The Effect of Aging on Human Skeletal Muscle Mitochondrial and Intramyocellular Lipid Ultrastructure

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The purpose of this study was to determine whether ultrastructural changes in intramyocellular lipid (IMCL) and mitochondria occur with aging. Muscle samples were analyzed from 24 young and 20 old, equally active, individuals for IMCL and mitochondria quantity and size as well as their association. Old men had larger IMCL droplets than all other groups in the total muscle area. Old individuals showed higher IMCL content in the subsarcolemmal area. Young participants had a greater number of mitochondria compared with old participants in both fiber regions and greater enzyme activities of cytochrome c oxidase and citrate synthase. The fraction of IMCL touching mitochondria was lowest in old women in the total area and in old men in the subsarcolemmal region. In summary, older adults have larger IMCL droplets, fewer mitochondria, and a lower proportion of IMCL in contact with mitochondria. These factors likely contribute to age-related reductions in mitochondrial function and lipid metabolism.

Key Words: Mitochondrial dysfunction—Oxidative stress—Insulin resistance—Subsarcolemmal.

The progression of aging is well known to result in a reduction in skeletal muscle mitochondrial content and whole-body muscle mass (sarcopenia), dramatically altering lipid metabolism. In accordance, the prevalence of diabetes in developed countries is highest in individuals older than 65 years (1). Skeletal muscle intramyocellular lipid (IMCL) content has been implicated in the development of the metabolic syndrome as higher IMCL in elderly individuals is associated with reduced mitochondrial ATP production and peripheral insulin resistance (2,3). However, trained endurance athletes also have elevated IMCL content (4), whereby an increased abundance of mitochondria likely mitigates the negative aspects of augmented intramuscular lipids. This paradox points to physical activity as a major determinant of the consequences of increased net muscle lipid uptake. Because older adults are typically more sedentary than young individuals, investigations studying the physiological effects of human aging must avoid the confounding effects of physical activity when interpreting their results. Currently, the hypothetical association between IMCL accumulation and insulin resistance with aging remains to be fully characterized in healthy adults who participate in an active lifestyle.

In combination with alterations in muscle lipids, mitochondrial content is well established to decline with advancing age (5,6). An overall diminished flux through metabolic pathways and chronic activation of inflammation, factors that are exacerbated in older individuals, have been implicated in the downregulation of mitochondrial biogenesis (7). Furthermore, a reduced efficiency of Lon protease, a mitochondrial specific protease, has been demonstrated across the life span in conjunction with an overall increase in proteosome dysfunction (8,9). Although investigations have described a reduced volume density of mitochondria in elderly human skeletal muscle (5,10,11), only one has simultaneously explored changes in the size and number of mitochondria (10), with conflicting results. Additionally, specifying subpopulations of mitochondria within the cell appears to be important when evaluating age-related changes in mitochondrial function (12). The content of subsarcolemmal mitochondria is lower in insulin-resistant type 2 diabetic individuals compared with lean individuals (13), and this fraction is particularly responsive to exercise in older adults (14). Thus, the simplistic view of diminished mitochondrial content in aged skeletal muscle may overshadow more specific aspects of mitochondrial dysfunction including subcellular localization.

Studies involving mitochondrial dynamics (fusion and fission) have recognized mitochondria as mobile organelles that can respond to diverse stimuli (15–17). The location of mitochondria within a cell is constantly changing, and the balance of fission and fusion results in the size, abundance, and location of these organelles (16). Recent work has shown that exercise training causes a shift in subcellular localization of IMCL, such that lipid droplets are more closely associated with mitochondria (18). As physical activity is reduced in older individuals, structural changes may interfere with mitochondrial access to IMCL and result in increased storage within skeletal muscle. Previous observations of IMCL accumulation with aging using 1H-NMR have been limited in their ability to discern changes in IMCL and mitochondrial morphology or their proximity to one another (3,19,20). Therefore, we sought to evaluate the alterations...
in mitochondria and IMCL structure that occur with natural human aging in skeletal muscle.

In this investigation, we assessed the independent and interactive effects of aging and gender on the size and abundance of IMCL and mitochondria, as well as their subcellular localization and association in human skeletal muscle. In addition, we evaluated basal messenger RNA (mRNA) content of transcripts involved in lipid metabolism, mitochondrial function, and cellular oxidative stress, as well as mitochondrial enzyme activities to assess mitochondrial content. We intentionally studied young and older adults who were equivalently active in order to discern changes in skeletal muscle that are inherently related to the aging process and not inactivity.

**METHODS**

**Participants**

Muscle samples were acquired from 24 young (12 men/12 women) and 20 older (10 men/10 women) adults at rest, at least 48 hours removed from strenuous physical activity. The samples from young participants are a subset from a recent investigation in our laboratory (21). Young adults were considered recreationally active and exercised an average of 3 hours per week in a variety of modes, including resistance and aerobic exercise, as well as recreational sports such as volleyball, soccer, or basketball. Physical activity details are shown in Table 1. The older adults are a subset of participants from a 26-week resistance exercise training study (22), and samples were acquired at the conclusion of the training program to try to match the physical activity patterns of the young and old participants. This program consisted of whole-body resistance exercise training twice per week as described in detail (22). All data presented in the current study from both age groups have not been previously published. Prospective participants were informed of the procedures and risks involved in the study and gave their informed consent, which was approved by the McMaster Research Ethics Board. Participants were screened by telephone, followed by a medical history evaluation and consent from their family physician. Criteria for exclusion from the study included evidence of one or more of the following: coronary heart disease, congestive heart failure, hypertension, chronic obstructive pulmonary disease, diabetes mellitus, major orthopedic disability, renal failure, and smoking. Young women were eumenorrheic and were biopsied during the mid-follicular phase of their menstrual cycle. Older women were postmenopausal and were not taking hormone replacement therapy.

**Experimental Design**

Participants arrived in the neuromuscular clinic after an overnight fast. After supine rest, blood was drawn for hormone and metabolite determination. All samples were collected into heparinized tubes, centrifuged at 1,200 g for 10 minutes, and the plasma fraction was aliquoted into 1.5-ml tubes and stored at −80°C for later analysis.

Following the blood draw, a biopsy was obtained from the vastus lateralis under local anesthetic as previously described (23). All visible fat and connective tissue were removed, and the samples were divided into sections for subcellular localization and association in human skeletal muscle. We intentionally studied young and older adults who were equivalently active in order to discern changes in skeletal muscle that are inherently related to the aging process and not inactivity.

**Table 1. Participant Characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th></th>
<th>Old</th>
<th></th>
<th>Age Effect</th>
<th>Gender Effect</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men</td>
<td>Women</td>
<td>Men</td>
<td>Women</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>23 ± 2</td>
<td>22 ± 1</td>
<td>71 ± 2</td>
<td>70 ± 2</td>
<td><strong>p &lt; .05</strong></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78 ± 3</td>
<td>63 ± 2</td>
<td>81 ± 3</td>
<td>65 ± 3</td>
<td>NS</td>
<td><strong>p &lt; .05</strong></td>
<td>NS</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>177 ± 1</td>
<td>166 ± 1</td>
<td>176 ± 2</td>
<td>160 ± 2</td>
<td><strong>p &lt; .05</strong></td>
<td><strong>p &lt; .05</strong></td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25 ± 1</td>
<td>23 ± 1</td>
<td>26 ± 1</td>
<td>25 ± 1</td>
<td><strong>p &lt; .05</strong></td>
<td><strong>p = .083</strong></td>
<td>NS</td>
</tr>
<tr>
<td>% Body fat</td>
<td>20 ± 2</td>
<td>29 ± 1</td>
<td>19 ± 1</td>
<td>36 ± 2</td>
<td><strong>p &lt; .05</strong></td>
<td><strong>p &lt; .05</strong></td>
<td><strong>p = .014</strong></td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>63 ± 2</td>
<td>45 ± 1</td>
<td>65 ± 2</td>
<td>41 ± 1</td>
<td><strong>p &lt; .05</strong></td>
<td><strong>p &lt; .05</strong></td>
<td><strong>p = .049</strong></td>
</tr>
<tr>
<td>% FFM</td>
<td>80 ± 2</td>
<td>71 ± 1</td>
<td>81 ± 1</td>
<td>64 ± 2</td>
<td><strong>p &lt; .05</strong></td>
<td><strong>p &lt; .05</strong></td>
<td><strong>p = .015</strong></td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>4.9 ± 0.1</td>
<td>5.0 ± 0.2</td>
<td>5.0 ± 0.3</td>
<td>4.8 ± 0.3</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Plasma insulin (μIU/ml)</td>
<td>6.5 ± 1.0</td>
<td>5.7 ± 1.0</td>
<td>5.7 ± 0.9</td>
<td>4.5 ± 0.4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.4 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Note: *p Values refer to significant main effects (p < .05) identified by two-way (age by gender) analysis of variance. See text for details. N = 14 (9 men/5 women) for young and N = 10 (6 men/4 women) for older adults for plasma insulin and glucose. Values are mean ± SE. AE = aerobic exercise; BMI = body mass index; FFM = fat-free mass; HOMA-IR = homeostasis model assessment of insulin resistance; NS = nonsignificant; RE = resistance exercise; Rec = recreational activity.
was snap-frozen in liquid N\textsubscript{2} and stored at \textasciitilde-80°C. Body composition was assessed using dual energy x-ray absorptiometry (QDR 1000W; Hologic, Waltham, MA) using whole-body software for adults (v5.63).

Analytical Methods

Metabolites and hormones.—Plasma glucose concentration was determined using a glucose analyzer (Model 2300 Stat Plus; Yellow Springs Instrument Co. Inc, Yellow Springs, OH). Insulin concentrations were determined using a commercially available radioimmunoassay kit (Coat-a-count, TKIN5; Diagnostic Products, Los Angeles, CA). Plasma samples were only available for 14 young (9 men/5 women) and 10 old (6 men/4 women) participants.

Enzyme activity.—Citrate synthase (CS) and cytochrome c oxidase (COX) maximal enzyme activities were determined following homogenization of 15–25 mg of wet muscle using a UV spectrophotometer (model 8453; Hewlett Packard, Wilmington, DE), as previously described by our group (24,25). Samples were run in duplicate. Due to a limited amount of sample, only 12 young (6 men/6 women) and 8 old individuals (5 men/3 women) were evaluated for enzyme activity.

Gene expression.—RNA was extracted, and samples were treated with DNase I for 25 minutes to remove any contaminating DNA. Primers and probes for each gene of interest were designed as previously described by our laboratory (26). Primer sequences are as follows: cytochrome c oxidase subunit IV (COX IV), forward-gcagcttgtcatcagctacac, reverse-tcaatgtcacccgcaactac, reverse-ccaggttctcgttgctgtact; Dynamin-related protein 1 (Drp1), forward-ttggtaaaatggcagtct ct catggtgaacagcaacta, reverse-gtccagtttacggcgatacat; citrate synthase (CS) and cytochrome c oxidase subunit IV (COX IV), forward-cgagcaatttc and correct identification was confirmed by reviewing mitochondria and subsarcolemmal areas of the muscle samples. The images were photographed using a 1-s exposure time and digitized using a white light illuminator (C-80 Epi-illumination UV Darkroom; Diamed, Mississauga, ON, Canada). Lipid and mitochondrial analysis was determined using a computerized image analysis system (Image Pro Plus, ver. 4.0; Media Cybernetics, Silver Spring, MD). Criteria were set for lipid droplet and mitochondrial identification, and correct identification was confirmed by reviewing 12 random micrographs with a pathologist trained in EM (Dr. K. Chorneyko, MD). Lipid droplets and mitochondrial fragments were circled and converted to actual size using a calibration grid. Values are reported as mean individual IMCL or mitochondrial size (\textmu m\textsuperscript{2}), total number of IMCL droplets or mitochondria per square micrometer of tissue (#/\textmu m\textsuperscript{2} tissue), and percentage IMCL or mitochondrial area density as previously described (18). Because of limitations in sample quantity, EM analysis for three participants from the older adult group (one man/two women) was not included in the data set.

EM analysis.—Muscle samples were fixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Spurr’s resin. Thin sections (70 nm) were cut using an ultramicrotome (Ultracut E; Reichert, Vienna, Austria), placed on Cu/Ph grids (200 mesh size), and stained for 5 minutes in uranyl acetate followed by 2 minutes in lead acetate. Sections were visualized at a magnification of \times 6500 using a transmission electron microscope (Jeol 1200 Ex; Jeol, Tokyo, Japan). Two micrographs each from four different muscle fibers (eight images per person) were taken randomly and sequentially while blinded to gender and age. Images were taken from the subsarcolemmal region (subsarcolemmal area) and another containing a portion of the subsarcolemmal region adjacent to the nucleus with most of this image containing the intermyofibrillar area (total area). Therefore, the total area is a combination of both the intermyofibrillar and subsarcolemmal areas of the muscle samples. The images were photographed using a 1-s exposure time and digitized using a white light illuminator (C-80 Epi-illumination UV Darkroom; Diamed, Mississauga, ON, Canada).

Lipid and mitochondrial analysis was performed on an iCycler real-time PCR system (BioRad Laboratories, Hercules, CA) in the One-step Taqman Master Mix reagents (Roche, Branchburg, NJ) according to the manufacturer’s instruction with the gene of interest and housekeeping gene (ß2-microglobulin) analyzed within the same run.

Statistical Analysis

All data were compared using a two-way analysis of variance with gender and age as the between group effects (Statistica, Version 5.0; Statsoft, Tulsa, OK). When significance was attained, Tukey’s honestly significant difference post hoc analysis was utilized to determine specific differences. Participant characteristics, mRNA content, and enzyme activities were compared to IMCL and mitochondrial size, number, and their association with one another using Pearson’s correlation. Data are expressed as mean \pm SE. Significance was set as \( \alpha = .05 \).
RESULTS

Descriptive Data

The participant characteristics are provided in Table 1. Older adults were significantly older, shorter, and had greater adiposity compared with young individuals ($p < .05$). Overall, men were taller and leaner than women ($p < .05$). There were no differences between groups for resting plasma glucose, insulin or calculated homeostasis model assessment of insulin resistance (HOMA-IR, $p > .05$). The total duration of physical activity did not differ between groups; however, older adults spent a greater proportion of time performing resistance exercise than young adults ($p < .05$) and less time engaged in recreational activities ($p < .05$). In addition, young women tended to spend a greater proportion of time performing aerobic exercise than all other groups ($p = .089$).

Enzyme Activities

Enzyme activities from young and old individuals are provided in Figure 3. The maximal activities of COX and CS were 43% and 33% lower, respectively, in older adults compared with young individuals ($p < .05$). Women tended to have a higher CS activity than men ($p = .053$). COX activity was positively correlated with the number of mitochondria ($\# / \mu m^2$, $r = .65$, $p = .002$) and mitochondrial area density ($r = .70$, $p < .001$) in the subsarcolemmal area (see below).

Electron Micrograph Data

An overview of the important morphological findings in old vs. young muscle is provided in Figure 1. Representative images of young and old muscle are shown in figure 2.

Intramyocellular lipid.—Table 2 summarizes results from our analysis of electron micrographs in muscle tissue. Older men had IMCL droplets that were larger than those of the other groups when considering the total fiber area ($p = .017$). However, older adults, as a group, had larger lipid droplets than young individuals in the subsarcolemmal area ($p = .009$). IMCL number in the subsarcolemmal area, expressed as $\# IMCL/\mu m^2$, was greater in women than in men ($p = .019$) but was not different between the young and old groups ($p > .05$). Lipid area density was significantly higher in older men compared with older women, young men, and young women in the total fiber area ($p = .017$). In contrast, women had a greater lipid area density in the subsarcolemmal area of the muscle fiber ($p = .019$) but was not different between the young and old groups ($p > .05$). Lipid area density was significantly higher in older men compared with older women, young men, and young women in the total fiber area ($p = .017$). In contrast, women had a greater lipid area density in the subsarcolemmal area of the muscle fiber ($p = .019$) but was not different between the young and old groups ($p > .05$). The fraction of IMCL droplets in direct contact with mitochondria was significantly lower in older women than in other groups ($p < .001$) when considering the total fiber area. However, in the subsarcolemmal region, older men had the lowest proportion of IMCL droplets in direct contact with mitochondria compared with all other groups; this was significantly higher in older women and greatest in young men and women ($p = .018$). Finally, the percent body fat of the participants was positively correlated with lipid area density within the subsarcolemmal region ($r = .439$, $p = .005$).

Mitochondria.—There were no significant effects of age or gender on mitochondria size in the total or subsarcolemmal fiber regions ($p > .05$). The number of mitochondria per square micrometer was greater in young adults compared with old in the total and subsarcolemmal regions (SS: 132%, $p < .001$; total: 132%, $p < .001$). The mitochondrial area density in the total fiber area tended to be highest in young men, followed by young women, and lowest in old men and women ($p = .062$). Young adults had a significantly higher mitochondrial area density in the subsarcolemmal region compared with the older adults (126%, $p < .001$).
mRNA Abundance

Lipid metabolism.—All graphs are provided in Figure 4. mRNA content of HSL was significantly higher in older adults compared with young individuals \((p < .05)\) and tended to be higher for SREBP-1 \((p = .057)\). HSL mRNA was inversely correlated with the fraction of mitochondria in contact with IMCL in the total fiber area \((r = −.33, p = .041)\). mRNA content of PPAR\(\alpha\), PPAR\(\gamma\), and UCP3 were significantly lower in the old compared with young \((p < .05)\). PPAR\(\alpha\) was negatively correlated with IMCL size in the subsarcolemmal area \((r = −.38, p = .015)\). In addition, there was a trend for PPAR\(\gamma\) mRNA to be greater in women \((p = .052)\) and for UCP3 to be greater in men \((p = .091)\). LCAD tended to be lower in women than in men \((p = .056)\).

Mitochondrial function.—mRNA content of COX IV, Lon protease, and Mfn2 mRNA was significantly lower in older adults compared with young individuals \((p < .05)\) and tended to be lower for Drp1 \((p = .096)\). Mfn2 was positively

Table 2. Summary of Mitochondrial and IMCL Morphology in Young and Old Men and Women

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
<th>Age Effect</th>
<th>Gender Effect</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMCL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean lipid size ((\mu m^2))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total area</td>
<td>0.269 ± 0.034</td>
<td>0.208 ± 0.019</td>
<td>0.219 ± 0.028</td>
<td>(p &lt; .05)</td>
<td>(p &lt; .05)</td>
</tr>
<tr>
<td>Subsarcolemmal area</td>
<td>0.173 ± 0.019</td>
<td>0.197 ± 0.023</td>
<td>0.292 ± 0.077</td>
<td>(p &lt; .05)</td>
<td>NS</td>
</tr>
<tr>
<td>No. of IMCL/(\mu m^2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total area</td>
<td>0.031 ± 0.005</td>
<td>0.040 ± 0.003</td>
<td>0.035 ± 0.004</td>
<td>0.038 ± 0.004</td>
<td>NS</td>
</tr>
<tr>
<td>Subsarcolemmal area</td>
<td>0.125 ± 0.014</td>
<td>0.211 ± 0.020</td>
<td>0.137 ± 0.029</td>
<td>0.168 ± 0.035</td>
<td>NS</td>
</tr>
<tr>
<td>Lipid area density (%)</td>
<td>0.732 ± 0.147</td>
<td>0.840 ± 0.102</td>
<td>1.679 ± 0.336</td>
<td>0.830 ± 0.140</td>
<td>(p = .061)</td>
</tr>
<tr>
<td>% of lipids touching mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total area</td>
<td>2.5 ± 0.5</td>
<td>4.1 ± 0.5</td>
<td>3.9 ± 1.3</td>
<td>7.3 ± 2.8</td>
<td>NS</td>
</tr>
<tr>
<td>Subsarcolemmal area</td>
<td>85.3 ± 8.5</td>
<td>85.7 ± 3.5</td>
<td>85.0 ± 1.5</td>
<td>32.5 ± 2.5</td>
<td>(p &lt; .05)</td>
</tr>
<tr>
<td>Mitochondria number</td>
<td>61.2 ± 6.1</td>
<td>55.1 ± 3.6</td>
<td>12.2 ± 3.8</td>
<td>35.0 ± 8.8</td>
<td>(p &lt; .05)</td>
</tr>
<tr>
<td>Mitochondria area density (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total area</td>
<td>7.9 ± 1.0</td>
<td>5.2 ± 0.5</td>
<td>2.9 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>(p &lt; .05)</td>
</tr>
<tr>
<td>Subsarcolemmal area</td>
<td>17.7 ± 2.0</td>
<td>18.8 ± 1.3</td>
<td>8.7 ± 1.6</td>
<td>9.3 ± 2.1</td>
<td>(p &lt; .05)</td>
</tr>
</tbody>
</table>

Note: \(p\) Values refer to significant main effects \((p < .05)\) identified by two-way (age by gender) analysis of variance. See text for details. \(N = 24\) (12 men/12 women) for young and \(N = 17\) (9 men/8 women) for older adults. Values are mean ± SE. IMCL = intramyocellular lipid; NS = nonsignificant.
correlated with the fraction of IMCL touching mitochondria 
when considering the total fiber area (r = .33, p = .033).
Sirt1 mRNA content was significantly greater in the old 
(p < .05). The only gender difference observed for mitochon-
drida-related genes was in COX IV mRNA, which was sig-
nificantly higher in men than in women (p < .05).

Antioxidant enzymes and the oxidative stress response.—
The mRNA content of FOXO1A was significantly greater in old compared with young individuals (data not shown, 
 p < .05), and this was inversely correlated with the number 
of mitochondria (μm², r = −.42, p = .009) and the fraction 
of mitochondria in contact with IMCL in the total fiber area 
(r = −.32, p = .046). The mRNA content of SOD1 and SOD2 
was significantly lower in older adults (data not shown, 
 p < .05) and was positively correlated with mitochondrial 
area density in the total fiber area (data not shown, SOD1: 
 r = .71, p = .001; SOD2: r = .32, p = .041). In addition, SOD1 
mRNA content was significantly greater in men compared 
with women and was negatively correlated with IMCL area 
density in the total fiber area (r = −.35, p = .031).

DISCUSSION

The present study confirms that older adults have higher IMCL content and provides novel morphological evidence that this is due to greater IMCL size (independent of quantity) combined with a reduced association with mitochondria. Furthermore, this is the first report, to our knowledge, to show that the decline in skeletal muscle mitochondrial content commonly observed with human aging is due to a reduction in the number but not the size of the existing mitochondria.

Previous studies have demonstrated elevated IMCL content in older individuals using ³H-NMR spectroscopy, although these investigations do not provide any insight regarding size, subcellular localization, or proximity relationships within skeletal muscle (3,19,20). The present results indicate that the age-related increase in IMCL area density is due to a greater size, but not abundance, of lipid droplets, and that this is specific to the subsarcolemmal region in women. Recent work from our laboratory has shown that aerobic exercise training results in an increased number of lipid droplets, presumably as an adaptation to maximize surface area for lipolysis during exercise (18). Because our results indicate that mitochondrial oxidative capacity was lower and IMCL were larger in elderly adults, it is likely the changes in IMCL content observed here reflect increased storage of excess free fatty acids as triglycerides within the IMCL. This would limit the accumulation of harmful lipid intermediates (diacylglycerol, ceramide) that have direct lipotoxic effects on cellular function (27,28). This is consistent with our observed elevation of mRNA transcripts involved in triacylglycerol synthesis (SREBP-1) and breakdown (HSL) in older participants. However, older adults also had diminished gene expression of PPARα and PPARγ, indicating a downregulation of transcripts involved in lipid transport and oxidation. Although we did not observe differences in the young and old participants in the HOMA-IR, perhaps related to the high relative fitness of the elderly participants, previous work in mice has demonstrated an age-related decrease in PPARγ related to reduced insulin sensitivity (29). Interestingly, PPARα was negatively correlated with IMCL size in the subsarcolemmal area but not to the other aspects of IMCL content: abundance and area density. The lack of a relationship between HOMA-IR and IMCL content in the current study combined with the lower mitochondrial activity and content of the older adults brings into question the hypothetical relationship between IMCL and insulin resistance put forth by others (3,20) with regard to the geriatric population. Moreover, this reinforces the importance of mitochondrial function in relation to IMCL content in the pathology of insulin resistance and highlights the incomplete data available when using whole-muscle measurements.

We have also confirmed that skeletal muscle from women contained a greater amount of IMCL than men, which appears to be retained with age when the entire fiber area is
taken into account. Women have a greater area density of IMCL (18,21) and oxidize more fat during endurance exercise (30,31). The augmented IMCL stores in female skeletal muscle may result from the influence of sex hormones, as has been shown in rats (32). The age of the older adults precludes this possibility as the older women were beyond menopause and estrogen would play a minimal role. It is tempting to speculate that the conservation of increased IMCL content in women with aging is due to greater adiposity in the old women compared with young women. However, old men had the highest IMCL content in the total fiber area despite having the lowest body fat of all groups. Furthermore, the weak correlation between body fat and IMCL area density is only significant in the young participants when they are separated from the older adults, indicating that body fat is not necessarily the best predictor of IMCL content and does not explain our observations with aging.

We have uniquely demonstrated that the decreased mitochondrial content commonly observed with human aging is due to a reduction in the number, but not size, of the existing mitochondria, concomitant with reductions in mitochondrial enzyme activities. These findings are similar to those recently observed in aged mice (33) and are in contrast to the reduced mitochondrial size seen in obesity and type 2 diabetes (34). Some authors have postulated that impairments in insulin sensitivity are due simply to reduced mitochondrial content that results in an imbalance of fatty acid

Figure 4. Gene expression of transcripts related to (A) lipid metabolism and (B) mitochondrial biogenesis and function in young and old individuals. CPT1 = carnitine palmitoyltransferase-1; HSL = hormone sensitive lipase; LCAD = long-chain acyl-CoA dehydrogenase; PPARα = peroxisome proliferator–activated receptor alpha; PPARγ = peroxisome proliferator–activated receptor gamma; PPARδ = peroxisome proliferator–activated receptor delta; TFPβ = trifunctional protein beta; SREBP-1 = sterol regulatory element–binding protein-1; UCP3 = uncoupling protein-3; COX IV = cytochrome c oxidase subunit IV; Mfn2 = mitofusin 2; Sirt1 = sirtuin 1; Drp1 = dynamin-related protein 1. N = 24 (12 men/12 women) for young and N = 20 (10 men/10 women) for older adults. Data are mean ± SE. *Significantly different from young individuals (p < .05). †Trend for a difference from young individuals (p < .10).
utilization, and thus accumulation of lipid, in young individuals (13,35). We did not find any associations between HOMA-IR and mitochondrial enzyme activity or morphology, indicating that deficits in muscle oxidative capacity may not be directly attributable to parameters of insulin sensitivity in older adults. We have previously shown that increased physical activity leads to an increase in the size, but not number, of mitochondria (18), and fewer mitochondria in the current study were not correlated with increased numbers of IMCL droplets, which collectively reinforces the fact that our current observations in older adults are not simply due to a lower activity level. Thus, the defects in mitochondrial content/morphology with aging appear to be distinct from those of insulin resistance and changes in physical activity.

Some prior investigations in humans have postulated that mitochondrial size decreases with aging (10,11). However, in the work of Orlander et al. the changes in mitochondrial number were limited to the subsarcolemmal region, and only when some age groups are excluded from their analysis were the differences significant. Because their youngest and oldest age groups were significantly more active than the other age groups, some of their conclusions may be questionable, especially considering that they did not detect any age-related changes in oxidative enzyme activities or IMCL content. Many studies concerning mitochondrial dysfunction with aging have failed to take into account the location of specific populations of mitochondria within skeletal muscle, which can differ with regard to oxidative capacity and adaptability to contractile activity (36,37). In the current study we considered a distinct portion of the muscle fiber, the subsarcolemmal area, as well as the total fiber area, which appear to have markedly different IMCL and mitochondrial ultrastructure.

The cause of reduced mitochondrial content has been extensively studied in relation to accumulations in DNA and protein damage resulting from reactive oxygen species (ROS) production. Higher levels of FOXO1A expression appear to contribute to an enhanced ROS response (38), and this may be due to an elevated level of oxidative damage in the current study that has been previously established to occur with aging (39). Although indirect, reduced gene expression of SOD1 and SOD2 could underlie increased oxidative stress due to an impaired antioxidant defense system. In support of this, SOD1 and SOD2 expression was positively correlated with mitochondrial number. Moreover, the age-related decline in the expression of Lon protease, a mitochondrial matrix enzyme, results in greater levels of oxidatively damaged proteins (8), compounding the influence of reduced cellular antioxidants. Age-related increases in DNA and protein damage also have negative effects on mitochondrial biogenesis (40), further contributing to a decreased number of mitochondria. Several investigations have reported “giant” mitochondria in aged skeletal muscle with malformed cristae and an extremely large size (41). We did not observe these mitochondria in older adults, potentially because they were healthy and active for their age.

Physical associations between IMCL and mitochondria with aging have not been previously investigated. We have ascertained that aging results in a reduced number of IMCL touching mitochondria and that this occurs in the subsarcolemmal region for men and throughout the entire fiber area in women. Aerobic exercise training produces the opposite effect, whereby mitochondria and IMCL become closely associated and are therefore more optimally situated for oxidation (18). Mitochondrial dynamics (fission and fusion) may explain changes in subcellular localization and associations with IMCL. It has been shown that proteins regulating mitochondrial fusion, mitofusins, are upregulated in response to acute exercise (15), while the inverse is demonstrated in obese individuals (42), indicating a potential regulation of mitochondrial dynamics by physical activity. The observed decrease in the gene expression of Mfn2 and Drp1 with aging, proteins involved in mitochondrial fusion and fission, respectively, could partially explain the diminished association of mitochondria with IMCL droplets. Indeed, we find a significant positive correlation between Mfn2 and the fraction of IMCL in contact with mitochondria. Thus, the aging process may downregulate fusion and fission events, retarding motility and reducing the metabolic need for mitochondria to be in close proximity with IMCL stores.

A potential shortfall of our findings is the inability to determine fiber type–specific differences in relation to mitochondria and IMCL ultrastructure. Although we cannot discount the influence of fiber type on our observations, increases in the proportion of type I fibers with aging are not likely to be the sole cause of the changes in IMCL and mitochondrial morphology. Previous studies have used the soleus muscle to demonstrate an age-related increase in IMCL content (3,19,20). Being primarily of the type I myosin heavy chain isoform in young adults and heavily recruited for posture and daily ambulation, this muscle would not be expected to present a dramatically different fiber composition in older adults. In addition, because type I fibers are more insulin sensitive (43) and contain a greater content of mitochondria (44), a larger fraction of these fibers in the elderly should serve to counter a decline in muscle oxidative capacity and insulin sensitivity, strengthening the current findings in relation to subcellular structure. Furthermore, inclusion of the changes that occur in the total area of the muscle fibers, independent of fiber type, are likely to be more representative of whole-muscle function and metabolism, which is of primary importance for the elderly population. While alterations in mRNA are not always representative of changes at the protein level, we do observe significant agreement between morphological assessments and several mRNA transcripts as well as mitochondrial enzyme activities. The aforementioned differences and relationships with age were observed in spite of both groups
being evenly matched for physical activity, and these changes would likely be exacerbated in sedentary older individuals.

In summary, we have demonstrated that reductions in the association of larger IMCL droplets with a fewer number of mitochondria occur with aging. These alterations appear to be independent of changes in insulin sensitivity and physical activity and are likely an inherent aspect of the aging process. Furthermore, structural changes to IMCL and mitochondria were correlated with transcriptional alterations related to aberrant lipid metabolism, mitochondrial dysfunction, and oxidative damage, implying that morphology is a critical component of cellular homeostasis with senescence. These findings have important implications in the rapidly expanding field of mitochondrial dynamics, as well as for exercise or pharmaceutical treatments that seek to attenuate the effects of aging on skeletal muscle.

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