerated production of GCLC at 14 days of HU contributes to the decline of GCL activity in muscles of old rats with prolonged stress.

Interestingly, the increase of GCLM protein content with stress occurs later (14 days) than the increase of GCLC protein content in aging muscles (3,7 days), suggesting an uncoupling response of the two subunits of GCL to hindlimb unloading (**Figure 9**). This uncoupling of GCLC and GCLM is also reported in aging brain and liver with increased oxidative stress (104, 179). The mechanism of the uncoupled response in muscle is likely because GCLC and GCLM are encoded by genes on different chromosomes that can be regulated differently.

The increase of GCLM protein expression in aging muscles with prolonged stress (14 days of HU) found in the current study is likely a compensatory adaptation to rescue GCL activity because as noted earlier, GCLM increases the catalytic function of GCLC. Experiments where the GCLC content is maintained showed that addition of GCLM protein increases GCL activity and conversely, reduced GCLM content decreases GCL activity (91, 173, 179). However, we found that the increased GCLM content does not increase GCL

activity. This finding is likely due to the significantly lower content of GCLC at 14 days of HU (**Figure 9**). Collectively, these results suggest that the influence of GCLM on the changes of GCL activity with stress is likely dependent on the changes of GCLC content.

Possible mechanism of the reduced GCLC expression with prolonged stress –

Many mechanisms such as oxidative stress and cysteine deprivation (low protein diet) have been proposed to increase GCLC protein content (91, 103). However, little is known about the mechanism underlying the decreased GCLC protein expression. One potential factor related to the decreased GCLC expression is transforming growth factor- β_1 (TGF- β_1). Studies show that TGF- β_1 down-regulates GCLC expression at the transcription level and causes a decline of GCL activity in a dose- and time-dependent manner (4, 70). TGF- β_1 protein expression has been determined in skeletal muscles where it increased in muscles with 7 days of disuse (56). Importantly, the same study also showed that the timing of the activation of GCLC gene activator and suppressor in disused muscles is different. The activation of TGF- β_1 was later than the activation of tumor necrosis factor- α (TNF- α), a cytokine that induces GCLC transcription, that increased at 3 days of muscle disuse (56). Thus, it is possible that the increased TNF- α is related to the initial increase of GCLC protein expression in aging muscles with stress, and the later increased TGF- β_1 regulates the decrease of GCLC protein expression in aging muscles with prolonged stress. Further investigation is needed to test this hypothesis.

Two other enzymes that are related to GSH replenishment, GR and GGT, do not change in aging muscles under stress. This finding of the enzyme-specific responses with stress is similar to the finding of Lapenna et al. where severe ischemia-reperfusion of myocardium affected GCL activity but not GR and GGT activities (88). The underlying mechanism of the enzyme-specific responses is

probably due to the different signal pathways induced by oxidative stress that affect the regulation of GCL, GR and GGT.

GSH utilization— Increased GSH utilization is another possible mechanism that depletes GSH level in aging muscles with prolonged stress. In the current study, we found that the increased GST activity (increased GSH utilization) was accompanied by the unaltered GSH levels. Additionally, the decreased GST activity (decreased GSH utilization) is accompanied by decreased GSH levels instead of increased GSH level. This finding of the coordinate reductions of GST activity and GSH level indicate that the decrease of GST activity is the reflection of the decreased content of GSH, which is the substrate of GST. Consequences of the reduced GSH level and GST activity are likely increasing the susceptibility of cells to oxidative damage.

Conclusion

The decrease in GCL activities is at least partially responsible for the decline of GSH levels in aging muscles with disuse. This decreased GCL activity is associated with the reduction of GCLC protein expression. The impairment in GSH homeostasis may render aging muscle more susceptible to the ongoing oxidative damage from stress.

Chapter 4

Carbonic Anhydrase III and Four-and-a-Half LIM Protein 1 are preferentially oxidized with muscle unloading

In the previous two studies, we found that the ability of aging muscles to cope with an additional oxidative stress associated with muscle disuse is compromised. Thus, in the study presented in this chapter, I investigated whether the global accumulation of oxidized proteins in the unloaded muscles is greater in older animals. In addition, I identified the proteins that show disuse-related changes in the contents of oxidative modifications, and further investigated whether the alterations of these target proteins with disuse are age-related.

The content in this chapter is unmodified from the published journal article: Chen C-N, Ferrington DA, Thompson LV. Carbonic Anhydrase III and Four-and-a-Half LIM Protein 1 are preferentially oxidized with muscle disuse. *J Appl Physiol.* 2008; 105: 1554-61. Copyright permission is granted by the Journal of Applied Physiology.

In this study, I was responsible for the experimental design, data collection and analysis, and the writing of the manuscript with the assistance of LaDora Thompson. Deborah Ferrington provided consultation in the proteomic methodologies used in the study as well as the revision of the manuscript.

I would like to thank David Durand and Nicole Fugere for the technical assistance. I would like to acknowledge Sheng Zhong and Janice Shoeman for the assistance of animal care.

I. Abstract

The identities of proteins that show disuse-related changes in the content of oxidative modification are unknown. Furthermore, it is unknown whether the global accumulation of oxidized proteins is greater in the aged animals with muscle disuse. The purposes of this study are (a) to identify the exact proteins that show disuse-related changes in the oxidation levels, and (b) to test the hypothesis that the global accumulation of oxidized proteins with muscle disuse would be greater in the aged animals. Adult and old rats were randomized into 4 groups: weight bearing, 3 days, 7 days and 14 days of hindlimb unloading (HU). Soleus muscles were harvested to investigate the protein oxidation with unloading. Slot blot, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot were used to detect the accumulation of 4-hydroxy-2-nonenol (HNE) and nitrotyrosine (NT) modified proteins. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and MS/MS were used to identify modified proteins. We found the global HNE- and NT-modified proteins accumulate significantly with aging but not with muscle unloading. Two HNE- and NT- target proteins, four-and-a-half LIM protein 1 (FHL1) and carbonic anhydrase III (CAIII), show changes in the oxidation levels with muscle unloading. The changes in the oxidation levels happen to adult rats but not old rats. However, old rats had higher baseline levels of HNE-modified FHL1. In summary, the data suggest that the muscle unloading-related changes of protein oxidation are more significant in specific proteins and the changes are age-related.

II. Introduction

Muscle disuse due to immobilization, bed rest, spaceflight, unloading and mechanical ventilation results in loss of muscle mass (atrophy). Studies show that the disuse-related muscle atrophy is associated with increased oxidative stress (79, 129, 130). Increased oxidative stress results in protein oxidation and the oxidized proteins are subsequently degraded by the ubiquitin-proteasome system. Support for the increased protein oxidation and degradation with muscle disuse is found in investigations using adult animals. These studies report a global accumulation of oxidized proteins (83, 140, 141, 145, 177) and ubiquitinated proteins (62, 163) using multiple models of muscle disuse. However, despite knowing the global accumulation of oxidized proteins with muscle disuse, the identity of the proteins that show disuse-related changes in the content of oxidative modification is currently unknown.

Aging is associated with the accumulation of oxidative stress-induced damage in many tissues. Studies demonstrate that older organisms have greater protein oxidation compared to younger counterparts (19, 96). The accumulation of oxidized proteins with aging has been associated with increased oxidant generation (7) and decreased capacity for elimination of oxidized proteins (6, 41, 60). Organisms respond to the age-related increase of oxidative stress by a compensatory increase of antioxidant capacity (20, 49, 55, 57, 93, 105). However, the ability of aged organisms to adapt to additional stressors appears to be compromised (87, 93). For example, Leeuwenburgh and colleagues reported that antioxidant capacity of muscles increases with exercise training in young rats, but not in old rats (93). Our previous study found that while the levels of antioxidant glutathione (GSH) remained stable with muscle unloading in adult rats, the levels in old rats decrease dramatically (20). The age-dependent difference in response of the antioxidant system to stimuli raises the possibility that the accumulation of oxidized proteins with muscle disuse would be greater in the aged animals.

The aims of the study are: (a) to investigate whether the global accumulation of oxidized proteins is greater in the aged animals with muscle disuse; (b) to identify proteins that show changes in the oxidation levels with muscle disuse. Based on the age-related changes of oxidant generation, antioxidant adaptation as well as proteolytic capacity, we hypothesized that the global accumulation of oxidized proteins with muscle disuse would be greater in the aged animals.

III. Methods

Animals and hindlimb unloading

Forty-nine male Fischer 344 rats aged 13 months (100% strain survival) (n = 24) and 26 months (25% strain survival) (n = 25) were purchased from the Minneapolis Veterans Administration Aged Rodent Colony that was maintained by the University of Minnesota. The rats were randomized into four experimental groups: normal weight bearing (control), and hindlimb unloading for 3 days (3d), 7 days (7d), and 14 days (14d). The durations of hindlimb unloading (3, 7 and 14 days) were chosen because there is evidence of age-dependent changes in the antioxidant capacities of the soleus with 7 and 14 days of unloading (20). In addition, muscles of adult rats following 7 days of cast immobilization and 14 days of hindlimb unloading have increased oxidized proteins (83, 140, 141).

The hindlimb unloading intervention was achieved by attaching the tail of the rat to a swivel mounted at the top of the cage. The height of the suspension was adjusted to prevent the hindlimbs from contacting the floor. This arrangement permits animals to move round with their fore-limbs while hindlimbs are unloaded (178). All the animals were housed in a research animal facility and were checked daily for any abnormal response to tail suspension. The protocol of this study was approved by the University of Minnesota Institutional Animal Care and Use Committee.

Overall experimental strategy to determine oxidized proteins

In order to determine if the age of the rat influences the accumulation of oxidized proteins in unloaded muscle and to identify the modified proteins, we selected the soleus muscle. The soleus muscle is composed of predominantly type I fibers that are affected significantly by unloading and show age-related changes (20, 59, 157). The muscle proteins within the soleus were separated experimentally into two fractions, soluble and myofibrillar protein fractions (159).

Subsequent individual protein separation (sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE), extent of oxidized proteins (Western Immunoblotting), and protein identification (mass spectroscopy) are facilitated when the muscles proteins are subfractionated (159).

The global or total accumulation of oxidized proteins in the unloaded muscles was evaluated by Western blot analysis using two experimental approaches; Slot blot (total proteins) and SDS-PAGE (proteins separated by molecular weight). 4-hydroxy-2-nonenol (HNE) and nitrotyrosine (NT) were chosen as two specific markers of protein oxidation and represent two different forms of oxidation. HNE is formed from lipid peroxidation and can modify cysteines, lysines and histidines (35). NT is the product of tyrosine nitration by peroxynitrite (106). Both of these modifications have been shown to render proteins dysfunctional (22, 61, 164). Mass spectroscopy was used to identify individual proteins that show changes in the content of HNE- and NT-modifications with muscle unloading.

Tissue preparation

Muscle tissue was prepared as described (159). Briefly, the rats were anesthetized with pentobarbital sodium (35 mg/kg body weight) after the intervention. Soleus muscles were harvested, weighed, and immediately frozen in liquid nitrogen. The frozen soleus muscles were stored in a -80°C freezer until processing. Muscles were separated into soluble and myofibrillar fractions that contain mainly the cytosolic and myofibrillar proteins, respectively (111). Specifically, a small piece of frozen soleus muscle was pulverized with a mortar and a pestle. The pulverized tissue was then homogenized with a glass homogenizer (Kontes Duall) in buffer containing 20mM imidazole, 2mM EDTA and 0.25mM PMSF (phenylmethylsulfonyl fluoride) (pH 7.4). The supernatant that contained the extracted proteins was collected after centrifuge at 12,000g for 30 minutes at 4°C. Buffer containing 2% CHAPS (3[3-chloamidoprophyl]

dimethylammonio-2-hydroxy-1-propanesulfonate) and 4M urea was added into the collected supernatant to ensure complete protein solubilization and prevent aggregation. This fraction of homogenate is called the soluble fraction (159). The pellet was homogenized in buffer containing 10mM TCEP (tris[2-carboxyethyl] phosphine) and 10% TFA (trifluoroacetic acid). The supernatant that contained the extracted proteins was collected after centrifuge at 12,000g for 30 minutes at 4°C. This fraction of homogenate is called the myofibrillar fraction. The homogenates were stored in a -80°C free zer. Protein concentrations were determined using bicinchoninic acid (BCA) protein assay kit (Pierce) with bovine serum albumin as the standard.

Evaluation of the global accumulation of oxidized proteins

The global accumulation of HNE and NT modified proteins in the soluble and myofibrillar fractions of soleus muscles were evaluated by Slot blot and SDS-PAGE followed by Western blot. In slot blots, an equal amount (soluble fraction: 5ug for HNE and 2.5ug for NT; myofibrillar fractions: 10ug for HNE and 5ug for NT) of protein was absorbed to polyvinylidene difluoride (PVDF) membranes by using Bio-Dot[®] SF microfiltration apparatus (BIO-RAD) and following the manufacturer's instructions. These protein loads were experimentally determined to be in the linear range of responses for each antibody and fraction.

In SDS-PAGE, an equal amount (soluble fraction: 9ug for HNE and 20ug for NT; myofibrillar fractions: 15ug for HNE and 18ug for NT) of protein was loaded onto 5 or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and separated by electrophoresis. The separated proteins were transferred from gels to PVDF membranes using Transblot SD semidry transfer cell (BIO-RAD) at 800 mA for 35 minutes. In order to control equal loading, each sample was loaded into two gels and the two gels were run in parallel. While one gel was

used for Western blotting, the other gel was silver stained using Silver Stain Plus Kit (BIO-RAD) to confirm the equal loading of each sample.

In both slot blot and SDS-PAGE, a standard sample (internal control) was loaded and transferred on each blot. The intensity of immune responses of all samples was normalized to the intensity of the standard sample, thus, permitting the comparison of samples across multiple blots.

Western blot for HNE and NT—The protein bound membranes were incubated overnight at 4°C with polyclonal HNE antibody (Alpha Diagnostics, San Antonio, TX, 1:2500) or polyclonal NT antibody (Cayman Chemical, Ann Arbor, MI, 1:2000). Membranes probed with HNE antibody and NT antibody were then incubated with biotinylated goat anti-rabbit secondary antibody (BIO-RAD, Hercules, CA, 1:3000) at room temperature for 1 hour. After the incubation with secondary antibodies, membranes probed with either HNE or NT antibody were incubated with a streptavidin signal amplification solution (Sigma, St. Louis, MO, 1:3000) and biotinylated alkaline phosphatase (BIO-RAD, 1:3000) at room temperature for 1.5 hours. Last, substrate BCIP-NBT (5-bromo-4-chlor-3'-iodolyl phosphate p-toluidine/nitro blue tetrazolium chloride) was used to visualize the immunoreaction on membranes. Membranes were imaged using a GS-800 Calibrated Densitometer (BIO-RAD) and intensity (slot area and whole lane for Slot blot and SDS-PAGE, respectively) (optical density, OD) was quantified by densitometry using Sigma Scan Pro (Systat, Point Richmond, CA) (47, 159).

Identification of proteins

The overall strategy to identify proteins is the same as described previously (47, 159). Briefly, we matched the individual bands on Western blot that showed HU-related changes in intensity of immune reaction with the bands on the silver stained gels. After the match, corresponding protein bands on silver stained gels were excised for mass spectroscopy. In mass spectroscopy, initial protein

identification was done by measuring peptide mass using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), and obtaining an initial identification using MASCOT and the NCBI nr (National Center for Biotechnology Information) rattus database. Peptide tandem mass spec (MS/MS) was used to confirm the initial protein identification. If needed, Western blot with protein antibody was used to reconfirm the identified protein from mass spectroscopy.

Mass Spectroscopy

The cysteine residues of the excised protein were reduced and alkylated by 10mM dithiothreitol (DTT) and 55mM iodoacetamide. Trypsin (0.00125%) was added to digest the protein to peptides. To extract the peptides, 25mM ammonium bicarbonate, acetonitrile and 5% formic acid were added. The extracted peptides were then rehydrated, concentrated and desalted using Millipore C18 Zip Tips following the protocol of the manufacturer. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (QSTAR XL, Applied Biosystems Inc., Foster City, CA) was used to obtain peptide mass fingerprints with CCA (alpha-cyano-4-hydroxy-trans-cinnamic acid) as the matrix. To obtain the initial protein identification, the peaks of peptides were submitted to MASCOT (<http://www.matrixscience.com>) and matched to the NCBI nr (National Center for Biotechnology Information) rattus database using the peptide tolerance of 100 ppm. Positive protein identification was based on the significant MOWSE (Molecular Weight Search) Score. Confirmation of the initial protein identification was done by sequencing peptide mass with peptide tandem mass spec (MS/MS). A peptide tolerance of 100 ppm was used in the searching (47, 159).

Western blot for four-and-a-half LIM protein 1 (FHL1)— To reconfirm the protein identity, four-and-a-half LIM protein 1 (FHL1) monoclonal antibody (Abnova, Taiwan, 1:1000), goat anti-mouse alkaline phosphatase-conjugated secondary

antibody (1:3000), BCIP-NBT and GS-800 Calibrated Densitometer were used. The procedures were the same as described in section *Western blot for HNE and NT*.

Statistics

Data were presented as mean \pm SE. Two-way analysis of variance (ANOVA) was used to determine the effect of aging and hindlimb unloading on the total amount of HNE- and NT- modification of soluble and myofibrillar proteins in soleus muscles. Tukey-Kramer Multiple Comparison Test was used as a post-hoc test when the main effect reached significance. One-way ANOVA was used to determine the effect of hindlimb unloading on the relative content of individual proteins and levels of modification of individual proteins in each age group. Tukey-Kramer Multiple Comparison Test was used as a post-hoc test. Independent t test was used to compare the baseline difference between adult and old rats. Significant difference was considered achieved when $p < 0.05$.

IV. Results

Age-independent effects of HU on the total protein oxidation

To investigate whether there are age-related differences in protein oxidative modifications with muscle unloading, soleus muscle proteins (soluble and myofibrillar fractions) were analyzed for the content of HNE and NT using a slot blot immunoassay. **Figure 1** shows a summary of the densitometry of the immune reactions for HNE and NT. There was no change in the content of HNE- and NT-modified proteins with HU in either soluble or myofibrillar fractions. The results were further confirmed by measuring the density of HNE- and NT-immunoreaction in whole lanes (SDS-PAGE) on Western blot (data not shown).

Soleus muscles from old animals have more HNE-modified proteins in both soluble fraction ($p=0.002$) (**Figure 1A**) and myofibrillar fraction ($p=0.017$) (**Figure 1C**). In addition, more NT-modified proteins were found in the soluble fraction of muscles from aged animals ($p=0.003$) (**Figure 1B**).

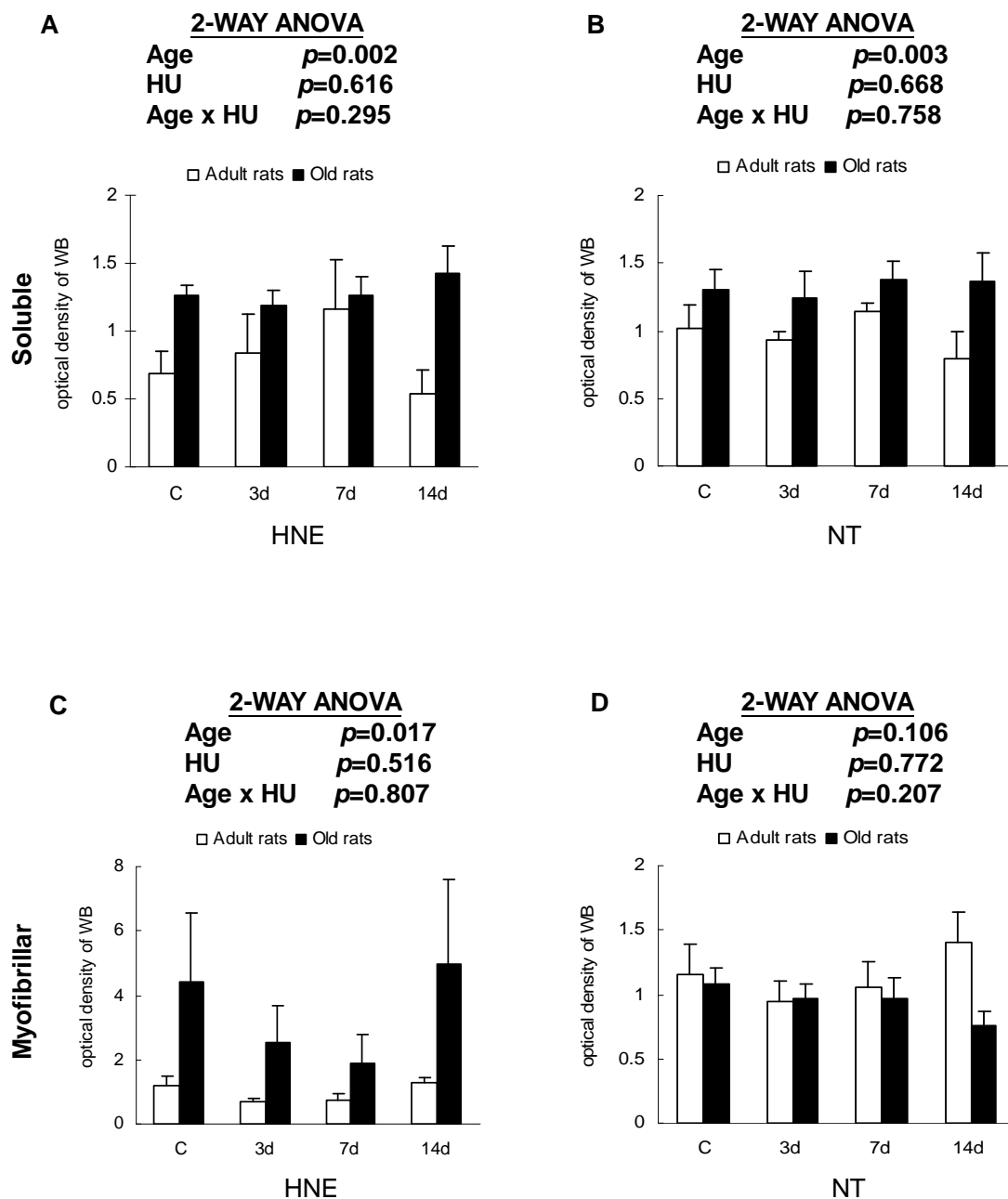


Figure 1. Content of HNE- and NT- modified proteins in soleus muscles of adult and old rats. Densitometric analysis is shown of immune reactions (slot blots) from muscles of rats with weight bearing (C), 3 days (3d), 7 days (7d), and 14 days of hindlimb unloading (14d). **A:** content of HNE-modified proteins in soluble fraction. **B:** content of NT-modified proteins in soluble fraction. **C:** content of HNE-modified proteins in myofibrillar fraction. **D:** content of NT-modified proteins in myofibrillar fraction. Data are presented as mean \pm SE. $n=5-8$ /group.

Protein-specific changes in oxidation with HU: Identification of proteins

Although no change was detected in the total content of HNE- and NT-modified proteins with HU, three individual immunoreactive bands on Western blots show significant densitometric changes with HU (described below). As shown in **Table 1**, mass spectrometry analysis identified band #1 as carbonic anhydrase III, and bands #2 and #3 were the four-and-a-half LIM domain protein 1 (FHL1).

Figure 2A shows the peptide mass fingerprints from band #2. The mass of 8 peptides matched to FHL1. The sequence of the peptide 1180 *m/z* corresponds with the sequence in FHL1 that further confirms the protein ID (**Figure 2B**). Similar results of MS were found for band #3. Because two closely migrating bands (band #2 and #3) were identified as FHL1 by mass spectrometry, we confirmed the result by Western blot with FHL1 antibody. **Figure 2C** shows the protein bands modified by HNE and NT correspond with the immunoreaction for FHL1.

Table 1. Identification of three HNE-or NT-immunopositive bands that show significant densitometric changes with HU									
Band no.	Protein identified	subfraction	Accession number ^a	MW (Da)	MALDI-TOF MS			MS/MS peptides (n) ^e	Modification
					Score ^b	sequence coverage (%) ^c	Peptides ^d		
#1	carbonic anhydrase III	Soluble	gi 31377484	29703	65*	28	7	2	HNE
#2	four and a half LIM domain protein 1	myofibrillar	gi 81907626	33821	73*	32	8	1	HNE, NT
#3	four and a half LIM domain protein 1	myofibrillar	gi 81907626	33821	74*	33	9	5	HNE, NT

* $p < 0.05$

^a NCBI nr accession number

^b Score and probability were determined based on MOWSE scoring algorithm

^c Percentage of amino acids matched in the identified protein

^d Number of identified peptides from MALDI-TOF MS mass fingerprint

^e Number of peptides sequenced using MS/MS

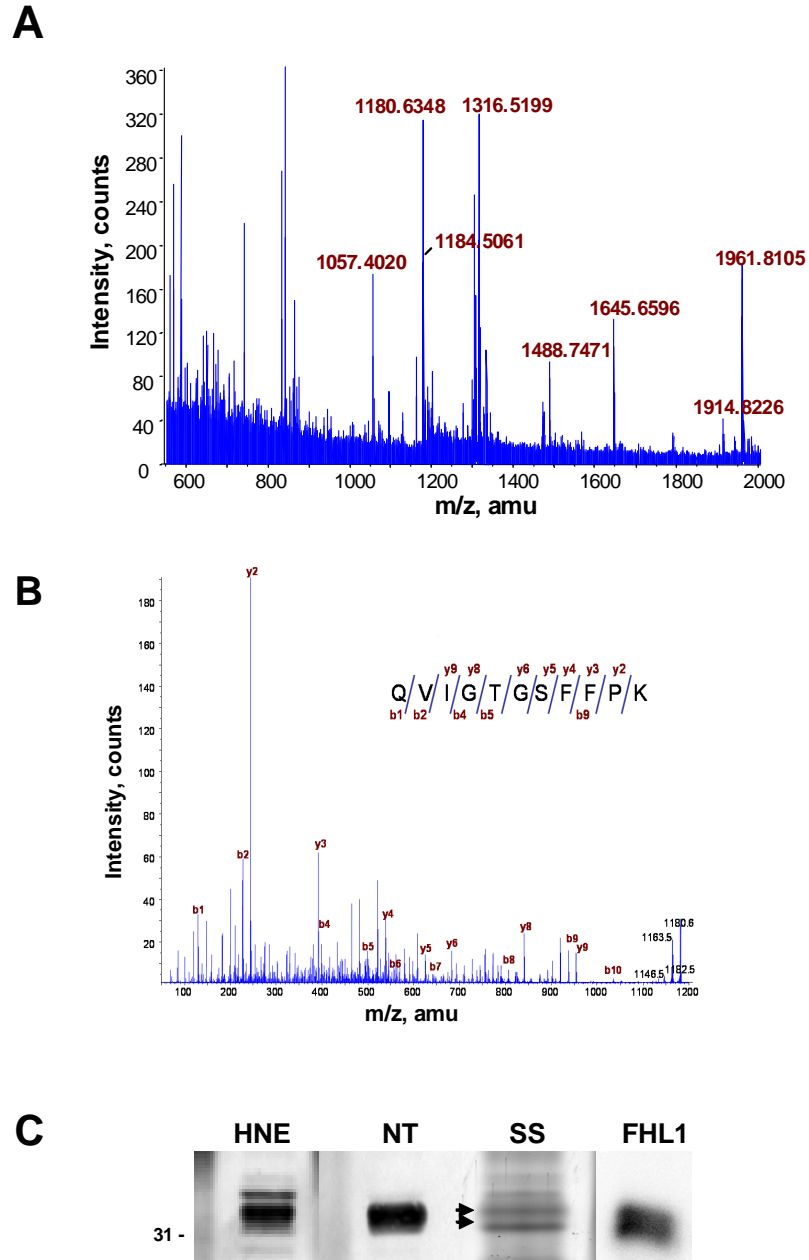


Figure 2. FHL1 identification and confirmation. (A) Peptide mass fingerprints of FHL1 by MALDI-TOF MS. The spectrum shows the 8 peaks that matched the theoretical m/z values for peptides from FHL1. (B) The MS/MS ion spectrum of peptide 1180 m/z corresponds with QVIGTGSFFPK, a peptide sequence that matches FHL1. The sequence is displayed above the spectrum. The y- and b- ions found experimentally are noted above the corresponding peak in the spectrum. amu: atomic mass units. (C) Left two panels show the immune reaction of FHL1 to HNE and NT antibodies, respectively. The third panel shows two corresponding protein bands (arrows pointed) on the silver stained (SS) gel that were both identified as FHL1 from mass spectrometric analysis. Right panel shows the immune reaction of protein to the FHL1 antibody. Position of molecular mass marker at 31 kDa is shown on left.

Carbonic anhydrase III and unloading — **Figure 3** shows the representative immunoblots as well as summary of the optical density of HNE-modified carbonic anhydrase III in soleus muscles from adult and old rats with different days of HU. The amount of HNE-modified carbonic anhydrase III in soleus muscles changes through the time course of HU in adult rats (ANOVA, $p=0.034$), but not in old rats (ANOVA, $p=0.088$).

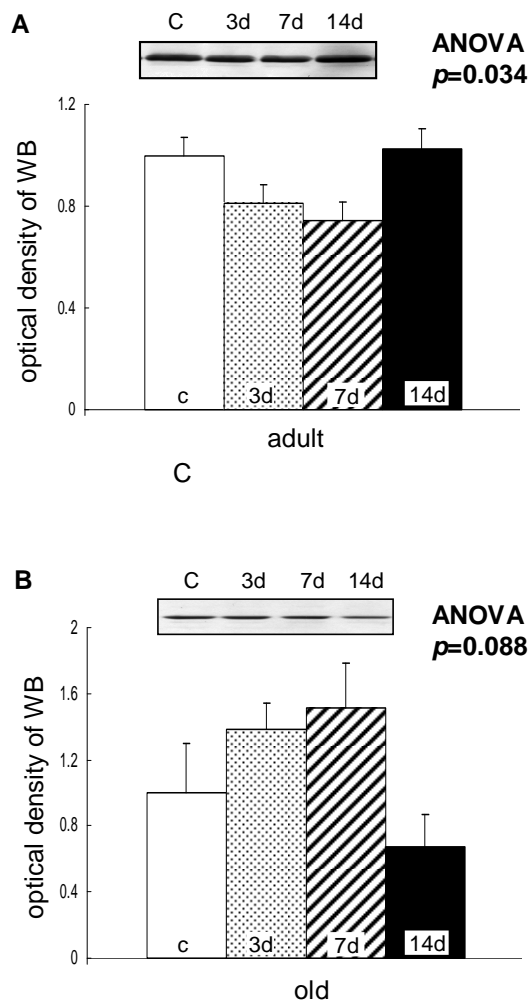


Figure 3. HU effect on the HNE-modified carbonic anhydrase III. Representative immunoblots and densitometric analysis of immune reactions from the soluble fraction of rats with weight bearing (C), 3 days (3d), 7 days (7d), and 14 days of hind-limb unloading (14d). A: HNE-modified carbonic anhydrase III in soleus muscles of adult rats. B: HNE-modified carbonic anhydrase III in soleus muscles of old rats. Values are normalized to the weight bearing control in each age group. Data are presented as mean \pm SE. $n=5-8$ /group.

Four-and-a-half LIM domain protein 1 (FHL1) and unloading — **Figure 4** shows the representative immunoblots and densitometric analysis of HNE- and NT-modified FHL1 from adult and old rats. In adult rats, the content of HNE- and NT-modified FHL1 in soleus muscles changes during the time course of HU (**Figure 4A and 4B**). Tukey-Kramer Multiple Comparison Test indicated that the content of NT-modified FHL1 in muscles from rats with 7 days of HU is 8 times higher than the content in muscles from rats with weight bearing and 14 days of HU. In old animals, no significant changes were detected with HU in the content of HNE- and NT-modified FHL1 (**Figure 4C and 4D**).

To clarify whether the changes in the content of HNE- and NT-modified carbonic anhydrase III and FHL1 with HU are associated with changes in the relative content of individual proteins, optical density of silver stained carbonic anhydrase III and FHL1 was determined. No changes in the relative content of carbonic anhydrase III and FHL1 with HU were found in either adult or old rats (data not shown).

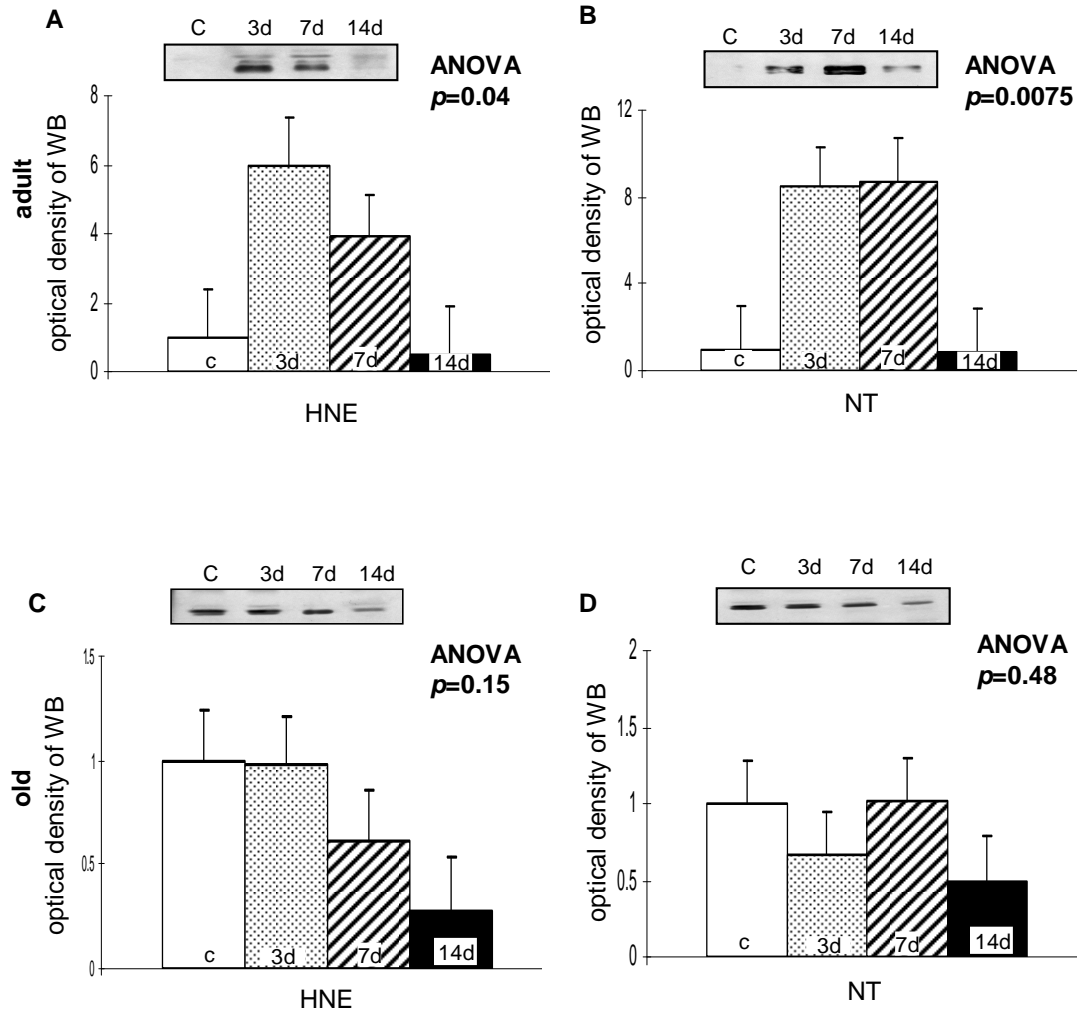


Figure 4. HU effect on the HNE- and NT-modified FHL1. **A:** Representative immunoblots and densitometric analysis of immune reactions (including both bands) from the myofibrillar fraction of rats with weight bearing (C), 3 days (3d), 7 days (7d), and 14 days of hindlimb unloading (14d). **A:** HNE-modified FHL1 in soleus muscles of adult rats. **B:** NT-modified FHL1 in soleus muscles of adult rats. **C:** HNE-modified FHL1 in soleus muscles of old rats. **D:** NT-modified FHL1 in soleus muscles of old rats. Values are normalized to the weight bearing control in each age group. Data are presented as mean \pm SE. $n=5-8$ /group.

Age-related changes of oxidative modification in carbonic anhydrase III and FHL1

To further understand whether the oxidative modification of carbonic anhydrase III (CAIII) and FHL1 are age-related, the content of HNE- and NT-modified CAIII and FHL1 was compared between adult and old rats with weight bearing (control). Except for HNE-modified FHL1, the content of NT-modified FHL1 and HNE- and NT- modified CAIII does not show age-related changes (data not shown). The content of HNE-modified FHL1 is 5 times greater in muscles from old rats than from adult rats ($p=0.04$) (**Figure 5**).

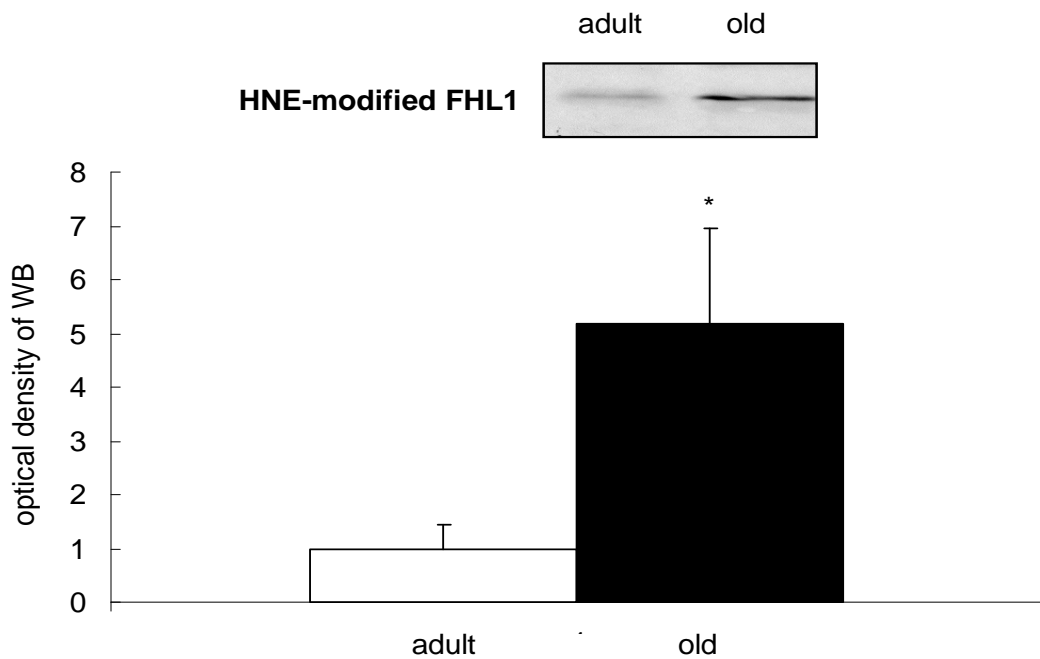


Figure 5. Age effect on the levels of HNE-modified FHL1. Representative immunoblots (upper panel) and densitometric analysis of immune reactions (lower panel) for HNE-modified FHL1 in muscles from adult and old rats. Values are normalized to the adult value. Data are presented as mean \pm SE. $n=6$ per group. * Significantly different than muscles from adult rats.

V. Discussion

Overview of main findings

This study had two major objectives: (a) to investigate whether aging influences the global accumulation of oxidized proteins in the unloaded soleus muscles; and (b) to identify proteins that show disuse-related changes in the content of oxidative modification. The findings, using two-way ANOVA, reveal that the age of the rat does not play a role in the global accumulation of HNE- and NT-modified proteins with unloading. Using mass spectrometry we identified two proteins, FHL1 and CAIII, which show changes in the oxidation levels with muscle unloading. The changes in the oxidation levels happen to adult rats but not old rats. The old rats had 5 times higher baseline levels of HNE-modified FHL1 compared to adult rats.

Description of the experimental model

In order to investigate both age- and disuse-related protein oxidation in skeletal muscles, Fischer 344 rats were chosen because this rat strain is a well-established rodent model for mammalian aging (98). Many animal models of disuse (limb immobilization, hindlimb suspension, mechanical ventilation and cordotomy) have been developed to mimic various conditions that lead to disuse induced muscle atrophy in humans. This study selected hindlimb suspension as the disuse model because it has been reported as the corresponding best model for mimicking conditions of bed rest and spaceflight in humans (130). Hindlimb suspension unloads the muscles of the lower limbs, but permits free movement of the lower limbs and maintains the neural input of the hindlimb muscles.

The age of the rat does not influence the global accumulation of oxidized proteins with unloading

We hypothesized that there would be an age-dependent global accumulation of oxidized proteins with muscle unloading because (a) oxidant generation in

skeletal muscles from aged animals is greater compared to young animals (7); (b) proteasome functions decrease with aging (6, 41, 60); and (c) our previous study found that antioxidant responses to muscle unloading are age-related (20). Specifically, the glutathione (GSH) levels in adult rats are maintained stable whereas the levels are decreased dramatically in old rats with muscle unloading (20). In the current study, we found no global accumulation of oxidized proteins with muscle unloading. This finding suggests that the balance of the oxidized protein generation and removal is maintained even though the antioxidant system in old rats responds differently to unloading than adult rats.

Aging — Aging-induced muscle atrophy and weakness have been associated with oxidative stress (20, 118). The results of this study show that the overall HNE- and NT- modified proteins in soleus, type I muscles, accumulate with aging (Figure 1). Previous research shows a greater content of protein carbonyls in type II muscles of older rats, monkeys and humans. Increased levels of HNE- and NT- modified proteins are reported in type II muscles of older rats and monkeys (18, 123, 175). Collectively, the results suggest that increased protein oxidation with aging occurs in both type I and type II muscles.

Unloading — In the current study, we found that the global accumulation of HNE- and NT- modified proteins does not occur with muscle unloading in both adult and old rats. One interpretation of this finding is that the HNE- and NT- modified proteins are efficiently removed from the unloaded muscles. Indeed, previous studies have shown that the ubiquitin-proteasome system that degrades oxidized proteins is up-regulated with muscle unloading (32, 62, 63, 153, 174). For instance, there is increased mRNA of ubiquitin (32, 62, 153), mRNA of specific ubiquitin-conjugating enzymes (E2) (62, 153), mRNA of proteasome subunits (32, 62, 63) and the level of ubiquitin conjugation (174) with muscle unloading. Most likely, the up-regulated ubiquitin-proteasome

system efficiently degrades the HNE- and NT- specific modified proteins of the unloaded soleus muscles.

An alternative interpretation of the finding is that lipid peroxidation and subsequent generation of HNE do not increase with muscle unloading. Evidence for this is reported in studies using thiobarbituric acid (TBAR), a specific marker of lipid peroxidation. The levels of TBARS in soleus muscles were unchanged with 7 days of cast immobilization in rats (84), with 14 days of hindlimb unloading in rats (83), and with 21 days of cast immobilization in rabbits (99). The results along with our finding of unchanged HNE-modified proteins with muscle unloading suggest that the reported accumulation of protein oxidative-modification may not originate from lipid peroxidation.

Global accumulation of oxidized proteins determined by protein carbonylation is reported in muscles from disuse experimental models (83, 145, 177). Protein carbonylation is a generic marker of protein oxidation and occurs through two major mechanisms, metal catalyzed oxidation and reaction of nucleophilic amino acid side chains with lipid oxidation products such as HNE. In the former mechanism, metals such as copper and iron catalyze the formation of highly-reactive, short-lived hydroxyl radicals that modify nearby amino acids, like proline, arginine, lysine, and threonine (152). In the latter mechanism, lipid peroxidation leads to the generation of aldehyde-containing byproducts, which covalently modify nucleophilic amino acid side chains in proteins, such as cysteine, histidine and lysine (152). Thus, carbonylation reflects the overall status of oxidized proteins whereas proteins with HNE adducts would be a subpopulation of the carbonylated proteins. Therefore, our results suggest that HNE does not contribute to the overall increase of carbonyls previously reported.

In contrast to the hindlimb unloading model of disuse, cast immobilization does result in an increase of HNE- and NT-modified proteins in muscles. Studies of Selsby and colleagues found that HNE- and NT- modified proteins increased 22%-33% and 35%-50%, respectively, after 7 and 8 days of limb immobilization (140, 141). This finding would suggest mechanisms of protein oxidation may be model specific.

Oxidation of FHL1 and CAIII changes with muscle unloading in adult but not old rats

Although several studies have reported global accumulation of oxidized protein with muscle disuse, the identification of the proteins has not been provided. The novel finding in the current study is that FHL1 and CAIII, identified by mass spectrometry, show changes in the levels of oxidative modification in the unloaded soleus muscles. Moreover, the changes are seen in adult rats but not in old rats.

FHL1 — Protein FHL1 (**F**our and a **H**alf **L**IM protein **1**), consisting of four complete LIM domains and a N-terminal half LIM domain, is one member of LIM-only protein family. The LIM domain is a cysteine-rich double zinc finger protein-binding motif and is involved in protein-protein interaction. FHL1 is highly expressed in skeletal muscles, especially in type I and type IIa muscle fibers (92, 102, 116), and is found predominantly at I-band of mature skeletal muscles (113). The functions of FHL1 are not fully characterized, yet this protein appears to be a regulator of myogenesis and muscle growth (113, 114, 116, 135). For instance, the mRNA expression of FHL1 has been found associated with the muscle size of rats (102) and levels of myogenic factors in C2C12 skeletal muscle cells (116). FHL1 is thought to play a role in the assembly of the sarcomere by interacting with myosin-binding protein C (113).

The abundance of cysteine residues in FHL1 makes this protein susceptible to HNE modifications. Although old rats have greater basal levels of FHL1 oxidation, we found that HU caused an increase of oxidation in adult rats only. The influence of FHL1 oxidation on muscle function remains unclear. However, alterations of cysteine by missense mutation of FHL1 gene (and other mutations) result in protein conformation disruption, atrophy, decreased expression and myopathy in humans (132, 166). Collectively, these studies suggest that alterations of amino acids of FHL1 affect stability of the protein and may cause muscle dysfunctions. Further studies are needed to investigate the effect of oxidation on the stability of this key skeletal muscle protein.

CAIII — The other protein that shows changes of the oxidation levels with muscle unloading is carbonic anhydrase III (CAIII). CAIII, being the most abundant soluble protein in liver (5%), in slow-twitch muscles (8%) and in adipocytes (25%), functions as a carbonic anhydrase, esterase and phosphatase (17). Recent studies indicate that CAIII also functions as an antioxidant through thiol oxidation (17, 134, 180). Studies found that the content of CAIII dramatically decreases with aging (17), and cells that over-express CAIII have lower basal level of ROS and greater resistance to oxidative stress (134).

CAIII contains several cysteine, histidine and lysine residues that make it a target of HNE. While the oxidative modification of CAIII has only mild inhibition of enzyme activities (161), the covalent binding of CAIII by oxidants allows it to play a role in regulating the re-dox balance of a cell. Interestingly, we found the level of HNE-modified CAIII changes with muscle unloading in adult rats but not old rats. This finding suggests that CAIII in adult rats responds to the stimuli while the old rats do not, supporting the previous finding (20, 87, 93) that the ability of aged organisms to adapt to additional stressors appears to be compromised.

Summary

The global HNE- and NT-modified proteins accumulate significantly with aging but not with disuse. However, two HNE- and NT- target proteins, FHL1 and CAIII, show changes in the oxidation levels with muscle unloading. The changes in the oxidation levels happen to adult rats but not old rats.

Chapter 5

Conclusion

Sarcopenia, the age-related decline of muscle mass and strength, is inevitable with aging. The decline of muscle function increases the risk for disability and mortality of the elderly. Muscle disuse, a condition that reduces muscle mass and strength in all populations, exacerbates the ongoing decline of muscle function in the elderly. The decline of muscle function with disuse is greater in aging muscles. Since muscle disuse due to bed rest or surgery (such as joint replacements) is more common in the elderly, understanding the cellular mechanisms behind the accelerated decline of muscle functions with disuse in the older individual is important. The understanding will help optimize the present interventions that restore muscle function in the elderly following a period of muscle disuse.

Oxidative stress, a condition where the well-balanced control of oxidant production and antioxidant buffering capacity is disrupted, is a shared mechanism of muscle dysfunction related to aging and disuse. We tested the overall hypothesis that the ability of muscles to cope with the increased oxidative stress due to muscle disuse is age-dependent. The results of our first study show that the homeostasis of glutathione (GSH), an essential endogenous antioxidant, is disrupted in aging muscles with disuse. In aging muscles, the GSH levels drop dramatically with 14 days of disuse. In contrast, the homeostasis of GSH is maintained in adult muscles with disuse. The results suggest that the regulation of GSH metabolism of muscles with disuse may be impaired with aging. In the second study, we investigated the enzymes that are involved in GSH metabolism to further understand the mechanisms behind the decline of GSH levels in aging muscle with prolonged disuse. The results show that the decreased glutamate cysteine ligase (GCL) activity explains the decline of GSH levels in aging muscles with prolonged disuse. In addition, the

decreased GCL activity is associated with the reduction of catalytic subunit of GCL (GCLC). Collectively, results from my first two studies indicate that the ability of aging muscles to maintain GSH levels with prolonged oxidative stress is impaired. The decline of GSH levels with prolonged oxidative stress in aging muscles is associated with the failure in maintaining the accelerated production of GCLC. Further investigations are needed to determine the changes of GCLC at the transcriptional level to understand the mechanisms of the decreased GCLC content in aging muscles with disuse. Understanding the mechanisms behind the impaired GSH responses in aging muscles with prolonged stress will facilitate the development of the treatment strategies.

The impaired up-regulation of the antioxidant system in aging muscles with disuse prompted the investigation of the accumulation of oxidized proteins. We found that the global 4-hydroxy-2-nonenol (HNE)- and nitrotyrosine (NT)-modified proteins accumulated significantly with aging but not with muscle disuse. Regarding the age-related differences, two HNE and NT target proteins change in the oxidation levels in adult rats but not in old rats. The two proteins are carbonic anhydrase III (CAIII) and four-and-a-half LIM protein1 (FHL1). In fact, old rats have greater baseline levels of oxidized FHL1. Because the normal function of FHL1 in muscles remains unclear, further studies comparing the muscle function between muscle cells with and without oxidized FHL1 may increase the understanding of this protein.

In summary, my studies provide evidence for compromised ability of the antioxidant system in aging muscles to adapt to disuse (Studies 1 and 2). This finding suggests interventions that supplement the decreased antioxidant capacity during the longer period of muscle disuse may help in the elderly population. In study 3, I identified two proteins that show changes in the oxidation level with disuse and found that the changes only occur in adult

muscles but not aging muscles. This finding increases the knowledge in the field of muscle biology.

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