Muscle mTORC1 suppression by IL-6 during cancer cachexia: a role for AMPK

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Cancer cachexia is associated with the unintentional loss of body weight that includes skeletal muscle mass loss (12). The disruption of anabolic and catabolic processes that regulate muscle protein turnover are a primary mechanism for the loss of muscle (31). The underlying perturbations causing altered protein turnover during cachexia appear complex and can vary with the stage and severity of body weight loss. Cachexia’s multifactorial nature produces systemic disorders that include chronic systemic inflammation, insulin resistance, anemia, hypogonadism, and anorexia (15). Fasting rates of muscle protein synthesis are suppressed with cachexia in rodents (38) and humans (24). Although the suppression of muscle protein degradation has been a focus of anticachexia therapies, the restoration of muscle protein synthesis during cachexia remains elusive (9, 17, 38). Further investigation is warranted to determine mechanisms contributing to the suppression of muscle anabolic signaling during cachexia.

An imbalance in muscle protein turnover is well established during cancer cachexia (16). The mTOR complex 1 (mTORC1) exerts regulatory control over muscle protein synthesis, and regulation of this complex by insulin/IGF-I signaling has been widely examined (18). We have reported that there is progressive reduction in fasted IGF-I/mTORC1 signaling during cachexia development in ApcMin/+ mice (38). In other rodent cachexia models, exogenous insulin or IGF-I treatment has not attenuated muscle wasting (11, 30) and suggest that mTOR suppression during cachexia is complex. There is also evidence that cachectic muscle can have a diminished capacity for anabolic signaling, as perturbations in muscle protein turnover have been reported in cancer patients in response to feeding (24, 40). The capacity to activate skeletal muscle mTOR signaling during the progression of cachexia in the ApcMin/+ mouse has not been determined.

5’-Adenosine monophosphate-activated protein kinase (AMPK) is an established mTOR inhibitor (4, 26) and a known upstream target of IL-6 signaling (19, 35). AMPK can also activate atrogen expression in skeletal muscle (32). These facts make AMPK worthy of investigation for the disruption of protein turnover regulation with cachexia (28). With the progression of cachexia, muscle AMPK activity increases in the ApcMin/+ mouse (27, 38). Interestingly, we have shown that exercise training can attenuate the development of cachexia in conjunction with inhibition of IL-6-induced AMPK activation (27). Despite a strong association between AMPK activation and muscle wasting severity, the role of AMPK or other mTORC1 regulators during the progression of cancer cachexia is currently unknown. Further delineation of this regulation can lead to potential targets for anticachexia therapies.

There is strong evidence that muscle IL-6/STAT signaling activation has a regulatory role in muscle wasting with many mouse cancer cachexia models (5, 7), including the ApcMin/+ mouse (1). IL-6 acts through binding the IL-6 receptor (IL-6r), which binds the glycoprotein 130 receptor (gp130r), forming a complex that activates intracellular signaling. The JAK/STAT pathway is the best-understood IL-6 signaling pathway (14). STAT3 inhibition can prevent muscle wasting in tumor-bearing mice through protein degradation inhibition (5). We have demonstrated that systemic IL-6 signaling inhibition can suppress STAT3 activation during late-stage cachexia, but this treatment could not rescue suppressed muscle protein synthesis (38). These findings suggest that muscle mTOR signaling has additional regulation disrupted during IL-6-dependent cachexia. IL-6 signaling regulation of muscle protein synthesis during the progression of cancer cachexia is poorly understood and warrants further investigation. The purpose of this study was to examine mTORC1 plasticity during cancer cachexia and to determine IL-6 regulation of muscle mTORC1 activity. mTOR activation was examined in ApcMin/+ mice and in C2C12
myotubes. We hypothesized that cachectic muscle would have an IL-6-dependent suppression of mTOR activation and that it would be dependent on muscle AMPK activation and be independent of STAT3 signaling.

**METHODS**

**Animals.** ApcMin/+ mice on a C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the University of South Carolina’s animal resource facility as previously described (23). Fifteen ApcMin/+ mice were stratified into groups based on circulating IL-6 concentrations after 2 wk of IL-6 overexpression: None (n = 4), Moderate (n = 6) and High (n = 5). Two experiments were conducted to administer to mice a bolus of IL-6 overexpression. The first used 18-wk-old wild-type (n = 6/group) and ApcMin/+ mice (n = 6/group), and the second used 14-wk-old ApcMin/+ mice after 2 wk of IL-6 overexpression (n = 7/group) (see IL-6 overexpression below). A subset of wild-type and ApcMin/+ mice was exercised (see Treadmill protocol below) or served as cage controls (n = 6/group). 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 (Rodent Diet no. 8604; Harlan-Teklad, Madison, WI) and water ad libitum. All animal experimentation was approved by the University of South Carolina’s Institutional Animal Care and Use Committee.

**Glucose administration.** Glucose (2 mg/kg ip) was injected 30 min prior to euthanasia based on the peak insulin secretion during a glucose tolerance test (GTT) (27). Glucose measurements were taken before glucose injection and at death to ensure injection accuracy.

**IL-6 overexpression.** In vivo intramuscular electroporation of an IL-6 plasmid was used to increase circulating IL-6 levels in mice as previously described (27). The quadriceps muscle was used as a vessel to produce IL-6 and secrete it into circulation and was not used for any analyses in the study. The gastrocnemius muscle used in the study was not subjected to electroporation. Briefly, mice were injected with 50 μg of the IL-6 plasmid driven by the CMV promoter, or empty control vector, into the quadriceps muscle. Mice were anesthetized with a 2% mixture of isoflurane and oxygen (1 l/min). The leg was shaved, and a small incision was made over the quadriceps muscle. Fat was dissected away from the muscle, and the plasmids were injected in a 50-μl volume of phosphate-buffered saline (PBS). 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. Both vector control and IL-6 groups received the appropriate plasmid starting at 12 wk of age. Mice were euthanized after 2 wk after IL-6 overexpression.

**Plasma insulin and IL-6.** Fasting blood samples were collected under brief isoflurane anesthetization from the retroorbital eye sinus and centrifuged at 10,000 g for 10 min at 4°C. Plasma was collected and stored at −80°C until analysis. Commercial ELISA kits for insulin (Millipore, Billerica, MA) and plasma IL-6 levels (Biosource, Carlsbad, CA) were measured according to the manufacturer’s instructions.

**Glucose.** Blood glucose measurements were performed using a handheld glucometer (Bayer CONTOUR) according to the manufacturer’s instructions.

**Tissue collection.** Mice were given a subcutaneous injection of ketamine-xylazine-acepromazine cocktail (1.4 ml/kg body wt sc) before the gastrocnemius was dissected. The gastrocnemius muscles were rinsed in PBS, snap-frozen in liquid nitrogen, weighed, and stored at −80°C until further analysis.

**Treadmill protocol.** At 5 wk of age, mice were grouped into either exercise or cage control at which time they started their training as previously described (27) with 3 days of acclimation. Briefly, acclimation consisted of running at a 5% grade for a total of 20 min with gradual increase in speed starting at 10 m/min and increasing to 18 m/min. After the 3 days of acclimation, mice started on a training regimen that consisted of a 5-min warmup at 10 m/min at 5% grade followed by 55 min of running at 18 m/min at 5% grade. Mice were encouraged to run by gentle taps. Mice ran 6 days a week and were given 1 day of recovery. After electroporation at 12 wk, the mice received a 2-day break from exercise before starting again. Mice ran until 14 wk of age, when they were euthanized.

**Myofibrillar protein synthesis.** Myofibrillar protein synthesis measurements were performed as previously described (36, 38, 39). Gastrocnemius muscle samples were homogenized in 1 ml of water. Myofibrils and other insoluble proteins were pelleted by centrifugation, and the supernatants containing free amino acids were used to determine the ratio of free [3H]phenylalanine (m/z 239 fragment) to endogenous (unlabeled) phenylalanine (m/z 234 fragment). The ratios were determined by GC-mass spectrometric analysis of the r-butylidimethylsilyl derivatives of these amino acids. Myofibrillar proteins were washed, hydrolyzed, and analyzed for [3H]phenylalanine enrichment by monitoring the m/z 237 and 239 fragments.

The fractional rate of myofibrillar synthesis, percent per day, was calculated as the percent enrichment of tracer in the hydrolysate of myofibrillar protein divided by the tracer enrichment in the free amino acid pool of muscle tissue. Myofibrillar protein enrichment was determined from the m/z 237 and m/z 239 ions because the lightest isotopomer (m/z 234) saturated the MS detector. The myofibrillar-to-free enrichment ratio was multiplied by 48 to obtain percent per day values because tracer incorporation occurred over a period of 30 min.

**C2C12 cell culture.** C2C12 myoblasts purchased from the American Type Culture Collection (Manassas, VA) were cultured in DMEM supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Upon reaching confluence, myoblast differentiation was induced for 72 h in DMEM supplemented with 2% heat-inactivated horse serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. For IL-6 treatment, cells were differentiated for 72 h and then treated with recombinant IL-6 (Sigma) with 0, 5, or 20 ng/ml for 24 h. A subset of myotubes were treated with insulin (20 nM, Sigma) for 15 min after 24 h of IL-6 (20 ng/ml) treatment. The STAT inhibitor pyrrolidine dithiocarbamate (PTDC, 20 μM) was administered along with (20 ng/ml) IL-6 for 24 h. Experiments using the AMPK inhibitor Compound C (20 μM, Sigma) were also combined with (20 ng/ml) IL-6 for 24 h. Cells were harvested by washing with ice-cold PBS and then scraping in ice-cold lysis buffer [50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM NaF, 1 mM β-glycerophosphate, 1 mM NaVO_3_ and 1/200 protease inhibitor cocktail (Sigma, P8340, pH 8.0)]. After sonication, cell debris was removed by centrifugation, and the supernatant was stored at −80°C. Protein concentrations were measured by the Bradford assay (Bio-Rad), and the samples were used for Western blot analysis.

**RNA isolation, cDNA synthesis, and real-time PCR.** RNA isolation, cDNA synthesis, and real-time PCR were performed as previously described (37), 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, AmpErerase UNG, MgCl_2_), 0.1 μl of cDNA, RNase-free water, and 60 nM of each primer. Fluorescence labeled probes for IGFI-1 (FAM dye) and the ribosomal RNA 18s (VIC dye) were purchased from Applied Biosystems and quantified with TaqMan Universal Master mix. Data were analyzed by ABI software using the cycle threshold (Ct), which is the cycle number at which the fluorescence emission is midway between detection and saturation of the reaction.

**Western blotting.** Western blot analysis was performed as previously described (23). Briefly, frozen gastrocnemius muscle was homogenized in Mueller buffer, and protein concentration was determined by the Bradford method (8). Crude muscle homogenate (40 μg) was fractionated on 8–10% SDS-polyacrylamide gels. Gels were transferred to PVDF membranes overnight. Membranes were Ponceau stained before.
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 p-Akt (Ser473), Akt, p-eIF4E-binding protein-1 (4EBP-1) (Thr37/46), 4EBP-1, p-mTOR (Ser2448), mTOR, p-p70S6K (Thr389), p70S6K, p-AMPK (Thr 172), AMPK, GAPDH, atrogin-1, ubiquitin (Cell signaling), p-FoxO3a (Ser253) (Millipore), FoxO (Cell signaling), and myosin heavy chain (Santa Cruz Biotechnology) were diluted 1:1,000 to 1:500 in 5% milk in TBS-T followed by 1-h incubation with membranes at room temperature. Anti-rabbit 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 (ECL; 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).

Fig. 1. IL-6-induced suppression of muscle protein synthesis (MPS) and related mTOR complex 1 (mTORC1) signaling are associated with increased STAT3 and AMPK activation. A: body weight change during the 2-wk treatment period. Gastrocnemius muscle weight (B) and muscle protein synthesis (C) in ApcMin/+ mice stratified by circulating IL-6 concentrations. D, top: representative Western blot of total and phosphorylated mTOR, 4EBP-1, and p70S6K in the gastrocnemius; bottom: quantification of the ratio of phosphorylated to total forms of mTOR, 4EBP-1, and p70S6K. E. IGF-I mRNA expression. F: correlation between muscle protein synthesis and plasma IL-6. G: correlation between mTOR phosphorylation and muscle protein synthesis. H, top: representative Western blot of total and phosphorylated STAT3 and AMPK in the gastrocnemius; bottom: quantification of the ratio of phosphorylated STAT3 and AMPK. I: correlation between phosphorylated mTOR and phosphorylated AMPK. Values are means ± SE. Significance was set at P < 0.05. *Different from None group; †different from Moderate group.

Fig. 2. Effect of IL-6 dose on muscle protein turnover in C2C12 myotubes. A, top: representative Western blot of total and phosphorylated STAT3 protein expression of myotubes treated with 0, 5, and 20 ng/ml IL-6; bottom: quantification of phosphorylated STAT3. B, top: representative Western blot of total and phosphorylated Akt, mTOR, and 4EBP-1 protein expression; bottom: quantification of phosphorylated Akt, mTOR, and 4EBP-1. C, top: representative Western blot of total and phosphorylated AMPK protein expression; bottom: quantification of phosphorylated AMPK. D, top: representative Western blot of total and phosphorylated FoxO3a, atrogin-1, and myosin heavy chain; bottom: quantification of phosphorylated FoxO3a, atrogin-1, and myosin heavy chain in C2C12 cells. E: C2C12 myotube diameter. Significance was set at P < 0.05. *Difference from 0 ng/ml treatment group; †difference from 5 ng/ml treatment group.

Fig. 3. Effect of IL-6 dose on muscle protein turnover in C2C12 myotubes. A, top: representative Western blot of total and phosphorylated STAT3 protein expression of myotubes treated with 0, 5, and 20 ng/ml IL-6; bottom: quantification of phosphorylated STAT3. B, top: representative Western blot of total and phosphorylated Akt, mTOR, and 4EBP-1 protein expression; bottom: quantification of phosphorylated Akt, mTOR, and 4EBP-1. C, top: representative Western blot of total and phosphorylated AMPK protein expression; bottom: quantification of phosphorylated AMPK. D, top: representative Western blot of total and phosphorylated FoxO3a, atrogin-1, and myosin heavy chain; bottom: quantification of phosphorylated FoxO3a, atrogin-1, and myosin heavy chain in C2C12 cells. E: C2C12 myotube diameter. Significance was set at P < 0.05. *Difference from None group; †different from Moderate group.
throughout the 18-wk time course. Two-way ANOVA was used to determine significance for circulating glucose, insulin, and IL-6 and protein synthesis and related signaling in the glucose administration experiment. A two-way ANOVA was also used for all data from the exercise experiment. A one-way ANOVA was used to determine differences in all other experiments throughout the paper. A Pearson correlation test was used in all correlations shown throughout the paper. Post hoc analyses were performed with Student-Newman-Keuls methods. Significance was set at \( P < 0.05 \).

RESULTS

IL-6 levels correlate with reduction in muscle protein synthesis and suppression of mTORC1 signaling. IL-6 overexpression has been previously shown to accelerate the progression of cachexia in \( \text{Apc}^{\text{Min}+/+} \) mice (2, 27), and mTOR activity is suppressed during the progression of cachexia (38). The purpose of this experiment was to examine the effect of different circulating levels of IL-6 on muscle mTOR signaling. Circulating IL-6 was overexpressed in \( \text{Apc}^{\text{Min}+/+} \) mice, and the mice were stratified into three groups based on circulating IL-6 levels: no circulating IL-6 (None), Moderate (32.1 \pm 7.45 pg/ml), and High (115.2 \pm 14 pg/ml). The moderate- and high-expressing mice showed 4% and 14% losses in body weight, respectively (Fig. 1A) during the 2 wk of IL-6 overexpression, whereas \( \text{Apc}^{\text{Min}+/+} \) mice expressing no IL-6 were weight stable. The changes in body weight corresponded with a 7% (\( P = 0.1 \)) and a 16% decrease in gastrocnemius muscle mass (Fig. 1B) in moderate and high expressers, respectively, compared with mice with no circulating IL-6. Both myofibrillar protein synthesis (Fig. 1C) and muscle IGF-I expression (Fig. 1E) were decreased, by 46 and 29% in mice with moderate circulating IL-6, respectively. Muscle protein synthesis (Fig. 1C) and muscle IGF-I mRNA (Fig. 1E) were further decreased in mice with high IL-6. Mice with moderate circulating IL-6 levels had a reduction in mTOR phosphorylation along with mTOR targets p70S6K and 4EBP-1 (Fig. 1D). Phosphorylation of these proteins was reduced further in mice with high circulating IL-6 levels. There was a negative correlation between muscle protein synthesis and circulating IL-6 (Fig. 1F) and a positive correlation between mTOR phosphorylation and muscle protein synthesis (Fig. 1G). IL-6 signaling targets STAT3 and AMPK were increased in a dose-dependent manner; however, the induction of phosphorylated AMPK failed to reach significance in the moderate expressers (Fig. 1H). There was a strong negative correlation between phosphorylation of AMPK and phosphorylation of mTOR (Fig. 1I).

Effect of IL-6 on Akt/mTOR signaling in C2C12 myotubes. After observing an IL-6 dose-dependent reduction in muscle mTORC1 signaling in the \( \text{Apc}^{\text{Min}+/+} \) mice, we next wanted to examine direct effects of IL-6 on C2C12 myotubes. Activation of IL-6 signaling was evident by increased STAT3 phosphorylation in a dose-dependent manner, inducing a twofold increase with the low dose (5 ng/ml) and a roughly threefold change with the high dose (20 ng/ml) (Fig. 2A). Akt phosphorylation was unaffected by IL-6 treatment, whereas mTOR and 4EBP-1 phosphorylations were reduced by IL-6 (Fig. 2B). AMPK phosphorylation was increased nearly fourfold (Fig. 2C) with the high dose of IL-6, whereas FoxO3a phosphorylation was reduced 40% (\( P = 0.04 \); Fig. 2D) during IL-6 treatment. IL-6 treatment also induced the expression of the FoxO transcriptional target atrogin-1 and corresponded to a reduction in myosin heavy chain (Fig. 2D). The impairment in protein turnover during IL-6 treatment was further demonstrated by the reduction in myotube diameter after 24 h of IL-6 treatment at 20 ng/ml (Fig. 2E).

Cachectic mice have a diminished capacity to activate mTORC1 signaling after a bolus of glucose. We (38) previously showed a progressive reduction in mTORC1 signaling throughout the progression of cachexia associated with the induction in AMPK and STAT3 activation. The current experiment was performed to examine the capacity to activate mTORC1 signaling during cachexia. Cachetic \( \text{Apc}^{\text{Min}+/+} \) mice (18 wk) showed a reduction in body weight associated with an increase in circulating glucose, insulin, and IL-6 compared with wild-type mice (Table 1). In addition, \( \text{Apc}^{\text{Min}+/+} \) mice displayed impaired glucose clearance after a GTT (Table 1). A bolus of glucose was administered to activate mTORC1 signaling in wild-type and \( \text{Apc}^{\text{Min}+/+} \) mice. Glucose administration increased circulating glucose and insulin to a similar extent between wild-type and \( \text{Apc}^{\text{Min}+/+} \) mice (Table 1). At baseline, cachexia increased Akt phosphorylation (Ser473) and suppressed mTOR phosphorylation (Ser2448) (Fig. 3A), which was indicative of observed suppression of muscle protein synthesis (Fig. 3B). In response to systemic glucose administration, muscle insulin-related signaling was increased in wild-type mice as Akt, mTOR, and p70S6K phosphorylation increased roughly twofold, whereas no change was observed for FoxO3a phosphorylation (Fig. 3A). In cachetic mice, glucose administration was able to increase Akt, mTOR, and p70S6K phosphorylation. However, cachexia suppressed the extent of mTOR and p70 phosphorylation compared with the wild-type re-

Table 1. Glucose, insulin, and IL-6 concentrations before and after glucose injections in wild-type and \( \text{Apc}^{\text{Min}+/+} \) mice

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>( \text{Apc}^{\text{Min}+/+} )</th>
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<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>Glucose</td>
</tr>
<tr>
<td>Body weight</td>
<td>27.2 ± 1.4</td>
<td>27.5 ± 0.87</td>
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<tr>
<td>IL-6, pg/ml</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>GTT (AUC)</td>
<td>27.025 ± 3.019</td>
<td>31.394 ± 4.194</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>Pre 116 ± 15</td>
<td>112 ± 8</td>
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<tr>
<td></td>
<td>Post 124 ± 39</td>
<td>384 ± 28*</td>
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<tr>
<td>Insulin, ng/ml</td>
<td>Pre 0.56 ± 0.06</td>
<td>0.58 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Post 0.57 ± 0.07</td>
<td>1.4 ± 0.09*</td>
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Values are means ± SE; \( n = 5 \) in each group. GTT, glucose tolerance test. Significance was set at \( P < 0.05 \). *Different from the Pre group; †main effect of genotype when compared within same treatment.

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response. FoxO3a phosphorylation was reduced during cachexia and did not increase with glucose administration (Fig. 3A). Regardless of cachexia, the rate of muscle protein synthesis was not induced 30 min after glucose administration (Fig. 3B). Glucose administration deceased AMPK phosphorylation in both wild-type and Apc<sup>Min/+</sup> mice; however, AMPK phosphorylation remained elevated in the Apc<sup>Min/+</sup> mice after glucose administration compared with wild-type mice (Fig. 3C). A subset of Apc<sup>Min/+</sup> mice were given a glucose bolus after 2 wk of IL-6 overexpression to determine the activation capacity of mTORC1 in mice with initial stages of cachexia (<5%) body weight loss. Both glucose and IL-6 resulted in a similar increase in Akt activity (Fig. 3D), with no additive effects on Akt activity in mice overexpressing IL-6 and treated with glucose. IL-6 overexpression reduced mTOR phosphorylation; however, glucose administration increased mTOR phosphorylation back to wild-type levels. Phosphorylation of mTOR target S6K was not affected by IL-6 overexpression yet increased over wild-type values with glucose administration.

To further explore the effects of IL-6 on insulin-induced activation of mTORC1 signaling, we dosed insulin on C2C12 myotubes that were treated with or without IL-6. IL-6 stimulation of myotube STAT3 phosphorylation was not affected by insulin administration (Fig. 4A). The pretreatment of myotubes with IL-6 resulted in a similar increase in Akt activity (Fig. 3D), with no additive effects on Akt activity in mice overexpressing IL-6 and treated with glucose. IL-6 overexpression reduced mTOR phosphorylation; however, glucose administration increased mTOR phosphorylation back to wild-type levels. Phosphorylation of mTOR target S6K was not affected by IL-6 overexpression yet increased over wild-type values with glucose administration.
with IL-6 did not affect insulin stimulation of Akt/mTOR signaling (Fig. 4B).

IL-6-induced inhibition of mTOR signaling is returned with AMPK inhibition. To elucidate possible mechanisms involved in IL-6-induced suppression of mTORC1 signaling, we inhibited signaling through STAT3 and AMPK, two known downstream mediators of IL-6 action. The STAT3 inhibitor PDTC successfully blocked STAT3 phosphorylation during IL-6 treatment, while IL-6-induced activation of AMPK was not affected (Fig. 5A). IL-6 treatment repressed mTOR and 4EBP-1 phosphorylation, which was not rescued with STAT3 inhibition (Fig. 5B). Administration of compound C, an AMPK inhibitor, to myotubes attenuated IL-6-induced STAT3 activation and abolished AMPK phosphorylation during IL-6 treatment (Fig. 5C). Treatment with compound C had no effect on Akt phosphorylation; however, phosphorylations of mTOR, 4EBP-1, and FoxO3a were returned to control levels (Fig. 5D). In addition to relieving the suppression on anabolic signaling, AMPK inhibition reduced IL-6-induced atrogin-1 and ubiquitinated protein expression (Fig. 5E).

IL-6 overexpression and treadmill exercise. We have recently that observed treadmill exercise can attenuate muscle wasting during IL-6 overexpression in the ApcMin/+ mouse (27). Associated with the reduction in wasting was a decrease in phosphorylated AMPK. Therefore, we wanted to investigate whether the anti-cachexia effects of exercise were, in part, through the maintenance of mTORC1 activity. Systemic IL-6 overexpression in ApcMin/+ mice decreased muscle IGF-I mRNA expression by 57%, which was rescued by exercise training (Fig. 6A). In contrast to IGF-I expression, Akt phosphorylation increased 64 and 49% with IL-6 overexpression independently of exercise training (Fig. 6B). However, mTOR, p70S6K, and 4EBP-1 phosphorylation decreased 46, 51, and 56%, respectively, with IL-6 overexpression (Fig. 6B). Exercise training restored phosphorylation of all proteins back to that of sedentary control ApcMin/+ mice.

**DISCUSSION**

During wasting conditions, the inability to activate anabolic signaling could contribute to a negative balance in protein turnover and acceleration of muscle mass loss. There is a sequential inhibition of mTOR activity and myofibrillar protein synthesis as cachexia progresses in the ApcMin/+ mouse (38). There is also a corresponding increase in circulating IL-6, muscle STAT3 activity, and AMPK activity with cachexia progression (38). Our current study extends these observations with the novel finding that IL-6 exerts a dose-dependent suppression of mTOR signaling in skeletal muscle. We also report that IL-6-induced suppression of mTOR in C2C12 myotubes can be restored with insulin treatment. However, during cachexia, the interaction of insulin and IL-6 signaling in ApcMin/+ mice is dependent on the severity of the cachexia. Glucose administration to cachectic ApcMin/+ mice failed to restore mTOR signaling. Precachectic ApcMin/+ mice overexpressing circulating IL-6 maintained the capacity to activate mTOR signaling with glucose administration. Furthermore, we demonstrate that STAT3 activity in myotubes is not required for IL-6-regulated mTOR suppression and AMPK activation. However, AMPK inhibition can rescue IL-6 suppression of mTOR signaling in myotubes. Last, we report that exercise training, which has been previously shown to decrease muscle AMPK activity in ApcMin/+ mice, can rescue IL-6-induced suppression of muscle mTOR signaling independently of muscle STAT activity.

IL-6 is associated with increased AMPK activity, reduction in mTORC1 signaling, and increased atrognere expression. We have previously found that regulation of muscle mTORC1 signaling can evolve with cachexia progression in ApcMin/+ mice and that it coincides with increasing levels of circulating
IL-6 (38). Furthermore, we identified a possible role of AMPK to inhibit mTORC1 during the development of cachexia. While others have shown AMPK activation to suppress mTOR signaling in muscle (41), the role of AMPK during cachexia remains unclear. Here, we observed a dose-dependent increase in activation of IL-6 targets STAT3 and AMPK phosphorylation in previously weight-stable ApcMin/H11001 mice. These changes were associated with a reduction in muscle protein synthesis, IGF-I mRNA, and mTOR signaling. As we previously observed (38), the suppression of mTOR during cachexia is independent of Akt activation. Moreover, we report a negative correlation between AMPK activity and mTORC1 phosphorylation. These results indicate that IL-6 overexpression in a previously weight-stable mouse can mimic the cellular signaling pattern of what is seen during later stages of cachexia. We followed up the in vivo studies to show the effect of IL-6 on muscle in vitro. C2C12 myotubes treated with IL-6 showed myotube atrophy associated with an increase in STAT3 and AMPK phosphorylation and a reduction in mTORC1 signaling. Similar to what we observed in vivo, IL-6 had no effect on Akt phosphorylation. Our data support previous findings that show IL-6 can cause a reduction in muscle protein synthesis in

Fig. 5. AMPK inhibition rescues mTOR activation during IL-6 treatment. A, top: representative Western blot of total and phosphorylated STAT3 and AMPK phosphorylation in C2C12 cells treated with IL-6 or IL-6 with the STAT3 inhibitor pyrrolidine dithiocarbamate (PDTC); bottom: ratio of phosphorylated to total STAT3 and AMPK. B, top: representative Western blot of total and phosphorylated mTOR and 4EBP-1 in C2C12 cells treated with IL-6 or IL-6 with PDTC; bottom: ratio of phosphorylated to total mTOR and 4EBP-1. C, top: representative Western blot of total and phosphorylated STAT3 and AMPK phosphorylation in C2C12 cells treated with IL-6 or IL-6 with the AMPK inhibitor compound C; bottom: ratio of phosphorylated to total STAT3 and AMPK. D, top: representative Western blot of total and phosphorylated Akt, mTOR, 4EBP-1, and FoxO3a in C2C12 cells treated with IL-6 or IL-6 with compound C (CC); bottom: ratio of phosphorylated to total Akt, mTOR, 4EBP-1, and FoxO3a. E, top: representative Western blot of atrogin-1 and polyubiquitinated proteins in C2C12 cells treated with IL-6 or IL-6 with compound C (CC); bottom: protein expression of atrogin-1 and polyubiquitinated proteins. Values are means ± SE. Significance was set at *P < 0.05. *Difference from 0 ng/ml group.
humans (33) and suppression of growth in mice (3, 22). However, this is the first study to report IL-6-induced suppression of mTOR in both in vivo and in vitro systems.

In addition to the suppression of mTOR signaling, we report IL-6 treatment on C2C12 myotubes induced markers of ubiquitin-dependent degradation. IL-6-treated myotubes had decreased FoxO3a phosphorylation, increased atrogin-1 expression, and an increase in polyubiquitinated proteins. These data are consistent with previous reports, which showed C2C12 myotubes treated with IL-6 had increased lysosomal and ubiquitin-related proteins and enzymatic activity (13). The induction of muscle protein degradation can also be regulated by AMPK activation (29). While FoxO3a phosphorylation is a target of Akt signaling, we report that FoxO3a phosphorylation was reduced in cachectic muscle after glucose administration. The inability of Akt to regulate FoxO3a during the progression of cachexia would promote muscle wasting, supporting the concept of alternative regulation of FoxO3a in cachectic muscle (38).

Glucose-induced activation of mTOR signaling is suppressed during cachexia. Although mTORC1 signaling in the ApcMin/+ mouse is suppressed in the fasted state, a question remains as to the capacity of mTORC1 to be activated by feeding. We show a blunted response to a bolus of glucose during cachexia, and this was once again independent of Akt activation. In fact, glucose administration to ApcMin/+ mice resulted in a further increase in Akt activity compared with PBS-treated ApcMin/+ mice. Our results are consistent with the inability of cachectic patients to increase muscle protein synthesis and related mTOR signaling with feeding (40). In contrast, Williams et al. (40) showed no change in muscle protein synthesis or mTOR signaling in the postprandial state between cachectic and control patients. As we have previously published (38), the extent of cachexia has a direct effect on postprandial muscle protein synthesis. The extent of body weight loss in the study by Williams et al. (xx) was undetermined and therefore cannot be accurately compared against our current data. As previously shown in the ApcMin/+ mouse, hyperphosphorylation of AMPK is evident during the later stages of cachexia (38). We found the glucose-induced decrease of AMPK activity in ApcMin/+ mice to be blunted compared with wild-type mice. Thus, chronic elevation of AMPK, as well as an inability to acutely suppress AMPK activation, may contribute to the inhibition of mTOR signaling during the progression to more severe cachexia. Interestingly, while our study confirms suppressed myofibrillar protein synthesis with the progression of cachexia, we did not detect any significant differences in muscle protein synthesis 30 min after glucose administration. We acknowledge that there is a limitation to the interpretation of these data, as the assay was not optimized to detect muscle protein synthesis changes. Further study is clearly required to determine whether glucose-induced activation of mTOR is sufficient to activate muscle protein synthesis, and also to determine the role of AMPK/mTOR regulation on either the induction or repression of muscle protein synthesis during the progression of cachexia. These data suggest that Akt phosphorylation is responsive to a glucose stimulus during cachexia. Additionally, this also suggests that in the fasted state alternative pathways other than the PI3K pathway are responsible for the increased Akt phosphorylation in severely cachectic muscle and may be due to increased mTORC2 activation (21).

Interestingly, glucose administration to weight-stable ApcMin/+ mice overexpressing systemic IL-6 could increase mTORC1 signaling similar to wild-type values. This proposes the idea of additional factors than just that IL-6 is responsible for the inhibition of mTOR signaling during cachexia. These factors could include, but are not limited to, increased FoxO3a phosphorylation, increased atrogin-1 expression, and an increase in polyubiquitinated proteins, which are consistent with previous reports (13). The induction of muscle protein degradation can also be regulated by AMPK activation (29). While FoxO3a phosphorylation is a target of Akt signaling, we report that FoxO3a phosphorylation was reduced in cachectic muscle after glucose administration.

Insulin activates mTOR signaling in C2C12 myotubes independently of IL-6 treatment. To determine whether we could see these effects in vitro, we treated C2C12 myotubes with insulin and/or IL-6 to ascertain what pathway would ultimately regulate mTOR activity. We found that insulin stimulation of Akt/mTORC1 signaling is maintained in the presence of high IL-6 levels, and IL-6 activation of STAT is not inhibited by insulin. The ability for insulin signaling to override IL-6-induced mTOR inhibition is similar to what we observed in
weight-stable ApcMin/+ mice overexpressing IL-6, once again suggesting differential interaction between insulin and IL-6 signaling during late-stage cachexia. The interaction between IL-6 and insulin signaling is complicated due to differential effects dependent on duration of IL-6 dose. Evidence suggests that transient increases in circulating IL-6, observed with an exercise bout, improves insulin sensitivity (10), whereas chronic IL-6 is associated with insulin resistance (20). Further work is warranted to determine what factor(s) regulates IL-6 biology during health and disease.

IL-6-induced suppression of mTOR is rescued with AMPK inhibition. IL-6-induced reduction in mTOR activation observed in vivo and in vitro has consistently been associated with increased STAT and AMPK activity. Recently, STAT inhibition has been shown to inhibit muscle atrophy during cachexia (6); however, the effect of STAT inhibition on anabolic signaling has not been described. To this end, we examined whether the canonical IL-6 pathway was necessary for the IL-6-induced repression of mTOR signaling in C2C12 myotubes. STAT3 inhibition did not rescue IL-6 suppression of mTOR signaling. IL-6-induced AMPK activation was also independent of STAT3 signaling. We have recently shown that inhibition of IL-6 signaling through an IL-6 receptor antibody attenuated the progression of cachexia in the ApcMin/+ mouse without rescuing the decrease in mTOR signaling despite an attenuation in STAT3 and AMPK activation (38). Interestingly, inhibition of AMPK was able to rescue mTOR signaling in C2C12 myotubes. As it relates to cachexia, these data suggest that activation of AMPK by high IL-6 levels could lead to the inhibition of mTOR signaling. In addition to rescuing mTOR signaling, AMPK inhibitor returned FOXO phosphorylation and decreased markers of ubiquitin-dependent proteolysis. Further study is warranted to examine whether IL-6 can inhibit mTOR through a STAT3-independent mechanism involving AMPK activation during the progression of cachexia in vivo.

Exercise increases mTOR signaling in the ApcMin/+ mouse during IL-6 overexpression. We have recently shown that treadmill exercise reduces AMPK activation in conjunction with attenuated loss in body weight and muscle mass during IL-6-induced cachexia (27). Furthermore, exercise training can preserve muscle mass despite increased STAT3 activation (27). The effect of exercise on mTOR activation during cachexia is unclear. In the current study, we show that endurance-based treadmill exercise could increase muscle IGF-I mRNA expression along with mTORC1 signaling, while Akt phosphorylation was increased during IL-6 overexpression regardless of exercise. Treadmill exercise has previously shown to reduce muscle protein degradation, yet it failed to improve muscle protein synthesis in a mouse model of chronic kidney disease (34). However, the same study reported an increase in Akt and FoxO phosphorylation in exercised muscle despite inactivation of mTOR and p70 (34). Moreover, exercise training did prevent muscle wasting in old rats, which was associated with increased mTOR phosphorylation (25). Together, the effect of exercise on muscle anabolic signaling remains equivocal, and further work is needed to determine whether exercise training during the initial onset of cachexia can improve mTOR signaling directly through a reduction in AMPK activity or indirectly through an alternative mechanism.

In conclusion, we report that IL-6 induces a dose-dependent suppression of mTOR signaling in skeletal muscle and cultured myotubes. Cachectic skeletal muscle also has a reduced capacity to increase anabolic signaling after glucose administration, which is independent of Akt activation and corresponds to an inability to suppress AMPK activation. In cultured myotubes, IL-6 does not affect insulin stimulation of Akt/mTOR signaling. Interestingly, IL-6 suppression of mTOR is independent of STAT3 activation and requires activation of AMPK. Last, treadmill exercise training was able to improve muscle mTOR signaling in tumor-bearing mice overexpressing systemic IL-6, and this effect was independent of muscle STAT3 activity.

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