AGING DIFFERENCES IN MECHANISMS OF HUMAN SKELETAL MUSCLE HYPERTROPHY

by

David J. Kosek

Marcas M. Bamman, Committee Chair
Louis Dell’Italia
Timothy Garvey
Joseph Messina
Douglas Weigent

A DISSERTATION

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AGING DIFFERENCES IN MECHANISMS OF HUMAN SKELETAL MUSCLE HYPERTROPHY

DAVID J. KOSEK

CELLULAR AND MOLECULAR PHYSIOLOGY

ABSTRACT

Sarcopenia, the age-associated loss of muscle mass, affects all individuals to varying degrees as years advance, and leads to decreases in strength, power and agility that contribute to increased frailty, the risk for falls and fractures, and morbidity. Of all the interventions tested, progressive resistance training has proven to be the most effective for increasing muscle mass, strength, and power, and enhancing functional parameters that are important for completion of tasks common in daily life.

The genetic and molecular mechanisms utilized by skeletal muscle to hypertrophy and/or atrophy are not well defined, and better understanding of these processes is required to develop more efficacious interventions aimed at decreasing/reversing the effects of sarcopenia. We examined the effects of progressive resistance training via percutaneous vastus lateralis biopsies on young (20-35 years) and older (60-75 years) men and women, and measured responses to training 24 hours after initial acute (1 full training sequence) and final training bouts of a 16 week program compared to a pretraining baseline.

We examined skeletal muscle cell cross sectional area and myosin heavy chain (MHC) expression to elucidate any differences in myofiber size and type distribution of MHC I, IIa and IIx among our subject groups. This study also included mRNA and protein examination of the myogenic regulatory factors
(MRFs). A blunted hypertrophic response was measured in older adults, particularly in older men, while young men possessed the greatest magnitude of growth. No coordination between MRF transcript and protein was found, however, myf-6 protein upregulation was measured in men and may be important for myofiber growth.

Expression of select Dystrophin-Associated Protein Complex (DAPC) components was measured in young and older men, and reflects responsiveness in this scaffold to resistance training, primarily in young. Phosphorylated serine 1417 on nNOS and Mitogen-Activated Protein Kinase (MAPK) signaling proteins were also measured as indicators of activation (nNOS), proliferation/differentiation (Extracellular signal-Regulated Kinases (ERK1/2)) and cell stress/damage (p38) response. The measured alterations in phosphorylation states of nNOS serine 1417 and p38 in older men may indicate elevated cell stress/damage associated with training.
DEDICATION

For my family and friends, who never let me give anything less than my best effort in all areas of life. I celebrate my accomplishments with all of you, who are so close to my heart.
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An immeasurable debt of gratitude is owed to Dr. Marcas Bamman for his devotion and endless effort in developing my abilities as both a scientist and professional in the field of biomedical research. I am extraordinarily fortunate to have had the opportunity to train under someone with as much intelligence, energy, pride, and class as he exhibits on a daily basis. Thanks are owed to him as a mentor, friend, teammate, and golf coach. Special thanks are also due to Dr. Jeong-su Kim and Dr. John Petrella, post-doctoral fellows in the Bamman lab who have since moved on in their professional careers. Their insight and knowledge have been integral to my work and golf swing, as well. Thanks also to my friend and fellow student in the Bamman lab, David Mayhew. He has been a sounding board and voice of reason for many ideas and will make a fantastic MD/Ph.D. Melissa Baker, lab technician and manager, and S. Craig Tuggle, exercise physiologist, are owed a great debt of gratitude; as without them, there is no study. Also thanks to my committee members: Dr. Louis Dell'Italia, Dr. Tim Garvey, Dr. Joseph Messina, and Dr. Douglas Weigent for their enthusiastic support and very thought-provoking critique of my studies. I also acknowledge the strong leadership of Dr. Dale Benos, chair of the Department of Physiology and Biophysics, and the tireless work of the Cellular and Molecular Physiology Program Directors, Dr. Lisa Schwiebert and Dr. Peter Smith.
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LIST OF ABBREVIATIONS

bHLH  basic helix-loop-helix
CAPON  carboxy terminal PDZ ligand of nNOS
Cdk2  cyclin-dependent kinase 2
DAPC  dystrophin-associated protein complex
DHEA  dihydroepiandrosterone
eNOS  endothelial-derived nitric oxide synthase
ERK1/2  extracellular signal-regulated kinases 1 and 2
FA  focal adhesion complex
FGF  fibroblast growth factor
GH  growth hormone
GHRH  growth hormone releasing hormone
GLUT4  glucose transporter 4
HGF  hepatocyte growth factor
Id  inhibitor of differentiation
IGF-I  insulin-like growth factor I
IGFR  insulin-like growth factor receptor
kDa  kilodalton
L-NAME  Nω-nitro-L-arginine methyl ester
MADS  MCM1, agamous, deficiens, serum response factor
<table>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<tr>
<td>MCK</td>
<td>muscle-specific creatine kinase</td>
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<td>MEF2</td>
<td>myocyte enhancer factor</td>
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<td>myosin heavy chain</td>
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<td>Myf-6</td>
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<td>MyoD</td>
<td>myogenic differentiation factor D</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>nNOS</td>
<td>neuronal-derived nitric oxide synthase</td>
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<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
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<td>PDZ</td>
<td>post-synaptic density protein-95, discs large, zonula occludins</td>
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<td>PFK-M</td>
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<td>Rb</td>
<td>retinoblastoma</td>
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<td>RDA</td>
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<td>SU</td>
<td>syntrophin unique</td>
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TGFβ  transforming growth factor β
Sarcopenia

Sarcopenia, the loss of muscle mass with advancing age, primarily targets myofibers expressing the glycolytic myosin heavy chain (MHC) type II isoforms (60; 149). It is characterized by decreases in strength and power (166), and studies show that healthy adults in their seventh and eighth decades exhibit 20-40% declines in these parameters compared to younger individuals (60). Sarcopenia is also associated with decreases in metabolic rate (136), protein synthesis and motor unit loss, with concomitant alterations in motor unit configuration in remaining muscle cells, e.g. myofiber type grouping (60; 136; 149). Accelerated muscle deficits occur beyond the age of 50 at a rate of 1-2% per year (166), with a 30% decrease in whole muscle size between the ages of 50 and 80 yr (57). These changes contribute to overall frailty of individuals, hindering their mobility and independence as age progresses (60). Age-related skeletal muscle detriments are most noticeable in the muscles of the lower limbs, as weaknesses here result in increased risk of falls and fractures in the elderly (166; 197). Beyond risk of injury, loss of muscle mass also causes decreased ability to perform daily functional tasks such as stair climbing and completion of household chores (190).
Increased susceptibility to contraction-induced damage (129; 170) followed by a slowed rate of recovery (37; 115) has been shown in older subjects. Nutrition is also of importance for adequate amino acid intake (136) and has been shown to decrease with age (60; 149), and therefore this becomes a contributing factor to both muscle breakdown and dysfunctional protein synthesis in older individuals. Lowered secretion of anabolic hormones in aged adults (60; 136; 149) also may contribute to decreased muscle mass and responsiveness to loading. It is clear that there are many factors that may contribute to age-related differences in muscle mass and function, as well as aging muscle’s responsiveness to resistance training.

**Sarcopenia Interventions**

Several interventions aimed at reducing the effects of sarcopenia have been studied, including testosterone (33; 209), dihydroepiandrosterone (DHEA) (209), and growth hormone (GH) (33; 209) replacement therapies, nutrional supplementation (209), and resistance training (33; 209). Conflicting evidence exists for the efficacy of testosterone replacement therapy, as some show that it produces modest myofiber hypertrophy with increased lean mass, while these changes have many times not been accompanied by increases in strength (33). Others state that increased mass and strength does occur in response to testosterone supplementation (60), while some contend that only modest strength increases may be achieved (101). The differences among these viewpoints may likely be the result of different subject group parameters and/or
dosages utilized in the various studies. Testosterone supplementation in lieu of ineffective estrogen replacement therapy aimed at maintaining/increasing muscle mass in women has also been suggested (84); however, long-term risks associated with administration of this hormone have not been assessed in either elderly women or men.

DHEA has also been hypothesized to increase the ratio of testosterone to cortisol in circulation (33), thus tipping the balance from inflammation, known to induce muscle breakdown (149), towards muscle anabolism. In terms of the affect of DHEA on muscle growth and strength increases, results are mixed (84). Previous work has shown that resistance loading-induced upregulation of pro-inflammatory cytokines is attenuated by anabolic steroid administration (212). However, concerns over the effect that administration of testosterone and other anabolic steroids has for increasing the risk of cancer in individuals brings into question the efficacy of this therapy as a treatment for sarcopenia (30; 159).

GH replacement studies have been aimed at inducing an increase in circulating IGF-I expression in elderly subjects, and has also shown mixed results for increasing muscle mass and strength (81; 84), as has administration of growth hormone releasing hormone (GHRH) (33). Thomas points out that GH administration in pharmacological doses increases muscle mass, but not strength (209). Large numbers of adverse events have also been reported in GH studies involving elderly men and women (33; 84; 209) and thus the drop-out rate in these studies was high.
Nutritional supplementation has also been controversial (33; 209), as some studies show gains in muscle mass without strength increases, while other studies suggest that strength increases do occur (33). Elderly individuals are thought to consume less than the recommended daily allowance (RDA) of 0.8g/kg of protein (33; 60; 208). Still, decreases in muscle mass have also been shown to occur in older individuals adhering to the RDA, whereas another study showed that doubling the RDA in elderly people produced a net positive nitrogen balance, necessary for protein synthesis (33). It seems that nutritional supplementation may provide the molecular “building blocks” necessary for muscle growth, but based on the equivocal data published to date, an additional stimulus may be required to maximize the utilization of these additional amino acids for muscle protein synthesis. An alternative program to pharmacologic or dietary intervention is thus sought, that will provide a growth stimulus and prevent/reverse the effects of sarcopenia.

Resistance training has shown great promise as a prescription for combating and/or reversing the sarcopenic disease state (67; 101; 149). This type of physical exercise is known to elicit improvements in muscle mass, strength, and power (58; 101), and enhances muscle protein synthesis (84; 229), increases insulin-dependent glucose metabolism (139; 231; 232), and supports maintenance and/or improvement of bone density, primarily in the elderly (58; 190). Resistance exercise has been shown to provide a greater stimulus for acute increases in hormone production (e.g. testosterone, GH) (34; 89; 119; 120), and satellite cell activation (77; 164) in young compared to old.
Therapies that combine resistance training and one of the abovementioned interventions have also been examined. Yarasheski and colleagues found that GH administration combined with resistance training did not augment the magnitude of muscle growth measured in elderly men compared to age-matched individuals who received resistance training, alone (230). The cost of therapy using recombinant GH also deters many from its prescription (84), and studies such as that of Yarasheski bring into question its necessity when resistance training induces muscle growth equally with or without GH therapy. Several studies were reviewed by Borst (33) that combine nutritional supplements with weight training and varied results were reported. This variation may be the result of differing study groups, ages, and study conditions such as muscle groups tested, amount of protein supplementation, or length of study. Work by Esmarck and colleagues (65) indicates that timing of protein supplementation is important for muscle growth, as only administration from immediately, to two-hours post-exercise contributes to increases in muscle mass. Still, another study administered protein supplementation immediately before or after resistance training in older men (59-76 years) and found no effect on muscle mass or strength gains (40). Determination of aging differences in the load-mediated growth response has not been thoroughly documented; however, ample evidence exists to establish the efficacy of resistance training over pharmacological or dietary intervention against sarcopenia (33; 60; 149).
Myogenic Regulatory Factors

The effects of aging and resistance training on components of skeletal muscle are far reaching, and much work has been done to examine these influences on the myogenic regulatory factors (MRFs). MRFs are a family of basic helix-loop-helix (bHLH) transcription factors responsible for specifying mesodermal precursor cells to the myogenic lineage (45) and upregulating transcription of muscle-specific genes (115) such as MHC, muscle-specific creatine kinase (MCK) and α-actin. Muscle growth and repair is largely dependent on myonuclear addition to mature myofibers through activation of satellite cells (87; 226); and the coordinated series of events that occur for satellite cells to ultimately donate nuclei to terminally differentiated myofibers is controlled by several processes, including the temporal expression of the MRFs (45; 95; 101; 226). MRFs also possess the ability to convert non-muscle cells to the muscle lineage, suggesting that they have the ability to act on otherwise repressed chromatin in target genes (128).

MRF Function in Myogenic Transcription

Upregulation of myogenic differentiation factor D (MyoD) and myogenic factor (myf)-5, or ‘early’ MRFs, control the conversion of muscle precursor cells into myoblasts (45; 95). At this point, expression of two ‘late’ MRFs, myogenin and myf-6 (MRF4), terminally differentiate myoblasts into myocytes (45; 186), earmarking them as nuclear donors for repair/regeneration and growth.
Myocytes will then fuse either with each other to form new myotubes, or with existing myofibers for growth and/or repair.

The MRFs form heterodimeric complexes with other bHLH proteins of the E2 gene family (95) and specifically bind DNA at E-box recognition sites (CANNTG) (201) found within the promoters of many muscle-specific genes (186). MRF homodimers are unable to bind the E-box, thus making dimerization with the E2 family a requirement for function (128). One particular regulatory mechanism for inhibiting MRF activity is through expression of inhibitor of differentiation (Id) proteins. Id proteins act in a dominant negative manner, heterodimerizing with E proteins and preventing their association with the MRFs (186).

The process of transcription upregulation is further complicated by the myocyte enhancer factor-2 proteins (MEF2), important for providing optimum MRF functioning (128), potentially by regulating correct MRF expression patterns (155). The MEF2 family cannot activate muscle genes on their own (128), but potentiate MRF activity by associating the MRF basic region with the MEF2 MADS (MCM1, agamous, deficiens, serum response factor) domain (128; 140; 228).

Upregulation of these transcription factors occurs in response to increased muscle load (115; 169), growth factors (71), and injury (45), and some report that they are also elevated in older adults (21; 173). For example, Hameed and colleagues (91) measured increased MyoD mRNA levels in older muscle compared to young. Others suggest that MRF responses to resistance training
are attenuated in the muscle of aged animals (131; 202), and Hameed also reports an increase in MyoD mRNA expression in young, but not old, in response to a single bout of loading (91; 101). It therefore may be possible that the magnitude of change from resting to load-induced MRF expression is paramount to successful repair/regeneration and growth processes.

**MAP Kinases and the MRFs**

Studies show that the MAP kinases have varying effects on the MRFs. p38 MAPK directly phosphorylates myf-6 at serines 31 and 42 of the amino-terminal transactivation domain, repressing the transcription of specific genes at the terminal stages of muscle differentiation (128; 201). Other studies show that p38 activates MyoD by either directly phosphorylating MEF2A and MEF2C (228), or by directly or indirectly phosphorylating MyoD, itself (128). Based on these findings it appears that *in vitro*, p38 has varied effects on the MRFs, and that its action may be time dependent, as MyoD is activated early in the myogenic program, while myf-6 function occurs later.

Interesting evidence supports that Extracellular signal-regulated kinases 1 and 2 (ERK1/2) have opposing effects depending on tissue maturity, as one study shows that ERK inhibits transcription in myoblasts, retaining their proliferative state, but contributes to myogenic transcription in myotubes (228). This work by Wu and colleagues (228) suggests that ERK expression in proliferating myoblasts is important for initially repressing myogenic transcription and maintaining their undifferentiated state, and this may be done so at least in
part by inhibiting myogenin expression (8). Reduced ERK activity then occurs as p38 levels increase, which induces muscle-specific transcription in differentiating myocytes by reducing cyclin D1 and increasing p21 (228). Once the differentiative program is initiated, ERK and p38 are thought to cooperate in promotion of functions within terminally-differentiated myotubes. The function of MAP kinases in metabolism and mechanotransduction upstream of satellite cell activation and MRF control is discussed in detail below.

**Further Inquiry of MRF Function**

Single fiber culture experiments have illustrated that myf-6 expression is dependent on the expression of MyoD, and that MyoD−/− satellite cells exhibit deficient differentiation and myocyte fusion (53). Elevated proliferation of MyoD−/− satellite cells occurs, along with increased myf-5 expression; however, inhibited induction of myogenin and myf-6 suggests that these cells are retained in a proliferative state and unable to differentiate (185). It therefore appears that MyoD is essential for initiating and completing the myogenic program in satellite cells (143). Similar experiments utilizing myf-6−/− have not been reported, and myogenin and myf-5-null mouse experiments are unable to be conducted, as these mice are not viable (161).

**Satellite Cells**

A subset of myoblasts fail to express differentiative markers and remain in a quiescent state located outside of the muscle sarcolemma, but within the
basement membrane of mature muscle cells (45; 108; 207). This subset of myoblasts, termed satellite cells, are skeletal muscle specific (95) and do not possess detectable levels of any MRFs when quiescent (45; 186). Normally dormant, satellite cells are activated to enter the G1 phase of the cell cycle in response to stretch (207), exercise (109), growth factors (87; 95) and muscle damage (15; 87; 95). Satellite cells remove themselves from the cell cycle by upregulating the p21 protein (45), a cyclin-dependent kinase inhibitor, in part via action by myostatin (138). This sufficiently arrests the progression of myoblasts at both G_1 and G_2 phases (138). Myostatin (growth and differentiation factor-8, GDF-8) has been shown to reduce cellular levels of cyclin-dependent kinase-2 (Cdk2) (211), thought to phosphorylate retinoblastoma (Rb) and allow entry into the S phase of the cell cycle. In doing so, myostatin effectively inhibits the MRF-driven progression of satellite cells to fusion-competent myoblasts.

Though small amounts of growth in terminally-differentiated skeletal muscle cells has been shown to occur as a result of increased protein synthesis (130), it appears that a greater magnitude of growth is accomplished through activation of satellite cells and subsequent myonuclear incorporation into myofibers (164). Without satellite cell activation, Anderson states that strength declines, muscles atrophy, and disability ensues (17). Further, the addition of myonuclei for significant growth induction in response to increased load appears to be requisite (6; 11; 164; 181). Aging results in decreased satellite cell number (45; 194), and may contribute to a lack of regenerative capacity and/or inhibited growth response in aged muscle.
Satellite Cell Activation

Figure 1 illustrates the MRF-mediated process of myonuclear incorporation via satellite cell activation, and modulators that are pertinent to this study are shown. Upon activation, satellite cells express MyoD and myf-5 which assigns them to a myogenic fate (45; 52), and MyoD has been shown to possess an integral role in precursor cell differentiation (143). MyoD−/− mice present a reduced regenerative potential whereby precursor cells do not differentiate (53; 143; 185), and overexpress myf-5 mRNA (186) but also fail to upregulate myf-6 (53). Results from these studies suggest that myf-5 upregulation does not completely compensate for a lack of MyoD, and that decreased myf-6 expression retains precursor cells in a proliferative state, unable to differentiate for eventual assimilation into skeletal muscle in response to growth and/or damage repair. Meanwhile, a distinct role for satellite cell self-renewal has been suggested for myf-5 (45). In this manner, the available pool of satellite cells is not depleted with each cycle of activation, and myf-5 functions to replenish the precursors that have progressed through differentiation and fusion.

After the proliferative phase, satellite cells upregulate expression of myogenin and myf-6, thus initiating the steps for terminal differentiation (45). These MRFs enhance the transcription of muscle specific genes until signaled to cease their actions. It also appears that the extracellular milieu is important to satellite cells for their activation, as a study by Conboy et al. (50) established via
FIGURE 1. MRF-mediated myonuclear addition via satellite cell activation and modulators of system activity. Precursor cells are converted to the myogenic lineage upon expressing MyoD and/or myf-5. Factors that activate this process include HGF, IGF-I, FGF, IL-6 and NO; whereas the TGF-β family of growth factors is inhibitory. p38 has been shown to activate MyoD expression for proliferation; and dual-expression of MyoD and myf-5 primes these cells for differentiation, which occurs upon expression of myogenin and myf-6. Myf-5 expression has been suggested to be a mechanism by which the quiescent satellite cell pool is replenished. Proliferation is halted by increases in p21 and other cell cycle inhibitors. The differentiative process can be inhibited by growth factors, known to induce proliferation, and elevated ERK levels. When p38 expression increases at this stage, ERK levels decrease, overriding their inhibitory effects on myoblast differentiation. Upon initiation of the differentiative program, ERK works in concert with p38 to enhance differentiation. Fully differentiated, fusion-competent myoblasts then fuse either together to form new myotubes, or with existing myofibers for damage repair and/or growth.
parabiosis that the ability of aged muscle to regenerate itself is possible when exposed to factors present in the serum of young mice.

*Autocrine/Paracrine Affectors of Satellite Cell Activity*

Satellite cells are acted upon by a large variety of factors from multiple origins that either inhibit (*e.g.* transforming growth factor beta (TGF-β) family) or enhance (*e.g.* fibroblast growth factor (FGF), insulin-like growth factor-I (IGF-I), hepatocyte growth factor (HGF), nitric oxide (NO)) their activity (45; 95), (Fig. 1). The great assortment of factors that influence satellite cell function is necessary to ensure normal muscle architecture in the face of the many demands that are placed on this tissue (45). An aging mouse model suggests that the older myofiber does not produce sufficient growth-promoting agents to induce satellite cell activation (194), and these findings are in line with those of Conboy and her parabiosis study mentioned previously (50).

Though multiple factors affect satellite cell activation and proliferation, particular attention will be paid to HGF, due to its activation by NO which therefore provides a link between muscle hypertrophy and the dystrophin-associated protein complex (DAPC) discussed in detail below. However, other factors shown to influence satellite cell activity are briefly discussed here. The TGF-β family of cytokines is recognized as inhibitors of both proliferation and differentiation in myoblasts (45), as well as inhibitory of satellite cell activation (138). The most notable TGF-β family member, myostatin, has been briefly described above. Animal models expressing myostatin mutations present a phenotype of profound muscle hypertrophy and resistance to body fat
accumulation (45; 141; 142). Myostatin mutation has since been found in one human (189) and resulted in substantial muscle growth. Myostatin maintains quiescence of satellite cells (200), and myostatin knockout mice possess increased proliferation of satellite cells compared to wild type (219). Myostatin mRNA has been shown to be decreased in human subjects in response to an acute bout of resistance loading (114), and further examination will help to determine the effect of repetitive loading on long term myostatin expression.

FGF has been described as an activator of satellite cell proliferation and inhibitor of differentiation, similar to HGF (45). Of the nine known FGF isoforms, FGF-6 is expressed exclusively in skeletal muscle (72). Sheehan and Allen demonstrated that FGF-1, 2, 4, 6, and 9 induce cell proliferation, and that addition of HGF to FGF-2, 4, 6, and 9 potentiated the action of FGF on satellite cells (191). However, contradicting studies exist regarding the functional role of FGF-6 in skeletal muscle regeneration (95). One group reports that FGF-6 knockouts have impaired satellite cell proliferation with subsequent defective muscle regeneration (72), while another suggests that mutating the FGF-6 locus produces no defects in response to either crush or pharmacologically-induced injury (notexin) (69).

IGF-I is important for the regulation of many GH-mediated responses throughout the body (5), including regulation of muscle growth (5; 45; 95). Binding of the ligand to its IGF receptor (IGFR) activates a MAPK cascade that culminates in satellite cell activation, as well as phosphatidylinositol-3 kinase (PI-3K) pathway activation which causes myotube fusion, among other functions
Increased muscle stretch and/or load has been shown to upregulate IGF-I expression, and increased IGF-I results in muscle hypertrophy (5; 45; 95; 196). While increasing IGF-I expression enhances muscle growth/regeneration (5; 45), IGF-I upregulation/overexpression may require muscle loading to produce the desired hypertrophic effect (55) in old, as responsiveness to IGF-I is lost with age (196).

Entry of satellite cells from quiescence into G1 appears to be triggered by a signaling cascade initiated by NO release, which in turn activates HGF to bind c-met (45; 205; 227) (HGF receptor on satellite cells), and this HGF may be expressed by satellite cells for autocrine functioning (192; 207). What is known, is that HGF/c-met induces satellite cell entry into G1 from quiescence (45; 192), that NO mediates this event (193; 206), and that HGF inhibitors neutralize satellite cell activation (59; 206). However, at later stages of the satellite cell maturation process, HGF is seen to inhibit differentiation (45; 78; 145; 192), suggesting that it functions more as an activator of satellite cells than as a growth factor after cell cycle entry.

Studies have shown that NO induces HGF/c-met interactions important for satellite cell activation (14; 145; 206; 225), and Anderson has suggested that membrane-bound nNOS is the most likely source for this NO signal (15) due to its location at the cell membrane and proximity to quiescent myogenic precursors. The proteins that allow membrane nNOS localization (15; 35; 220) and regulate its function (79; 118; 217) within the Dystrophin-Associated Protein Complex (DAPC) are also important for controlling this process. Based on the
DAPC’s known mechanosensitivity (23; 24; 99; 177; 216) it is highly likely that nNOS (198), and therefore HGF/c-met signaling, are responsive to resistance training.

MAPK Signaling and Satellite Cells

Halevy and Cantley (90) studied the opposing effects of IGF-I and HGF on MAPK/ERK and PI3K pathways and their involvement in proliferation versus differentiation mechanisms. Although HGF and IGF-I activate both pathways, HGF inhibits myoblast differentiation (PI3K-dependent) while inducing proliferation (MAPK/ERK-dependent), and IGF-I enhances both processes. Their conclusions demonstrate the opposing effects that HGF and IGF-I have on satellite cell activity, and illustrate the importance of MAPK/ERK in the satellite cell response to hormonal regulation.

The p38 family of MAPKs has previously been shown to induce satellite cell proliferation, while inhibition renders satellite cells quiescent (106; 128). This same study by Jones (106) also points out that p38 is required for MyoD induction; MyoD being a transcription factor widely used as an indicator of activated and/or proliferating satellite cells (95). ERK1/2 were also measured by Jones and colleagues using reverse transcriptase polymerase chain reaction (RT-PCR) (106) in differentiating MM14 cells. Although the presences of ERK1/2 were expected in proliferating cells, mRNA levels of these markers were not changed upon differentiation. This same study (106) suggests that ERK1/2 are necessary for myoblast proliferation, and ERK1/2 activation by HGF/c-met has
been documented (113). It should be noted that p38 has been shown to be activated by HGF in other cell types (124; 125; 174) and it is suggested to be activated by HGF in satellite cells (106). The HGF/c-met signaling pathway has been shown to induce satellite cell proliferation via ERK1/2 (106), while it is thought that p38 is important for satellite cell differentiation, and that p38 may also play a role in proliferation. However, little is known regarding the upstream mechanotransduction processes that directly respond to increased load or stretch.

Aging and Satellite Cells

Aging muscle has a smaller myonuclear domain than that of younger individuals, suggesting that myofiber atrophy precedes myonuclear loss, similar to that observed during unloading (77). Regenerative potential appears to be limited in aging muscle (22; 77; 160). Data from our laboratory show no statistically significant difference in the pretraining pool of satellite cells based on age and/or gender (164), in agreement with Roth et al. (182), but contrary to the findings in other studies (110). This difference in satellite cell number based on age between the studies could result from the different muscles biopsied, or possibly from the difference in age or baseline health/fitness of the elderly subject groups.

A study by Williamson and colleagues (222) suggests that older men remain in a heightened state of stress at rest, leading to an altered response to resistance loading vs. young based on phosphorylation levels of MAPK-
associated signaling molecules. These findings are supported by other studies measuring aging and stress response (41; 107), and may help to explain any age difference in satellite cell activation. Pulsatile release of small amounts of NO may maintain satellite cell quiescence, while large bolus production and release causes activation; therefore it appears that change in magnitude of signal, and not necessarily the signal itself, induces activation (15).

The DAPC

The DAPC is a collection of proteins responsible for providing architectural support to fully developed myofibers for force generation in skeletal muscle (10; 86), and it has been shown that certain individual protein components of this scaffold contribute to intracellular signaling mechanisms (10; 79; 99). A representation of the DAPC is found in Figure 2. Satellite cell (206; 207; 226), metabolic (93; 153; 188) and protein synthetic pathways (213) are activated in response to mechanical perturbation, signaling of which occurs at least in part, via the DAPC. Indeed, much of a myofiber’s mechanotransduction machinery is found within either the focal adhesion (FA) complex (82; 99) or the DAPC (23; 99; 172).

Briefly, FA are locations along the cell membrane where the extracellular matrix is physically linked to the myofiber cytoskeleton. These are found in myotendinous and neuromuscular junctions, and above the z-line of myofibers, termed costameres (29; 99; 233). FA are made up of membrane-protein
Figure 2. Illustration of DAPC components
integrins that link the extracellular matrix with a large variety of intracellular proteins including vinculin, talin, and alpha-actinin (29; 195). Consequently, alpha-actinin provides a convenient link between FA and the DAPC (92), and thus affords the opportunity for communication between these two complexes. The function of alpha-actinin is described in further detail below.

Loss of protein within the DAPC decreases skeletal muscle performance and eventually leads to loss of muscle mass (24). With certain machinery within the protein scaffold either non-functional, missing or in low abundance, the structural integrity of the cell membrane is weakened (86; 210) and disruption occurs during contraction (10; 168). Further, signaling cascades necessary for such functions as growth, repair, metabolism, and excitation/contraction coupling are inadequately transmitted ultimately resulting in an inability of the muscle to respond to increased load (86). Any weakness within this transmembrane complex would potentially result in contraction-induced damage of the myofiber (86). Indeed, age-related declines in some DAPC components have been documented (177).

DAPC Components

Neuronal-derived Nitric Oxide Synthase (nNOS)

Mechanical activity of muscle can modulate NO production by affecting NOS activity in the short term, and in the long term by regulating NOS expression (214). NOS itself plays a prominent role in cell signaling as a member of the DAPC, and loss of protein components within the complex result in reduced
nNOS localized at the sarcolemma (36; 43), which leads to concomitant reduction in NO signaling capacity (215). This loss of signaling has far reaching implications in terms of affecting ion channel function (144; 215), myocyte fusion (123; 215), neuromuscular junction formation (105; 215), contractility (116; 117; 175; 176; 215), glucose transport (19; 116; 215), vasomodulation (210), and satellite cell activation (14; 15; 17; 206).

It has been suggested by Anderson (15) that membrane-bound nNOS is architecturally positioned for NO release, which is shown to rapidly mediate the entry of satellite cells into G1 from quiescence (15; 17). Membrane-bound nNOS is sensitive to mechanical changes in the muscle sarcolemma such as stretch (214), and absence of nNOS from the DAPC (35; 36) and pharmacological blockade (15) result in dysregulation of satellite cell activation. Assays for NOS activity revealed stimulation in satellite cells in response to stretch, and treatment with Nω-nitro-L-arginine Methyl Ester (L-NAME) blocked NO synthesis (205) and consequently, inhibited satellite cell activation (206). Further study is required to determine activation. However, NO synthesis and release is but one part of a multi-step process for stimulating satellite cell activation.

Tatsumi and colleagues also showed that stretch-induced NO production mediates HGF release from extracellular matrix stores (204; 205), which bind c-met receptors shown to be localized on satellite cells (12; 204; 207). HGF release is blocked if NOS is inhibited; conversely, in the presence of a NO donor, unstretched cells will release HGF. Therefore, it appears that HGF release and
c-met binding is mediated by NO synthesis in response to mechanical perturbation (203).

NO production and release to stimulate HGF/c-met binding appears to be an autocrine/paracrine function. It was shown that the active, heterodimeric form of HGF is present in uninjured muscle of rats (203), while it was also suggested that NO induces HGF separation from a binding protein, the heparin sulfate proteoglycan complex (205). In 1998 the original source of HGF sequestered in the extracellular matrix was not known (204); however, Sheehan et al. demonstrated that HGF is produced by satellite cells, themselves (192).

It has been demonstrated that skeletal muscle tonically produces NO, which modulates various forms of muscle activity including: vascular control (62; 175), largely influenced by NO released from endothelial-derived nitric oxide synthase (eNOS); muscle metabolism (18; 20; 38; 147; 175) and contractile function (144; 176). Low levels of NO maintain satellite cell quiescence, and upon stretch a large bolus of NO is released to directly serve as a stimulus for, among other functions, satellite cell activation (15; 17). During exercise, studies show that NOS activity is elevated (178), while NO release can be increased 50-200% during periods of repetitive isometric contraction (18; 175).

The transient nature of NO mandates that its site of activation be located near the site of origin (126; 183); and studies by Anderson and colleagues have shown that the architectural positioning of nNOS within the DAPC lends itself as the most likely source of NO to induce satellite cell activation for growth and repair. Membrane-bound nNOS and skeletal muscle satellite cells are close
enough in proximity to one another for release of such a transitory signaling molecule to achieve this task (15). However, some reports show that nNOS is also expressed by satellite cells, themselves (14; 16; 205). Further inquiry to determine the importance of NO release as a paracrine or autocrine factor may lead to new methods for stimulating satellite cell activation in poorly-functioning systems such as with aging.

*nNOS Activation*

The regulation of NO production at the sarcolemma requires examination, as mechanical perturbation (203; 206; 207) and biochemical phosphorylation (4; 151; 171) both regulate nNOS activity. Phosphorylation of serine 1417 (ser1417) on nNOS has been shown to activate NO synthesis in the kidney (151), and a study involving human subjects and a 30-second cycle ergometer “sprint” established that the identical site on nNOSμ (muscle-specific 34 base pair insert splice variant of nNOS, serine 1451) is phosphorylated in response to this protocol (47). Further examination of this site in skeletal muscle requires examination to determine any alteration of function based on aging, acute or long term resistance training.

*Caveolin-3*

Caveolin-3 is a muscle specific isoform of caveolin known to facilitate signal transduction within the DAPC (216; 217). The exchange between nNOS and caveolin-3 is of interest, as caveolin-3 has been shown to inhibit membrane-
bound nNOS production of NO (216; 217) by blocking L-Arginine binding (79) in a manner similar to L-Arginine-based NOS inhibitors (3; 46; 73). The traditional ERK signaling cascade is also shown to be inhibited by caveolin (26; 63), while caveolin-3 disruption elicits a hyperactivation of the ERK2 cascade, in particular (26; 223).

Changes in caveolin-3 expression have been shown to induce structural changes to the distribution of DAPC components and T-tubule abnormalities (75). Changes in T-tubule structure and function may also affect calcium regulation, and caveolin-3 has also been implicated in skeletal muscle’s requirement for tight control over calcium flux (56). Other studies illustrate that overexpression of caveolin-3 in skeletal muscle induces downregulation of dystrophin and other associated glycoproteins, resulting in a phenotype similar to that observed in Duchenne muscular dystrophy models (54; 61; 68; 76). High levels of caveolin-3 are also thought to inhibit myoblast fusion in culture (68; 218). Therefore, increased caveolin-3 expression at the sarcolemma may also contribute to structural instability, increasing the incidence of stretch or overload-induced damage, while also decreasing the muscle’s ability to repair itself by inhibiting NO production.

**Alpha-1 Syntrophin**

Localization of nNOS within the DAPC is largely mediated through PDZ (PSD-95, discs large, ZO-1) domain interactions with alpha-1 syntrophin (35; 220), a 58-59 kDa protein that is ubiquitously expressed throughout the
sarclemma (210) of all three skeletal myofiber types in humans (104). Three syntrophin isoforms exist in skeletal muscle: alpha I, beta I (expressed predominantly in type II myofibers in humans (104)), and beta II which is present both throughout the sarclemma and at the neuromuscular junction in humans, but in the mouse is mostly synaptic in association (104; 162). In addition to one PDZ domain, syntrophins contain two pleckstrin homology (PH) domains (9), through which binding of dystrophin (104), alpha-dystrobrevin, aquaporin-4 (7; 104), and voltage-gated sodium channels (80; 104) occurs. Syntrophins also possess one syntrophin unique (SU) domain, which allows for association with other dystrophin family members (9; 10). Viewed as “adaptor” proteins, syntrophins facilitate localization of other proteins at the sarclemma that initiate further intracellular signaling (10; 48; 93; 134; 235).

A study by Kameya and colleagues (112) showed that in alpha-1 syntrophin knockout mice, nNOS does not localize to the sarclemma; and that these mice display no histological or contractile changes in the muscle. However, a considerable amount of nNOS was still observed within the cytosol of the muscle cells. It should be pointed out that regenerative capacity of this muscle was not measured, as contractility was measured from fiber bundles dissected from mutant mice. Therefore, no dependence of satellite cell activation on DAPC-associated nNOS was observed, although a marked abundance of cytosolic nNOS points toward other necessary intracellular functions, such as opposition of contractile force generation (176), and modulation of cytotoxicity (157).
**Alpha-Dystrobrevin**

Alpha and beta dystrobrevin are members of the dystrophin family of proteins, with the alpha isoform predominating in skeletal muscle (104). Alternative splicing of alpha dystrobrevin yields several distinct isoforms, of which the two larger isoforms, alpha-1 and alpha-2, contain syntrophin and dystrophin binding sites (13; 104; 156; 163). Interactions with the syntrophins occur through two specific syntrophin binding domains located near dystrobrevin’s carboxy terminus (10), while anchoring of dystrobrevin to the DAPC occurs through association of C-terminus coiled-coil domains with dystrophin (61).

Association of these proteins via the afore-mentioned domains suggests that dystrobrevin plays a part in assembly of a signaling scaffold at the sarcolemma; further evidence is provided by tyrosine phosphorylation of alpha-1, which promotes kinase activity (10). Multiple splice variants of dystrobrevin exist, suggesting that expression of a specific variant promotes binding of a subset of proteins, facilitating a specified signaling response (10; 62). No studies are known concerning dystrobrevin and aging and/or exercise; and no definitive connection between dystrobrevin mutations and musculoskeletal diseases has been made to date. However, one study conducted in alpha-dystrobrevin knockout mice suggested that, although no sarcolemmal structural differences existed compared to wild type mice, it was likely that intracellular signaling was altered (83).
Dystrophin

Dystrophin has been widely studied for its central role in providing a linkage between cytoskeletal actin and the extracellular matrix (10; 64). It is a 427 kilodalton (kDa) protein consisting of an N-terminal actin binding domain, a central rod domain comprised of 24 triple helical coiled-coil repeats, and a cysteine rich C-terminus where binding of alpha-actinin (92), beta-dystroglycan, dystrobrevin, and the syntrophins occurs (61). Beta-dystroglycan, in turn, binds with alpha-dystroglycan, which then associates with laminin in the extracellular matrix (10).

As dystrophin allows association of multiple proteins within the DAPC, diseases conferring a lack of this protein contribute to a greatly weakened cell membrane, thereby significantly decreasing abundance of other DAPC components and, consequently, intracellular signaling (24). Indeed, dystrophin’s importance for maintenance of membrane structural integrity and contribution to intracellular signaling has been documented (31). There are several mutations resulting in sarcolemmal loss of dystrophin at least in part; the most well-known of which is Duchenne muscular dystrophy, where sarcolemmae lacking dystrophin are weakened, allowing for increased muscle damage and insufficient mechanotransduction, or load-sensitive biochemical signaling. The end result of this deficiency is myofiber necrosis, eventually in the diaphragm, where muscle loss culminates in asphyxiation. However, the dystrophin complex is not restricted to skeletal muscle, as expression is observed in the brain and studies
on muscular dystrophy patients reflect lower than normal IQs and mild mental retardation (10; 221).

Studies of mdx⁻/⁻ mice have shown that loss of dystrophin results in the inability of alpha-1 syntrophin to localize to the muscle cell membrane (2; 7), and consequently, nNOS also does not associate with the sarcolemma (36; 44; 88). Further, another study concluded that absence of dystrophin leads to a loss of all other DAPC components (24). A recent study examining strength gains and training adaptation of dystrophin in the vastus lateralis of 12 young men reports no significant change in dystrophin concentration through 8 weeks of resistance training (224).

**Alpha-actinin**

Alpha-actinin binding the C-terminus of dystrophin provides a linkage that associates the DAPC with the actin cytoskeleton and other costameric adhesion complex proteins such as vinculin, talin, and β1-integrin (92). This association provides both enhanced structural support and coordinated signaling via physical contact between two complexes known for providing mechanotransduction from the contractile machinery outwardly to the cell membrane. Indeed, MacArthur and North suggest that alpha-actinin helps to integrate several functional pathways in skeletal muscle for maintenance of contractility, signaling, and metabolism (132).

Two alpha-actinin isoforms exist in adult skeletal muscle, and are encoded by two different genes (25). Alpha-actinin 2 has been found in greater
abundance in oxidative myofibers, while the alpha-actinin 3 isoform is specific to fast-twitch fibers (146). Although actinin-3 null mutations exist, they do not seem to produce any histological or phenotypical changes to the muscle cell (49).

The DAPC and Muscle Metabolism

The DAPC is known for its sensitivity to mechanical stimuli, and its importance for mechanical induction of the growth response has been addressed. However, the DAPC is also partially responsible for ensuring that metabolic processes are in proper working order depending on the energy requirements of the muscle. Cytosolic nNOS is suggested to play integral roles in modulating cellular functions, such as glucose metabolism (137). Both exercise (28; 231; 232) and NO, as well as NO donors are thought to elicit GLUT-4 translocation (20; 66; 97; 137; 179) as well as glucose uptake and transport (20; 97; 137; 234). It has also been shown that NO mediates exercise-stimulated glucose transport in skeletal muscle utilizing a mechanism distinct from insulin-dependent signaling pathways (97; 137).

Cytosolic nNOS appears to have another effect on muscle metabolism, as muscle-specific phosphofructokinase (PFK-M) competes for PDZ domain association with the carboxy-terminal PDZ ligand of nNOS (CAPON) (70). This study suggests that PFK-M binding localizes nNOS to the cytosol, which may allow NO production to facilitate metabolic and other intracellular processes. The localization of nNOS to PFK-M within the cytosol may also confer resistance of NO-related toxicity to the muscle cell in a manner similar to that observed in
neurons (70; 199). Due to both resting pulsatile (214) and contraction-induced bolus release (225) of NO, it seems logical that tissues in the surrounding area would require protection.

Another study shows that PFK-M associates with caveolin-3, and is sensitive to extracellular glucose concentrations (188). This may be important for localizing PFK-M to the sarcolemma, ensuring its close proximity to the site of glucose entry for glycolytic activity. They go on to suggest that this interaction with caveolin-3 would recruit PFK-M to the cell membrane; however, interaction of PFK-M, caveolin-3, and nNOS at the sarcolemma has not been shown. Caveolin-3 expression is necessary for activation of the Insulin Receptor/PI3K pathway, integral for glucose transporter-4 (GLUT-4) translocation and glucose transport (68). This pathway is inhibited in caveolin-3 null mice, and these animals exhibit insulin resistance (68; 158). Decreased caveolin-3 may not hinder satellite cell activation or provide structural instability, but it may point to the DAPC’s importance for managing glucose transport; as low caveolin-3 levels could contribute to a decreased localization of PFK-M to the DAPC for glycolytic function, as well as decreased GLUT-4 translocation.

Alpha-1 syntrophin has also been suggested to play a part in controlling muscle metabolism, as Hasegawa shows that stress-activated protein kinase -3 (SAPK3, p38γ) and alpha-1 syntrophin associate with one-another in yeast two-hybrid experiments (93). Maintenance of basal glucose uptake and negative regulation of GLUT4 expression are thought to occur via alpha-1 syntrophin binding with SAPK, unlike other p38 isoforms shown to enhance GLUT4
translocation (98). Based on this, it seems possible that resistance training-mediated GLUT4 upregulation may become more tightly regulated by enhanced alpha-1 syntrophin protein.

DAPC, Mechanotransduction, and Aging

Very few studies have been conducted examining age-related differences in DAPC components; however, Rice and colleagues (177) have illustrated alterations in expression of dystrophin, β-dystroglycan, and α-sarcoglycan with increasing age; and suggest that these changes, along with diminished membrane integrity, play a role in skeletal muscle remodeling. The MAPK ERK1/2 and p38 pathways have been used as a measure of the mechanosensitivity of aged muscle compared to young (100; 150). Mechanosensitivity of p38 was documented in these two studies, one that measured the muscle's response to a 15% passive stretch stimulus (100), and the other measured in response to 10 days of immobilization followed by a set number of days of free ambulation (150). Together, these studies illustrate that the p38 signaling pathway is intact in aged muscle, that it does not differ in signaling capability compared to young, but that phosphorylation of p38 is increased after an atrophic stimulus. This seems logical, as p38 is thought to regulate satellite cell proliferation and differentiation as a means to induce muscle growth and/or repair. Another study measured increased basal phosphorylation of both p38 and ERK 1/2 of resting aged rats, with heightened stretch-induced ERK 1/2 phosphorylation (154). Stretch-mediated
phosphorylation of p38 occurred; however results varied based on muscle type as the primarily oxidative soleus muscle possessed heightened p38 activation in 36 month-old rats without any age difference in the more glycolytic extensor digitorum longus muscle (154).

Of all the DAPC complex proteins mentioned previously, nNOS and dystrophin have been the most closely examined. Capanni and colleagues demonstrated that nNOS increases in abundance with aging rats, both at the muscle membrane and in the cytoplasm (42). Many experiments have also been conducted to examine muscular dystrophy in humans, rats, and mice, but skeletal muscle dystrophin in healthy adults has gone virtually unnoticed. Though aged muscle is weakened, has a slower contractile velocity, and is more susceptible to structural injury, a loss of dystrophin expression within these subjects would seem unlikely; as loss of dystrophin has been shown to result in widespread DAPC component loss (24).

The effect of aging on mechanotransduction components and muscle growth in a human model has to our knowledge not been studied; however it has been suggested that in sarcopenic muscle, satellite cells are highly resistant to activation (17; 111). Further, others suggest the existence of gradual age-related declines in responsiveness of skeletal muscle repair mechanisms (51; 85; 194), protein synthetic rates (229), and available pool of satellite cells (133; 194).
Dissertation Objectives

The overall objective of these studies was to advance our understanding of the mechanisms leading to load-mediated muscle hypertrophy, and to determine whether age-related impairments in the hypertrophic response to mechanical load among old vs. young humans were driven by age effects on the targeted processes. We assessed, in an age-dependent manner, specific factors thought to initiate and/or facilitate key signaling events that lead to myofiber hypertrophy during long-term resistance exercise training.

Specific Aim 1: Myofiber CSA and MHC Distribution

We tested the hypothesis that myofiber hypertrophy as a result of resistance training would be impaired in older subjects compared to young. The study design explored any aging and gender differences in load-mediated changes in myofiber size and MHC distribution during a 16-wk (3 d/wk) progressive resistance training program in young (20-35 yr) and older (60-75 yr) women and men.

Specific Aim 2: MRF mRNA and Protein Expression

We tested the hypothesis that a more robust hypertrophic response to resistance training in young vs. older adults would be at least partially driven by greater load-mediated mRNA and protein expression of the myogenic regulatory factor (MRF) family of muscle-specific transcription factors (MyoD, myf-5,
myogenin, myf-6), known to be requisite for myonuclear addition and muscle-specific gene expression in support of myofiber growth.

**Specific Aim 3: DAPC Component Proteins**

Because the most substantial age difference in the magnitude of myofiber hypertrophy was noted among young vs. older men (Aim 1), and there were no notable age differences in MRF expression among the two groups of men (Aim 2), we tested the hypothesis that the blunted hypertrophic adaptation among older men (vs. young) may be at least partially explained by age differences in the dystrophin-associated protein complex (DAPC), the mechanosensitive transmembrane protein system responsible for mechanotransduction to initiate and/or drive muscle growth and repair processes. Specific components of the DAPC were examined, theorizing that this complex is more functionally responsive to resistance training in young men compared to old.

**Specific Aim 4: Resistance Training-Induced Cell Signaling**

We tested the hypothesis that early induction of mitogen-activated protein kinase (MAPK) signaling pathways, including p38 MAPK and extracellular regulated kinase (ERK1/2) would be acutely up-regulated more so in young than old following novel exposure to mechanical load, as these MAPKs have been shown to associate with DAPC components and play important roles in the proliferation/differentiation response of myogenic precursor cells. Further, we tested the hypothesis that long-term up-regulation of the MAPKs, particularly
p38, which is indicative of stress and muscle damage, would be greater in old than young, implicating damage susceptibility and/or impaired regenerative capacity in the blunted hypertrophic capacity of older muscle.
EFFICACY OF 3 D/WK RESISTANCE TRAINING ON MYOFIBER HYPERTROPHY AND MYOGENIC MECHANISMS IN YOUNG VERSUS OLDER ADULTS

by

DAVID J. KOSEK, JEONG-SU KIM, JOHN K. PETRELLA, JAMES M. CROSS
AND MARCAS M. BAMMAN


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ABSTRACT

Resistance training (RT) has shown the most promise in reducing/reversing effects of sarcopenia, although the optimum regime specific for older adults remains unclear. We hypothesized myofiber hypertrophy resulting from frequent (3 d/wk, 16 wk) RT would be impaired in older (O; 60-75 yr 12 women, 13 men), sarcopenic adults compared to young (Y; 20-35 yr; 11 women, 13 men) due to slowed repair/regeneration processes. Myofiber type distribution and cross sectional area (CSA) were determined at 0 and 16 wk. Transcript and protein levels of myogenic regulatory factors (MRFs) were assessed as markers of regeneration at 0, 24 h post-exercise, and after 16 wk. Only Y increased type I CSA 18% (P<0.001). O showed smaller type IIa (-16%) and type IIx (-24%) myofibers prior to training (P<0.05) with differences most notable in women. Both age groups increased type IIa (O, 16%; Y, 25%) and mean type II (O, 23%; Y, 32%) size (P<0.05). Growth was generally most favorable in young men. Percent change scores on fiber size revealed an age x gender interaction for type I fibers (P<0.05) as growth among Y (25%) exceeded that of O (4%) men. Myogenin and MyoD mRNAs increased (P<0.05) in Y and O, while myf-5 mRNA increased in Y only (P<0.05). Myf-6 protein increased (P<0.05) in both Y and O. The results generally support our hypothesis as 3d/wk training led to more robust hypertrophy in Y vs. O, particularly among men. However, this differential hypertrophy adaptation was not explained by age variation in MRF expression.
INTRODUCTION

It is well-established that muscle mass declines with age (termed sarcopenia). In the knee extensors (e.g. vastus lateralis) which are important for ambulation and weight-bearing function, a 30% decrease in whole muscle size occurs between the ages of 50 and 80 years (15,40). This whole muscle atrophy results from atrophy of type II myofibers (34) and apparent loss of both type I and type II motor units as evidence from cadaveric studies of vastus lateralis indicate the number of myofibers, regardless of fiber type, declines substantially between the 6th and 8th decades (38). In the US, $18.5 billion of total direct health care costs in 2000 were attributable to sarcopenia (31), and this will undoubtedly increase as the percentage of American adults 65 years and older is expected to increase from 1 in 9 to 20% of the adult population by 2030 (National Institute on Aging statistics). Resistance training has shown the most promise among interventions aimed to decrease the effects of sarcopenia, as it enhances strength, power, and mobility function, and induces varying degrees of skeletal muscle hypertrophy (29). Based on this recent review of findings in older adults, it is not clear whether resistance training prescriptions currently recommended for young adults provide the most effective hypertrophy stimulus for aging muscle (29). We suggest differences in the state of the muscle may be a factor (i.e. the regenerative/recovery rate), as older, sarcopenic adults (vs. young) may benefit from prolonged recovery periods between loading bouts.
Repair/regeneration and growth of skeletal muscle is largely dependent on the addition of myonuclei to existing, terminally differentiated myofibers, by activation of satellite cells (28). Upon injury or stress to muscle fibers, satellite cells are induced to proliferate and differentiate, ending with their incorporation into mature myofibers as myonuclei. These events are regulated by several local processes that appear to respond in a load-dependent manner. Myogenic regulatory factors (MRFs) are basic helix-loop-helix (bHLH) transcription factors specific to skeletal muscle that regulate satellite cell differentiation and induce transcription of skeletal muscle-specific genes such as creatine kinase, myosin light and heavy chains, troponin I, and desmin (10,44,49,53). Expression of the ‘early’ MRFs, myf-5 and MyoD, commit somitic cells to the myogenic lineage, while the ‘late’ MRFs, myf-6 and myogenin, terminally differentiate proliferative myoblasts toward formation of multinucleated myotubes in developing muscle (58), or into nuclear donors to developed myofibers for repair/regeneration and growth. Myogenin and MyoD are also implicated in regulating myosin heavy chain transitions (16,47,48).

Basal levels of some MRFs are upregulated in aging, sarcopenic muscle (7,34,49) and, based on recent findings in rodents, the magnitude of upregulation appears to be directly linked to the degree of sarcopenia (17), suggesting that sarcopenic muscles remain in a state of failing compensatory effort in an attempt to stave off degeneration and atrophy. Resistance loading up-regulates MRF mRNA and protein concentrations (34,53), presumably facilitating growth/regeneration mechanisms. However, our previous findings indicate that
acute mRNA responses to loading are generally most favorable in young men (compared to other age/gender groups) while the muscles of older, sarcopenic women are less responsive (33,34). Load-mediated satellite cell activation is impaired in aged muscle in vivo (8,13,21,51), and it has been shown that older adults possess a decreased basal rate of mixed muscle protein synthesis (4,65) which may (66) or may not (63) improve with resistance training. Recent insight from the overload (ablation) model in rodents demonstrates that impaired type II hypertrophy in old age is highly associated with elevated AMP-activated protein kinase (AMPK) phosphorylation, indicative of inhibited protein synthesis (62). As myofiber hypertrophy is dependent on both net muscle protein synthesis and satellite cell recruitment, overall these findings suggest resistance training-induced myofiber hypertrophy may be impaired in older vs. young adults.

The age-related slowing of regenerative and recovery processes in aged muscle following mechanical loading and/or damage are well-documented in animal models and are typically linked to blunted or slowed satellite cell activation (8,12,21,43). Existing data in humans are sparse but the 3 d/wk resistance training findings of Roth et al. indicate that older women are more susceptible to training-induced myofiber damage than their young counterparts (56), while older men (vs. young men) are not (57). While these data point to a possible gender difference, we suggest damage susceptibility may be more a function of the state of the muscle than gender per se. For example, in these two studies, myofiber size data were not provided but because older men possessed pre-training strength levels similar to young men (no significant difference) (57)
and older women were 31% weaker than young (56), it is quite possible that the older women were “sarcopenic” within gender while the older men were not. Based on the combined findings from animal and human models, we suggest a 3 d/wk program of sufficient loading intensity to activate regenerative pathways may be effective in young beginners but may not allow sufficient recovery between loading bouts to optimize the growth adaptation in older, sarcopenic muscles. Although limited, there are some published findings in support of this concept after 3 months of resistance training (64). In a study of young and older men and women, Roth et al. (55) found significant thigh muscle hypertrophy (determined via MRI) in all 4 age x gender groups after 6 months of training. The magnitudes of increase were not statistically different among the 4 groups and were quite similar in young and older men, while young women gained nearly twice the muscle area of older women.

The current recommendations set forth by the American College of Sports Medicine do not differentiate between young and older adults regarding frequency of resistance training, recommending 2-3 d/wk for all beginning adults (36). Further research is needed to better define optimal training frequencies for specific age groups. In our view, an important first step is to establish whether age influences the hypertrophic adaptation to a standardized frequency regimen while controlling for as many potential confounders between young and older subject cohorts as possible (e.g., initial training status, gender composition, etc).

The primary aim of this study was to test the hypothesis that myofiber hypertrophy as a result of frequent (3 d/wk) resistance training would be impaired
in older, sarcopenic adults compared to young subjects. Young (20-35 yr) and older (60-75 yr) untrained men and women completed 16 wk of 3 d/wk leg resistance training. In order to effectively test this hypothesis, care was taken to insure that the training program was of the same relative intensity and volume for all subjects in the 4 age-gender groups. Considering that age-dependent differences in hypertrophy may be mediated by a blunted regenerative capacity and/or slowed recovery rate in older muscle, we also evaluated MRF expression both early and late in the training program. Muscle biopsies were collected at 3 timepoints hereafter referred to as baseline pre-exercise, acute post-exercise, and 16-wk post-training. The acute sample was collected 24 h after each subject’s first full exercise bout, while the 16-wk sample was obtained 24 h after each subject’s final training bout.

Of clinical significance, we report after only 16 wk of resistance training, that older adults were capable of restoring the size of type II myofibers to the pre-training sizes found in adults ~35 years younger. However, the findings generally support our overarching hypothesis, as more robust hypertrophy was found in the younger cohort (particularly young men). While these data demonstrate that adults ~64 years of age obviously retain the ability to undergo load-mediated hypertrophy, we suggest alternate, age-specific training programs should be pursued to maximize the efficacy of this adaptation.
METHODS

Subjects

Forty-nine adults were recruited from the Birmingham, Alabama metropolitan area into two age groups. Age ranges were 60-75 yr for the older group (12 females, OF; 13 males, OM) and 20-35 yr for the younger group (11 females, YF; 13 males, YM). Subjects were free of any musculoskeletal or other disorders that might have affected their ability to complete testing and/or resistance training. Subjects were not obese (BMI<30) nor had any leg resistance training experience within the past five years. None of the subjects were being treated with exogenous testosterone or other pharmacologic interventions known to influence muscle mass. The study was approved by the Institutional Review Boards of both the University of Alabama at Birmingham and the Birmingham Veterans Affairs Medical Center. Each subject gave written informed consent prior to participation.

Progressive Resistance Training Program and Dynamic Strength Testing

The resistance training program focused on the knee extensors. Subjects trained 3 d/wk for 16 wk. Subjects warmed up on a cycle ergometer or treadmill for approximately 5 minutes or until warm (light sweat) prior to each training session. Resistance training consisted of 3 exercises including knee extension, leg press, and squats. Each exercise was performed for 3 sets at 8-12 repetitions using resistance exercise stations or plate loaded stations (barbell squats and linear 45º leg press). To accurately compare training and testing
loads between weight stack and free weight stations, actual resistances were determined for each weight stack using a load cell and regression procedures as we have described previously (52). The amount of rest between sets was standardized for all subjects at 90 seconds.

Subjects completed two familiarization sessions to receive instruction on proper technique and to practice the dynamic strength testing protocols (52). Subjects returned to the laboratory 2-3 days after the second familiarization session for knee extension, leg press and squat one-repetition maximum (1RM) assessments using established methods. Attempts of 1RM with progressively increasing load were performed with each attempt separated by 90-120 s rest intervals. 1RM was defined as the highest load lifted through a full range of motion prior to 2 failed attempts at a given load. Verbal encouragement was provided during all 1RM attempts.

After dynamic strength testing to establish 1RM, subjects initiated the training protocol by performing 2 sets of each exercise for 2-3 sessions. Once completed, subjects began the full training protocol of 3 sets per exercise and this session was considered the start of the 16 week training period. Initially, training loads were based on 80% of baseline 1RM strength. As training progressed, resistance was incremented when a subject completed 12 repetitions for at least two of the three total sets at a given resistance while maintaining proper form. The typical increase in resistance was 2.3 kg for knee extension, and approximately 5% for leg press and squat. The goal of this progression was to induce volitional fatigue in the 8-12 repetition range for each
subject throughout the training program. 1RM testing was repeated at mid-training (wk 8) and post-training (wk 16). Actual load intensities and repetitions per set were statistically evaluated and are summarized in Results.

Body Composition

Thigh lean mass, total body lean mass, and body fat percentage were determined by dual energy X-ray absorptiometry (DEXA) using a Lunar Prodigy (model #8743, GE Lunar Corporation, Madison, WI) and enCORE 2002 software (version 6.10.029) according to the manufacturer’s instructions.

Muscle Biopsy and Tissue Preparation Procedures

All muscle biopsies were performed in the Pittman General Clinical Research Center at UAB. Muscle samples were collected from m. vastus lateralis by percutaneous needle biopsy using a 5 mm Bergstrom biopsy needle under suction as previously described (18). The baseline biopsy was taken from the left leg, the acute post-exercise biopsy was taken from the right leg 24 h after the first full bilateral loading bout in order to avoid any residual effects of the baseline biopsy, and the third, 16-wk post-training biopsy was obtained 24 h after the final training session. At the bedside, visible connective and adipose tissues were removed with the aid of a dissecting microscope. Portions of each sample to be used for RNA and protein isolation were immediately weighed and snap-frozen in liquid nitrogen. A separate portion for immunohistochemistry was mounted cross-sectionally on cork in OCT mounting medium mixed with
tragacanth gum, and frozen in liquid nitrogen-cooled isopentane. All samples were stored at -80º C until analyses.

**Total RNA Isolation**

The procedure of RNA isolation has been described in detail previously (33). Briefly, frozen muscle samples (average 35 mg) were homogenized, and total RNA was extracted using the TRI Reagent (Molecular Research Center, Cincinnati, OH), followed by precipitation with isopropanol, 2 ethanol washes, drying and suspension in nuclease-free water at a ratio of 0.8 µl per mg muscle. Fluorometric analysis (TD-700, Turner Designs, Inc. Sunnyvale, CA) was performed to determine RNA concentration using the RiboGreen RNA Quantitation Kit (Molecular Probes, Inc. Eugene, OR) as described previously (33). RNA samples were stored at -80ºC.

**RT-PCR**

One µg of RNA was reverse transcribed in a total volume of 20µl using SuperScript II Reverse Transcriptase (Invitrogen, GIBCO-BRL, Carlsbad, CA) with a mix of oligo(dT) (100ng/reaction) and random primers (200ng/reaction). After 50 min incubation at 45ºC, the RT reaction mixtures were heated at 90ºC for 5 min to discontinue the reaction and then stored at -80ºC for subsequent PCR analyses.

A relative RT-PCR method using 18S ribosomal RNA as an internal standard (Invitrogen, Life Technology, GIBCO-BRL, Carlsbad, CA) was used to
determine relative expression levels of the target mRNAs. The specific primer sets used to amplify myogenin, MyoD, myf-5, and myf-6 mRNAs were published previously (34). Primers were designed using the Primer Select computer program (DNASTar, Madison, WI) and custom-made by Invitrogen (GIBCO). Interaction tests between target mRNA primers and the alternate 18S primers were conducted and primer sets were selected for use after confirming no interaction.

For each PCR reaction, 18S (with a 324-bp product) was coamplified with each target cDNA (mRNA) to express each as a ratio of target mRNA/18S. The 18S primers were mixed with competimers to insure that 18S and each target mRNA coamplified in the linear range. The ratio of this primer:competimer mixture was optimized in preliminary experiments, and ranged from 1:15 to 1:50 depending on the abundance of the target mRNA.

Based on the number of PCR reactions, a PCR pre-mix was prepared as described previously (33). For each PCR, 1 μl of RT product (cDNA) was added into 24 μl of pre-mix and topped with 50μl mineral oil (Sigma-Aldrich, St. Louis, MO). PCR was carried out in a DNA Engine® (PTC-200™) Peltier Thermal Cycler (MJ Research, Waltham, MA) with an initial denaturing step of 3min at 96°C, followed by specific cycles (30-34 cycles depending on the results of linearity tests for each target mRNA and 18S) of 1 min at 96°C, 45 sec at specific annealing temperatures (57-62°C depending on primers), 45 sec at 75°C, and a final step of 3 min at 72°C. Immediately following PCR, 25 μl of PCR product (22 μl of the reaction mixture diluted with 3 μl loading buffer) were separated by
electrophoresis (100V constant) in a 2% agarose gel for 1.5 or 2 hours (depending on the band separation between 18S (324 bp) and the specific target gene due to the size of each mRNA product). Gels were run with molecular weight markers (100 bp Hyper Ladder IV, Genesee Scientific, San Diego, Ca) to confirm the expected size of each mRNA. To eliminate age group or gender bias, each 20-well gel contained samples for subjects within each group (i.e., YM, YF, OM, OF) with the different subject groups loaded in random order on each gel. Ethidium bromide (0.1 μg/ml) was pre-mixed in the 2% agarose gel, and images were captured under UV in a BioRad ChemiDoc imaging system (Hercules, CA). Band densitometry was performed using BioRad Quantity One software. Parameters for image development were described in detail previously (33).

Protein Immunoblotting

Immunoblotting was conducted to assess muscle protein lysate concentrations of MyoD, myogenin, and myf-6. In pilot experiments we were unable to verify a reliable primary antibody against myf-5. Frozen muscle samples (30-40 mg) were homogenized and supernatant assayed for total protein as described in detail (7). Briefly, samples were powdered using a liquid nitrogen-cooled mortar and pestle and homogenized in 3 μl/mg muscle of ice cold lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.5% NP-40, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 100 μM PMSF, and 0.5x protease inhibitor cocktail containing bestatin, leupeptin, aprotinin (P2714,
Sigma-Aldrich, St. Louis, MO). Following two centrifugation steps (15,000xg for 20 min at 4°C), supernatant samples were assayed for total protein using the BCA technique with BSA as a standard (5). Twenty μg of total protein diluted in Laemmli buffer were separated by SDS-PAGE (120 V constant) in 12% mini-gels (Bio-Rad MP3, Bio-Rad Laboratories, Hercules, CA). Proteins were transferred at constant current (1 mA per cm² x 30 min) to PVDF membranes using a semi-dry transfer cell (Trans-Blot SD, Bio-Rad Laboratories, Hercules, CA). Within subjects, baseline, acute post-exercise, and 16-wk post-training samples were loaded in adjacent lanes. To control for age or gender bias, each 10- or 12-lane gel contained samples for one subject from each of 3 different age-gender groups, with the different subject groups loaded in random order on each gel. Equal loading across lanes and equal transfer were verified by staining all gels (after transfer) with Coomassie blue and staining randomly selected membranes with Ponceau-S.

Primary antibody specificities, optimal blocking conditions, and target antigen migration patterns (i.e. molecular weights) were confirmed in control experiments and in our previous work (7). Immunoblotting was carried out using rabbit polyclonal antibodies against MyoD (1:1000, sc-760), myogenin (1:1000, sc-576), and myf-6 (1:1000, sc-301). Membranes were blocked with 2% BSA, 2% milk in PBST, while primary and HRP-conjugated goat anti-rabbit secondary (1:50,000) antibodies were diluted in 0.5% BSA, 0.5% milk in PBST. Membranes were blocked overnight at 4°C under gentle agitation followed by incubation in primary and secondary antibodies for 1 h each at room temperature, with a PBST
rinse protocol (1 quick rinse, 1 x 15 min, 2 x 5 min) following each treatment. All bands were visualized by chemiluminescence (Super West Dura kit, Pierce Biotechnologies, Rockford, IL) in a BioRad ChemiDoc imaging system and band densitometry was performed using BioRad Quantity One software as we previously described in detail (7). For each imaging session, serial imaging ceased at the first point of saturation on the developing image. This standardization, combined with equal and random distribution of the four age-gender groups across the membranes, enabled us to accurately test for age, gender, and resistance training effects.

Immunofluorescence Microscopy

We recently detailed our methods for myofiber typing based on myosin heavy chain (MHC) isoform immunoreactivity (34). Briefly, 6 μm sections were fixed for 45 min at room temperature in 3% neutral-buffered formalin. Following fixation, sections were washed 2 x 5 min with 1x PBS (all subsequent PBS wash steps were 3 x 5 min). Sections were blocked with 5% goat serum in PBS for 20 min at room temperature followed by a wash step. Primary and secondary antibodies were diluted in 1% goat serum in PBS. Anti-MHCI primary antibody (mouse mAb NCL-MHCs, NovoCastra Laboratories, 1:100) was applied for 30 min at 37°C. After a wash step, sections were incubated with ALEXA 594-conjugated goat anti-mouse secondary Ab (Pierce Biotechnologies, 1:200) for 30 min at 37°C. Sections were washed and again blocked (5% goat serum in PBS) for 20 min at room temperature. To locate sarcolemmæ for myofiber sizing, a
wash step was followed by incubation with anti-laminin mouse mAb (VP-L551, NovoCastra Laboratories, 1:80) for 30 min at 37°C, a wash step, and incubation with ALEXA 488-conjugated goat anti-mouse secondary Ab (Pierce Biotechnologies, 1:200) for 30 min at 37°C. Slides were then washed and subjected to a third and final block (5% goat serum in PBS) for 20 min at room temperature. After a wash step, sections were incubated with the final primary antibody (anti-MHCIIa mouse mAb, U. of Iowa Hybridoma Bank, 1:80) for 30 min at 37°C, washed, and incubated with ALEXA 488-conjugated goat anti-mouse secondary Ab (Pierce Biotechnologies, 1:200) for 30 min at 37°C. Nuclei were revealed by a Hoechst 33258 DNA counterstain (Molecular Probes, 1:10,000 in PBS) for 2 minutes at room temperature. Slides were mounted with 1% paraphenylene diamine, 90% glycerol in PBS. Slides and cover slips were bound together by use of nail polish, and stored protected from light at -20°C.

High-resolution (48-bit TIFF) fluorescent images were captured at 10x and 20x, and image analysis was performed using Image-Pro Plus 5.0 software. All analyses were conducted by a single analyst blinded to age, gender, and timepoint (before or after 16 wk of training) of each sample. Myofiber type distribution was determined from 928 ± 37 myofibers per sample before training and 815 ± 33 after training. We confirmed MHC isoform specificities of these mAbs by Western blot (Figure 1). Myofibers positive for MHCI and negative for MHCIIa were classified as type I, fibers positive for MHCIIa and negative for MHCI were classified as type IIa, and fibers negative for both MHCI and MHCIIa were classified as type IIx (Figure 3). With this technique, hybrid myofibers (e.g.,
co-expression of I/IIa or IIa/IIx) are revealed by both color and intensity. Myofibers co-expressing more than one MHC isoform were excluded from analyses. For cross-sectional area (CSA) measurements, each myofiber was manually traced along its laminin-stained border. CSA in $\mu m^2$ was calibrated using a stage micrometer and only those fibers determined to be cross-sectional based on a roundness factor <1.639 were included in the analysis (roundness = perimeter$^2$/4$\pi$area; perfect circle = 1.0, pentagon 1.163, square 1.266, equilateral triangle 1.639). The number of randomly selected myofibers by type included in CSA analyses were 60 ± 2 type I, 63 ± 2 type IIa, and 49 ± 2 type IIx (pre-training only). Three subjects did not have sufficient type IIx distribution to obtain IIx CSA or IIx area distribution before training. Due to the well-documented MHCIIx to MHCIIa shift induced by resistance training, this increased to 41 subjects after training who lacked a sufficient number of of IIx myofibers for CSA assessment. Training-induced changes in myofiber size were therefore restricted to type I and type II myofibers, with type II CSA being a weighted average based on the relative distributions of IIa and IIx myofibers.

Statistical Analysis

Data are reported as mean ± SE. Between groups differences in pre-exercise descriptive variables were tested using age x gender ANOVA. All variables measured before and after loading were analyzed using age x gender x load repeated measures ANOVA. MRF protein and mRNA concentrations were assessed across all 3 biopsy timepoints and were thus analyzed via 2 x 2 x 3
Figure 3. Myosin heavy chain (MHC) isoform specificities of monoclonal antibodies used for myofiber typing were confirmed by immunoblot (left). Lane A; 8% SDS-PAGE (50:1 acrylamide:bis) of myofibrillar protein stained with Rapid Coomassie Blue (upper band MHCIIx, middle band MHCIIa, lower band MHCI). Lane B; MHCIIa-specific mAb A4.74 (University of Iowa Hybridoma Bank). Lane C; MHCI-specific mAb NCL-MHCs (Novocastra Laboratories). Sample immunostain (right) displays MHCI-positive myofibers in rust, MHCIIa-positive myofibers in green, and MHCIIx myofibers are dark (negative for both MHCI and MHCIIa). Sarcolemmae were revealed by anti-laminin mAb (VP-L551, Novocastra Laboratories) and nuclei via Hoechst DNA counterstain.
repeated measures ANOVA. Myofiber size and distribution were assessed at baseline and post-training and were thus tested via 2 x 2 x 2 repeated measures ANOVA. For each ANOVA model with a significant main or interaction effect, Tukey HSD tests were performed post hoc to localize the effect(s). Statistical significance was accepted at \( P < 0.05 \) for all tests.

RESULTS

Subject Characteristics

Body composition and strength results for each of the 4 age-gender groups are shown in Table 1. Within age group, men and women were of similar age. Typical gender differences (\( P<0.05 \)) were noted for height, weight, lean mass, and strength, with higher values among the men. Significant main effects confirmed the expected age and gender differences in percent body fat (lower in men, lower in young, \( P<0.05 \)) but these were accompanied by an age x gender interaction (\( P<0.05 \)), as percent body fat among young women was relatively high and not different from older women. Main age effects (\( P<0.05 \)) were noted for all 3 dynamic strength measures. Across the 3 strength tests, 1RM strength at baseline was 29% lower in older vs. young men and 19% lower in older vs. young women.

During resistance training, a minimum adherence rate of 83.3% (5/6 sessions) was required and the average adherence rate was 90%. There were no differences in adherence rates between groups based on age or gender (young women 91%, young men 90%, older women 92%, older men 89%). We
Table 1. Descriptive characteristics and effects of training on body composition and strength.

<table>
<thead>
<tr>
<th></th>
<th>Young Females (n=11)</th>
<th>Young Males (n=13)</th>
<th>Older Females (n=12)</th>
<th>Older Males (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>27.9 ± 1.1</td>
<td>26.2 ± 1.4</td>
<td>63.3 ± 0.9</td>
<td>64.5 ± 1.1</td>
</tr>
<tr>
<td>Height (cm)‡</td>
<td>164.5 ± 2.4</td>
<td>179.7 ± 2.0</td>
<td>161.8 ± 2.2</td>
<td>178.5 ± 1.9</td>
</tr>
<tr>
<td>Weight (kg)‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>67.6 ± 3.4</td>
<td>79.1 ± 2.4</td>
<td>65.5 ± 3.0</td>
<td>87.9 ± 3.0</td>
</tr>
<tr>
<td>16 weeks</td>
<td>68.2 ± 3.4</td>
<td>80.3 ± 1.8</td>
<td>64.8 ± 3.3</td>
<td>87.7 ± 3.1</td>
</tr>
<tr>
<td>Body Fat (%)†§‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>36.8 ± 1.8</td>
<td>21.8 ± 2.5</td>
<td>39.1 ± 1.4</td>
<td>31.7 ± 1.6</td>
</tr>
<tr>
<td>16 weeks</td>
<td>36.3 ± 2.1</td>
<td>20.8 ± 2.3</td>
<td>37.7 ± 1.7</td>
<td>30.4 ± 1.6</td>
</tr>
<tr>
<td>Total LM (kg)†‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>40.1 ± 1.1</td>
<td>59.0 ± 1.3</td>
<td>37.5 ± 1.6</td>
<td>57.3 ± 1.6</td>
</tr>
<tr>
<td>16 weeks</td>
<td>40.8 ± 1.1</td>
<td>60.0 ± 1.3</td>
<td>38.1 ± 1.6</td>
<td>58.1 ± 1.7</td>
</tr>
</tbody>
</table>

Bilateral 1RM Strength Assessments (N)

<table>
<thead>
<tr>
<th></th>
<th>Knee Ext. †§‡ξ</th>
<th>Squat †§‡Φ</th>
<th>Leg Press †§‡ξ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>397 ± 27</td>
<td>592 ± 34</td>
<td>928 ± 47</td>
</tr>
<tr>
<td>16 weeks</td>
<td>547 ± 33*</td>
<td>921 ± 54</td>
<td>1538 ± 90</td>
</tr>
<tr>
<td></td>
<td>853 ± 50*</td>
<td>1265 ± 50*</td>
<td>2121 ± 50*</td>
</tr>
<tr>
<td></td>
<td>415 ± 31*</td>
<td>683 ± 30*</td>
<td>754 ± 45</td>
</tr>
<tr>
<td></td>
<td>609 ± 29*</td>
<td>927 ± 40*</td>
<td>1181 ± 55</td>
</tr>
</tbody>
</table>

Values are mean ± SE. LM, lean mass; TLM, thigh lean mass; 1RM, One-repetition maximum voluntary strength. *Within groups training effect, †Main training effect, §Main age effect, ‡Main gender effect, ξGender x training interaction, αAge x training interaction, ΦAge x gender interaction, P<0.05.

conducted analyses of training program design variables post hoc to test whether the program differed by age or gender in volume or intensity (data not displayed).

No differences by age or gender were found in the number of repetitions performed per set for any of the 3 exercises, with overall averages of 10.6, 10.9, and 10.2 repetitions per set for the squat, leg press, and knee extension, respectively. Squat training intensity did not differ by age or gender and
averaged 74% 1RM at 8 wk and 75% 1RM at 16 wk. Leg press training intensity was unexpectedly different by gender (higher in women, P<0.01), averaging 84% 1RM at 8 wk in women vs. 78% in men. This gender difference remained at 16 wk; 83% in women vs. 76% in men (P<0.01). Knee extension training intensity did not differ by age or gender at 8 wk but was significantly higher in older adults (68% 1RM) vs. young (63% 1RM) by 16 wk (P<0.05). We presume subjects required relatively lower loads to achieve the target number of full range of motion repetitions during knee extension training because of fatigue since this movement was always performed last.

Strength levels for each of the 1RM tests improved substantially after 16 wk of RT in all 4 groups (P<0.05). Across groups, strength increases averaged 38-49% (leg press), 34-44% (knee extension), and 28-37% (squat). Relative strength gains were similar among men and women. On absolute strength data, gender x training interactions for each strength test (P<0.05) were driven by larger absolute gains in men.

Myofiber Type Distribution and Size

Myofibers were classified based on myosin heavy chain (MHC) isoform expression as type I (MHCI only), IIa (MHCIIa only), or IIx (negative for both MHCI and MHCIIa). Hybrid myofibers (I/IIa or IIa/IIx) were excluded from distribution and size measurements. Fiber type distribution results as shown in Table 2 were determined on 928 ± 37 myofibers per sample before training and 815 ± 33 fibers per sample after training. The distribution of type I myofibers did
### Table 2. Myofiber cross-sectional area, type distribution, and type area distribution

<table>
<thead>
<tr>
<th></th>
<th>All Young (n=23)</th>
<th>All Older (n=24)</th>
<th>Young Females (n=10)</th>
<th>Young Males (n=13)</th>
<th>Older Females (n=11)</th>
<th>Older Males (n=13)</th>
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<tbody>
<tr>
<td></td>
<td>CSA, µm²</td>
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<td><strong>Myofiber Cross-Sectional Area</strong></td>
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<tr>
<td>Type I †θ</td>
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<tr>
<td>Baseline</td>
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<td>4083±244</td>
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<td>2713±174</td>
<td>4537±365</td>
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<td>5905±307*</td>
<td>4613±319*</td>
<td>4853±265</td>
<td>6714±373*</td>
<td>3611±201</td>
<td>5461±449</td>
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<td>4552±323*</td>
<td>4823±270*</td>
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<tr>
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<td><strong>Myofiber Type Distribution (%)</strong></td>
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<tr>
<td>Baseline</td>
<td>37.8 ± 2.9</td>
<td>35.0 ± 1.8</td>
<td>38.8 ± 2.9</td>
<td>37.0 ± 4.8</td>
<td>38.5 ± 2.4</td>
<td>32.1 ± 2.3</td>
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<tr>
<td>16 weeks</td>
<td>35.8 ± 2.8</td>
<td>35.9 ± 2.2</td>
<td>38.4 ± 4.2</td>
<td>33.8 ± 3.8</td>
<td>38.2 ± 2.8</td>
<td>33.4 ± 3.0</td>
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<tr>
<td>Baseline</td>
<td>47.2 ± 2.4</td>
<td>50.2 ± 1.7</td>
<td>45.8 ± 2.6</td>
<td>48.2 ± 4.0</td>
<td>49.1 ± 2.3</td>
<td>51.3 ± 2.3</td>
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<td>16 weeks</td>
<td>63.2 ± 2.8*</td>
<td>62.8 ± 2.2*</td>
<td>61.1 ± 4.1*</td>
<td>64.7 ± 4.0*</td>
<td>60.0 ± 2.6</td>
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<tr>
<td>Baseline</td>
<td>15.1 ± 1.9</td>
<td>14.8 ± 2.0</td>
<td>15.4 ± 3.0</td>
<td>14.8 ± 2.6</td>
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<td>16 weeks</td>
<td>1.0 ± 0.5*</td>
<td>1.3 ± 0.5*</td>
<td>0.5 ± 0.2*</td>
<td>1.5 ± 0.8*</td>
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<td><strong>Myofiber Type Area Distribution (%)</strong></td>
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<tr>
<td>Type I †‡</td>
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<tr>
<td>Baseline</td>
<td>37.6 ± 2.8</td>
<td>41.4 ± 2.2</td>
<td>41.5 ± 2.4</td>
<td>34.5 ± 4.5</td>
<td>48.1 ± 3.0</td>
<td>35.7 ± 2.2</td>
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<tr>
<td>16 weeks</td>
<td>32.7 ± 2.6</td>
<td>39.4 ± 2.6</td>
<td>37.7 ± 3.8</td>
<td>28.9 ± 3.4</td>
<td>45.7 ± 3.5</td>
<td>34.0 ± 3.1</td>
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<tr>
<td>Baseline</td>
<td>50.5 ± 2.4</td>
<td>48.5 ± 1.8</td>
<td>47.2 ± 1.9</td>
<td>53.0 ± 4.0</td>
<td>44.9 ± 2.2</td>
<td>51.7 ± 2.4</td>
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<tr>
<td>16 weeks</td>
<td>66.5 ± 2.6*</td>
<td>60.3 ± 2.5*</td>
<td>62.3 ± 3.8*</td>
<td>69.8 ± 3.4*</td>
<td>53.4 ± 3.1</td>
<td>66.1 ± 3.1*</td>
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<tr>
<td>Baseline</td>
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<td>12.6 ± 2.5</td>
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<tr>
<td>16 weeks</td>
<td>0.8 ± 0.5*</td>
<td>0.4 ± 0.3*</td>
<td>0.0 ± 0.0*</td>
<td>1.4 ± 0.9*</td>
<td>0.8 ± 0.6</td>
<td>0.0 ± 0.0*</td>
</tr>
</tbody>
</table>

Values are mean ± SE. CSA, cross-sectional area. *Within groups training effect, †Main training effect, §Main age effect, ‡Main gender effect, θAge x gender x training interaction, P<0.05.
not differ among the groups and did not significantly change with training, as the overall average distribution was 36.4 ± 1.7% at baseline and 35.9 ± 1.7% after training. However, taking into account changes in fiber size, a main training effect revealed an overall decline in the area distribution of type I myofibers after training (39.5 ± 1.8% pre to 36.1 ± 1.9% post, P<0.05). A main gender effect (P<0.005) revealed that women had a substantially higher type I area distribution than men both before (45.0 ± 2.0% vs. 35.1 ± 2.5%) and after (41.9 ± 2.7% vs. 31.4 ± 2.3%) training.

The resistance training program resulted in the IIx to IIa MHC shift typically found after exercise training. The distribution of MHCIIa (type IIa) myofibers increased markedly after training (P<0.001) in both women (47.4 ± 1.7% to 60.1 ± 2.3%, P<0.001) and men (49.8 ± 2.3% to 65.3 ± 2.5%, P<0.001). Similar increases were also noted by age (P<0.001). No significant age or gender effects in type IIa distribution were found. Post hoc tests within groups showed that older women were the only age-gender group not to significantly increase the distribution of MHCIIa myofibers (P=0.118) Overall, the area distribution of type IIa myofibers increased robustly with training (49.5 ± 1.5% pre to 63.3 ± 1.9% post, P<0.001). A main gender effect (P<0.005) was driven primarily by a significantly higher post-training value in men (67.9 ± 2.3%) vs. women (57.7 ± 2.6%). Within age-gender groups, only elderly women did not significantly increase type IIa area distribution, while young men exhibited the most pronounced change.
The distribution of MHCIIX myofibers (MHCIIXa and MHCI negative fibers) fell markedly in all groups after training with an overall drop from 14.9 ± 1.4% to 1.2 ± 0.3% (main training effect, P<0.001). All 4 groups responded similarly to training with a robust decline in these IIx myofibers as no age or gender differences were found. As expected, a main training effect was also observed for the area distribution of type IIx myofibers (P<0.001). Within groups, the area distribution of MHCIIX myofibers declined significantly in all but older women (P=0.158). Presumably this lack of effect was due in part to the extremely small type IIx myofibers among older women.

Results of cross-sectional area (CSA) measurements by fiber type are displayed in Table 2. As a consequence of the IIx-to-IIa MHC shift, the numbers of MHCIIX myofibers post-training were not sufficient to quantify type IIx CSA after training. Training effects for CSA were therefore evaluated for type I, type IIa, and a combined measure of all type II myofibers (weighted by percent distribution of MHCIIXa and MHCIIX fibers). For type I myofiber CSA, a main training effect (P<0.001) and an age x gender x training interaction (P<0.05) were observed. Overall, type I CSA increased 12% from 4521 ± 171 μm² to 5080 ± 166 μm². This was driven by a significant 18% (778 μm²) increase (P<0.001) in young adults, as type I hypertrophy was not found among older subjects. Within age-gender groups, type I hypertrophy was found only in young men (26% or 1081 μm², P<0.005). No significant main effects of age or gender were noted.

Main age and gender effects (P<0.005) indicated that type IIa myofibers were overall 19% smaller in older adults and, across age, 31% smaller among
women compared to men. The IIa fibers of older women were significantly smaller than all other groups. Resistance training resulted in an overall main training effect (P<0.001) indicating 21% type IIa hypertrophy (4345 ± 195 μm² to 5245 ± 239 μm²). Collapsed across gender, young adults realized 25% (1179 μm², P<0.001) type IIa hypertrophy while significant but somewhat lesser growth (16% or 633 μm², P<0.05) was noted in older adults, resulting in a trend toward age group x training interaction (P=0.10). However, it is noteworthy that type IIa size after training in older adults was not significantly different from young prior to training and, in fact, the two values were nearly matched (P=0.98). Within groups, only young men significantly increased type IIa myofiber size (1494 μm² or 29%, P<0.001).

Analysis of type II CSA (weighted by percent distribution of MHCIIa and MHCIIx fibers) yielded significant main effects for age (P<0.005), gender (P<0.001), and training (P<0.001) and a tendency for an age x training interaction (P=0.084). Type II fibers were smaller in older versus young adults and in women compared to men. Overall, type II CSA increased 28% from 4068 ± 191 μm² to 5197 ± 241 μm². Young adults realized 32% (1419 μm², P<0.001) type II hypertrophy compared to 23% (851 μm², P<0.005) among older subjects. However, type II fiber size after training in older adults was restored to the young value prior to training (P=0.99). Within age-gender groups, resistance training induced type II hypertrophy in young men and young women, as well as in older men (P<0.05). Young men realized the largest absolute (1713 μm²) and relative (35%) increases in type II CSA, which represented roughly twice the growth seen
among older men (869 μm² or 19%). Type II myofiber CSA in young women increased 1035 μm² (27%). The numerical change of 831 μm² among older women did not reach significance (P=0.14) even though the small average type II fiber size at baseline appeared to expand 31%.

In a separate analysis, we tested whether individual percent change scores in fiber size differed among the 4 groups. We found an age x gender interaction for type I fibers (P<0.05) as the mean percent growth among individual young men (25%) exceeded that of older men (4%). No significant group differences were noted for percent change scores in type II fiber size.

Between groups ANOVA was tested on pre-training CSA data to assess age-related atrophy in MHCIIx fibers (Table 2). Marked group differences were noted for the CSA of type IIx myofibers, as main age (P<0.01) and gender (P<0.001) effects were found. Overall, type IIx myofibers were 24% smaller in older compared to young subjects and 40% smaller in women (2186 ± 187 μm²) versus men (3650 ± 238 μm²). Age-related type IIx atrophy among older women was indicated by a striking 44% smaller IIx CSA compared to young women. In contrast, type IIx myofiber size did not differ significantly between young and older men.

mRNA and Protein Levels of Myogenic Regulatory Factors

MRF mRNA and protein results by age and gender are shown in Figures 4-7. mRNA data are plotted by age in Figure 4 and by gender in Figure 5, and protein data are plotted by age in Figure 6 and by gender in Figure 7 (age and
Figure 4. Relative RT-PCR results for myogenin, myf-6, MyoD, and myf-5 mRNA levels by age across 3 timepoints (baseline, acute loading, 16 wk training). Values are mean ± SE. *Significant change from baseline within group, P<0.05. Overall main and interaction effects from the age x gender x training ANOVA models are described in the Results text.
gender were the independent variables in the repeated measures ANOVA models). We did not plot results for each age-gender within-groups cohort separately as no 3-way interactions (age x gender x training) were found. We recognize that transcript and protein levels vary over time and likely in response to each loading bout; thus there are no lines connecting the biopsy timepoints on each figure.

### MRF mRNA Levels

These results are shown by age group in Figure 4 and by gender in Figure 5. A main training effect for myogenin mRNA concentration indicated an overall increase of 52% from baseline to wk 16 (P<0.001), with the bulk of the increase occurring in response to the initial loading bout (48% acute response, P<0.001). No main age or gender effects were noted in the overall ANOVA model for myogenin mRNA levels. However, as shown in Figure 4, the acute response was not significant among older adults as post hoc tests revealed that myogenin mRNA expression increased at this early timepoint in young subjects only (55%, P<0.001). Myogenin mRNA levels did not increase significantly above baseline in elderly subjects until the 16 wk timepoint (47%, P<0.05). When collapsed across age and displayed by gender, men and women responded as shown in Figure 5. A significant, early acute elevation of myogenin mRNA occurred only among men (66%, P<0.005), while in women a significant increase was not detected until after 16 wk of training (49%, P<0.005). These differential responses by age (Figure 4) and gender (Figure 5) were primarily driven by
Figure 5. Relative RT-PCR results for myogenin, myf-6, MyoD, and myf-5 mRNA levels by gender across 3 timepoints (baseline, acute loading, 16 wk training). Values are mean ± SE. *Significant change from baseline within group, P<0.05. Overall main and interaction effects from the age x gender x training ANOVA models are described in the Results text.
young men as they saw the largest load-mediated elevation of myogenin mRNA and, in fact, were the only age-gender cohort to show significant within-groups increases (71% acute response, P<0.01; 68% overall increase from baseline to wk 16, P<0.05).

No main effect of the training program was found for myf-6 mRNA concentration. However, myf-6 expression tended to be higher in older adults (P=0.078) across the 3 timepoints (Figure 4). A gender x training interaction (P=0.05) was noted, as myf-6 mRNA levels tended to fall in women by wk 16 (P=0.051) but did not change among the men (Figure 5). A main training effect for MyoD mRNA levels resulted from an overall increase of 69% from baseline to wk 16 (P<0.001). MyoD expression significantly rose 90% in young adults (P<0.005) and 54% in older subjects (P<0.05) (Figure 4). Parsed by gender (Figure 5), a significant increment in MyoD mRNA was noted only among women from baseline to 16 wk (P<0.001) and from the acute timepoint to 16 wk (P<0.01). This effect was primarily attributed to young women as they exhibited remarkably low levels of MyoD mRNA prior to training and were the only age-gender cohort to show a significant within-groups increase after 16 wk of training (5-fold, P<0.005). Young women were also responsible for a significant age x gender interaction (P<0.05) as levels of MyoD mRNA expression were significantly lower in young versus older women (no age difference was noted in men). The underlying cause of these low MyoD transcript levels in young women is not known but the finding has been consistent in our laboratory (34). For myf-5 mRNA concentration, a main age effect (P<0.01) was noted as older adults
expressed 28% more myf-5 at baseline (Figure 4). An overall training effect for myf-5 mRNA levels (15% increase, P<0.005) was driven almost entirely by young adults, as post hoc tests revealed a significant increase by wk 16 (27%, P<0.005) for myf-5 mRNA expression among young subjects only (Figure 4). Further, this response was driven more by young men than young women; of the four age-gender groups, only young males significantly enhanced transcript levels of myf-5 from 0 to 16 wk (34%, P<0.05). Parsed by gender (Figure 5), young men were responsible for the significant overall increase seen among men only (19%, P<0.05).

MRF Protein Levels

These results are shown by age in Figure 6 and by gender in Figure 7. For myogenin protein concentration, a main training effect (P<0.01) was found. Post hoc testing localized this to an overall increase from the acute timepoint to 16 wk (14%, P<0.005). No significant differences by age were noted (Figure 6). The rise from the second timepoint to 16 wk was driven by women (23%, P<0.05) after a tendency for myogenin protein concentration to fall acutely (Figure 7). A robust overall increase (77%) in myf-6 protein concentration was found (main training effect, P<0.001). Post-hoc analyses showed significant increases in myf-6 protein from baseline to 16 wk and from the acute timepoint to 16 wk (P<0.001). As shown in Figure 6, both age groups responded similarly with rather large increases in myf-6 protein concentration (64% in young; 93% in older adults). A main gender effect was observed (P<0.01), as myf-6 protein levels
Figure 6. Immunoblot results for myogenin, myf-6, and MyoD protein levels by age across 3 timepoints (baseline, acute loading, 16 wk training). Values are mean ± SE. *Significant change from baseline within group, P<0.05. Overall main and interaction effects from the age x gender x training ANOVA models are described in the Results text.
were on average 43% higher among the men (Figure 7). Further, the training response was mainly driven by men with nearly a 2-fold increase in myf-6 protein concentration by 16 wk (P<0.005) (Figure 7). Despite the findings at the transcript level, MyoD protein concentrations were not influenced by age or gender and remained unchanged across the 3 timepoints (Figures 6 and 7). We were unable to assess myf-5 protein concentrations as no antibody could be validated.

DISCUSSION

Age Differences in Resistance Training-Induced Myofiber Hypertrophy

A primary finding was that frequent 3 d/wk resistance training at a relatively high intensity (8-12RM) and volume (9 sets for knee extensors) induced greater myofiber hypertrophy in young vs. older adults. We found no significant increase in type I myofiber CSA among the older adults compared to 18% growth in young, and type II hypertrophy was less robust (851 µm² or 23%) compared to that seen among young subjects (1419 µm² or 32%). However, the fact that, after only 16 wk of resistance training, older adults restored type II fibers to the size of young subjects prior to training should not be overlooked. This is a clinically significant finding as age-related myofiber atrophy is known to be localized to type II myofibers (as supported by our pre-training data). Preferential type II hypertrophy (exceeding type I hypertrophy) is a typical resistance training adaptation and likely results from the relatively greater increase in type II motor unit recruitment. Nominal daily activities are accomplished primarily by
Figure 7. Immunoblot results for myogenin, myf-6, and MyoD protein levels by gender across 3 timepoints (baseline, acute loading, 16 wk training). Values are mean ± SE. *Significant change from acute timepoint (myogenin) or from baseline (myf-6) within group, P<0.05. Overall main and interaction effects from the age x gender x training ANOVA models are described in the Results text.
recruitment of the smaller, type I motor units (lower recruitment threshold) while type II motor units are typically not recruited during low-intensity daily activities but are rapidly recruited when contraction intensity increases (higher recruitment threshold). Thus, the type II myofibers realize the greatest relative increase in activity (compared to type I) during high-intensity contractions such as resistance exercise.

Young men clearly experienced the most marked adaptation as the only age-gender group with significant hypertrophy of both type I and type IIa myofibers, and the largest increase in the size of type II myofibers. By contrast, we did not detect significant growth of either fiber type within older women. Again though, we would argue that the trends toward myofiber growth in these older women hold clinical relevance, as type II myofiber size post-training was nearly restored to the baseline type II fiber size recorded in women 35 years younger. The muscles of older adults are clearly capable of hypertrophy in response to resistance training as shown in several prior studies (reviewed in (29)); however, because we found the response to be less robust compared to young subjects using this specific 3 d/wk program, we suggest alternate training programs for older adults should be pursued to improve the efficacy of myofiber growth. Maximizing growth of each myofiber is particularly important considering that some myofiber loss (~20-25%) occurs by the 7th decade (39). This compounds the degree of atrophy at the whole muscle level and, obviously, resistance training can only enhance myofiber size (not number).
One of two possible reasons must account for the noted age difference in 3 d/wk resistance training-mediated hypertrophy: 1) The training program design that is most effective for hypertrophy in older novice participants differs from what works well in young; or 2) Irrespective of training program design, young novice trainers will always adapt with superior hypertrophy compared to their older counterparts. While the latter cannot be ruled out based on the current shortage of published findings, we suggest the former explanation has merit. Several animal studies have demonstrated slowed repair/regeneration in older vs. young adult animals (8,12,21,43,61). If recovery time is rate-limiting in older muscle, one might expect a lesser hypertrophy adaptation in older vs. young adults subjected to the same fairly high loading frequency. There is some indirect evidence to support this concept in aging humans, as substantial myofiber hypertrophy has been detected in older women (26) at a reduced training frequency (2 d/wk). Further, in a recent survey of the literature we noted that older men and women both appear to adapt more favorably to 2 d/wk loading (29). Hakkinen et al. (25) studied both young and older men with ages similar to our cohort and found comparable gains in myofiber size between the two age groups; however, the program was periodized weekly, resulting in only 1 day per week of “hypertrophy training” at an intensity similar to our 3 d/wk program (8-10 RM), suggesting less frequent loading for hypertrophy might be beneficial.

While our overarching hypothesis focused on frequency of loading, another program design variable that should be considered is volume within each loading bout. It is certainly reasonable to expect the volume of loading within
each exercise bout to influence the repair/regeneration timecourse as well. For example, Roth et al. (55) found no age differences in MRI-determined muscle size gains after 6 months of 3 d/wk training. Their subjects completed only 4 sets of knee extensor work per exercise bout compared to 9 total sets in the present study. In another study of older men, 3 total sets of knee extensor training per bout led to significant myofiber hypertrophy on a 3 d/wk program (19). Further, we previously found that 4 sets of loading per bout induced robust myofiber hypertrophy in older men on a 3 d/wk training frequency (although growth remained impaired among the older women) (6). Based on these combined findings, the older adults in the present study may have realized greater myofiber growth by reducing loading volume even if frequency was maintained at 3 d/wk.

Effects of Age on Load-Mediated Changes in Regenerative Markers

MRFs are commonly studied markers of myogenic activities including transcription of muscle genes (42), activation/differentiation of satellite cells (50), and transitions in myofiber phenotype (32). There are several reports of MRF upregulation in response to various muscle perturbations including stretch overload (11,41), ablation (1,2), denervation (35), myotoxicity (43), and acute resistance loading (9,22,34,53,61). In a number of these studies, MRF responses and/or muscle repair mechanisms were found to be limited by old age (34,41-43,61). This may result from heightened basal levels of MRFs in the aging muscle (7,34,35,49) since MRF expression tends to increase as the degree of sarcopenia advances (17).
Elevated resting levels of MRFs suggest sarcopenic muscles remain in a state of failing regenerative effort; thus it stands to reason that MRF responses to a growth/regeneration stimulus such as resistance loading could be impaired. The studied group of older adults were, however, fairly healthy (based on the stringent exclusion criteria applied for this training study) and were “young-old” in light of the growing population of octogenarians. Type II atrophy indicative of sarcopenia was noted (driven primarily by older women) but, compared to animal models of senescent muscle, we do not consider the stage of sarcopenia in the ~64 yr old human muscles studied to be advanced. In fact, one could argue that older men were only in the beginning stages of decline (while older women had regressed further). This may explain why myf-5 mRNA was only 1 of 7 MRF transcript and protein measurements found to be significantly higher in the older muscles at baseline.

Our overarching hypothesis was that 3 d/wk resistance training would not provide sufficient recovery between bouts for older adults to adapt appropriately. We generally noted a lesser hypertrophy adaptation among older adults and followed that with a series of experiments to evaluate whether there was an associated age difference in regenerative drive based on the load-mediated expression of MRFs. At the transcript level, young adults responded with greater increases in myogenin and myf-5 while the MyoD response was similar in the two age groups. The responses were not remarkably different by gender. Barring protein level analyses, one might argue that the MRF-based myogenic potential tended to improve with training more in young than old. However, no distinct age
differences in responsiveness were found at the protein level. In fact, we report a
general lack of coordinated change between mRNAs and their translated protein
products.

Due to a number of posttranscriptional controls (e.g., RNA splicing, RNA
editing, blocked nuclear export, subcellular localization, negative translational
control), a close relationship between mRNA and protein would not be expected.
As reviewed recently by Moore (45), a number of factors including small
noncoding microRNAs and proteins that complex with a given mRNA to form a
messenger ribonucleoprotein particle (mRNP) largely dictate its fate. These
components offer myriad posttranscriptional control mechanisms that can lead to
large variation in the levels of agreement between mRNA and protein
abundances (45). As an example, it has previously been shown that an age-
related 80-fold elevation of myogenin mRNA corresponded to only a 6-fold
elevation at the protein level (35). Our reported differences by age and/or
training in MRF levels were on a much smaller scale (less than 1-fold), which
would likely limit the ability to detect coordinate mRNA and protein levels.
However, similar general trends between the two seemed plausible, particularly
at the later timepoint after several repeated exposures to loading (16 wk of
training). In our view, these findings underscore the strength in assessing both
mRNA and protein whenever possible.

Both myogenin and MyoD mRNA concentrations increased over time with
little to no change at the protein level. This suggests increased transcription of
myogenin and MyoD was largely futile, overridden perhaps by negative
translational control or some other form of feedback. The opposite occurred for myf-6 as we found robust increases in myf-6 protein with no change in transcript. Based on the numerous control mechanisms reviewed recently (45), it is possible that myf-6 mRNA existed in sufficient quantities in a sequestered cytosolic location and was transported to the ribosomal machinery for translation in response to training without requiring additional transcription. Why only myf-6 protein and not the other MRF proteins accumulated with resistance training is unknown. Of interest, however, is the finding in a recent resistance training study that changes in myf-6 protein expression correlated with changes in myofiber size while changes in myogenin and MyoD did not (27). The bulk of the increase in myf-6 protein was localized to men in the present study, and young men experienced the greatest myofiber hypertrophy. These data combined suggest myf-6 may play an important role in load-mediated myofiber growth.

Strength vs. Hypertrophy; Non-Muscle Mass Adaptations

A notable finding was that strength gains among the older subjects were sufficient to meet or exceed the pre-training strength levels recorded in subjects ~35 yr younger. It is generally accepted that increases in strength during the first few weeks of RT result from neural adaptations including enhanced motor unit recruitment and firing frequency of agonists, and/or reduced co-contraction of antagonists (23-25,46). Based on relative gains in strength versus myofiber size, the observed increases in strength appear to have been largely driven by neural adaptations. However, as relative strength improvements were similar across
groups, our data indicate that older adults relied more heavily on neural or other non-muscle mass adaptations than their younger counterparts. Combining older men and women, gains in 1RM strength ranged 33-49% across the 3 movements while type I myofibers did not hypertrophy (non-significant 7%) and type II CSA increased only 23%. Young adults combined increased 1RM strength 28-47% accompanied by type I and type II hypertrophy of 18% and 32%, while young men alone improved strength 34-38% matched by myofiber growth of 26-35%. Young men appear to have gained strength almost entirely as a consequence of hypertrophy, while in older subjects hypertrophy accounted for a lesser fraction of the improved strength.

A common criticism of RT-induced strength improvements among older subjects is that changes may be largely driven by factors other than physiologic adaptations such as increased confidence, leading to 1RM strength values prior to training that are underestimated. This is a valid criticism if familiarization is not sufficient prior to testing baseline strength. We standardized familiarization procedures across all subjects in an effort to avoid this problem and therefore obtain accurate baseline strength levels. With these procedures in place we have previously shown similar specific strength estimates among untrained men and women in the two age groups (52). Further, others have shown, with adequate familiarization, that voluntary vs. electrically evoked contractile force does not differ by age and it has been reported that both old and young can achieve 95-100% of electrically activated maximal force with voluntary effort (14,30,37,59). It is therefore unlikely that strength gains among the older
subjects were biased by underestimated baseline strength, leading us to presume that neural or other non-muscle mass adaptations improved with time more in old than young.

All subject groups showed a typical compensatory shift in myosin heavy chain isoform expression that is generally accepted to occur in response to both endurance (54) and resistance (3,6,20,60) training. There was a marked decrease in the number of histologically determined myofibers expressing exclusively MHCIIx in all groups, while the number of MHCIIa expressing myofibers significantly increased in all but elderly women. A sparse distribution of IIa/IIx and I/IIa coexpressing myofibers were observed but remained excluded from analyses. Type I myofiber distribution did not significantly change in any subject group. Because both young and older muscles underwent a similar degree of fiber type transition (yet differed significantly in hypertrophy), this provides further support that the mechanisms controlling muscle mass and myosin isoform expression are distinctly different (16).

Finally, we identified a subset of 3 older men with extremely large myofibers. Without these 3 men, we would have reported significant age-related type II atrophy in the 10 remaining older men compared to the young group before training. Compared to the remaining 10 older men, these 3 subjects had substantially larger type I (52%, 7209 vs. 4738 μm²), type IIa (45%, 6349 vs. 4370 μm²) and type IIx (72%, 5125 vs. 2973 μm²) myofibers prior to training. Their apparent changes with training followed trends similar to the other 10, as type II CSA increased while type I CSA did not. We spent a great deal of time
discussing whether or not these 3 men constitute a true representation of ~65 yr men in the population at large but we found no compelling reason to exclude their data. Based on DEXA results, thigh muscle mass for these 3 men (13.66 kg) did not appear different from the remaining 10 older men (13.22 kg). Although the DEXA results cannot be compartmentalized into specific muscles, the striking difference in fiber sizes leads us to speculate perhaps a lower vastus lateralis total myofiber number in these 3 men. These 3 men made up almost one quarter of our older male cohort; however, we do not know whether this high a fraction of ~65 yr men actually possess such large myofibers.

Conclusions

The functional benefits of resistance training for older adults cannot be overlooked. Further, we report after only 16 wk of resistance training, that older adults were capable of restoring myofibers to the pre-training fiber sizes found in adults ~35 years younger. Certainly these data support the work of others in demonstrating that adults ~64 years of age retain the ability to undergo load-mediated hypertrophy. However, in general we noted more robust hypertrophy in the younger cohort (particularly young men). We suggest alternate, age-specific training programs should be evaluated to maximize enhancements in both function and muscle mass to counteract sarcopenia. This might include direct comparisons between various manipulations of training frequency and/or volume to affect rest/recovery rates between training bouts in order to meet this goal.
ACKNOWLEDGMENTS

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MODULATION OF THE DYSTROPHIN-ASSOCIATED PROTEIN COMPLEX
DURING RESISTANCE TRAINING IN YOUNG AND OLDER MEN

by

David J Kosek and Marcas M Bamman

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ABSTRACT

The dystrophin associated protein complex (DAPC) is a scaffold of proteins linking the skeletal muscle contractile apparatus with the extracellular matrix that is integral to structural stability and localization of intracellular signaling components driving mechanotransduction. We determined whether progressive resistance training (PRT) would influence key DAPC components, and theorized that differential modulation of the DAPC may partially explain age differences in the efficacy of PRT-induced myofiber hypertrophy. Seventeen young (27 yr) and 13 older (65 yr) men completed 16 wk of PRT with muscle biopsies at baseline (T1), 24 h after bout 1 (T2), and 24 h after the final bout at wk 16 (T3). Myofiber hypertrophy in young (type I 31%, P<0.005; type II 40%, P<0.001) far exceeded hypertrophy in old (type II only, 19.5%, P<0.05). PRT altered protein expression for caveolin-3 (decreased 24% by T3, P<0.01); alpha-1 syntrophin (increased 16% by T3, P<0.05); alpha dystrobrevin (fell 23% by T3, P<0.01); and dystrophin (rose acutely (30% by T2, P<0.05) and returned to near baseline by T3). Changes in alpha dystrobrevin and dystrophin were driven primarily by young. Though expression of nNOS did not change, the phosphorylation state of nNOS (Ser$^{1417}$) decreased 70% (P<0.005) by T3, particularly in older men (81%). The phosphorylation state of p38 MAPK increased 2-fold by T3 in older men (P<0.01), indicating cell stress. These findings illustrate PRT-induced modulation of the DAPC and suggest that the hypertrophic response is blunted in older men due to altered DAPC protein adaptability that may stem from insufficient repair mechanisms.
INTRODUCTION

Sarcopenia, the well established loss of muscle mass with age, is characterized by atrophy of type II myofibers (54) as well as loss of type I and II motor units (57). Decreases in type II fiber size and total motor unit number accelerate beyond the sixth decade (57), and contribute to declines in strength and power necessary for functional performance and completion of everyday tasks (68). Resistance training has shown great promise in slowing and/or reversing the effects of sarcopenia (46). However, consideration of age-related differences in skeletal muscle phenotype and functional characteristics may be necessary when optimizing the exercise prescription for older individuals (55).

Our previous findings showed that young men (20-35 yr) possess the ability to achieve robust growth of type I and type II myofibers via 16 wk of progressive resistance training; whereas older men (60-75 yr) were less responsive to the same regimen, realizing a smaller magnitude of hypertrophy which was localized to type II fibers only (55). In subsequent work, we found this age difference in responsiveness linked to a failure among older men (vs. young) to recruit skeletal muscle satellite cells (SCs), as only young men increased SC number and the number of myonuclei per fiber (67). These data suggest upstream mechanotransduction events responsible for SC activation may be less effective in the muscles of older men.

The dystrophin-associated protein complex (DAPC) is a mechanosensitive system largely responsible for transmitting force production and initiating intracellular signaling; thus any differences in this complex based on age or its
Figure 8. Schematic representation of the DAPC. We report resistance training-induced modification of this complex via caveolin-3, alpha-1 syntrophin, alpha dystrobrevin, and dystrophin protein expression changes. Alpha-actinin and nNOS levels were not statistically altered by repetitive load and were no different based on age. Further studies should examine F-actin, vinculin, talin, and alpha-7 and beta-1 integrins, as well as the sarcoglycan complex (not shown).
modulation by resistance training may contribute to the observed age disparity in hypertrophic responsiveness. The DAPC is a scaffold of proteins responsible for providing architectural support to the mature myofiber for force production (6; 40), and several protein components of the DAPC are also known to be important for initiating signaling processes (6; 37; 44). Proteins within this complex that are either not expressed in adequate abundance, or that do not function properly, cause a weakened structural integrity of the muscle (40; 80) and membrane disruption occurs during contraction (6; 69).

Membrane-bound neuronal nitric oxide synthase (nNOS) is sensitive to mechanical load induced by stretch (82), and some studies show that absence of nNOS from the muscle cell membrane (17; 18) or pharmacological blockade (7) results in dysregulation of SC activation. The importance of SC activation for achieving robust myofiber growth has been shown (3; 43), and it is suggested that nNOS is architecturally positioned within the DAPC (7) via alpha-1 syntrophin linkage (17; 45; 86) to provide SCs with the necessary NO-mediated signal for activation (7; 74; 79). Loss of protein components within the DAPC result in reduced sarcolemmal nNOS (18; 20), which leads to a concomitant reduction in NO signaling capacity.

Caveolin-3 (cav-3) found within the sarcolemma is associated with the DAPC (83; 85). Importantly, cav-3 is thought to inhibit nNOS-mediated NO production by blocking L-arginine binding (37) in a manner similar to L-arginine-based NOS inhibitors (2; 22; 35). Dystrobrevin is a subsarcolemmal protein that has also been studied for its importance in connecting multiple other proteins for
signaling and structural strength at the muscle membrane. It is a member of the dystrophin family of proteins, with the alpha isoform predominating in skeletal muscle (44). Multiple splice variants of dystrobrevin exist, suggesting that expression of a specific variant promotes binding of a subset of proteins that facilitate a targeted signaling response (6; 31).

Dystrophin has been studied extensively, and is known for its subsarcolemmal role in providing a linkage between cytoskeletal actin and the extracellular matrix (6; 28; 32; 90). Binding of beta-dystroglycan, dystrobrevin, and the syntrophins (30) occurs at the C-terminus of dystrophin; while alpha-dystroglycan, in turn, associates with beta-dystroglycan intramembranously, and laminin at the extracellular matrix (6). Dystrophin knockout mice are unable to localize alpha-1 syntrophin to the sarcolemma (1; 4), resulting in a loss of nNOS from the muscle cell membrane (18; 21; 40). Deficiency of dystrophin expression has been shown to induce altered calcium channel function, which may then lead to membrane damage (69). Other studies show that dystrophin-deficient skeletal muscle is more susceptible to mechanically-induced damage than normal muscle (56), a deleterious effect revealed most obviously in Duchenne’s muscular dystrophy.

The C-terminal region of dystrophin also associates with alpha-actinin, which may provide a signaling link between the DAPC and costameric adhesion complexes (41). MacArthur and North suggest that alpha-actinin helps to integrate several functional pathways in skeletal muscle to maintain normal contractility, signaling, and metabolism (59). Two isoforms predominate in
skeletal muscle, encoded by two different genes (14). Alpha-actinin-2 has been found in greater abundance in oxidative myofibers, while alpha-actinin-3 is expressed mainly in glycolytic myofibers (65). Functional characteristics of alpha-actinin, regardless of isoform expression in skeletal muscle, warrant study of its expression in a training model.

Based on our previous resistance training findings that young men have a more robust hypertrophy adaptation than older men (55), apparently due to greater SC recruitment (67), further examination is required to determine upstream mechanotransduction mechanisms by which hypertrophy occurs, as well as age-related changes to these systems that may lead to age differences in growth potential. The DAPC acts via transmission of mechanical forces from intracellular contractile machinery outwardly to the basement membrane, activating signaling pathways necessary for the hypertrophic response (44; 81), and is thus an attractive target. We therefore examined key components of the DAPC that provide structural stability and intracellular signaling capacity, hypothesizing that age differences in this complex at baseline or in response to resistance training may explain the age discrepancy in the magnitude of myofiber hypertrophy.

Extracellular signal-regulated kinase (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) have previously been shown to associate with DAPC components and likely play important signaling roles in mechanotransduction (13; 42; 50). We therefore examined the expression and phosphorylation of ERK1/2 (primarily considered to be involved in cell division, growth, and
differentiation processes) and p38 MAPK (associated with cellular stress responses) (51) as an important component of our overarching aim toward unraveling the basis for age differences in the hypertrophic response.

METHODS

Subjects

Thirteen older (60-75 yr) and 17 young (20-35 yr) men were recruited from the Birmingham, AL metropolitan area, and were free of any musculoskeletal disorders that may have affected their ability to complete the resistance training regimen. No subjects were treated with pharmacological interventions known to influence muscle mass prior to or during the study. Volunteers were excluded if body mass index was not less than 30 kg/m² or for any leg resistance training undertaken within 5 years prior to the study. Each subject gave written informed consent before participation, and the study was approved by the Institutional Review Boards of both the University of Alabama at Birmingham and Birmingham Veterans Affairs Medical Center. Subject characteristics are shown in Table 3.

Progressive Resistance Training Program

The progressive resistance training program utilized in this study was previously described in detail (55). Briefly, subjects trained 3 days/wk for 16 wk in a program that focused on the knee and hip extensors. The regimen consisted of a warm-up on a cycle ergometer or treadmill for ~5 min. or until warm (light sweat), followed by three resistance training exercises including squat, leg press,
Table 3. Subject characteristics by age group at baseline.

<table>
<thead>
<tr>
<th></th>
<th>Young Men (n=17)</th>
<th>Older Men (n=13)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>26.5 ± 4.8</td>
<td>64.6 ± 3.9</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>178.4 ± 7.8</td>
<td>178.5 ± 6.8</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>81.4 ± 11.9</td>
<td>88.7 ± 10.5</td>
</tr>
<tr>
<td>Body Fat (%)*</td>
<td>24.4 ± 8.1</td>
<td>31.8 ± 5.6</td>
</tr>
</tbody>
</table>

*Age difference, P<0.01.

and knee extension. Each movement was completed at 8-12 repetitions per set for 3 sets, with 90 s rest periods between sets, using weight stack resistance exercise and/or plate-loaded stations (barbell squats and linear 45° leg press). Load cell and regression procedures described previously (68) were used to calculate actual resistances for comparing training and testing loads between weight stack and free weight stations.

After two familiarization sessions and practice strength tests, baseline strength was established by one-repetition maximum (1RM) testing using established procedures (12; 55). Subjects progressed to 3 sets of each exercise at 80% of 1RM during the first few sessions. This first session of 3 sets x 80%1RM marked the start of the 16 week training program. Resistance was increased as subjects became stronger through training, and this was signaled by a subject’s capacity to complete 12 or more repetitions for at least two of the three total sets in a given exercise. This progression resulted in an average of 10-11 repetitions per set throughout the program. Subjects underwent 1RM testing midway through (wk 8) and post-training (wk 16). We previously showed
that training intensity and program adherence (~90%) were not different between age groups (55).

**Muscle Biopsy and Tissue Preparation Procedures**

All muscle biopsies were performed in the Pittman General Clinical Research Center at UAB. Muscle samples were collected from m. vastus lateralis by percutaneous needle biopsy using a 5 mm Bergstrom biopsy needle under suction as previously described (33). A baseline biopsy was taken from the left leg, and an acute post-exercise biopsy was taken from the right leg 24 h after the first full bilateral loading bout in order to avoid any residual effects of the baseline biopsy. The third, 16-wk post-training biopsy was obtained 24 h after the final training session from the left leg. At the bedside, visible connective and adipose tissues were removed with the aid of a dissecting microscope. Portions of each sample (30-40 mg) to be used for RNA and protein isolation were immediately weighed and snap-frozen in liquid nitrogen. A separate portion for immunohistochemistry was mounted cross-sectionally on cork in OCT mounting medium mixed with tragacanth gum, and frozen in liquid nitrogen-cooled isopentane. All samples were stored at -80º C until analyses.

**Myofiber Cross-sectional Area (CSA)**

Using immunofluorescence microscopy techniques described previously (55), myofibers positive for myosin heavy chain (MHC) type I (MHCI) and negative for MHCIIa were classified as type I, fibers positive for MHCIIa and
negative for MHCI were classified as type IIa, and fibers negative for both MHCI and MHCIIa were classified as type IIx. Hybrid myofibers (e.g., co-expression of I/IIa or IIa/IIx) that were revealed by both color and intensity using this technique were excluded from analyses. For cross-sectional area (CSA) measurements, each myofiber was manually traced along its laminin-stained border. Myofiber type distribution and size were determined in blinded fashion by a single analyst as described previously (53; 55).

Protein Immunoblotting

Immunoblotting was conducted to assess muscle cell membrane protein lysate levels of nNOS, alpha-1 syntrophin, caveolin-3, alpha-dystrobrevin, dystrophin, and alpha-actinin. Protein lysate from the cytosolic fraction was also assessed for cytosolic nNOS content. Snap frozen muscle samples (30-40 mg) were homogenized and supernatant assayed for total protein as described in detail (12). Some samples taken from biopsies of subjects who entered the study in its initial stages were homogenized into whole tissue lysate; while samples from subjects entering the study later were divided into membrane and cytosolic fractions. Homogenization procedures were consistent within each subject across all 3 biopsy timepoints.

All samples were powdered using a liquid nitrogen-cooled mortar and pestle and homogenized in 3 µl/mg muscle of ice cold lysis buffer specific for either: whole tissue [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.5% NP-40, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 100 µM PMSF, 0.1 µM
Okadaic acid, 1 mM Orthovanadate, and 0.5x protease inhibitor cocktail containing bestatin, leupeptin, aprotinin (P2714, Sigma-Aldrich, St. Louis, MO); or cytosolic and membrane fractions: [whole tissue lysis buffer plus 25 mM Hepes (pH 7.4), 300 mM Sucrose, and 1 mM magnesium chloride]. Using cytosolic/membrane fraction lysis buffer, supernatant (cytosolic fraction) was removed following centrifugation at 110,000xg at 4°C for 90 min. Resuspension buffer [50 mM sodium chloride, 0.1% SDS, 5 mM EDTA, 100 μM PMSF, 0.1 μM Okadaic acid, 1 mM Orthovanadate, 25 mM Hepes (pH 7.4), 10% Sucrose, and 0.5x protease inhibitor cocktail containing bestatin, leupeptin, aprotinin (P2714, Sigma-Aldrich, St. Louis, MO)] was then added at 2 μl/mg muscle to the remaining pellet which was rehomogenized and centrifuged at 100,000xg at 4°C for 10 min to yield the membrane fraction. All muscle lysates (whole tissue, cytosolic or membrane fraction) were assayed for total protein using the BCA technique with BSA as a standard (10).

Twenty μg of total protein diluted in Laemmli buffer were separated by SDS-PAGE (120 V constant) in 4-15% Tris/HCl mini-gels (Bio-Rad MP3, Bio-Rad Laboratories, Hercules, CA) for all proteins of molecular weight below 120 kDa. Those proteins of molecular weight over 120 kDa (nNOS and dystrophin) were separated by SDS-PAGE (120 V constant) in 5% Tris/HCl mini-gels (Bio-Rad MP3, Bio-Rad Laboratories, Hercules, CA). Transfer from 4-15% gels was conducted at constant current (1 mA per cm² x 30 min) to PVDF membranes using a semi-dry transfer cell (Trans-Blot SD, Bio-Rad Laboratories, Hercules, CA). 5% gels were transferred to PVDF membranes using a wet transfer cell, 14
hours, 20 minutes at 10V constant current in a 4°C cold room followed by one hour at 100V in an ice cold circulating water bath. Within subjects, baseline, acute post-exercise, and 16-wk post-training samples were loaded in adjacent lanes. To control for age bias, each 12-lane gel contained samples for at least one subject from each age group, with subject groups loaded in alternating fashion on each gel. Equal loading across lanes and equal transfer were verified by staining all gels (after transfer) with Coomassie blue, staining randomly selected membranes with Ponceau-S, and immunoblotting for β-actin.

Primary antibody specificities, optimal blocking conditions, and target antigen migration patterns (i.e. molecular weights) were confirmed in control experiments. Immunoblotting was carried out using: rabbit polyclonal antibodies against alpha-1 syntrophin (1:1000, Sigma, S4688), caveolin-3 (1:1000, Abcam, ab2912), alpha-actinin (1:2000, Sigma, A2543), p38 MAPK (1:1000, Cell Signaling Technology), ERK1/2 (1:1000, Cell Signaling Technology), and phosphorylated Ser\textsuperscript{1417} nNOS (1 μg/ml, Upstate Signaling Solutions, 07-544); a goat polyclonal antibody against alpha-dystrobrevin (1:500, Santa Cruz, sc-13812); and mouse monoclonal antibodies against dystrophin (1:250, Vector, VP-D508), nNOS (1:1000, BD Bioscience, 610308), phosphorylated Thr\textsuperscript{180}/Tyr\textsuperscript{182} p38 (1:2000, Cell Signaling Technology), and phosphorylated Thr\textsuperscript{202}/Tyr\textsuperscript{204} ERK2 (p44) (1:2000, Cell Signaling Technology). Membranes were blocked simultaneously with primary antibody incubation, and blocking solution varied depending on clonality of the primary antibody. Membranes treated with polyclonal primary antibodies were blocked with 2% BSA, 2% milk in PBST (1x
PBS with 0.1% Tween-20), while HRP-conjugated goat anti-rabbit (1:50,000) or donkey anti-goat (1:50,000) secondary antibodies were diluted in 0.5% BSA, 0.5% milk in PBST. Membranes treated with monoclonal primary antibodies were blocked with 5% goat serum in PBST, while HRP-conjugated goat anti-mouse (1:50,000) secondary antibody was diluted in 1% goat serum in PBST. The combined blocking solution/primary antibody incubation was performed overnight at 4°C under gentle agitation followed by incubation in secondary antibodies for 1 h at room temperature, with a PBST rinse protocol between treatments (1 quick rinse, 1 x 10 min, 2 x 5 min).

All bands were visualized by chemiluminescence (Super West Dura kit, Pierce Biotechnologies, Rockford, IL) in a BioRad ChemiDoc imaging system and band densitometry was performed using BioRad Quantity One software as we previously described in detail (12). For each imaging session, serial imaging ceased at the first point of saturation on the developing image. This standardization, combined with equal and random distribution of the two age groups across the gels (PVDF membranes), enabled us to accurately test for age and resistance training effects.

**Statistical Analysis**

Data are reported as mean ± SE. Between groups differences in pre-exercise descriptive variables were tested using independent t-tests. All variables measured across 16 wk of training were analyzed using age x time repeated measures ANOVA. Lysate levels of specific proteins were assessed
across all 3 biopsy timepoints and were thus analyzed via 2 (age group) x 3 (timepoint) repeated measures ANOVA. For each ANOVA model with a significant main or interaction effect, Tukey HSD tests were performed post hoc to localize the effect(s). Statistical significance was accepted at P < 0.05 for all tests. Muscle tissue lysate was fractionated into membrane and cytosolic fractions for 17 of the 30 subjects. The nNOS analysis was limited to these 17 subjects (11 young, 6 old) since nNOS is located in both compartments, making the whole tissue lysate nNOS data impossible to interpret. This restriction enabled us to test differential changes in cytosolic and membrane-bound nNOS across training. Protein levels of caveolin-3, alpha-dystrobrevin, alpha actinin, and phosphorylated and total p38 were determined on all 30 subjects (17 young, 13 old). Technical problems resulted in fewer subjects for alpha-1 syntrophin (13 young, 10 old), dystrophin (14 young, 10 old), and phosphorylated and total ERK1/2 (17 YM, 12 OM).

RESULTS

Myofiber Cross-sectional Area (CSA)

Figure 9 illustrates the results that 16 weeks of progressive resistance training had on myofiber growth in young and older men. Data are displayed as magnitudes of myofiber growth (measured in $\mu m^2$) for type I and type II myofibers in young and older men. Due to the well-known shift of myosin heavy chain (MHC) IIx to MHCIIa expression induced by resistance training, an insufficient number of IIx myofibers for CSA assessment were found at the 16 wk timepoint.
Figure 9. Magnitude change of myofibers as measured by cross-sectional area (CSA) of type I and type II myofibers in young (black bars) and older (white bars) men. Data are mean ± SEM μm² change. *Different from baseline within group, P<0.05. Young men produced significant growth of both type I (1300 μm² or 31%, P<0.0005) and type II (1992 μm² or 40.1%, P<0.001) myofibers, while older men achieved significant hypertrophy of type II myofibers only (888 μm² or 19.5%, P<0.05).
Training-induced changes in myofiber size were therefore restricted to type I and type II myofibers, with type II CSA being a weighted average based on the relative distributions of IIa and IIx myofibers as we have described (55).

A main training effect (P<0.001) and training x age group interaction (P<0.005) were found for type I myofiber size. No pretraining differences were noted between young and older men. However, progressive resistance training resulted in an overall 17% increase (P<0.0005) in CSA that was driven by young men, who gained over 1300 μm² in type I myofiber area (31% increase, P<0.0005). Older men showed no significant training-induced type I myofiber growth.

Progressive resistance training elicited significant growth of type II myofibers. A main age effect was observed (P<0.05), as young men possessed 19% larger type II fibers overall compared to their older counterparts. A main training effect (P<0.0001) revealed a 32% increase in type II CSA. Post hoc analysis showed that young men enhanced CSA of type II myofibers by 1992 μm² (over 40%, P<0.001), more than twice the type II hypetrophy seen in old (888 μm² or 19.5%, P<0.05). The age difference in hypertrophy response was revealed by an age group x training interaction (P<0.05) in light of the significant type II myofiber growth by both age groups.

Components of the Dystrophin-Associated Protein Complex (DAPC)

A schematic of the DAPC in Figure 8 shows the associations among DAPC proteins bound to one another, the contractile apparatus, and other
costameric proteins. DAPC protein results displayed in Figures 10 and 11 are presented below based on regional localization within the DAPC.

Sarcolemmal Region of the DAPC

Levels of the transmembrane protein, caveolin-3, are shown in Figure 10A. A main training effect was found, as caveolin-3 levels dropped by wk 16 (P<0.005). Post hoc Tukey analysis revealed a 20% decline from 0 to 16 wk (P<0.05) and a 24% reduction from the acute timepoint to wk 16 (P<0.01). Because caveolin-3 inhibits nNOS (37; 75; 85), we also analyzed the ratio of caveolin-3 to membrane nNOS; however, no significant changes across time were noted for either age group (data not shown). Older men expressed 34% more caveolin-3 (P=0.051) than their young counterparts prior to training.

Subsarcolemmal Region of the DAPC

Alpha-1 syntrophin protein levels are shown in Figure 10B. No significant difference in expression existed between young and older men, but a main training effect (P<0.05) was found, as alpha-1 syntrophin expression increased 16% from baseline to wk 16. Because caveolin-3 has been shown to inhibit nNOS function while alpha-1 syntrophin is necessary for localizing this enzyme to the sarcolemma, we analyzed the ratio of caveolin-3 to alpha-1 syntrophin. This ratio was assessed as a method for indirectly determining any modulation of the DAPC that would contribute to nNOS inhibition or potential enhancement as a result of training. Results illustrated a main training effect (P<0.001), whereby subjects decreased the ratio of caveolin-3 to alpha-1 syntrophin 39% by wk 16.
Figure 10. Western analysis results for A) caveolin-3, B) alpha-1 syntrophin, C) membrane nNOS, and D) phosphorylated Ser^{1417} membrane nNOS in young (black bars) and older (gray bars) men. E) Representative Western blot of each protein from both subject groups across 3 timepoints (pretraining (PT), acute, and post-16 wk). †Main training effect, P<0.05; Brackets over bars = post-hoc results of main training effects; *Different from baseline within group, P<0.05. Data are mean ± SEM.
The effect was driven primarily by older men, who decreased this ratio 48% over the same time interval (P<0.05).

Protein levels of nNOS were determined in cytosolic and membrane-bound fractions separately. Membrane fraction nNOS protein levels are shown in Figure 10C. There was a strong trend toward an age x training interaction (P=0.054), as membrane nNOS tended to increase by wk 16 among older men while remaining relatively stable in young. Cytosolic nNOS expression did not differ by age group and was unaltered by resistance training (data not shown).

We also examined the phosphorylation state of an activating phosphorylation site on nNOS. Phosphorylation of nNOS at Ser\textsuperscript{1417} up-regulates NO synthesis in the kidney (66); thus we examined this activation site in skeletal muscle (Figure 10D). The phosphorylation state was assessed by the ratio of phosphorylated to total membrane nNOS. This yielded a striking main training effect (P<0.005), as nNOS Ser\textsuperscript{1417} phosphorylation was 70% lower at wk 16 compared to baseline. Among older men, this ratio dropped 81% by wk 16 (P<0.05) while young men showed no significant changes.

Alpha-dystrobrevin protein data are shown in Figure 11A. A main training effect (P<0.05) was found, as expression tended to rise acutely (NS) followed by a significant 23% decline by wk 16 (P<0.01). Post hoc analysis revealed that a significant 28% decrease from the acute timepoint to 16 wk in young men (P<0.05) drove the main effect. Young men tended to express more alpha-dystrobrevin prior to training (37%, P=0.088). Dystrophin results are presented in Figure 11B, and immunoblotting using an antibody specific for the rod domain of
Figure 11. Western analysis results for A) alpha-dystrobrevin, B) dystrophin, and C) alpha-actinin in young (black bars) and older (gray bars) men. A representative Western blot of each protein from both subject groups across 3 timepoints (pretraining (PT), acute, and post-16 wk) is shown alongside each graph. †Main training effect, P<0.05; Brackets over bars = post-hoc results of main training effects; ‡Different from acute within group, P<0.05. Data are mean ± SEM.
dystrophin detected two bands that may result either from proteolysis, or from presence of two dystrophin isoforms (personal communication, Technical Service, Vector Laboratories). The results presented in Figure 11B represent both bands combined. A main training effect ($P<0.01$) was brought on by a 30% acute increase ($P<0.05$) followed by a significant decrease back to near baseline levels by 16 wk (-20%, $P<0.05$). A trend toward a training $\times$ age group interaction ($P=0.07$) was noted, as only young men significantly decreased dystrophin levels from the acute timepoint to wk 16 (-32%, $P<0.05$). When analyzed separately, each band yielded statistical results identical to these data (data not shown for each band separately). Figure 11C illustrates the stability of alpha-actinin in response to 16 wk of resistance training, as well as the similarity in expression between age groups. No significant change was recorded for alpha-actinin for either age group at any timepoint in the training regimen.

Intracellular MAP Kinase Signaling

Total and phosphorylated levels of extracellular signal-related kinase (ERK1/2, p42/44) and p38 mitogen activated protein kinase (MAPK) were assessed since these kinases have shown activation in response to mechanical stretch (64) and exercise (5) and associate with DAPC components (13; 42; 56). Figures 12A and 12B display the ratios of phosphorylated to total ERK and p38 MAPK, respectively. Representative Western blots are shown in Figure 12C for each age group across the 3 timepoints. Cytoskeletal $\beta$–actin provided a loading
Figure 12. Western analysis results for ratios of phosphorylated to total A) ERK1/2 and B) p38 MAPK in young (black bars) and older (gray bars) men. C) Representative Western blot of each protein from both subject groups across 3 timepoints (pretraining (PT), acute, and post-16 wk). †Main training effect, P<0.05; #Training x age group interaction, P<0.05; Brackets over bars = post-hoc results of main training effect; *Different from baseline within group, P<0.05; ‡Different from acute within group, P<0.05. Data are mean ± SEM.
control. β–actin levels were quite stable, as no age differences or training-mediated changes were found (Figure 12C). ERK1/2 results revealed no significant age differences or training-induced changes in phosphorylated or total protein levels. However, results for p38 MAPK illustrated a main training effect for total protein (P<0.05), as p38 MAPK expression fell roughly 22% from the acute timepoint through wk 16 (P<0.05). A main training (P<0.01) effect and training x age group (P<0.05) interaction were found for the phosphorylation state of p38 MAPK. Post hoc analysis revealed an overall 65% increase in the phosphorylation state from the acute timepoint through wk 16 (P<0.01). The interaction was caused by a marked increase among older men, who doubled the relative amount of phosphorylated p38 MAPK by wk 16 as compared to both the pretraining (P<0.01) and acute (P<0.05) timepoints.

DISCUSSION

While it is generally understood that mechanical load-mediated hypertrophy of skeletal myofibers requires up-regulation of the protein synthesis machinery (87; 92) and is facilitated by myonuclear addition (49; 67), the mechanotransduction events sparking these processes are as yet unclear. The DAPC is a mechanosensitive structure that may in fact be responsible for providing the spark; yet until now load-mediated DAPC modulation has not been studied in humans. This novel investigation of the singular and combined influences of aging and resistance training on the DAPC and associated MAPKs is thus an important first step toward understanding mechanosensitive processes that lead
to successful or impaired hypertrophy. To our knowledge, we are the first to: 1) Assess DAPC modulation by resistance training; 2) Determine whether age differences in DAPC modulation may help explain differential rates of hypertrophy among young vs. older adults; and 3) Examine age differences during long-term resistance training in DAPC-associated signaling pathways activated by mechanical stress, inflammation, and/or damage.

The present data and our previous findings (55) demonstrate that progressive resistance training 3 d/wk for 16 wk induces type II myofiber hypertrophy to help combat the effects of sarcopenia among older men; however, clearly a greater magnitude of myofiber hypertrophy is achieved by young vs. older men with this prescription. Young men were capable of inducing 2x the growth of their older counterparts in type II myofibers, and were the only group with type I myofiber growth. In the face of a blunted hypertrophic response among older men, several components of the DAPC were altered (caveolin-3, alpha-1 syntrophin, alpha-dystrobrevin, and dystrophin). Perhaps the most striking age differences in DAPC modulation were the marked decline in the caveolin-3 to alpha-1 syntrophin ratio among older men only, and the acute rise and subsequent fall of dystrophin and alpha-dystrobrevin seen only in young men. In addition, older men realized the greatest changes in protein activation, driving the fall in nNOS phosphorylation and the rise in p38 MAPK phosphorylation. Previous resistance training work from our laboratory (67), showed that only young men possessed the ability to proliferate skeletal muscle satellite cells and to incorporate additional nuclei into mature myofibers during
progressive resistance training. A disconnect in aged muscle between the loading stimulus and the mechanism by which satellite cells are activated to respond is suggested, and we surmise that an age difference in mechanotransduction, the communication of a mechanical signal into a biochemical response, exists.

Advantages and Limitations of the Model

High-intensity resistance exercise is perhaps the most potent, non-invasive mechanical perturbation of human muscle in vivo. The high resistance loads demand the contracting muscle(s) to generate tremendous internal forces, particularly in limb muscles that act on class three levers such as the knee and elbow. These extreme muscle tensions will in all likelihood maximize the activation of any mechanotransduction processes that signal muscle growth and structural remodeling. Resistance training consists of repeated bouts of high-intensity contractions separated by lengthy rest periods. As is the case with any exercise treatment, the long term “training” adaptations can be viewed as the cumulative result of acute responses to each loading bout. It is well-accepted that acute resistance loading stimulates muscle transcript (52; 53; 70) and protein (89) expression as well as the transient phosphorylation of proteins (88) involved in regeneration and growth, and that the acute responses to a single bout of resistance loading can differ markedly in trained vs. untrained muscle (11; 47; 55). We have captured a number of substantial changes in mRNA and
protein expression using a 24 h post-loading timepoint for tissue collection in both untrained and trained muscle (11; 12; 52; 53; 55; 67).

The primary focus of this study was on age-specific changes in DAPC remodeling with resistance training; thus we followed the same 24 h post-exercise timeline in order to maximize our ability to assess acute and chronic changes in the expression/abundance of DAPC proteins. Intracellular signaling events such as protein kinase cascades presumed to “prime” the myofiber’s transcriptional and translational machinery must precede these processes, and there is evidence that the “normal” signaling response is a rapid and short-lived activation of kinases and other proteins following mechanical load (29; 36; 88) or high-intensity continuous exercise (23). Obviously our model of 24 h post-exercise sampling was not optimally designed to capture these transient protein activation responses and this was not our intent. However, based on the transient nature of the “normal” signaling timecourse, one might expect a prolonged change in intracellular signaling (i.e. detected 24 h after mechanical load) to be abnormal and indicative of a failing compensatory effort. This is certainly the case in studies of aging animals, as they demonstrate an impaired and prolonged regeneration/recovery timecourse following muscle injury (26; 62; 63). As discussed later, prolonged or delayed (24 h) changes in the phosphorylation states of signaling proteins in our model were most apparent in older men, which we interpret to be indicative of failing compensation.
DAPC Protein Component Expression

Though the myotendinous junction, neuromuscular junction, DAPC, and other costameric proteins probably all play important roles in mechanotransduction (40; 83), the DAPC was examined due to the suggested necessity of nNOS for stretch-mediated satellite cell activation (8; 9) and importance of other DAPC components for structural stability and additional intracellular processes (34; 61; 72; 84). Any weakness within this transmembrane complex would potentially result in contraction-induced damage of the myofiber (40), and others suggest the existence of gradual age-related declines in responsiveness of skeletal muscle repair mechanisms (27; 39; 73), protein synthetic rates (91), and the available pool of satellite cells (60; 73). Indeed, age-related declines in some DAPC components have also been documented (71). Our novel findings demonstrate that the DAPC is altered by training, and select components that act on upstream effectors of satellite cell activation and cell growth are modulated in an age-dependent manner.

Caveolin-3

Current literature shows that decreasing levels of caveolin-3 allow heightened nNOS enzyme activity (37; 85), as caveolin-3 expression disrupts NO synthesis (37) without affecting nNOS expression (77). Though membrane-bound nNOS protein levels did not significantly change, the overall decrease in caveolin-3 expression could potentially alter the ratio of these two proteins to a point where heightened production of NO occurs. This shift may be necessary
for increased signaling to targets for satellite cell activation (7; 9; 79) and other intracellular processes. The strong trend (P=0.051) for older men to possess higher caveolin-3 levels than young subjects prior to training may be compensatory to the well known increased oxidative stress with aging. Because caveolin-3 inhibits membrane nNOS, a higher basal level of caveolin-3 expression would potentially limit NO-induced cellular damage. As adaptations against oxidative stress develop in training muscle (48), exercise-mediated decreases in caveolin-3 may strike a balance between caveolin-3-dependent intracellular signaling and protection against cytotoxicity, while boosting nNOS function to produce the load-mediated NO transients that promote satellite cell activation.

**Alpha-1 Syntrophin**

Initially we theorized that alpha-1 syntrophin expression levels would increase in response to training, offering more structural stability to growing myofibers and providing increased linkage for nNOS (and theoretically, enhanced NO signaling) at the sarcolemma. Increased expression would also promote signaling by alpha 1-syntrophin itself, as others have shown its phosphorylation by CaM kinase II (61), binding of phosphotidylinositol-4,5-bisphosphate (25), as well as serine phosphorylation by stress-activated protein kinase 3 (SAPK3, or p38γ) (42). Levels of alpha-1 syntrophin did indeed increase in response to resistance training, which may have enhanced both structural strength of the DAPC and signaling capacity. The ratio of caveolin-3 to alpha-1 syntrophin was
assessed to gauge the degree of nNOS inhibition (by caveolin-3) versus DAPC nNOS association (bound to alpha-1 syntrophin), with a lower ratio expected to favor load-mediated nNOS activity and thus NO transients to spark growth signaling processes. Our data illustrate a significant training-induced decrease in the ratio, primarily in older men, which resulted from the fall in caveolin-3 concomitant with alpha-1 syntrophin enhancement. If in fact a decrease in this ratio favors nNOS-mediated NO production, our findings suggest the muscles of older men may have adapted via DAPC modulation in an attempt to promote NO signaling. On the other hand, the rise in alpha-1 syntrophin may have been in response to membrane over-stress and heightened p38 activity, as p38γ binds the PDZ domain of alpha-1 syntrophin resulting in phosphorylation (42).

**Alpha-Dystrobrevin and Dystrophin**

We found marked age differences in the modulation of alpha-dystrobrevin and dystrophin, with strikingly similar age-specific responses among the two proteins. Among young men, both proteins tended to increase acutely after a single resistance loading bout, followed by significant returns to baseline expression or below in the trained state (wk 16). By contrast, levels of both proteins showed no significant changes among older men. The functional significance of the age differences are not known, but the acute responses to unaccustomed resistance exercise support the concept that the DAPC may be more mechanosensitive to novel load exposure in young men. The linkage of alpha dystrobrevin with dystrophin points to a possible dependence of
dystrobrevisin on the abundance of dystrophin and its modulation with training, and 
this close association could potentially explain the similar changes in both of 
these targets. Loss of dystrophin leads to concomitant losses in other DAPC 
components (13) and it is likely that, since dystrobrevisin does not provide 
structural support so much as signaling capacity (38), it would be among the first 
proteins decreased. Alpha-dystrobrevisin levels tended to be lower in old at both 
the pretraining and acute timepoints but these differences were not statistically 
significant (P=0.088 and P=0.081, respectively). However, it is noteworthy that 
sufficient alpha-dystrobrevisin is required for both nNOS localization to the 
membrane and NO-mediated signal transduction (38); thus the apparently low 
levels in old may have influenced signaling processes involved in growth and 
repair.

The importance of dystrophin for maintaining skeletal muscle structural 
integrity and contributing to intracellular signaling has been well documented 
(15), with a great deal of work being done to research the muscular dystrophies. 
We initially suspected that, in accordance with previous findings (90), no 
significant long-term changes would occur for the expression of dystrophin 
protein due to its essential role in linking the extracellular matrix with the actin 
cytoskeleton (6). However, the changing expression pattern of dystrophin across 
the training program among young men primarily drove both the acute rise and 
16 wk fall, nearly resulting in a training x age group interaction (P=0.07). The 
acute increase may have been in response to damage inflicted on the muscle 
during the initial training bout. Dystrophin protein production is upregulated in an
attempt to retain muscle mass and provides a buffering mechanism against load-induced mechanical stress to individuals experiencing dramatically increased loads (13). Conversely, dystrophin deficiency leads to loss of the entire DAPC (13), and culminates in muscle cell death (15). It would thus be reasonable to expect that previously unaccustomed muscle would acutely respond to a loading stimulus by increasing the amount of “shock absorption”; and once familiarity with such a load is established, return expression levels to those measured at baseline. However, comparatively less mechanosensitive muscle, which we propose includes the older muscles in this study, may either remain in a state requiring increased abundance of such a protein, or be unable to modulate protein abundance. If in fact the acute rise in dystrophin was in response to damage or over-stress, the return of dystrophin levels to baseline by wk 16 among young men suggests acclimation to the repetitive stimulus, and indicates additional dystrophin is not necessary (in normal healthy muscle) for the poorly understood adaptations that protect muscle from damage with repeated loading bouts.

Membrane nNOS Expression and Phosphorylation

It is apparent that absence of nNOS from the sarcolemma (17; 18) and pharmacological inhibition of NO (7) result in dysregulation of satellite cell activation. Anderson and colleagues (8; 9) propose that the primary source of NO for such a task is membrane-bound nNOS, due to its proximity with satellite cells, as well as for the possibility of NO production in response to stretch on the sarcolemma during contraction. NO has been shown to enhance hepatocyte
growth factor (HGF)-mediated satellite cell activation by initiating HGF binding to its receptor (c-met) on satellite cells (78; 79). We predicted that membrane-bound nNOS expression would increase in young men in response to training, based on the hypothesis that this would provide enhanced NO production for the purposes of activating satellite cells (78; 79) and contributing to other processes. We also expected this effect to be less robust in older men, affording some explanation for their inability to activate these quiescent stem cells. Contrary to our hypothesis, membrane-bound nNOS protein content did not change. This finding indicates that the NO-producing enzyme is adequately available at the sarcolemma in both age groups, pointing to the possibility of an age difference in nNOS function rather than abundance.

We pursued this question by assessing the phosphorylation state of Ser$^{1417}$. Phosphorylation of this residue has been shown to induce NO production in the kidney (66) but to our knowledge the present study is the first to assess membrane-bound nNOS activation via Ser$^{1417}$ phosphorylation in human skeletal muscle during resistance training (Chen et al. assessed this phosphorylation site in human whole tissue homogenates immediately following a brief bout of high-intensity cycling (23)). Pretraining levels between young and older men were not statistically different, indicating potential similarities in the amount of basal NO production at the sarcolemma. Stretch activation of nNOS has been documented (79). In our model, nNOS phosphorylation was not elevated following novel mechanical load (acute response) and, in fact, a substantial reduction was noted by wk 16 particularly among older men. NO-
mediated satellite cell activation has been proposed to occur as a result of brief NO transients (7). The prolonged suppression of nNOS phosphorylation noted primarily among old lends support to the concept of failing compensation. Inhibitory phosphorylation and alternative activation sites may give a clearer picture of nNOS regulation in future investigations of old vs. young, particularly if studied at earlier timepoints following a mechanical loading stimulus.

Alpha-Actinin

Alpha-actinin binding the C-terminus of dystrophin provides a linkage that associates the DAPC with the actin cytoskeleton and other costameric adhesion complex proteins such as vinculin, talin, and β1-integrin (41). Not only does this provide enhanced structural support to myofibers, but it allows for signaling between two complexes thought to be important in mechanotransduction from the contractile machinery outwardly to the cell membrane. Initially, we postulated that alpha-actinin would be upregulated in response to training, an adaptation to enhance structural stability and increase crosstalk among the mechanosensitive DAPC and α7/β1-integrin complex (19). However, our data show no change based on age or training, and illustrate alpha actinin as a very stable protein, suggesting adequate levels of alpha-actinin exist for structural support and any possible role in mechanotransduction. We did not differentiate alpha-actinin isoforms; thus further studies of isoform expression in a resistance training model might be revealing, as well as biochemical assessment of signal transduction by alpha-actinin.
MAP Kinase Signaling

MAP kinase signaling is upregulated in response to stress such as exercise (5; 93) and muscle stretch (47; 64), thus providing skeletal muscle with a contraction-mediated method for regulating a host of intracellular processes including transcription of muscle-specific genes, the initiation of growth processes, as well as the activation of protein degradation. Based on these studies and others showing DAPC protein association with MAP kinase signaling components (13; 42; 56), we examined protein expression and phosphorylation of ERK1/2 and p38 MAPK to determine if either of these two kinase signaling pathways was altered 24 h after unaccustomed resistance loading (acute) or 24 h post-training (wk 16). No prolonged phosphorylation was seen in either age group after one loading bout. As training progressed to 16 wk, however, a 2-fold increase in the phosphorylation state of p38 MAPK was found only among old. As stated earlier, based on the current literature, such a prolonged or delayed state of heightened phosphorylation is abnormal and suggests failing compensation.

Phosphorylation of p38 MAPK in skeletal muscle is induced by mechanical stress and damage (13; 16), and is associated with muscle atrophy (24). In fact, p38 phosphorylation has been shown to be required for cytokine-mediated expression of the muscle-specific E3 ubiquitin ligase MAFbx/atrogin-1 (58), which is associated with increased proteasome activity and muscle protein degradation. Further, heightened activation of p38 MAPK has been shown to suppress
myogenesis via phosphorylation (inhibition) of the myogenic transcription factor, myf-6 (76). Myf-6 is one of 4 members of the myogenic regulatory factor (MRF) family governing the coordinate processes of satellite cell differentiation and muscle-specific gene transcription during successful muscle regeneration and growth. Phosphorylation of myf-6 via p38 MAPK represses myf-6 transcriptional activity and therefore disrupts late-stage myogenesis (76). We have previously reported robust increases in myf-6 protein abundance during resistance training in both young (64%) and older (93%) adults even though myofiber hypertrophy was blunted in old (55). Based on the current finding of a robust and prolonged induction of p38 MAPK phosphorylation among older men, it is entirely possible that the induction of myf-6 protein expression among elderly in our prior study represented a futile attempt to progress myogenesis. Likewise, we suspect the impaired rate of hypertrophy among older men in the present work was at least partially mediated by the deleterious effects of prolonged p38 MAPK activation, leading to failing compensation.

CONCLUSIONS

We have shown for the first time in humans that several constituent proteins comprising the mechanosensitive DAPC are modulated by acute resistance loading and/or long-term resistance training. Importantly, age differences found in the magnitude and timecourse of changes in expression of key DAPC components (e.g., alpha-dystrobrevin, dystrophin, and the caveolin-3 to alpha-1 syntrophin ratio) suggest age-dependent modulation of the DAPC may
play a role in the impaired rate of resistance training-mediated myofiber hypertrophy among old. Changes in the phosphorylation states of membrane nNOS and p38 MAPK were also biased by age and may be indicative of a failing compensatory effort among old during attempted myogenesis. The marked rise in p38 MAPK phosphorylation among old after 4 months of 3 d/wk high-intensity resistance training points to the possibility that the muscles of older men experienced an over-stress throughout training and hence an impaired growth rate. Overall, these novel findings should ignite further research to identify the specific mechanotransduction process(es) that drive myofiber growth in young, and slow the rate of hypertrophy among older adults. Only then can we begin to design the most efficacious resistance training treatment for sarcopenia.
REFERENCES


DISCUSSION

It is known that skeletal muscle growth results from increased loading, while atrophy occurs as a result of unloading, disease state, and aging (57; 60; 103). However, the coordinated molecular events that follow these varying stimuli have not been very well defined. This study was undertaken as an effort to better understand mechanisms of muscle growth in response to progressive resistance training, a highly effective treatment for sarcopenia, the age-mediated loss of muscle mass. We determined key changes in muscle growth pathways and associated mechanosensitive architecture that may contribute to muscle hypertrophy. Further, downregulation of phosphorylated ser1417, as well as increased phosphorylation of p38 in response to resistance training in older men compared to young may indicate cell stress/damage in the aged model, leading to blunted myofiber growth.

Myofiber CSA and MHC Isoform Distribution

We initially assessed differences in skeletal muscle cell cross-sectional area and MHC isoform expression based on age and gender. These measures gave us an indication of the effect of sarcopenia in our older subject groups compared to young, and changes to these parameters with resistance training illustrated the efficacy of the training stimulus for reducing/reversing the disease state. In the young subject groups, measurements for these parameters following long-term resistance training provided us with comparative gender-matched groups thought to function more efficiently than their older counterparts.
in terms of producing muscle growth. Differences between young men and women elucidated gender-specific variation in baseline myofiber size and responsiveness to the training stimulus.

The major finding of this initial work was that the potential for greater hypertrophic response resides in the myofibers of young versus older adults, but that older adults still retain a certain lower capacity for growth. Of clinical importance is the finding that older adults were able to produce a hypertrophic response that restored the size of type II fibers to those measured in pretrained young subjects, indicating remission of the sarcopenic phenotype and a possible turn towards functional capacity similar to that observed in young. This confirms the effectiveness of the resistance training program as an intervention against sarcopenia, since type II myofibers are preferentially targeted by both sarcopenia and resistance training. The observed MHC isoform shift away from the glycolytic fibers expressing MHC IIx toward more oxidative and fatigue-resistant fibers expressing MHC IIa expression is a known training-induced modification (74), and further supports this premise.

Functional parameters such as muscle power and contractile velocity were previously measured in our laboratory (166) and show that older adults possess velocity impairments and decreases in concentric power. These age-related impairments were largely reversed by our training program (165).

Women possessed smaller type II myofibers than men at baseline; and this finding is not novel (74). This result was primarily driven by older women, who possessed type II myofibers roughly half the size of those found in young
men; however, the afore-mentioned rescue of muscle phenotype occurred in the aged females. Though the magnitude of type II growth in older women was not significant, they increased the cross-sectional area of these fibers 31% by week 16, suggesting responsiveness to the training stimulus. The greatest magnitude of hypertrophy was found in young men, as gains in size of both type I and type II myofibers were significant. Compared to older men, who exhibited hypertrophy of type II fibers only, we show that young men produced roughly twice the growth of their aged counterparts; and this indicated a blunted hypertrophy response in our older male subjects. Based on these findings we then evaluated the load-driven regenerative capacity in all four subject groups, as determined by examining mRNA and protein expression levels of the MRFs.

Myogenic Regulatory Factors

Upon establishing baseline fiber size data for all four subject groups and measuring training-induced alterations in cross sectional area of their fibers, we examined the MRFs, a family of transcription factors whose upregulation is necessary for assigning myogenic precursor cells (satellite cells) to the myogenic lineage for growth and repair, and that upregulate synthesis of muscle-specific mRNAs (27; 115). We theorized that measuring these targets at baseline and in response to both acute and long-term resistance training would elucidate age and gender differences in muscle-specific gene transcription, and the responsiveness of this axis to mechanical load may at least partially explain the varied hypertrophic response.
Results of greatest consequence from this study were that examination of MRF protein indicated no coordinated change from mRNA to protein expression with training, and that myf-6 protein increased significantly by week 16 in both male subject groups. The multitude of post-transcriptional controls in place within the cell may have contributed to this discrepancy between message and protein synthesis (e.g. negative translational control, RNA splicing, inhibited nuclear export), and a review by Moore explains in greater detail the fates awaiting gene regulation and synthesized mRNA (148). The increase in myf-6 expression may be meaningful when considering that young men presented the largest magnitude of myofiber growth. In support of this notion, one study suggested that increased myf-6 protein expression correlated with increases in myofiber size, unlike that of MyoD and myogenin (96). The importance of MRF expression for myofiber growth and repair has been documented (45; 95; 101; 226); however, based on our findings we suggested that study of the MRFs did not sufficiently explain the observed differences in the myofiber growth response.

Therefore our laboratory examined satellite cells, muscle-specific precursor cells expressing and affected by MRF expression. This particular study (164) examined satellite cell activation and subsequent myonuclear addition to mature myofibers. These studies were undertaken to more clearly assess both the results of MRF expression and the ability of all subject groups to respond to training by incorporating myonuclei. This study illustrated that, although small increases in myofiber growth may occur without myonuclear addition, satellite cell activation and myonuclear incorporation is requisite to
achieve robust hypertrophy; and the only subject group to accomplish these tasks were young men.

The conclusions from the Petrella study (164) led to the hypothesis that differences upstream of satellite cell activation pathways may be altered with age. It also seemed as though sufficient MRF expression was available for activation of satellite cells with their subsequent fusion into mature myofibers. These earlier experiments confirmed that young men were most capable of producing robust hypertrophy in response to training. As such, young men were examined as a model of relative “optimum growth functioning” compared to gender-matched older individuals who reflected the lowest magnitude change of type II myofiber growth of all four subject groups, and this provided a comparison of hypertrophic response alterations with age.

The DAPC

Next we examined the DAPC as a likely target of mechanotransduction events contributing to the growth response, and ultimately myofiber hypertrophy. Since NO has been shown to be important for satellite cell activation in response to muscle stretch (15; 17; 205; 206; 225), and nNOS is suggested as the primary source of NO for this response (15; 206), nNOS and the membrane complex to which it is associated were studied. Other DAPC protein components have also been implicated in mechanotransduction (86; 102; 121), and this complex is regarded as integral for maintenance of membrane stability and intracellular signaling (23; 184). Young men showed the greatest responsiveness to
increased load based on myofiber growth, satellite cell activation and myonuclear addition, therefore DAPC studies were conducted on them and our older men to determine any aging and/or training-dependent differences within that system that may contribute to blunted growth. Any age-related changes within this complex, or inability of older men to modulate DAPC components through training would suggest an association of this system with blunted growth and other phenomena commonly observed in aging individuals (180), such as decreased glucose metabolism (187) and elevated stress and inflammatory pathways (152; 167).

**DAPC Component Expression**

The protein expression of several DAPC components was altered in response to our training stimulus, and is summarized in Figure 13. Load-mediated alterations were measured for caveolin-3, alpha-1 syntrophin, alpha-dystrobrevin, and dystrophin. Young men in particular demonstrated significant changes to alpha-dystrobrevin and dystrophin, which drove those main training responses, reflective of responsiveness to the training that was not observed in their older counterparts. A very strong trend was observed for older men to possess higher expression of caveolin-3 than young, followed by training-induced lowering of its expression. This suggested exercise-mediated modulation of its expression possibly to affect its association with membrane-bound nNOS as an attempt to increase NO output.
Figure 13. Summary of resistance training-mediated DAPC protein modification in young and older men. Four out of six DAPC targets altered expression in response to resistance training. However, alpha-dystrobrevin and dystrophin were the only two targets to show preferential responsiveness in young men.
Intracellular Signaling-Mediated Inhibition of Myofiber Hypertrophy

Though no changes in nNOS expression were measured, the significant decrease in serine 1417 phosphorylation with training in older men suggests a need for this subject group to tighten control over NO output. Excessive NO production contributes to the generation of reactive oxygen species that inflict cell damage (152), and NO is released in response to muscle stretch (18; 175). Therefore, it seems logical that if muscle is damaged in response to repetitive load and stretch, that further production of NO would only potentiate the injury. Although NO is a trigger for HGF-mediated satellite cell activation, necessary to produce large-scale myofiber hypertrophy, this mechanism in older individuals could potentially be blunted as an attempt at decreasing the level of damage and inflammation caused by resistance training (135).

Inflammation decreases muscle protein synthesis, reduces muscle mass, and inhibits growth (122), and studies have shown p38 activation to be a marker of cell stress and damage (32; 127); accordingly, our data reflect elevated levels of phosphorylated compared to total p38 in older muscle. Training-induced increases in alpha-1 syntrophin may be exacerbating the p38-mediated response in older adults, as it is phosphorylated by p38γ (93), potentially to affect downstream stress signaling targets.

Upregulation of p38 by training indicates muscle damage inflicted in older adults, and it is possible that decreased phosphorylation of serine1417 on nNOS function together to signal and diminish inflammation. Indeed, a study utilizing
both alpha-sarcoglycan-null and mdx mice showed that treatment of these animals with HCT1026, a non-steroidal anti-inflammatory drug (NSAID) that releases NO, contributes to both decreased inflammation and maintenance of muscle mass and satellite cell pool (39). However, our study does not involve the use of NSAIDs, and therefore inflammation is elevated and NO production may be harmful to the muscle, versus beneficial to its growth.

We theorize that baseline elevated caveolin-3 levels in older adults aid in decreasing nNOS production of NO for the purposes of diminishing reactive oxygen species-mediated damage. Training-induced decreases in caveolin-3 expression thus provide muscle with the means to strike a balance between protecting the muscle from NO-derived damage and NO-mediated satellite cell activation for growth. However, elevated inflammation as marked by p38 phosphorylation, and decreased serine 1417 phosphorylation of nNOS in older adults stifles NO synthesis, and growth does not occur.

MAPK Signaling and MRF Function

Results from the satellite cell, MRF, and MAP Kinase studies presented herein are summarized in Figure 14. MAP Kinase signaling has been implicated in varying areas of muscle physiology, but we examined ERK1/2 and p38 as markers of proliferation/differentiation and cell damage, or stress. ERK1/2 have been shown to play a dual role in muscle repair and regeneration by inhibiting proliferating cells from differentiating (in part, by inhibiting myogenin expression (8)) until p38 expression increases, and then by working in conjunction with p38
Figure 14. Summary of satellite cell, myogenic regulatory factor, and MAPK data. The data presented herein reflect aging differences in the resistance training-induced responses of various afore-mentioned targets known for contributing to the myogenic process, which may at least partially explain the differential hypertrophy response measured in young and older men.
to enhance differentiation signaling (228). No changes were observed for ERK1/2, suggesting that our biopsy timecourse missed the point at which elevated expression of total and/or phosphorylated ERK1/2 could be measured. Without the knowledge that satellite cells were activated and that myonuclear addition occurred in young men to produce the magnitude to myofiber growth observed herein, one could argue that ERK1/2 upregulation did not occur. However, since ERK1/2 is important for proliferation of precursor cells (90; 106) and young men displayed that this indeed occurred, this seems a logical assumption.

Upregulation of p38 has been shown to affect MyoD expression (128; 228). More importantly, p38 has been shown to phosphorylate myf-6, decreasing its transcriptional functions (128; 201). We measured significant increases in myf-6 protein for both young and older men. However, only young men incorporated additional myonuclei in response to training, and displayed robust myofiber growth. This suggests that p38 activity inhibited the transcriptional activity of the upregulated myf-6, thus contributing to older subjects’ impaired ability to add myonuclei to facilitate myofiber enlargement.

Though the p38 antibodies used in our study were not isoform specific, it is likely that the increased phosphorylation occurred in p38α and p38β, as they are responsible for mediating p38’s myogenic properties (128). The activation of those two isoforms would thus inhibit myf-6 activity, and blunted myofiber growth in older men was observed in concert with p38 phosphorylation and upregulated myf-6 expression. Also, p38γ (SAPK-3) may have been activated in response to
elevated stress and damage placed on the muscle by our training stimulus, as resistance training is known to induce muscle damage and inflammation (122; 135), and this may have some effect on increased alpha-1 syntrophin expression (93). That older individuals possess elevated phosphorylation of p38 compared to total protein suggests that, beyond inhibition of myogenic processes, a higher degree of cell damage compared to young exists, and that this proposed stress response intensifies with continued training.

Summary

Sarcopenia is a disease that, in some ways, we hope will affect all of us, as to be afflicted one must have lived a considerable number of years. To not have experienced the effects of sarcopenia means that one’s days have been ended prematurely; however, for those of us who become afflicted, we live in hopes that the twilight of our time here is spent as active members of society. Though no cure currently exists for age-related muscle loss, progressive resistance training is the most efficacious prescription known to alleviate and/or reverse its effects. Not only does it stimulate muscle growth and regeneration, resistance training provides benefits to a great portion of the body, including processes that control circulation, bone health, and mental acuity (1), as well as glucose metabolism (231; 232). Perhaps most importantly, resistance training provides functional enhancement (94) for completion of daily tasks and increased overall quality of life.
The findings presented herein illustrate differences between young and older men that contribute to the varied response to the same resistance training stimulus. It is evident that the DAPC is important for mechanotransduction processes that are altered in aging muscle, and that the altered expression of DAPC components may result from increased inflammation and damage that is not resolved. The apparent inability of damage repair mechanisms to function in older muscle seemingly nullifies MRF upregulation, and may blunt NO production necessary for HGF-mediated satellite cell activation. Conversely, young muscle appears responsive to the weight training stimulus based not only on the magnitude of myofiber growth, but also on myf-6 upregulation, satellite cell activation, myonuclear incorporation, modification of DAPC protein expression, and apparently normal function of membrane nNOS and MAPK signaling.

*Future Directions*

Studies involving modified contraction paradigms and longer rest periods for older adults in between training sessions may prove to minimize training-induced damage and enhance damage repair, and may improve the efficacy of resistance training in these subjects. Also, power training (e.g. low weight and high velocity contraction) programs would potentially improve functional measures in older individuals and provide continued advancement toward the optimum sarcopenia intervention.

On the molecular level, further study of MRF regulation is required to further elucidate not only the expression of all involved proteins such as Id, MEF
and E2 families, but also to biochemically determine their timing of expression, interplay, and control elements, including relevant MAPK signaling. Mechanotransduction studies of other costameric proteins such as vinculin and talin, as well as examination of the myotendinous and neuromuscular junctions are warranted. Further study of the NO/HGF/c-met signaling axis will allow a better understanding of satellite cell activation, and may provide insight into nNOS function at the sarcolemma.

While there are still many questions unanswered when considering the optimum resistance training program to maximize the underlying molecular control processes that drive muscle growth, so far most signs seem to point toward resistance exercise in some form as the most efficacious intervention against sarcopenia.
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APPENDIX

UAB Institutional Review Board Approval
Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by
the Departments and Agencies adopting the Common Rule (56FR28033, June 18, 1991)
unless the activities are exempt from or approved in accordance with the Common Rule. See
section 101(b) of the Common Rule for exemptions. Institutions submitting applications or
proposals for support must submit certification of appropriate Institutional Review Board (IRB)
review and approval to the Department or Agency in accordance with the Common Rule.

1. Request Type
   [ ] ORIGINAL
   X CONTINUATION
   [ ] EXEMPTION

2. Type of Mechanism
   [ ] GRANT
   [ ] CONTRACT
   [ ] FELLOWSHIP
   [ ] COOPERATIVE AGREEMENT
   [ ] OTHER:

3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.

4. Title of Application or Activity
Maximizing Mechanisms of Muscle Hypertrophy to Combat Sarcopenia in Older Adults (Muscle
Plasticity With Resistance Training and Detraining in Older Adults)

5. Name of Principal Investigator, Program Director, Fellow, or Other
BAMMAN, MARCAS M

6. Assurance Status of this Project (Respond to one of the following)
   [X] This Assurance, on file with Department of Health and Human Services, covers this activity:
      Assurance Identification No. FWA0003960, the expiration date 02/14/09, IRB Registration No. IRB00000196
   [ ] This Assurance, on file with (agency/department), Assurance No. , the expiration date , IRB Registration/Identification No. (if applicable)
   [ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review
      and approval upon request.
   [ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph ______

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)
   [X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
      by: [X] Full IRB Review (date of IRB meeting) 06/20/2007 or [ ] Expedited Review (date) ______
      [ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects
         covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments
   Protocol subject to Annual continuing review.
   HIPAA Waiver Approved? No
   Title F9610627014
   Maximizing Mechanisms of Muscle Hypertrophy to Combat Sarcopenia in Older Adults (Muscle Plasticity With Resistance Training and Detraining in Older Adults)

IRB Approval Issued: 7-17-07

9. The official signing below certifies that the information provided above is
correct and that, as required, future reviews will be performed until study
closure and certification will be provided.

10. Name and Address of Institution
    University of Alabama at Birmingham
    701 20th Street South
    Birmingham, AL 35224

11. Phone No. (with area code) (205) 934-3789
12. Fax No. (with area code) (205) 934-1301
13. Email: smoore@uab.edu

14. Name of Official
    Ferdinand Uthaler, M.D.
15. Title
    Chairman, IRB

16. Signature
    Ferdinand Uthaler, M.D.

17. Date
    7-17-07

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