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Muscle oxidative capacity during IL-6-dependent cancer cachexia

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White JP, Baltgalvis KA, Puppa MJ, Sato S, Baynes JW, Carson JA. Muscle oxidative capacity during IL-6-dependent cancer cachexia. Am J Physiol Regul Integr Comp Physiol 300: R201–R211, 2011. First published December 9, 2010; doi:10.1152/ajpregu.00300.2010.—Many diseases are associated with catabolic conditions that induce skeletal muscle wasting. These various catabolic states may have similar and distinct mechanisms for inducing muscle protein loss. Mechanisms related to muscle wasting may also be related to muscle metabolism since glycolytic muscle fibers have greater wasting susceptibility with several diseases. The purpose of this study was to determine the relationship between muscle oxidative capacity and muscle mass loss in red and white hindlimb muscles during cancer cachexia development in the Apc<sup>Min/+</sup> mouse. Gastrocnemius and soleus muscles were excised from Apc<sup>Min/+</sup> mice at 20 wk of age. The gastrocnemius muscle was partitioned into red and white portions. Body mass (−20%), gastrocnemius muscle mass (−41%), soleus muscle mass (−34%), and epididymal fat pad (−10%) were significantly reduced in severely cachetic mice (n = 8) compared with mildly cachetic mice (n = 6). Circulating IL-6 was fivefold higher in severely cachetic mice. Cachexia significantly reduced the mitochondrial DNA-to-nuclear DNA ratio in both red and white portions of the gastrocnemius. Cytochrome c and cytochrome-c oxidase complex subunit IV (Cox IV) protein were reduced in all three muscles with severe cachexia. Changes in muscle oxidative capacity were not associated with altered myosin heavy chain expression. PGC-1α expression was suppressed by cachexia in the red and white gastrocnemius and soleus muscles. Cachexia reduced Mfn1 and Mfn2 mRNA expression and markers of oxidative stress, while Fis1 mRNA was increased by cachexia in all muscle types. Muscle oxidative capacity, mitochondria dynamics, and markers of oxidative stress are reduced in both oxidative and glycolytic muscle with severe wasting that is associated with increased circulating IL-6 levels.

mitochondria; wasting; mitofusin-2 protein; fission 1 protein

Cachexia is a condition of whole body wasting that develops with chronic diseases such as AIDS, chronic obstructive pulmonary disorder, and cancer, particularly cancers in the gastrointestinal tract and lung (3, 36, 55). Although chronic inflammation and insulin resistance are associated with many types of cachexia, the diverse chronic diseases that induce skeletal muscle catabolism may have both common and unique regulatory mechanisms related to the wasting process. There is evidence that muscle phenotype differentially regulates muscle catabolism with many wasting diseases (30), with primarily fast glycolytic muscle mass being more susceptible to loss compared with slow oxidative muscle (8, 32, 59). These results differ from disuse-induced muscle atrophy (i.e., bed-rest, spaceflight) where slow-oxidative muscle fibers initially undergo a greater atrophy (12). Specifically related to cancer cachexia, tumor-implanted mice have a reduction in mass of primarily fast-type gastrocnemius and tibialis anterior muscles, while maintaining slow-oxidative soleus muscle mass (1). During the development of cachexia in the Apc<sup>Min/+</sup> mouse, a genetic model of colon cancer (39), there is a greater loss of gastrocnemius type IIb fiber cross-sectional area compared with type Ila fiber (8). This selectivity to wasting stimuli may be related to oxidative muscle fibers having an increased tolerance for systemic inflammation and metabolic alterations that occur with many wasting diseases (56). Alterations in Apc<sup>Min/+</sup> mouse muscle oxidative capacity with cachexia and its relationship to wasting susceptibility have not been previously examined.

Muscle oxidative capacity reflects the cellular energy demands. The mitochondria’s role in energy production, apoptotic processes, and production of reactive oxygen species (ROS), make it an important regulator of muscle mass loss with wasting diseases. Muscle mitochondrial content appears to be compromised during wasting conditions including chronic obstructive pulmonary disorder (28, 46), diabetes (24), and congestive heart failure (18). With diabetes, mitochondrial protein loss is associated with the extent of muscle wasting in both slow and fast muscles (24). Muscle mitochondria are also in a dynamic state of equilibrium and can adapt to metabolic signaling and environmental stimuli (27). An increased turnover of mitochondrial proteins and/or a suppression of mitochondrial biogenesis could contribute to this loss of muscle oxidative capacity. The transcriptional cofactor peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a critical regulator of skeletal muscle mitochondrial biogenesis (26), and its expression has been shown to correspond with alterations in muscle mass. Rat gastrocnemius muscle catabolism with diabetes, uremia, and tumor implantation is associated with a reduction in PGC-1α mRNA expression (48). Mitochondrial function requires the coordination of mitochondria fission/fusion processes, which are referred to as mitochondrial dynamics (20). Fusion proteins mitofusin-1 (Mfn1) and -2 (Mfn2) promote mitochondrial elongation and activity. In contrast, the outer mitochondrial membrane protein fission 1 (Fis1) has been shown to induce mitochondrial fragmentation (29). While alterations in muscle Fis1 expression during cachexia is not well understood, type 2 diabetic (5, 25) and obese patients (5) overexpress Fis1. There is evidence that Fis1 expression is associated with apoptosis (29, 31, 43). Increased ROS are also regulators of lysosomal-mediated protein degradation related to the autophagy process in...
the development of cancer cachexia in the mass loss in primarily red and white hindlimb muscles during voluntary activity wheels compared with wild-type (WT) thase activities and functional capacity related to distance run previously weight-stable, mice were group housed and killed at 20 wk of age.

ApcMin+/ mouse is related to the spontaneous development of intestinal/ severe symptoms; ApcMin+/H9251 mouse is dependent on increasing levels of circulating IL-6 (6). Systemic IL-6 overexpression in precachectic ApcMin+/ mice lacking IL-6 do not develop cachexia, and when IL-6 is systemically overexpressed in these mice, cachexia develops (6). Systemic IL-6 overexpression in precachectic ApcMin+/ mice can accelerate the progression of cachexia, including muscle mass loss. Although both glycolytic and oxidative skeletal muscles in the ApcMin+/ mouse waste, primarily glycolytic muscle demonstrate greater wasting (38). We previously demonstrated that tumor bearing, weight-stable ApcMin+/ mice have similar gastrocnemius muscle citrate synthase activities and functional capacity related to distance run in voluntary activity wheels compared with wild-type (WT) mice. (39). The purpose of this study was to determine the relationship between muscle oxidative capacity and muscle mass loss in primarily red and white hindlimb muscles during the development of cancer cachexia in the ApcMin+/ mouse. We hypothesized that muscle mass loss would be associated with a decrease in muscle oxidative capacity regardless of primary metabolic phenotype.

MATERIALS AND METHODS

Animals. All WT and ApcMin+/ mice used in this study were on a C57BL/6 background and were originally purchased from Jackson Laboratories (Bar Harbor, ME). All mice were bred at the University of South Carolina’s Colon Cancer Research Center Mouse Core breeding facility that is housed in the University’s Animal Resource facility, as previously described (6, 8). For all mice in the study the room was maintained on a 12:12-h light-dark cycle with the light period starting at 0700. Mice were provided standard rodent chow (cat. no. 8604 Rodent Diet; Harlan Teklad, Madison, WI) and water ad libitum. Body weights and food intake were measured weekly. All animal experimentation was approved by the University of South Carolina’s Institutional Animal Care and Use Committee. Two separate groups of mice were used for the two main experiments in the study.

Experiment 1 examined WT (n = 8) and ApcMin+/ (n = 14) mice during the development of cachexia until 20 wk of age. The 14 ApcMin+/ mice allowed for stratification of cachexia severity (n = 8 severe symptoms; n = 6 mild symptoms), since cachexia development in this mouse is related to the spontaneous development of intestinal/colon polyps, which has considerable interanimal variability. These mice were group housed and killed at 20 wk of age.

Experiment 2 accelerated cachexia in 16-wk-old, precachectic, previously weight-stable, ApcMin+/ mice and WT mice, by increasing circulating IL-6 levels through in vivo electroporation of an IL-6 overexpression vector, as previously described (6, 8). The gastrocnemius muscle masses, body masses, and plasma IL-6 levels have been previously published for the mice in experiment 2 (8). All other data related to experiment 2 in the current paper is novel and have not been previously published. For experiment 2, 16-wk-old ApcMin+/ mice (control; n = 6 and + IL-6; n = 6) overexpressed IL-6 or control for 4 wk (See procedure below), and were then killed. WT mice (control; n = 6 and + IL-6; n = 6) overexpressed IL-6 for 10 wk and were then killed at 26 wk of age.

Determination of cachexia symptom severity. The inherent variability in cachexia development between mice is a strength of the ApcMin+/ mouse model, and ApcMin+/ mice cachexia symptom severity was assigned at the study’s end as in previously published studies (6, 7). Mice were classified as having mild, moderate, or severe cachexia based on their body mass, gastrocnemius muscle mass, and epididymal fat pad mass. Mice were categorized as having mild cachexia, if all of these variables were within 1 SD of the mean of age-matched WT mice. Mice with severe cachexia were > 2 SDs away from the means of age-matched WT mice.

IL-6 overexpression. The body and muscle weights for mice used in the electroporation experiments have been previously published (8). In vivo intramuscular electroporation of an IL-6 plasmid was used to increase circulating IL-6 levels in mice as previously described (6, 8). The electroporated quadriceps muscle was not used for any analyses in the study, and the gastrocnemius muscle analyzed in the study was not subjected to electroporation. Briefly, mice were injected with 50 μg of the IL-6 plasmid driven by the cytomegalovirus promoter, or empty control vector, into the quadriceps muscle. Mice were anesthetized with a 2% mixture of isoflurane and oxygen (1 l/min). A series of eight 50-ms, 100-V pulses was used to promote uptake of the plasmid into myofibers, and then the incision was closed with a wound clip. Control, + IL-6 groups within ApcMin+/, and WT mice received the appropriate plasmid starting at 16 wk of age. Preliminary data demonstrated that circulating IL-6 levels can substantially decline 2 and 3 wk following electroporation, and others have reported similar findings related to overexpression with this procedure (21). To maintain circulating IL-6 levels, the entire plasmid injection and electroporation procedure was repeated at 2-wk intervals, alternating between the right and left quadriceps muscles.

Plasma IL-6. Levels of plasma IL-6 were determined as previously described (6). Plasma IL-6 levels were measured with a mouse-specific ELISA (Biosource, Carlsbad, CA) by taking blood samples under brief isoflurane anesthesia from the retroorbital eye sinus at 10, 14, and 20 wk of age.

Polyp counts. Polyp counts were done as previously described (39). Formalin-fixed intestinal sections from all animals were briefly stained with 0.1% methylene blue, and they were counted by the same investigator who was blinded to the treatments. Polyps were counted under a dissecting microscope, using tweezers to pick through the intestinal villi and identify polyps. Polyps were categorized as large (>2 mm in diameter), medium (1–2 mm), or small (<1 mm) in the small and large intestines.

Tissue collection. Mice were given a subcutaneous injection of ketamine/xylazine/acepromazine cocktail (1.4 ml/kg body wt). Gastrocnemius and soleus muscles, epididymal fat pads, and tibias were excised. At the time of dissection the gastrocnemius muscle was cut into the visually red lateral head and the white intermediate portion of the muscle located between the medial and lateral gastrocnemius heads (supplemental Fig. 2). Tibia length was measured as an indicator of animal body size and a correction factor for skeletal muscle and adipose tissue weights. The gastrocnemius muscles were rinsed in PBS, snap frozen in liquid nitrogen, weighed, and stored at −80°C until further analysis.

mtDNA PCR. DNA was isolated using DNAzol reagent (Invitrogen). Briefly, muscle (20–30 mg) was homogenized in 1 ml MDNazol, pelleted with 100% ethanol, and resuspended in 8 mM NaOH. Quantitative real-time PCR analysis was carried out in 25-μl reactions consisting of 2× SYBR green PCR buffer (AmpliTaq Gold DNA Polymerase, Buffer, dNTP mix, AmpErase UNG, MgCl2), 0.150 μg DNA, deionized water, and 60 nM of each primer. PCR was run with the DNA sample with cytochrome B forward: 5′-ATT CCT TCA TGT CGG AAG CG-3′; cytochrome B reverse: 5′-ACT GAG AAG CCC CCT CAA AT-3′; Gapdh forward: 5′-TTG GGT TGT ACA TCC AAG CA-3′; Gapdh reverse: 5′-CAA GCA AAG GAG
CTG AG-3’. Samples were analyzed on an ABI 7300 Sequence Detection System. Reactions were incubated for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles consisting of a 15-s denaturing step at 95°C and 1-min annealing/extension step at 60°C. Data were analyzed by ABI software by using the cycle threshold (Ct), which is the cycle number at which the fluorescence emission is midway between detection and saturation of the reaction. The \( 2^{-\Delta\Delta Ct} \) method (34) was used to determine changes in gene expression between cytochrome B with Gapdh Ct as the correction factor. The ratio between mtDNA and nuclear DNA genes was normalized to the red mild group and used as an index of mitochondrial content. This method has been modified from a previously used technique to determine mitochondrial content in muscle (11).

**Gastrocnemius succinate dehydrogenase staining.** Succinate dehydrogenase (SDH) staining was performed as previously described to characterize mitochondrial enzyme function in the gastrocnemius muscle (42). Sectioning of muscle was performed the same as described previously (38). Briefly, transverse sections (10 μm) were cut from the midbelly of the medial gastrocnemius on a cryostat at −20°C, and slides were stored at −80°C until SDH staining was performed. The frozen sections were dried at room temperature for 10 min. Sections were incubated in a solution made up of 0.2 M phosphate buffer (pH 7.4), 0.1 M MgCl₂, 0.2 M succinic acid, and 2.4 mM nitroblue tetrazolium at 37°C for 45 min. The sections were then washed in deionized water for 3 min, dehydrated in 50% ethanol for 2 min, and mounted for viewing with mounting media. Digital photographs were taken from each section at a ×200 magnification with a Nikon spot camera, and fibers were traced with imaging software (Scion Image, Frederick, MD). Approximately 200 fibers/animal were traced at a ×20 magnification in a blinded fashion. The percentage of SDH positive fibers was then determined based on a criteria integrated optical density value and categorized as stained or nonstained.

**RNA isolation, cDNA synthesis, and real-time PCR.** RNA isolation, cDNA synthesis, and real-time PCR was performed as previously described (6), using reagents from Applied Biosystems (Foster City, CA). Quantitative real-time PCR analysis was carried out in 25-μl reactions consisting of 2× SYBR green PCR buffer (AmpliTaq Gold DNA Polymerase, Buffer, dNTP mix, AmplErase UNG, MgCl₂), 0.1 μl cDNA, 0.5 μM forward, and 60 μM of each primer. The sequence for the primers were as follows: PGC-1α forward: 5′-AGG ACG GAT TGC CCT CAT TT-3′; PGC-1 reverse: 5′-AGT GCT AAG ACC GCT GCA TT-3′; 18S forward: 5′-AAA CCG CTA CCA CAT CCA AG-3′; 18S reverse: 5′-CCC TCT TAA TCA TGG CCT CA-3′. Fluorescence-labeled probes Sirt1 (FAM dye), Mn1 (FAM dye), Mn2 (FAM dye), Fix1 (FAM dye), MHC IIA (FAM dye), MHC IIB (FAM dye), catalase (FAM dye), mnSOD (FAM dye), and the ribosomal RNA 18s (VIC dye) were purchased from Applied Biosystems and quantified with TaqMan Universal mastermix. The \( 2^{-\Delta\Delta Ct} \) method (34) was used to determine changes in gene expression between treatment groups with the 18S Ct as the correction factor.

**Western blot analysis.** Western blot analysis was performed as previously described (38). Briefly, frozen gastrocnemius muscles were homogenized in Mueller buffer and protein concentration determined by the Bradford method (13). Crude muscle homogenate (40 μg) was fractionated on 8%-12% SDS-polyacrylamide gels. Gels were transferred to PVDF membranes overnight. Membranes were Ponceau stained to verify equal loading of each gel. Membranes were blocked overnight in 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T). Primary antibodies for cytochrome-c oxidase complex subunit IV (Cox IV; 17 kDa), cytochrome c (14 kDa), Stat3, phospho Stat3 (86 kDa) (Cell Signaling), PGC-1α (90 kDa) (Santa Cruz Biotechnology), UC3P (33 kDa) (Thermo Scientific), 4-hydroxynonenal (4-HNE; Alpha Diagnostic), Sirt1 (120 kDa) (Santa Cruz Biotechnology), and nitrotyrosine (Sigma) were diluted 1:500 to 1:1,000 in 5% milk in TBS-T followed by a 1-h incubation at room temperature. Anti-rabbit or anti-mouse IgG horseradish peroxidase conjugated secondary antibodies (Cell Signaling) were incubated with the membranes at 1:2,000 dilutions for 1 h in 5% milk in TBS-T. Enhanced chemiluminescence (GE Healthcare Life Sciences, Piscataway, NJ) was used to visualize the antibody-antigen interactions. Images were digitally scanned, and blots were quantified by densitometry using scientific imaging software (Scion Image, Frederick, MD).

**Slot blot.** To detect nitrated proteins in muscle, a slot blot technique was used as previously described (15). Briefly, 5 μg of whole muscle homogenate was transferred to a PVDF membrane using a Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA) following the manufacturer’s instructions. The membrane was then probed with a nitrotyrosine antibody as described above.

**Statistical analysis.** A two-way ANOVA was used to determine differences between cachexia severity (body weight × mild × severe) and muscle fiber type (red gastrocnemius × white gastrocnemius × soleus). A repeated-measures, two-way ANOVA was used to determine differences in body weight and plasma IL-6. Post hoc analyses were performed with Student-Newman-Keuls methods. A Pearson correlation was used to draw correlations between the mtDNA-to-nuclear DNA ratio in the red gastrocnemius muscle and percentage body weight loss in ApcMin/+ mice. In addition, correlations were used to show the relationship between circulating IL-6, body weight, and large polyphs. One-way ANOVAs or independent Student’s t-tests were used to determine significance for all other variables. Significance was set at \( P < 0.05 \).

## RESULTS

**Body, muscle, and fat pad weight.** ApcMin/+ mice undergo variable body weight, muscle mass, and fat mass loss, which is initiated between 12 and 14 wk of age, and the loss is proportional to the animal’s intestinal/colon tumor burden and circulating IL-6 levels (6). We report that the current cohort of ApcMin/+ mice in this study also demonstrated variable body weight loss. While WT mice and mildly cachectic ApcMin/+ mice had similar body weights throughout the study (supplemental Fig. 1), ApcMin/+ mice that developed severe cachexia had significantly reduced body weights by 16 wk of age and continued to weigh less throughout the remainder of the 20-wk study (supplemental Fig. 1). However, tibia lengths were not different between WT, mildly cachetic, or severely cachetic ApcMin/+ mice (Table 1). At 20 wk of age, the severely cachetic mice had a 20% body weight reduction from their 12-wk body wt (Table 1), while the mild group remained weight stable, and the WT mice were gaining body mass. The gastrocnemius muscles from severely cachetic mice were reduced 41% \( (P < 0.001) \), and the soleus muscles were reduced by 34% \( (P = <0.001; \text{Table 1}) \) compared with mildly cachetic mice. Severely cachetic mice also had a complete loss of epididymal fat pad weight \((P < 0.001; \text{Table 1})\).

**Muscle oxidative metabolism.** The red portion of the lateral head of the gastrocnemius muscle, a white portion of the gastrocnemius muscle, and soleus muscles from WT and ApcMin/+ mice were examined for markers of oxidative metabolism. The gastrocnemius muscle was cut into red and white portions at the time of dissection. \( \text{(See } \text{Tissue collection in methods).} \) There was not sufficient soleus muscle available to examine mitochondrial DNA content, so only the red and white portions of the gastrocnemius were examined. As expected, there was a main effect of muscle type on the mitochondria DNA-to-nuclear DNA ratio, with the red gastrocnemius muscle having approximately three- to fourfold greater mitochondrial content than the white gastrocnemius muscle, regardless...
of cachexia status (Fig. 1A). There was also a main effect of cachexia severity (WT vs. mild vs. severe) on mitochondrial content across the red and white gastrocnemius muscles. Muscle mitochondrial protein expression was also examined. Cytochrome c and Cox IV protein expression was highest in the soleus muscle from WT and mildly cachectic mice, and the white gastrocnemius muscle had threefold lower expression in these mice (Fig. 1, B and C). Cytochrome c and Cox IV Protein expression was significantly reduced in all muscles from severely cachectic mice, with cachectic soleus expression being reduced to levels comparable to the white gastrocnemius muscle. The red gastrocnemius mitochondrial DNA-to-nuclear DNA ratio from all ApcMin/H11001 mice was significantly correlated with the degree of body weight loss from 12 to 20 wk of age (Fig. 1D). Sections across the midbelly of whole gastrocnemius muscles indicated that there was a 50% reduction in the frequency of SDH-positive fibers with severe cachexia (Fig. 1E). Interestingly, these metabolic changes were independent of alteration in frequency of gastrocnemius IIA and IIB expressing fibers (supplemental Fig. 3) or the expression of myosin IIA or IIB mRNA expression (supplemental Fig. 4, A and B). These data demonstrate that cancer alone is not sufficient to initiate a reduction in muscle oxidative capacity in ApcMin/H11001 mice, but there is a strong relationship between the

Table 1. Body weight change, muscle weights, and epididymal fat pad weights in 20-wk-old wild-type and ApcMin/+ mice

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>12 wk, g</th>
<th>20 wk, g</th>
<th>Change, %</th>
<th>Gastroc, mg</th>
<th>Soleus, mg</th>
<th>Epi Fat, mg</th>
<th>TL, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>8</td>
<td>25.3 ± 0.05</td>
<td>28.8 ± 0.01</td>
<td>9.2 ± 0.08</td>
<td>150 ± 3</td>
<td>9.6 ± 0.03</td>
<td>445 ± 32</td>
<td>17.5 ± 0.02</td>
</tr>
<tr>
<td>Mild</td>
<td>6</td>
<td>26.5 ± 1.1</td>
<td>25.8 ± 1.2</td>
<td>0.7 ± 1.1†</td>
<td>135 ± 7</td>
<td>9.6 ± 0.06</td>
<td>334 ± 50†</td>
<td>17.3 ± 0.01</td>
</tr>
<tr>
<td>Severe</td>
<td>8</td>
<td>26.4 ± 0.07</td>
<td>18 ± 0.05*</td>
<td>−20 ± 3*</td>
<td>80 ± 5*</td>
<td>6.3 ± 0.03*</td>
<td>0 ± 0*</td>
<td>17.1 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE. Mild, ApcMin/+ mice having mild cachexia symptoms at 20 wk of age; Severe, ApcMin/+ mice having severe cachexia symptoms at 20 wk of age; Gastroc, gastrocnemius muscle; soleus, soleus muscle; epi fat, epididymal fat pad; TL, tibia length. Significance was set at P < 0.05. †Different from wild-type group; *different from all other groups.

Fig. 1. Muscle oxidative metabolism in wild-type (WT, Wt) and ApcMin/+ mice with varying severity of cachexia. A: ratio of mitochondrial and nuclear DNA from either the red or white portion of the gastrocnemius muscle. Data are normalized to WT red gastrocnemius. R Gas, red gastrocnemius muscle; W Gas, white gastrocnemius muscle; ME, main effect. B, top: representative Western blot of cytochrome c protein expression in red and white portions of these mice (Fig. 1A and B). Cytochrome c and Cox IV Protein expression was significantly reduced in all muscles from severely cachectic mice, with cachectic soleus expression being reduced to levels comparable to the white gastrocnemius muscle. The red gastrocnemius mitochondrial DNA-to-nuclear DNA ratio from all ApcMin/+ mice was significantly correlated with the degree of body weight loss from 12 to 20 wk of age (Fig. 1D). Sections across the midbelly of whole gastrocnemius muscles indicated that there was a 50% reduction in the frequency of SDH-positive fibers with severe cachexia (Fig. 1E). Interestingly, these metabolic changes were independent of alteration in frequency of gastrocnemius IIA and IIB expressing fibers (supplemental Fig. 3) or the expression of myosin IIA or IIB mRNA expression (supplemental Fig. 4, A and B). These data demonstrate that cancer alone is not sufficient to initiate a reduction in muscle oxidative capacity in ApcMin/+ mice, but there is a strong relationship between the
degree of body weight loss with cancer and the loss of skeletal muscle oxidative capacity. This loss in muscle oxidative capacity with severe cachexia appears independent of the muscles' initial phenotype, as both red and white muscles were affected.

Mitochondrial biogenesis. Muscle PGC-1 and Sirt1 expression were examined as indices of mitochondrial biogenesis. Muscle PGC-1 mRNA and protein expression were highest in the soleus muscle from WT and mildly cachectic mice, and the white gastrocnemius muscle had significantly lower levels of expression (Fig. 2A and B). In all muscles from severely cachectic mice, PGC-1 mRNA expression was significantly reduced. PGC-1 protein followed the same pattern as the mRNA expression, except that protein PGC-1 protein levels were not significantly reduced in severely cachectic white gastrocnemius muscle, which may be related to the already extremely low level of expression. Sirt1 mRNA expression demonstrated a main effect of muscle type, with the soleus muscle having approximately sixfold greater mRNA expression than the white gastrocnemius muscle regardless of cachexia status (Fig. 2C). There was also a main effect of cachexia severity (WT vs. mild vs. severe) on Sirt1 mRNA expression. Sirt1 protein expression was not different between WT or mildly catechetic in any muscle type. However, Sirt1 protein expression was significantly reduced in the severely cachectic red and white gastrocnemius muscle. Interestingly, there was no reduction in Sirt1 mRNA or protein expression in severely cachectic white gastrocnemius muscle, which was distinct from the pattern of expression of other oxidative metabolism markers.

IL-6 and cachexia. We have previously published that the development of cachexia in the ApcMin/+ mouse is an IL-6-dependent process (6). The present study reports that circulating IL-6 levels at 14 wk of age were significantly elevated in ApcMin/+ mice destined to have severe cachexia, and IL-6 levels continued to increase through 20 wk of age (Fig. 3A). Plasma IL-6 levels were not detectable in WT mice at any age (data not shown), and ApcMin/+ mice classified with mild cachexia symptoms did not significantly increase plasma IL-6 levels during the study (Fig. 3A). Circulating IL-6 levels were significantly inversely correlated with ApcMin/+ mouse body weight at 20 wk of age (Fig. 3B), and also significantly correlated with the ApcMin/+ mouse intestinal tumor burden (Fig. 3C). There was no effect of muscle phenotype on the induction of muscle Stat3 phosphorylation with cachexia. However, muscle Stat3 phosphorylation was induced with severe cachexia across all three muscle types examined (Fig. 3D; main effect of severity). Muscle IL-6 mRNA expression was not induced in any muscle type examined, regardless of cachexia severity or underlying intestinal tumor burden (Fig. 3E).

We have previously published that systemic IL-6 overexpression in ApcMin/+ mice exhibiting stable body weight will accelerate cachexia development (6, 8). This includes the induction of body weight loss, gastrocnemius muscle mass loss, and a reduction in gastrocnemius IIB fiber cross-sectional area. The body and muscle weight values for the IL-6 overexpressing ApcMin/+ and WT mice used in this experiment have been previously published (6). ApcMin/+ overexpressing IL-6 for 4 wk experienced body weight loss of 11%, while control ApcMin/+ mice not overexpressing IL-6 had no loss in body weight over the same 4 wk period. The IL-6 accelerated cachexia was a more moderate body weight loss compared with the mice in our study classified with severe cachexia and having >20% weight loss. This experiment enabled the examination of muscle oxidative proteins in ApcMin/+ mice.
with less severe cachexia. Four weeks of IL-6 overexpression in Apc<sup>Min</sup> mice significantly reduced whole gastrocnemius muscle PGC-1α protein by 44%, Cox IV protein by 57%, and cytochrome c protein by 39% compared with vector control gastrocnemius muscle. Data are normalized to WT red gastrocnemius, white gastrocnemius, and soleus muscles from WT and Apc<sup>Min</sup> mice. Muscle PGC-1, Cox IV, or cytochrome c protein expression were not different in WT mice overexpressing IL-6 for 10 wk (Fig. 3G).

Mitochondrial dynamics. The regulation of mitochondrial fission and fusion is critical to the maintenance of mitochondrial function in muscle (20). The mRNA expression for muscle fusion proteins Mfn1 and Mfn2 and fission protein Fis1 were examined in the red gastrocnemius, white gastrocnemius, and soleus muscles from WT and Apc<sup>Min</sup> mice having different severities of cachexia at 20 wk of age. There was a main effect of muscle type and cachexia severity on Mfn1 and Mfn2 mRNA expression. Basal muscle Mfn1 and Mfn2 mRNA expression was lowest in the red gastrocnemius and highest in the soleus muscle. Muscle Mfn1 and Mfn2 mRNA expression was decreased by cachexia severity across all muscle types (Fig. 4, A and B; main effect of severity). Muscle Mfn1 and Mfn2 mRNA expression was not different

Fig. 3. Circulating IL-6, muscle IL-6 mRNA expression, and muscle STAT3 signaling in WT and Apc<sup>Min</sup> mice with varying cachexia severity. A: plasma IL-6 levels at 10, 14, and 20 wk of age in Apc<sup>Min</sup> mice exhibiting mild or severe cachexia at 20 wk of age. B: correlation between plasma IL-6 level and body weight at 20 wk (n = 9). C: correlation between large polyps and plasma IL-6 (n = 14). D: top; representative Western blot of phosphorylated Stat3 (pStat3; TYR 705) and total Stat3 protein expression in red gastrocnemius, white gastrocnemius, and soleus muscles from WT and Apc<sup>Min</sup> mice. Bottom: pStat3-to-Stat3 ratio normalized to WT red gastrocnemius muscle. E: IL-6 mRNA expression in red gastrocnemius, white gastrocnemius, and soleus muscles from WT and Apc<sup>Min</sup> mice. Data are normalized to WT red gastrocnemius. F: effect of circulating IL-6 overexpression on gastrocnemius muscle oxidative (OXPHOS) protein expression in Apc<sup>Min</sup> mice. Top: representative Western blot of PGC-1, Cox IV, and cytochrome c. Bottom: PGC-1, Cox IV, and cytochrome c protein expression normalized to vector control. Values are means ± SE. ND, not detectable; †different from mild group; ‡different from all other weeks within the severely cachectic groups; $different from control vector.

Mitochondrial dynamics. The regulation of mitochondrial fission and fusion is critical to the maintenance of mitochondrial function in muscle (20). The mRNA expression for muscle fusion proteins Mfn1 and Mfn2 and fission protein Fis1 were examined in the red gastrocnemius, white gastrocnemius, and soleus muscles from WT and Apc<sup>Min</sup> mice having different severities of cachexia at 20 wk of age. There was a main effect of muscle type and cachexia severity on Mfn1 and Mfn2 mRNA expression. Basal muscle Mfn1 and Mfn2 mRNA expression was lowest in the red gastrocnemius and highest in the soleus muscle. Muscle Mfn1 and Mfn2 mRNA expression was decreased by cachexia severity across all muscle types (Fig. 4, A and B; main effect of severity). Muscle Mfn1 and Mfn2 mRNA expression was not different
between WT and mildly cachectic Apc<sup>Min/+</sup> mice. Mitochondrial fission protein Fis1 mRNA did not demonstrate altered expression related to muscle type, as was found with Mfn1/ Mfn2. However, there was a main effect of cachexia severity across all muscle groups to increase muscle Fis1 mRNA expression (Fig. 4C).

**Muscle oxidative stress.** 4-HNE and nitrotyrosine protein modifications were examined to determine oxidative stress in the red gastrocnemius, white gastrocnemius, and soleus muscles during cachexia. There was a significant main effect of muscle type on the expression of 4-HNE modified proteins, with the red gastrocnemius having the highest expression regardless of cachexia (Fig. 5A). There was no effect of muscle type on the expression of nitrotyrosine modified proteins, across all muscle types. The expression of these modified proteins was reduced with severe cachexia (Fig. 5A and B).

Cachexia severity reduced uncoupling protein 3 (UCP-3) protein expression in all muscle types (Fig. 5C). Although red and white gastrocnemius muscle UCP-3 expression did not differ between WT and mild Apc<sup>Min/+</sup> mice, UCP-3 protein expression was reduced in the mild Apc<sup>Min/+</sup> mouse soleus muscle compared with WT. We also measured the expression of catalase and MnSOD mRNA in the different muscle types (Fig. 5, D and E). There was a main effect of muscle type on catalase and MnSOD expression, but no effect of cachexia severity.

**DISCUSSION**

Many chronic illnesses that induce wasting also reduce muscle oxidative capacity. However, the implications of this altered metabolic capacity in the overall wasting process remain poorly defined. Skeletal muscle’s capacity for oxidative metabolism has been implicated as a factor for determining a muscle’s sensitivity to inflammatory, metabolic, and aging-related challenges (4, 19, 32, 59). To our knowledge, this is the first study to report that during IL-6-dependent cancer cachexia, skeletal muscle oxidative capacity is reduced, and this occurs in both glycolytic and oxidative skeletal muscles. We further demonstrate that the loss of oxidative capacity is associated with repressed signaling related to mitochondrial biogenesis, and an altered expression of mitochondrial fission/fusion mRNA. Although increased production of ROS is thought to be an important regulator of muscle protein catabolism with some wasting diseases, we demonstrate that indicators of skeletal muscle oxidative stress are suppressed during the progression of cancer cachexia. We also report that the reduction in muscle oxidative capacity is not dependent on altered myosin expression, which suggests that these metabolic changes with cachexia are not aligned with an overall shift in muscle phenotype.

An important question related to muscle wasting with several disease states is how a muscle’s capacity for oxidative metabolism influences its catabolic susceptibility (32, 59). We report here that the reduction of muscle mass in our tumor-bearing mice is associated with a loss of overall muscle oxidative capacity in both primarily red and white hindlimb skeletal muscle. Thus, the initial, precachectic oxidative capacity of muscle does not appear protective against the loss of oxidative metabolism with the progression of cancer cachexia. This change with cachexia does not appear to be due to any inherent defects in Apc<sup>Min/+</sup> mouse muscle metabolism. Citrate synthase activity in skeletal muscle from 12-wk-old Apc<sup>Min/+</sup> mice is similar to WT mice, and these mice show normal exercise-induced increases in citrate synthase activity after several weeks of treadmill or wheel-running exercise (39). It is important to understand the contribution of disuse and inactivity brought on by the disease vs. disease-related mechanisms that specifically target mitochondria loss. We have previously found that Apc<sup>Min/+</sup> mouse voluntary wheel-running activity is gradually reduced just prior to and during the acceleration of cachexia (6). This suggests that increased muscle contractile activity could be beneficial for maintaining muscle oxidative capacity and possibly muscle mass during early stages of cancer cachexia. It is likely that decreased activity contributes in some way to the reduction in oxidative capacity. However, the decreased mitochondrial content in glycolytic muscle, combined with the lack of change in muscle myosin isofrom expression, presents evidence for the possibility of direct cancer-related mechanisms acting to reduce muscle oxidative...
capacity. Future work is warranted to understand specific metabolic changes occurring in type I muscle fibers with the onset and progression of cancer cachexia.

The mitochondrion is an organelle that has been reported to be in a perpetually dynamic state, balancing between biogenesis, fission/fusion dynamics, and autophagy (26). This dynamic balance appears to be required for the maintenance of proper mitochondrial function (61). A disruption of any or a combination of these processes during cachexia could serve to diminish muscle oxidative capacity. PGC-1α’s function as a transcription factor involved in regulating muscle oxidative capacity and mitochondrial biogenesis has been well described (47, 49). PGC-1α has recently been shown to be involved in the regulation of various other cellular processes that are directly or indirectly related to cellular energy status. These processes are related to protein degradation (48), mTOR activity (17), apoptosis (2), and regulation of ROS (53). We report that PGC-1α expression is suppressed in cachectic hindlimb muscles with both oxidative and glycolytic phenotypes, and this coincides with a reduction in muscle oxidative capacity and muscle wasting. Decreased PGC-1α expression has been found in many conditions that produce muscle wasting (40). A reduction in PGC-1α was observed in muscle from patients with diabetes (40, 44, 50, 60) and congestive heart failure (57). Reductions in PGC-1α are also seen in humans and mice fed a high-fat diet (16, 52) and obese mice (16). PGC-1α knockout mice have reduced mitochondrial content in white and red muscles, altered mitochondrial function, and increased susceptibility to apoptosis (2). Further work is needed to determine whether PGC-1α expression is a critical regulatory event for wasting of glycolytic and oxidative skeletal muscle. PGC-1α activity is enhanced by deactylation...
involving Sirt1. Interestingly, when examining the soleus muscle, we found that Sirt1 mRNA expression was not as clearly associated with reduced oxidative capacity and muscle wasting. Sirt1 mRNA expression was not repressed in the wasting soleus that also had a reduction in oxidative capacity. PGC-1α inhibition of FOXO-induced expression of atrogenes atrogin1 and murf1 has an important impact on muscle degradation processes (48). We have previously reported an increase in atrogin1 during cachexia in the \(\text{Ap}^\text{Min+/−}\) mouse (8). Together, the loss of PGC-1α during muscle wasting may lead to several catabolic pathways, which means PGC-1α could be a critical target for maintenance of muscle mass during wasting conditions.

The coordination of mitochondrial fission and fusion, referred to as mitochondrial dynamics, has been recently identified as critical components of mitochondrial function, morphology, and distribution (20). Fusion proteins Mfn1 and Mfn2 can promote mitochondrial elongation and activity, and regulate mitochondrial membrane potential and glucose oxidation in cultured cells (45). Our data show a reduction in Mfn1 and Mfn2 mRNA expression in severely cachectic \(\text{Ap}^\text{Min+/−}\) mice, regardless of initial muscle oxidative capacity. A reduction in Mfn2 has been observed in muscle from type 2 diabetic (5, 25) and obese patients (5). However, patients with chronic heart failure and weight loss did not show a change in muscle Mfn2 (23). TNF-α and IL-6 can reduce Mfn2 expression in cultured cells (5). Interestingly, Mfn2 gene expression has been shown to be controlled by PGC-1α and PGC-1β in conjunction with the estrogen-related receptor-α (33, 51). In the present study, the reduction in PGC-1α seen in our severely cachectic mice may be responsible for the decrease in Mfn1 and Mfn2 expression. Furthermore, the loss of fusion proteins may predispose the mitochondria to undergo fragmentation and become predisposed to apoptosis (54). Related to the regulation of mitochondrial fission, our data shows an increase in Fis1 mRNA expression in hindlimb muscles from severely cachectic mice. Fis1 overexpression is proapoptotic (29, 31, 43). Apoptosis during wasting diseases is currently unknown, but Fis1 overexpression is proapoptotic (29, 31, 43). Apoptosis in skeletal muscle is commonly observed during cancer cachexia (10, 22), and we have also seen apoptosis induced in cachectic \(\text{Ap}^\text{Min+/−}\) mouse hindlimb muscle (7). In the \(\text{Ap}^\text{Min+/−}\) mouse, muscle Fis1 expression may be inducing apoptosis providing an additional mechanism for linking muscle mass loss to mitochondrial content.

A major focus of mitochondria-associated cachexia research has centered on the generation of ROS and their role in the regulation of muscle wasting (41). There is a prevailing theory that glycolytic fibers preferentially waste because of increased oxidative stress occurring may be insufficient to produce oxidative damage. Although it remains to be determined whether the generation of ROS is important for the initiation of cachexia, a role for ROS in late stages of severe muscle mass loss during IL-6-dependent cancer cachexia has not been established.

**Perspectives and Significance**

We demonstrate that cancer-induced muscle mass loss coincides with a reduction in the expression of proteins related to oxidative metabolism and mitochondrial biogenesis in both red and white mouse hindlimb muscles. Therefore, inherent oxidative capacity is not protective against the loss of either mass or oxidative metabolic capacity with cancer. Furthermore, the loss of muscle oxidative metabolic capacity is related to the degree of wasting incurred with cancer. Clinical application of our data supports the examination of pharmaceutical, nutritional, and/or contractile activity treatments for the maintenance of muscle oxidative capacity. The use of these treatments may have potential as a preventative or therapeutic measure for patients to attenuate cancer-induced muscle wasting. In addition, preservation of muscle oxidative capacity could lead to increased tolerance for daily physical activity, which would improve their quality of life. Future mechanistic work is also needed to determine whether the loss of oxidative capacity is a key mediator of cancer cachexia-induced muscle mass loss and whether it plays a regulatory role for the induction of myonuclei apoptosis and protein degradation processes.

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**DISCLAIMER**

The contents of this paper are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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