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Inflammatory signalling regulates eccentric contraction-induced protein synthesis in cachectic skeletal muscle

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Abstract

Background Skeletal muscle responds to eccentric contractions (ECC) with an anabolic response that involves the induction of protein synthesis through the mechanistic target of rapamycin complex 1. While we have reported that repeated ECC bouts after cachexia initiation attenuated muscle mass loss and inflammatory signalling, cachectic muscle's capacity to induce protein synthesis in response to ECC has not been determined. Therefore, we examined cachectic muscle's ability to induce mechano-sensitive pathways and protein synthesis in response to an anabolic stimulus involving ECC and determined the role of muscle signal transducer and activator of transcription 3 (STAT3)/nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling on ECC-induced anabolic signalling.

Methods Mechano-sensitive pathways and anabolic signalling were examined immediately post or 3 h after a single ECC bout in cachectic male $Apc^{Min/+}$ mice (n = 17; 16 ± 1% body weight loss). Muscle STAT3/NFκB regulation of basal and ECC-induced anabolic signalling was also examined in an additional cohort of $Apc^{Min/+}$ mice (n = 10; 16 ± 1% body weight loss) that received pyrrolidine dithiocarbamate 24 h prior to a single ECC bout. In all experiments, the left tibialis anterior performed ECC while the right tibialis anterior served as intra-animal control. Data were analysed by Student's t-test or two-way repeated measures analysis of variance with Student-Newman-Keuls post-hoc when appropriate. The accepted level of significance was set at $P < 0.05$ for all analysis.

Results $Apc^{Min/+}$ mice exhibited a cachectic muscle signature demonstrated by perturbed proteostasis (Ribosomal Protein S6 (RPS6), P70S6K, Atrogin-1, and Muscle RING-finger protein-1 (MuRF1)), metabolic (adenosine monophosphate-activated protein kinase, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and Cytochrome c oxidase subunit IV (COXIV)), and inflammatory (STAT3, NFκB, extracellular signal-regulated kinases 1 and 2, and P38) signalling pathway regulation. Nonetheless, mechano-sensitive signalling pathways (P38, extracellular signal-regulated kinases 1 and 2, and Protein kinase B (AKT)) were activated immediately post-ECC irrespective of cachexia. While cachexia did not attenuate ECC-induced P70S6K activation, the protein synthesis induction remained suppressed compared with healthy controls. However, muscle STAT3/NFκB inhibition increased basal and ECC-induced protein synthesis in cachectic $Apc^{Min/+}$ mice.

Conclusions These studies demonstrate that mechano-sensitive signalling is maintained in cachectic skeletal muscle, but chronic STAT3/NFκB signalling serves to attenuate basal and ECC-induced protein synthesis.

Keywords $Apc^{Min/+}$; Cancer cachexia; Eccentric contractions; Interleukin-6; Muscle protein synthesis

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Introduction

Skeletal muscle mass depletion associated with cancer cachexia contributes to increased patient morbidity and mortality.[1,2] Skeletal muscle size is influenced by the dynamic balance between the rates of protein synthesis and breakdown,[3,4] and disrupted protein turnover accompanies cancer cachexia.[5–7] While our understanding of suppressed basal protein synthesis and activated breakdown during cachexia has increased dramatically,[8,9,7] we have a more limited understanding of how the cachectic environment affects skeletal muscle responsiveness to anabolic stimuli, which is clinically relevant for the treatment of the cachectic cancer patient. Resistance exercise is a potent anabolic stimulus that stimulates muscle hypertrophy through the activation of mechanistic target of rapamycin complex 1 (mTORC1) in healthy adults[10–12] and can attenuate skeletal muscle mass loss in several muscle wasting conditions.[13–15] Despite the clinical significance of maintaining or improving muscle mass during cancer, limited information currently exists on the cachectic muscle's anabolic response to resistance exercise.

Eccentric contractions (ECC) induced by high-frequency electrical stimulation have been used to examine signalling associated with muscle hypertrophy in rodents[16–19] and have demonstrated great utility for improving our mechanistic understanding of contraction-induced protein synthesis and mTORC1 signalling.[20,18,21,22] Related to cancer cachexia, evidence suggests that increased loading by synergist ablation or ECC can maintain muscle mass in tumour-bearing mice.[23–26] Indeed, we have reported that repeated ECC bouts after the initiation of cachexia can attenuate muscle mass loss through reduced inflammatory signalling[24]; however, cachectic muscle's capacity to induce protein synthesis in response to ECC has not been determined and warrants further investigation. We have found that mechano-activation of protein synthesis in stretched myotubes can be disrupted by conditioned media from Lewis lung carcinoma cells,[27] suggesting that tumour-derived cachectic factors can interfere with mechanical signalling inducing protein synthesis *in vitro*. While these studies demonstrate that skeletal muscle from tumour-bearing animals may be responsive to exercise training or loading, the regulation of protein synthesis by muscle contraction in the presence of a systemic cachectic environment requires further investigation.

Suppressed muscle protein synthesis and mTORC1 signalling are associated with interleukin-6 (IL-6) induction of signal transducer and activator of transcription 3 (STAT3), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and 5'-adenosine monophosphate-activated protein kinase (AMPK) in tumour-bearing mice.[28,29] Indeed, we have previously demonstrated an inverse relationship between plasma IL-6 and muscle protein synthesis during cachexia progression, and systemic IL-6 overexpression can suppress mTORC1 signalling in tumour-bearing mice.[28] In contrast, blocking muscle IL-6 signalling through STAT3 inhibition or glycoprotein 130 (gp130) receptor loss attenuated wasting in tumour-bearing mice.[30,31] Additionally, acute and chronic muscle STAT3/NFκB inhibition improved mTORC1 signalling in cachectic mice.[29,32] Lastly, extracellular signal-regulated kinases 1 and 2 (ERK1/2) and P38 mitogen-activated protein kinase (MAPK) inhibition restored myotube stretch-induced protein synthesis in the presence of Lewis lung carcinoma-derived cachectic factors.[27] Thus, there is a clear rationale that muscle inflammatory signalling involving STAT3/NFκB can disrupt basal and mechanical-induced regulation of protein synthesis during cancer cachexia. However, it is currently unknown if suppressed muscle protein synthesis and mTORC1 signalling can be activated in the cachectic environment. Therefore, we examined cachectic muscle's ability to induce protein synthesis in response to an anabolic stimulus involving ECC and determined the role of muscle STAT3/NFκB signalling on ECC-induced anabolic signalling. Interestingly, we report that mechano-sensitive signalling is maintained in cachectic skeletal muscle, but STAT3/NFκB signalling attenuates basal and ECC-induced protein synthesis.

Methods

Animals

Male $Apc^{Min/+}$ mice on a C57BL/6 background were originally purchased from Jackson Laboratories and bred at the University of South Carolina's Animal Resource Facility. Mice used in the current study were obtained from the investigators breeding colony in the Center for Colon Cancer Research Mouse Core. Mice were individually housed, kept on a 12:12 h light-dark cycle, and had access to standard rodent chow (cat#8604 Rodent Diet; Harlan Teklad) and water *ad libitum*. Body weight and food measurements were taken weekly, and the percentage body weight loss from peak body weight was calculated. Mice lacking the $Apc^{Min/+}$ mutation (C57BL/6) served as controls for all experiments. The University of South Carolina's Institutional Animal Care and Use Committee approved all animal experimentation in this study.

Experimental designs

Male C57BL/6 (n = 15) and $Apc^{Min/+}$ (n = 27) mice (20 weeks of age) were used to determine cachectic muscle's ability to induce mechano-sensitive pathways and protein synthesis in response to a single ECC bout. In the first experiment, C57BL/6 (n = 6) and $Apc^{Min/+}$ (n = 7) mice were sacrificed immediately following a single ECC bout. In the second experiment, C57BL/6 (n = 9) and $Apc^{Min/+}$ (n = 10) mice were sacrificed 3 h after a single ECC bout. In the third experiment,

an additional cohort of cachectic $Apc^{Min/+}$ mice (n = 10; 16 ± 1% body weight loss) received a single pyrrolidine dithiocarbamate (PDTC) treatment (10 mg/kg body weight; cat#: P8765; Sigma Aldrich) 24 h prior to a single ECC bout. We have previously found that this treatment paradigm can sufficiently lower muscle inflammatory signalling prior to muscle contraction.[32] In the current study, a single PDTC treatment did not alter total tumour number (86 ± 4; P = 0.91) or plasma IL-6 levels (44 ± 5; P = 0.71), as we have previously observed following short-term treatment after the initiation of cachexia.[29] In all experiments, mice were fasted 2 h prior to contraction and remained fasted until sacrifice (immediately or 3 h post). There were no differences in cachexia indices (e.g. body weight, muscle mass, and fat loss) between $Apc^{Min/+}$ mice in all experiments; therefore, general animal characteristics from each cohort are summarized in *Table* 1. Additionally, protein expression in the non-contracted, control leg from $Apc^{Min/+}$ mice in Experiments 1 and 2 was used to determine the cachectic muscle phenotype (*Figure* 1).

Eccentric contractions

Eccentric contractions of the left tibialis anterior (TA) muscle was induced by high-frequency electrical stimulation of the sciatic nerve as previously described with slight modifications.[16,24] Mice were anaesthetized via isoflurane (2% in O_2 with 1.5% maintenance), the left leg/hip region was shaved, and two needle electrodes were placed subcutaneously on the left leg to stimulate the sciatic nerve. Tetanic muscle contractions were generated using a Grass Stimulator (Model S88, Grass Instruments, Quincy, MA, USA) for 10 sets of six repetitions (100 Hz, 6–12 V, 1 ms duration). Ten seconds of rest was given between repetitions, and 50 s of rest was given between sets. The stimulation protocol recruits all motor units and results in net plantar flexion of the ankle.[33,34] The dorsiflexors (TA and EDL) undergo ECCs while the plantar flexors (gastrocnemius, soleus, and plantaris) perform concentric muscle contractions. In all experiments, the left TA performed ECC while the right TA served as intra-animal, non-contracted control. Our laboratory and others have demonstrated repeated ECC bouts, but not concentric contractions, can induce muscle and myofiber growth in rodents.[23,16,24,33] Thus, the TA was examined in all experiments. Mice were given an intraperitoneal injection of warm saline following the stimulation procedure and returned to cages upon complete recovery.

Table 1 C57BL/6 and $Apc^{Min/+}$ mice that performed a single eccentric contraction bout

	C57BL/6	$Apc^{Min/+}$
No. of mice	15	27
Body weight, g		
Peak	26.7 ± 0.7	24.7 ± 0.4
ECC	26.7 ± 0.7	20.7 ± 0.4[a, b]
% change	0 ± 0	−16 ± 0.6[a]
Tibialis anterior, mg	49 ± 0.7	28 ± 0.9[a]
Epididymal fat, mg	361 ± 44	3 ± 2[a]
Spleen, mg	71 ± 4	506 ± 21[a]
Testes, mg	201 ± 7	103 ± 8[a]
LABC, mg	88 ± 2	31 ± 2[a]
Seminal vesicle, mg	261 ± 8	32 ± 3[a]
Plasma IL-6, pg/mL	0 ± 0	43 ± 3[a]
Tumour number	0 ± 0	87 ± 4[a]
Tibia length, mm	16.9 ± 0.1	16.8 ± 0.1

Data are means ± standard error. There were no differences between control and stimulated TA muscles; therefore, the average is presented. A two-way repeated measures analysis of variance was used to determine differences between body weight at peak and immediately prior to ECC. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Student's t-test was used to determine differences in all other variables. Statistical significance was set at $P < 0.05$. ECC, eccentric contraction; g, grams; IL-6, interleukin-6; LABC, levator ani/bulbocavernosus muscle; mg, milligrams; mL, millilitre; mm, millimetre; No., number; pg, picogram.
[a]Significantly different from C57BL/6.
[b]Significantly different from difference from peak body weight.

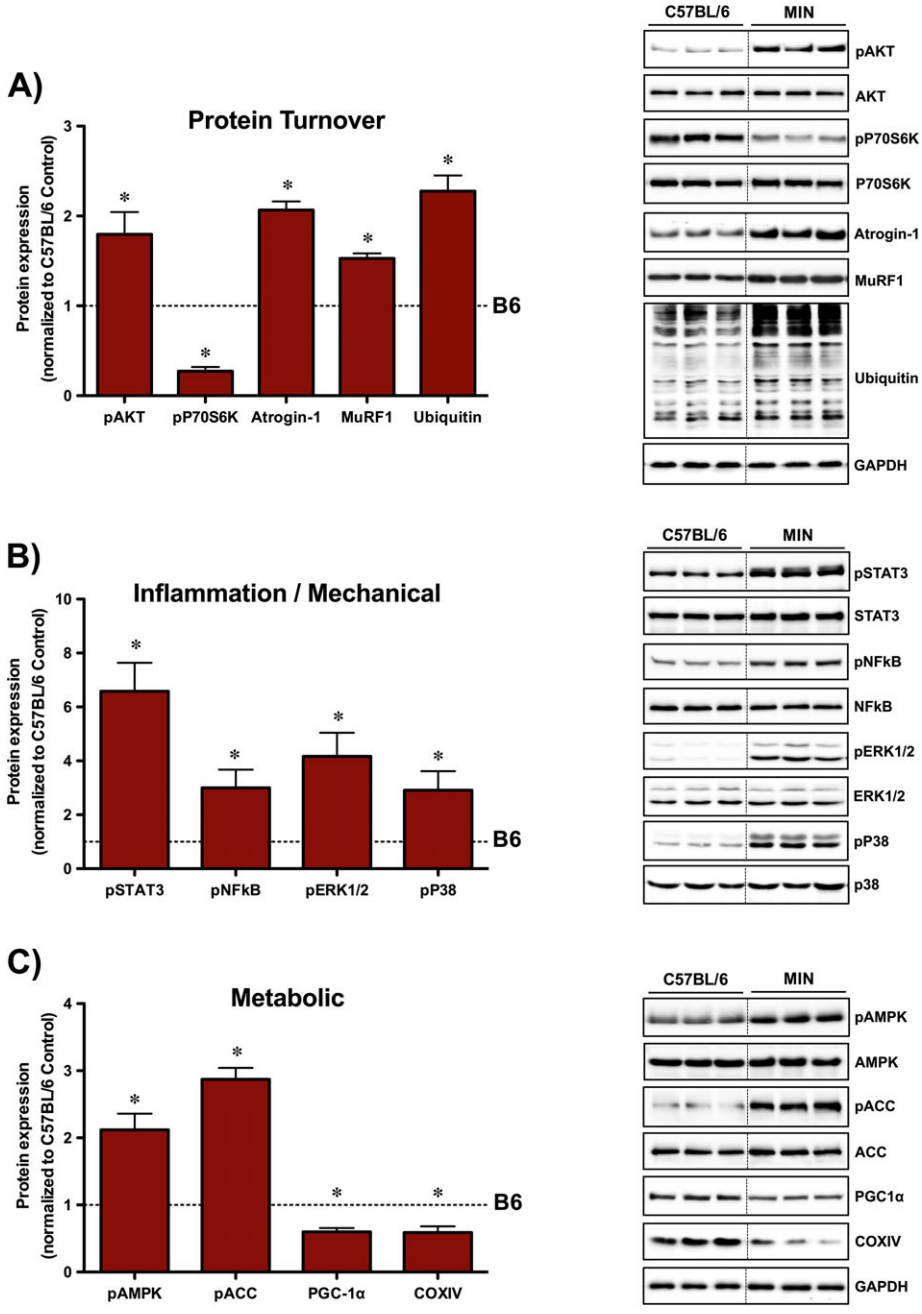
Tissue collection

Mice received an intraperitoneal injection of puromycin (0.04 μmol/kg body weight) 30 min prior to sacrifice.[35,29] Mice were anaesthetized with a subcutaneous injection of ketamine/xylazine/acepromazine cocktail (1.4 mL/kg body weight) at the time of sacrifice. Muscles and organs were rapidly excised, cleared of excess connective tissue, rinsed in phosphate-buffered saline, dried on blotting paper, weighed, and snap frozen in liquid nitrogen. Immediately prior to dissection, blood was collected via retro-orbital sinus with heparinized capillary tubes, placed on ice, and centrifuged (10 000 × g for 10 min at 4°C). The supernatant was removed and stored for plasma IL-6 analysis. Plasma and tissue samples were stored at −80°C until analysis.

Western blotting

Western blot analysis was performed as previously described.[36] Briefly, frozen TA muscle was homogenized in Mueller buffer, and protein concentration was determined by the Bradford method. Crude TA muscle homogenates were fractionated on 7–15% sodium dodecyl sulphate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were stained with Ponceau red to verify equal loading and transfer. Membranes were then blocked at room temperature for 1–2 h in 5% non-fat milk Tris-buffered saline with 0.1% Tween-20 (TBST). Primary antibodies for puromycin (Millipore, cat#MABE343, 1:2000), phospho P70S6K (T389) (cat#9205, 1:1000), total P70S6K (cat#2708, 1:1000), RPS6 (S240/244) (cat#2215, 1:500), total RPS6 (cat#2708, 1:1000), phospho Akt (S473)

Figure 1 Cachectic muscle phenotype. (A) Protein turnover regulation in C57BL/6 and $Apc^{Min/+}$ mice. (B) Inflammatory and mechano-sensitive pathways in C57BL/6 and $Apc^{Min/+}$ mice. (C) Metabolic signalling regulation in C57BL/6 and $Apc^{Min/+}$ mice. Tibialis anterior protein expression was examined in the non-contracted, control muscle. The activation of signalling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to C57BL/6 Control values. Dotted lines indicate that images were cropped for representative purposes. Data are means ± standard error; n = 15; C57BL/6, n = 17; $Apc^{Min/+}$. Student's t-test was used to determine differences between C57BL/6 and $Apc^{Min/+}$ mice. Statistical significance was set at $P < 0.05$. *, Significantly different from C57BL/6; ACC, Acetyl-CoA carboxylase; AMPK, adenosine monophosphate-activated protein kinase; ERK1/2, extracellular signal-regulated kinases 1 and 2; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; STAT3, signal transducer and activator of transcription 3.



(cat#4060, 1:1000), total Akt (cat#9272, 1:2000), phospho NFκB (S563) (cat#3033, 1:500), total NFκB (cat#4764, 1:2000), phospho STAT3 (Y750) (cat# 9145, 1:1000), total STAT3 (cat#4904, 1:2000), phospho AMPK (T172) (cat#, 1:2000), total AMPK (cat#2603, 1:1000), phospho Acetyl-CoA carboxylase (ACC) (S59) (cat#3661, 1:1000), total ACC (cat#3662), phospho ERK1/2 (T202/Y204) (cat#4370,1:1000), total ERK1/2 (cat#4695, 1:1000), PGC-1α (Abcam,

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cat#ab54481, 1:1000), COXIV (cat#4844, 1:1000), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (cat#2118, 1:10000), MuRF1 (ECM Biosciences, cat#MP3401, 1:2000), Atrogin-1 (ECM Biosciences, cat#AP2041, 1:5000), and ubiquitin (cat#3933, 1:2000) were incubated overnight in 5% TBST milk. We have previously validated the specificity of this PGC-1α antibody in tibialis anterior skeletal muscle through somatic gene transfer of empty or PGC-1α overexpression plasmid (data not shown). Membranes were then incubated in 5% milk-TBST containing anti-rabbit (cat#7074, 1:5000) or anti-mouse (cat#7076, 1:5000) IgG horseradish-peroxidase conjugated secondary antibodies for 1 h at room temperature. Exceptions to the aforementioned procedures were that for puromycin incorporation 1% BSA-TBST was used for primary antibody and horseradish-peroxidase conjugated rabbit anti-mouse IgG2a antibody (LifeTechnologies, cat#610220, 1:5000) in 5% milk-TBST was used for secondary antibody. All antibodies were from Cell Signalling Technology unless otherwise stated. Tibialis anterior protein extracts from a mouse that did not receive puromycin at sacrifice were included on all puromycin gels as a negative control. Enhanced chemiluminescence (GE Healthcare Life Sciences) was used to visualize the antibody-antigen interactions. Immunoblot images were collected using a digital imager (G:BOX Chemi XX6, Syngene, Frederick, MD, USA) and quantified by densitometry using imaging software (Image J; NIH). Each gel contained samples from all groups, and data were normalized to the respective control group (e.g. C57BL/6 control).

Plasma interleukin-6 concentration

Plasma IL-6 concentrations were determined as previously described.[37] A commercially available IL-6 enzyme-linked immunosorbent assay kit was obtained from BD Biosciences, and the manufacturer's protocol was followed. Briefly, clear 96-well plates were coated and incubated overnight with an IL-6 capture antibody. The next morning, the plate was blocked with assay diluent buffer and washed, and equal volumes of standards and plasma samples were added in duplicate. After a 2 h incubation, the plate was washed, and sAV-HRP reagent was added to each well. After several washes, TMB substrate was added, and the reaction was developed for 20 min. The reaction was stopped with sulfuric acid, and absorbance was read at 450 nm using an iMark microplate absorbance reader (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Results are reported as the means ± standard error. A repeated-measures two-way analysis of variance was performed to determine differences between cancer cachexia and eccentric contractions in C57BL/6 and $Apc^{Min/+}$ mice. Post-hoc analyses were performed with Student-Newman-Keuls methods when appropriate. Student's *t*-test was used to determined differences between two groups when appropriate. The accepted level of significance was set at $P < 0.05$ for all analysis. Statistical analysis and figure generation were performed using Prism 5 for Mac OS X (GraphPad Software Inc, La Jolla, CA, USA).

Results

Systemic and muscle cachectic phenotype

In order to determine if wasting skeletal muscle could respond to a novel ECC bout, we first established the cachectic phenotype in two separate cohorts of male $Apc^{Min/+}$ mice. $Apc^{Min/+}$ mice displayed several key features of severe cachexia that included body weight loss, muscle atrophy, adipose tissue depletion, high tumour burden, elevated plasma IL-6 levels, and hypogonadal features (levator ani-bulbocavernosus and seminal vesicle atrophy) (*Table* 1). Body weights at sacrifice were lower when compared with C57BL/6 mice, and significant body weight loss from peak measurement was observed within $Apc^{Min/+}$ mice. Body weight loss was accompanied by reduced TA muscle mass and epididymal fat loss in $Apc^{Min/+}$ mice. Cachexia increased spleen weight and plasma IL-6 compared with C57BL/6 mice (*Table* 1). There were no differences in tibia length between C57BL/6 and $Apc^{Min/+}$ mice. To further characterize the cachectic phenotype, protein expression related to protein turnover, inflammation, and metabolism was examined in the non-contracted control TA muscle (*Figure* 1). Cachectic muscle demonstrated perturbed protein turnover regulation by disrupted mTORC1 signalling (increased Akt and decreased P70S6K), E3 ligase expression (Atrogin-1 and MuRF1), and total ubiquitin (*Figure* 1A). Moreover, cachexia activated inflammatory/mechanical related proteins (STAT3, NFκB, ERK1/2, and P38) (*Figure* 1B). Lastly, cachexia altered the expression of proteins related to mitochondrial content (PGC-1α and COXIV) and metabolic regulation (AMPK and ACC), respectively (*Figure* 1C). Overall, these findings demonstrate that severe cachexia in this cohort of mice was associated with high circulating IL-6, enhanced muscle inflammatory signalling, and disrupted anabolic and metabolic regulation.

Mechanical signalling response to eccentric contractions

Having established the severe cachectic phenotype in this cohort of male $Apc^{Min/+}$ mice, we then examined mechanosensitive pathways immediately following a single ECC bout

(*Figure* 2A). First, we examined two MAPKs that have been shown to be activated immediately post-ECC.[18,21] While cachexia increased basal P38 and ERK1/2 phosphorylation in the non-contracted control TA muscle, ECC induced their activation irrespective of cachexia (*Figure* 2B). While absolute P38 and ERK1/2 phosphorylation were greater following ECC during cachexia, there were no differences in the degree of activation from the non-contracted TA muscle. Given that ECCs are a potent stimulator of Akt/mTORC1 signalling,[18,21,38] we next determined Akt/mTORC1 activation through phosphorylation of Akt and the downstream mTORC1 target P70S6K. While cachexia increased basal Akt phosphorylation in the non-contracted control TA muscle, ECC induced its activation irrespective of cachexia (*Figure* 2C). Interestingly, while cachexia decreased basal P70S6K phosphorylation in the non-contracted control TA muscle, ECC induced its activation irrespective of cachexia (*Figure* 2C). While absolute P70S6K phosphorylation was decreased following ECC during cachexia, there were no differences in the degree of activation from the non-contracted control TA muscle. We further validated P70S6K activation through the phosphorylation of the direct P70S6K target RPS6. Similarly, cachexia decreased basal RPS6 phosphorylation in the non-contracted control TA muscle, while ECC induced its activation irrespective of cachexia (*Figure* 2C). Collectively, these findings demonstrate that mechano-sensitive pathways were induced immediately post-ECC despite disrupted basal Akt/mTORC1 signalling in cachectic $Apc^{Min/+}$ mice.

Proteolytic, metabolic, and inflammatory signalling response to eccentric contractions

Given that the ubiquitin-proteasome pathway has been implicated in the proteolytic response to acute resistance exercise,[39] we examined the expression of two E3 ligases immediately following a single ECC bout. While ECC induced Atrogin-1 and MuRF1 protein expression in C57BL/6 mice, this was not observed in $Apc^{Min/+}$ mice (*Figure* 2D). Given the potential role of metabolic stress to inhibit anabolic processes, we next examined AMPK and its direct downstream target ACC in response to ECC. Interestingly, while the absolute AMPK and ACC phosphorylation were greater immediately post-ECC during cachexia, there were no differences in the degree of activation from the non-contracted TA muscle (*Figure* 2E). Lastly, we examined muscle inflammatory signalling pathways that have been implicated in cachexia and muscle contraction. Interestingly, STAT3 and NFκB were activated immediately post-ECC in C57BL/6 (*Figure* 2F). While basal STAT3 and NFκB were induced by cachexia, only NFκB was further induced immediately post-ECC in $Apc^{Min/+}$ mice (*Figure* 2F). Collectively, these findings demonstrate that mechano-sensitive pathways remained intact and was not associated with an exacerbated metabolic and proteolytic response immediately post-ECC in cachectic $Apc^{Min/+}$ mice.

Cachectic muscle anabolic signalling response to eccentric contractions

Having established the cachectic muscle's mechano-sensitive response to a single ECC bout, we then determined if cachexia disrupted ECC-induced protein synthesis and mTORC1 signalling. Therefore, anabolic signalling was examined 3 h after a single ECC bout (*Figure* 3A). While cachexia suppressed protein synthesis, ECC activated protein synthesis irrespective of cachexia. Although the relative induction by ECC was not altered by cachexia, the absolute protein synthesis rate remained suppressed relative to C57BL/6 mice (*Figure* 3B). We then examined several upstream regulators and downstream targets implicated in the mechanical activation of mTORC1 signalling. Interestingly, while cachexia increased basal P38 and ERK1/2 phosphorylation, these mechano-sensitive signalling molecules were not altered 3 h post-ECC irrespective of cachexia (*Figure* 3C). We then examined Akt/mTORC1 activation in response to ECC. While Akt was not altered by ECC regardless of cachexia, there was a strong trend ($P = 0.07$) for ECC to decrease Akt phosphorylation in C57BL/6 mice (*Figure* 3D). While cachexia decreased the phosphorylation of P70S6K, ECC induced its activation irrespective of cachexia (*Figure* 3D). ECC also increased RPS6 phosphorylation irrespective of cachexia (*Figure* 3D). Collectively, these findings demonstrate that protein synthesis and mTORC1 signalling was induced 3 h post-ECC in severely cachectic $Apc^{Min/+}$ mice.

Cachectic muscle proteolytic, metabolic, and inflammatory response to eccentric contractions

Muscle protein breakdown, metabolic dysfunction, and enhanced inflammatory signalling have established roles during cancer-induced muscle wasting, and these same signalling pathways are perturbed in response to muscle contraction.[40–43] ECC did not alter the expression of Atrogin-1 and MuRF1 irrespective of cachexia (*Figure* 4A). We have previously shown that the sustained activation of AMPK coincided with suppressed mTORC1 signalling 3 h after a single bout of concentric muscle contractions.[32] Interestingly, while AMPK and ACC were activated by cachexia, ECC decreased their activation irrespective of cachexia (*Figure* 4B). Lastly, we found that both STAT3 and NFκB phosphorylation were induced by cachexia and were further increased by ECC irrespective of cachexia (*Figure* 4C). Collectively, these data demonstrate that muscle metabolic and

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inflammatory signalling molecules were sensitive to both cachexia and ECC.

Muscle inflammatory signalling regulation of basal and eccentric contractions-induced protein synthesis

Given that muscle STAT3 and NFκB signalling was induced by cachexia and ECC, we next examined its involvement in the regulation of basal and ECC-induced protein synthesis in cachectic $Apc^{Min/+}$ mice. To accomplish this, we used an established pharmacological approach to lower basal muscle inflammatory signalling prior to ECC.[29,32] This experimental paradigm has previously been used by our laboratory to lower muscle STAT3/NFκB signalling prior to a single bout of low-frequency electrical stimulation.[32] Therefore, we administered PDTC 24 h prior to a single ECC bout in cachectic $Apc^{Min/+}$ mice (*Figure* 5A). As expected, PDTC decreased basal STAT3 and NFκB phosphorylation in the non-stimulated control TA

Figure 2 Muscle mechano-sensitive signalling immediately post-eccentric contractions (ECC). (A) Experimental design. C57BL/6 and $Apc^{Min/+}$ mice were sacrificed immediately post-ECC. Mice were fasted 2 h prior to contraction. (B) Muscle mitogen-activated protein kinase signalling regulation by ECC in C57BL/6 and $Apc^{Min/+}$ mice. (C) Muscle Akt/mechanistic target of rapamycin complex 1 signalling regulation by ECC in C57BL/6 and $Apc^{Min/+}$ mice. (D) Muscle proteolytic regulation by ECC in C57BL/6 and $Apc^{Min/+}$ mice. (E) Muscle metabolic signalling regulation in C57BL/6 and $Apc^{Min/+}$ mice. (F) Muscle inflammatory signalling regulation by ECC in C57BL/6 and $Apc^{Min/+}$ mice. The activation of signalling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to C57BL/6 Control values. Dotted lines indicate that images were cropped for representative purposes. Data are means ± standard error; $n = 6$; C57BL/6, $n = 7$; $Apc^{Min/+}$. A two-way repeated measures analysis of variance was used to determine differences between treatment groups. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at $P < 0.05$. Different letters are statistically different. &, Main effect of ECC; #, main effect of $Apc^{Min/+}$; ERK1/2, extracellular signal-regulated kinases 1 and 2; TA, tibialis anterior.

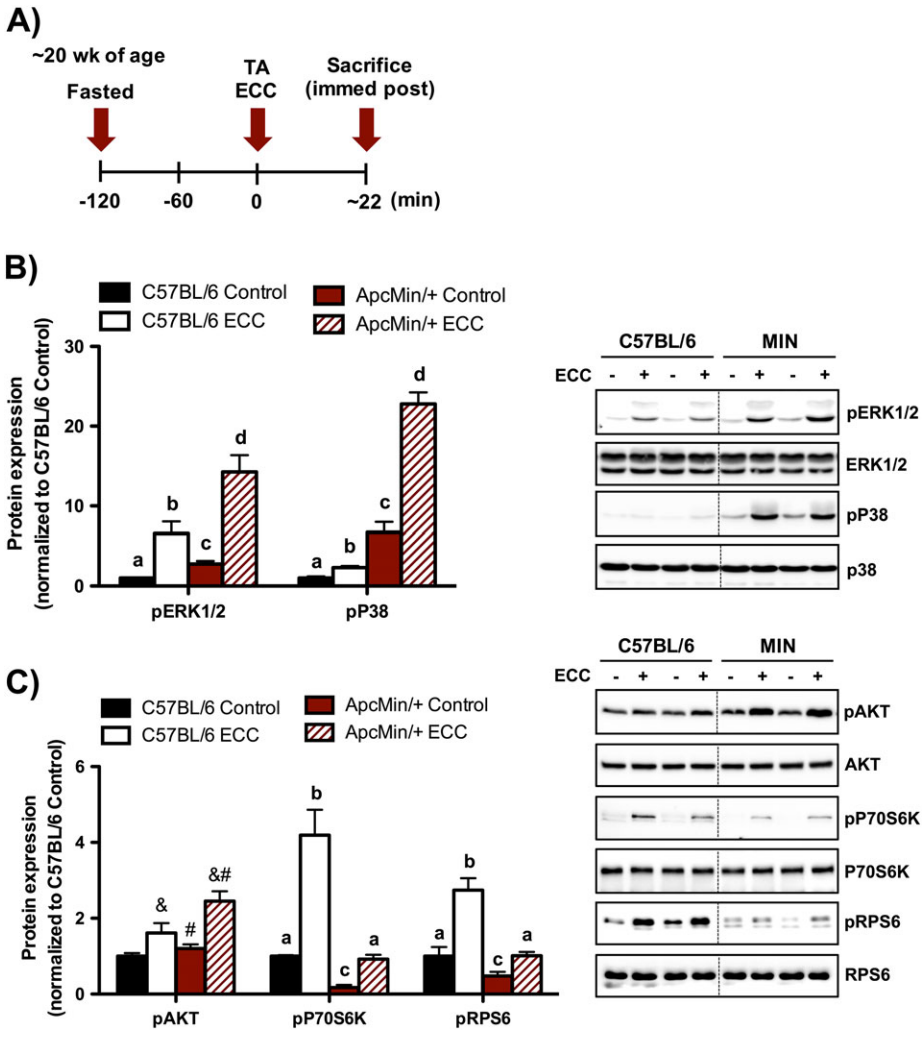

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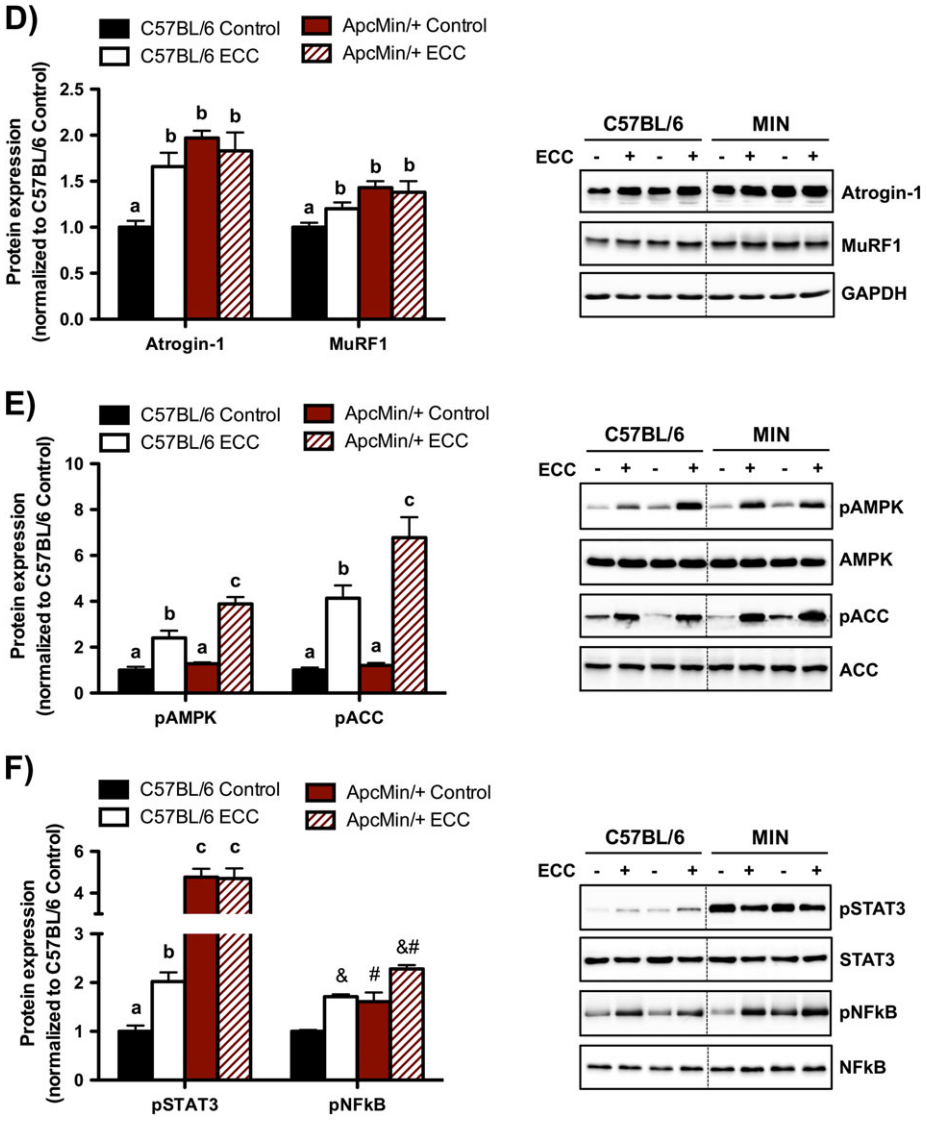
Figure 2 (Continued)



muscle (*Figure* 5B). There was no effect of PDTC on basal P38, ERK1/2, or Akt in cachectic skeletal muscle (data not shown). Interestingly, PDTC induced basal protein synthesis in cachectic muscle and was further increased 3 h post-ECC (*Figure* 5C). While there was a trend ($P = 0.08$) for PDTC to increase basal P70S6K phosphorylation, there was a robust induction 3 h post-ECC. Similarly, PDTC increased basal RPS6 phosphorylation and was further increased 3 h post-ECC in cachectic skeletal muscle (*Figure* 5D). As previously observed, the activation of mechano-sensitive pathways (P38, ERK1/2, and Akt) were not altered 3 h post-ECC (data not shown). Collectively, these data demonstrate that acute muscle STAT3 and NFκB inhibition improved basal and ECC-induced protein synthesis in cachectic $Apc^{Min/+}$ mice.

Proteolytic, metabolic, and inflammatory signalling response to pyrrolidine dithiocarbamate and eccentric contractions

Lastly, we examined the proteolytic, metabolic, and inflammatory signalling response to PDTC and ECC. Neither PDTC or ECC altered the expression of Atrogin-1 and MuRF1 in $Apc^{Min/+}$ mice (*Figure* 5E). While PDTC did not alter basal AMPK and ACC activation in cachectic skeletal muscle, these molecules were further suppressed 3 h post-ECC (*Figure* 5F). Interestingly, while PDTC suppressed basal muscle STAT3 and NFκB signalling, it did not block the induction 3 h post-ECC (*Figure* 5G). Altogether, these data demonstrate that improved ECC-induced protein synthesis by PDTC corresponded to suppressed metabolic

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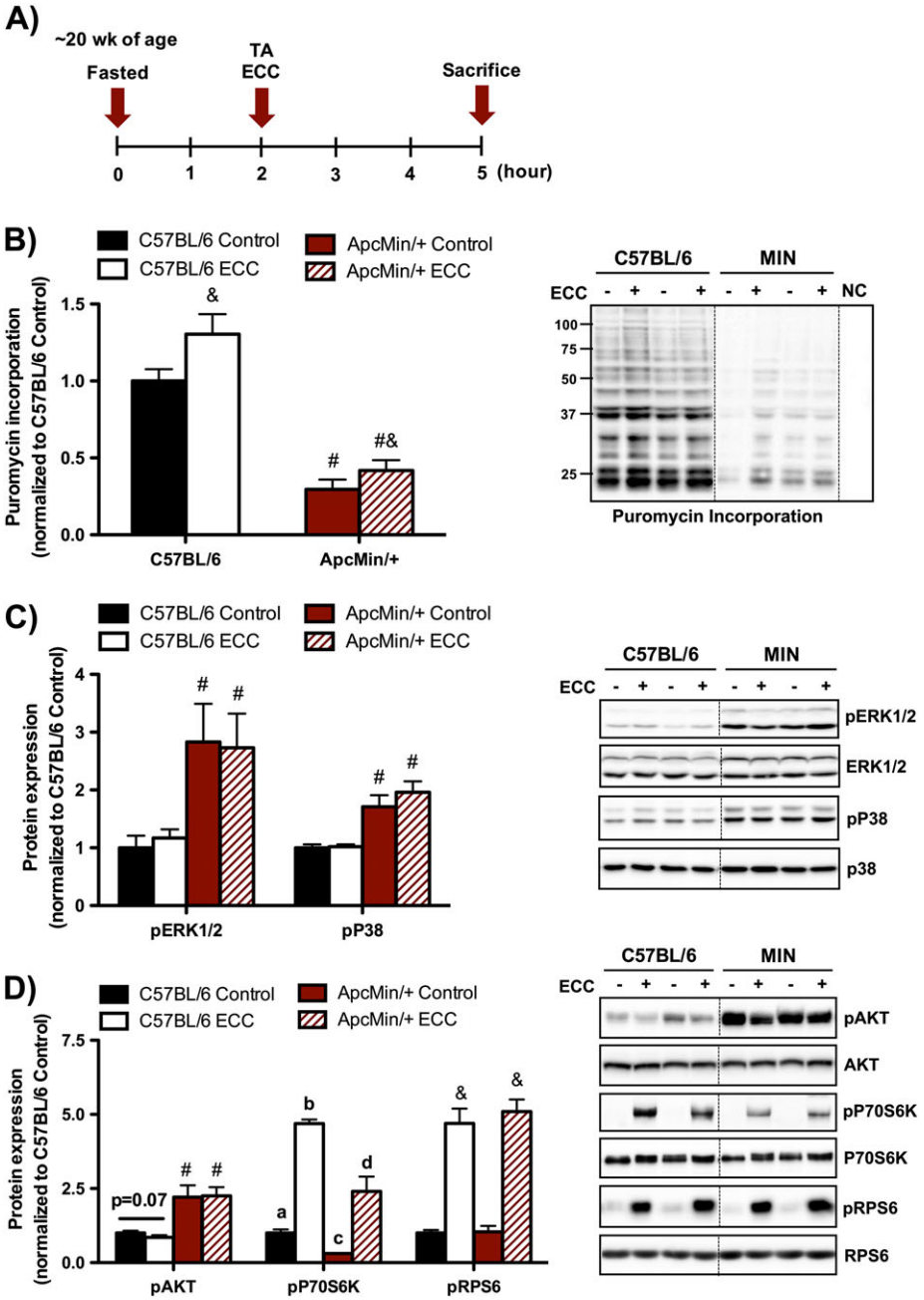
Figure 3 Muscle protein synthesis and mechanistic target of rapamycin complex 1 signalling 3 h post-eccentric contractions (ECC). (A) Experimental design. C57BL/6 and $Apc^{Min/+}$ mice were sacrificed 3 h post a single bout of eccentric contractions. Mice were fasted 2 h prior to contraction and remained fasted during the 3 h recovery until sacrifice. Mice were injected with puromycin 30 min prior to sacrifice. (B) Muscle protein synthesis regulation by ECC in C57BL/6 and $Apc^{Min/+}$ mice. (C) Muscle mitogen-activated protein kinase signalling regulation by ECC in C57BL/6 and $Apc^{Min/+}$ mice. (D) Muscle Akt/mechanistic target of rapamycin complex 1 signalling regulation by ECC in C57BL/6 and $Apc^{Min/+}$ mice. The activation of signalling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to C57BL/6 Control values. Dotted lines indicate that images were cropped for representative purposes. Data are means ± standard error; n = 9; C57BL/6, n = 10; $Apc^{Min/+}$. A two-way repeated measures analysis of variance was used to determine differences between treatment groups. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at $P < 0.05$. Different letters are statistically different. &, Main effect of ECC; #, main effect of $Apc^{Min/+}$; ERK1/2, extracellular signal-regulated kinases 1 and 2; TA, tibialis anterior.



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signalling but was independent to altered proteolytic E3 ligase expression in cachectic $Apc^{Min/+}$ mice.

Discussion

Healthy skeletal muscle stimulates protein synthesis in response to anabolic stimuli associated with daily living, which can include physical activity and feeding. While basal muscle protein synthesis and mTORC1 signalling is suppressed in tumour-bearing mice and some human cancer patients,[7,44] the capacity for cachectic muscle to respond to an anabolic stimulus is not well understood. This knowledge could have significant ramifications for the treatment of the cachectic cancer patient. Resistance exercise consisting of ECC is a potent stimulator of protein synthesis and muscle growth.[11,12,45]

Figure 4 Muscle proteolytic, metabolic, and inflammatory signalling 3 h post-eccentric contractions (ECC). (A) Muscle proteolytic regulation by ECC in C57BL/6 and $Apc^{Min/+}$ mice. (B) Muscle metabolic signalling regulation by ECC in C57BL/6 and $Apc^{Min/+}$ mice. (C) Muscle inflammatory signalling regulation by ECC in C57BL/6 and $Apc^{Min/+}$ mice. The activation of signalling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to C57BL/6 Control values. Dotted lines indicate that images were cropped for representative purposes. Data are means ± standard error; n = 9; C57BL/6, n = 10; $Apc^{Min/+}$. A two-way repeated measures analysis of variance was used to determine differences between treatment groups. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at $P < 0.05$. Different letters are statistically different. &, Main effect of ECC; #, main effect of $Apc^{Min/+}$; ACC, Acetyl-CoA carboxylase; AMPK, adenosine monophosphate-activated protein kinase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; STAT3, signal transducer and activator of transcription 3.

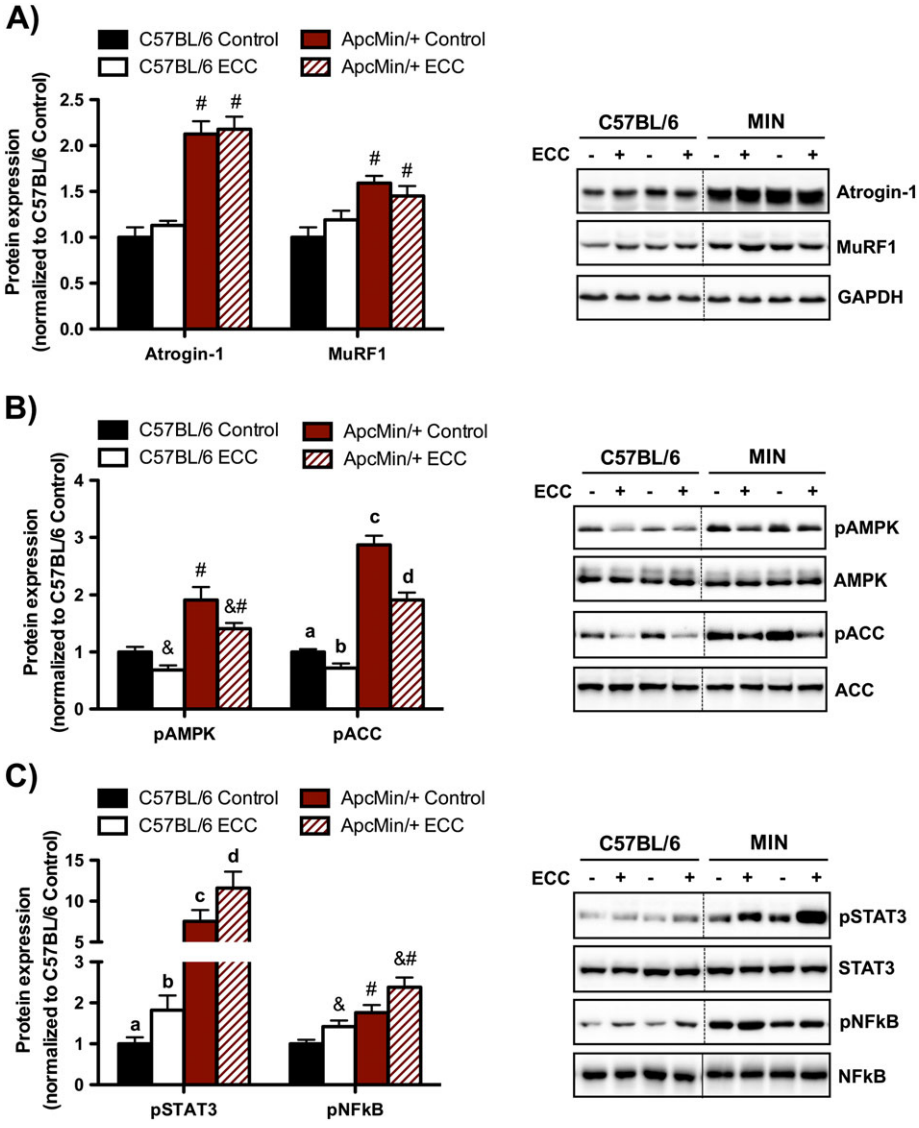

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Figure 5 Muscle inflammatory signalling regulation of eccentric contractions (ECC)-induced protein synthesis in $Apc^{Min/+}$ mice. (A) Experimental design. $Apc^{Min/+}$ mice were sacrificed 3 h post-ECC. A cohort of $Apc^{Min/+}$ mice was given a single pyrrolidine dithiocarbamate (PDTC) treatment (10 mg/kg body weight) 24 h prior to ECC. Mice were fasted 2 h prior to ECC and remained fasted during the 3 h recovery until sacrifice. Mice were injected with puromycin 30 min prior to sacrifice. (B) Muscle signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling in the non-contracted, control muscle following a single PDTC treatment. (C) Muscle protein synthesis regulation by PDTC and ECC in $Apc^{Min/+}$ mice. (D) Muscle mTORC1 signalling regulation by PDTC and ECC in $Apc^{Min/+}$ mice. (E) Muscle proteolytic regulation by PDTC and ECC in $Apc^{Min/+}$ mice. (F) Muscle metabolic signalling regulation by PDTC and ECC in $Apc^{Min/+}$ mice. (G) Muscle inflammatory signalling regulation by PDTC and ECC in $Apc^{Min/+}$ mice. The activation of signalling molecules was determined by the phosphorylated and total ratio when appropriate. For protein expression, values were corrected for equal protein loading using GAPDH. All samples were run on the same gel and normalized to $Apc^{Min/+}$ Control values. Dotted lines indicate that images were cropped for representative purposes. Data are means ± standard error; n = 10; $Apc^{Min/+}$, n = 10; $Apc^{Min/+}$ PDTC. A two-way repeated measures analysis of variance was used to determine differences between treatment groups. Post-hoc analyses were performed with Student-Knewman-Keuls methods when appropriate. Statistical significance was set at $P < 0.05$. Different letters are statistically different. &, Main effect of ECC; #, main effect of PDTC; TA, tibialis anterior.

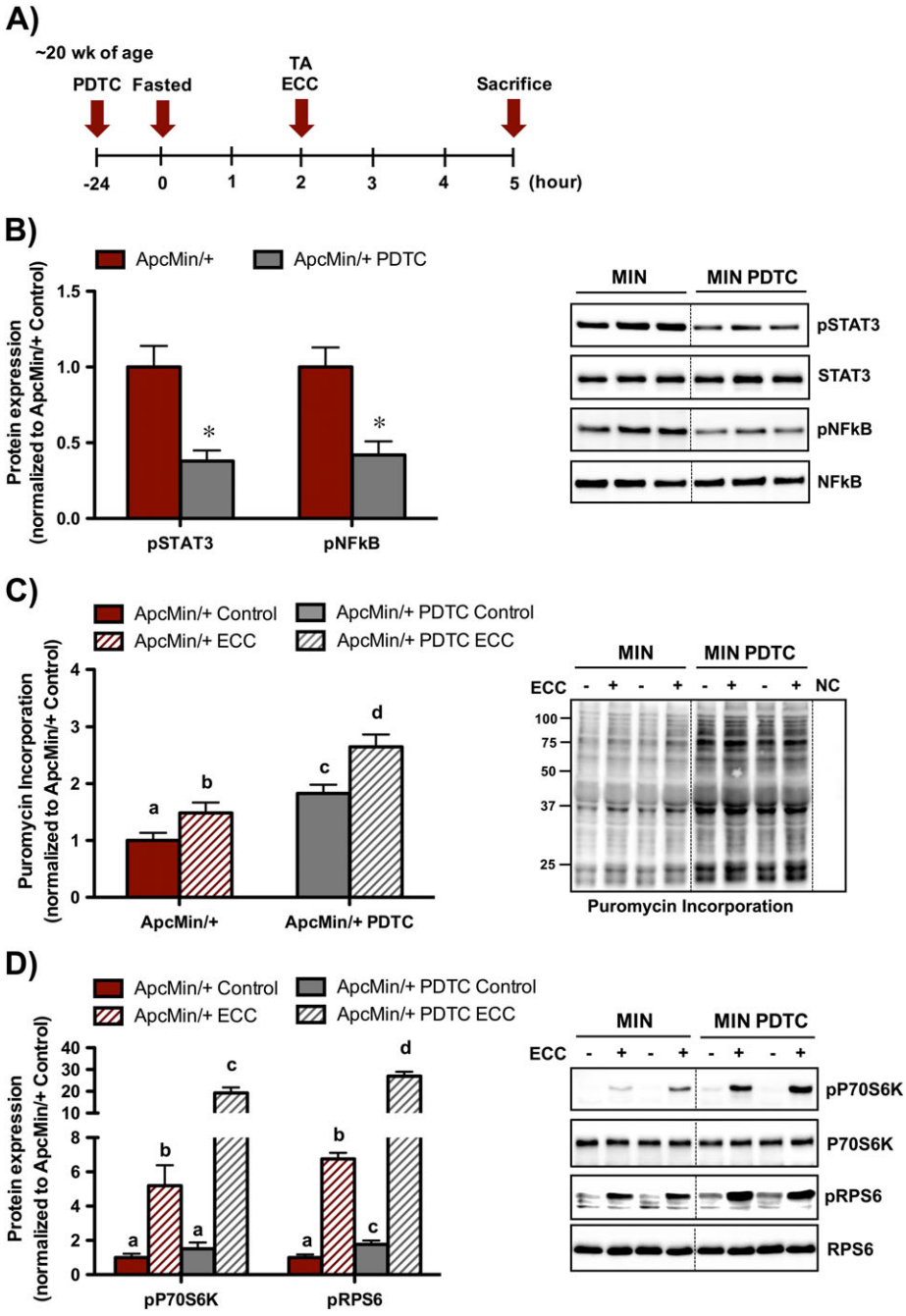

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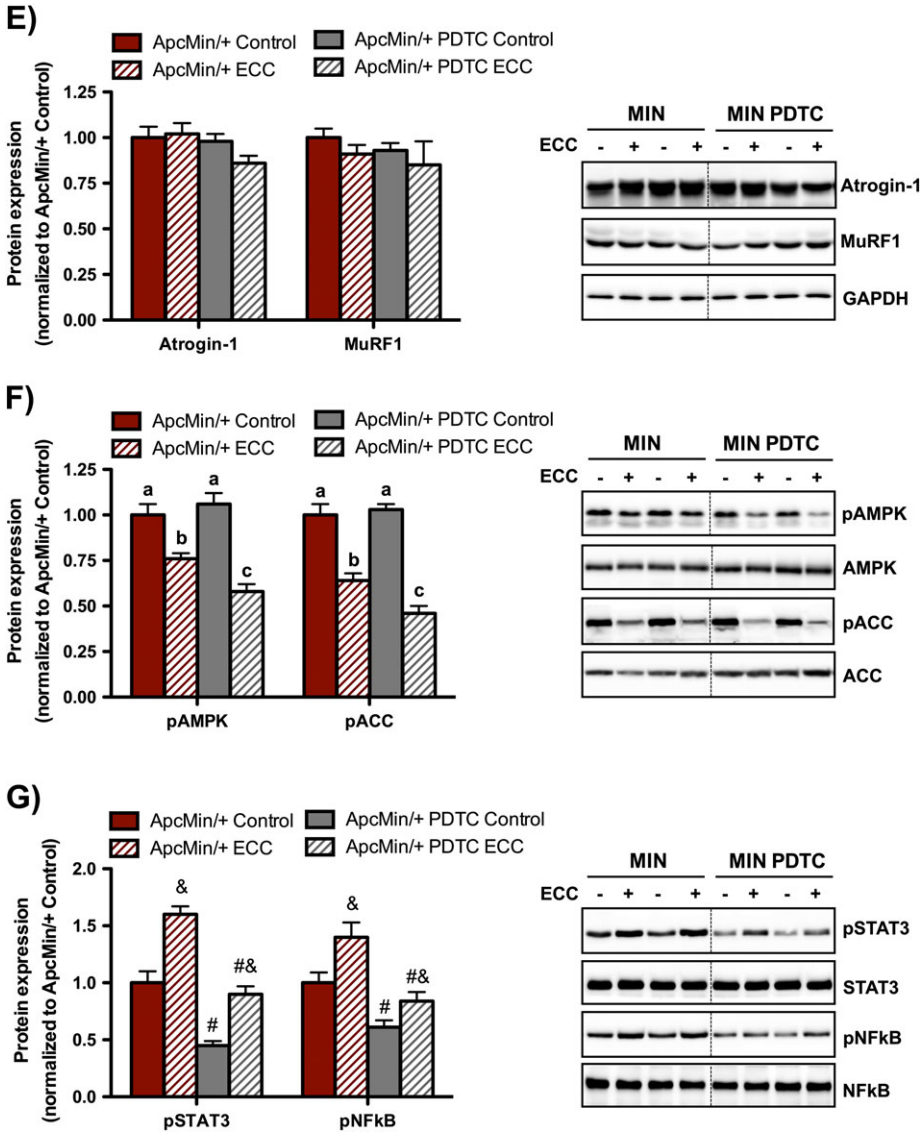
Figure 5 (Continued)



We have previously found that repeated ECC bouts after the initiation of cachexia attenuated myofiber atrophy and was accompanied by suppressed muscle inflammatory signalling. However, the capacity to activate mTORC1 signalling and protein synthesis by ECC has not been investigated. Therefore, we examined if cachectic muscle maintained the ability to induce mechano-sensitive pathways and protein synthesis in response to a single ECC bout. We report that ECC-induced mechanical signalling was maintained in cachectic muscle, but the capacity for increased protein synthesis was attenuated. This finding demonstrates an unexpected uncoupling between the activation of known mechano-sensitive regulators of anabolic signalling and the absolute protein synthesis induction. Therefore, we also examined if the cachectic environment involving muscle inflammatory signalling could regulate ECC-induced protein synthesis. Interestingly, both cachexia and ECC induced muscle STAT3 and NFκB signalling. However, muscle STAT3/NFκB inhibition by PDTC increased basal and ECC-induced protein synthesis in cachectic $Apc^{Min/+}$ mice. These findings demonstrate that mechano-sensitive signalling is responsive to ECC and highlight muscle inflammatory signalling's role in the altered regulation of both basal and ECC-induced protein synthesis during cancer cachexia.

While exercise training has been discussed as a potential therapy to mitigate muscle atrophy during cancer cachexia, there is currently a limited understanding of the acute response and training adaptation to exercise. Whole-body treadmill exercise prevented muscle mass loss in tumour-

bearing mice[46–48] and blocked the disruption of muscle oxidative metabolism regulation at the initiation of cachexia.[49,50] However, the inability of severely cachectic mice to perform voluntary exercise remains a consistent barrier and has limited our understanding of the muscle response to exercise during refractory cachexia. To address this, we have reported that cachexia disrupted the metabolic and anabolic signalling response to a single bout of stimulated low-frequency concentric contractions,[32] which mimics low intensity, endurance type exercise. Given that exercise involves muscle contractions that can vary in overall intensity and metabolic demand, the molecular responses related to growth and metabolism are distinct between contraction types. Furthermore, the response of cachectic muscle to high force contractions is not well established. Therefore, we first examined the mechanical and metabolic response to a single ECC bout. We found that several contraction and mechano-sensitive kinases (MAPKs, Akt, and P70S6K) were induced by ECC in cachectic muscle. The activation of P38, ERK1/2, and Akt was transient, which extends previous observations in mouse skeletal muscle.[18] In addition, P70S6K activation by ECC remained elevated irrespective of cachexia, which is in contrast to our previous observations using concentric muscle contractions.[32] Interestingly, we also observed a transient AMPK induction by ECC, which was not associated with mTORC1 signalling inhibition. Collectively, we provide initial evidence that the mechanical and metabolic plasticity of muscle to ECC is maintained despite the presence of a systemic cachectic environment.

There is considerable interest in understanding the mechanisms that serve to repress cachectic muscle anabolic signalling. We previously reported that repeated ECC bouts performed after the initiation of cancer cachexia could attenuate myofiber atrophy[24]; however, this study did not determine whether these improvements were related to the induction of muscle growth or the attenuation of muscle breakdown. While our current study has extended these findings to demonstrate that mechano-signalling in cachectic muscle is maintained, the ability to synthesize protein remained dramatically suppressed. These findings further demonstrate that the capacity for either basal or contraction-induced muscle protein synthesis is suppressed by cachexia. The chronic activation of AMPK in cachectic skeletal muscle from tumour-bearing mice has been implicated as a potential mechanism for mTORC1 and protein synthesis suppression.[7,28] Interestingly, our current study demonstrates the induction of protein synthesis after ECC coincides with reduced AMPK activation in tumour-bearing mice. Furthermore, reduced AMPK activation after a single ECC bout has also recently been observed in castrated mice.[51] These findings are in contrast to our previous observations using low-frequency electrical stimulation and point to the differential regulation of muscle metabolic signalling by different types of contraction. We previously found a sustained AMPK activation following a single bout of low-frequency concentric contractions in cachectic skeletal muscle.[32] The specificity of the responses induced by different types of contractions may be related to metabolic stress as cachectic muscle develops mitochondrial dysfunction.[52–54] The cachectic muscle's anabolic and metabolic response to different contraction types will require further investigation. Nonetheless, our findings suggest that ECC may be a potential therapeutic treatment to promote muscle anabolism during cancer cachexia progression.

Muscle signalling related to inflammation, energy status, and proteostasis are disrupted during cachexia progression and have been implicated in the regulation of muscle wasting.[55,56] Interestingly, many of these same pathways are induced by muscle contraction and exercise.[57] IL-6 and muscle STAT3 signalling through the gp130 receptor are activated during the progression of cachexia[30,58,52] and are associated with mTORC1 and protein suppression in pre-clinical cachexia models.[7,28] Inhibition of muscle IL-6 signalling through either direct STAT3 inhibition or gp130 loss can attenuate wasting in mouse models of cachexia.[30,31] We have previously found that short-term PDTC treatment attenuated the suppression of mTORC1 signalling and protein synthesis while concomitantly reducing muscle STAT3 and NFκB activation in $Apc^{Min/+}$ mice.[32,29] We extend these findings by demonstrating a single PDTC dose improved basal protein synthesis. It has recently been suggested that intermittent cycles of pathway inhibition/activation may be required to combat muscle wasting during cancer cachexia.[59] Indeed, many cytokine-related signalling pathways have established roles in myogenesis[60] and load-induced muscle growth and remodelling.[42,61,41] Therefore, we utilized an experimental paradigm that lowered chronic muscle inflammatory signalling but did not block contraction-induced signalling.[32] Importantly, we found that PDTC treatment increased both basal and ECC-induced protein synthesis in cachectic muscle. These findings demonstrate that cachectic muscle retains the anabolic capacity to increase protein synthesis, and inflammatory signalling contributes to the suppression of these processes. Additional research is warranted to determine the specific mechanisms related to STAT3 and NFκB that serve to diminish the capacity for protein synthesis in cachectic muscle. Moreover, further research is also required to determine the effect of muscle inflammatory signalling on metabolic remodelling in response to repeated contraction bouts, which could dramatically impact health outcomes related to exercise.

Conclusions

In summary, we examined cachectic muscle's ability to induce protein synthesis in response to ECC and determined the role of muscle inflammatory signalling involving STAT3 and NFκB on ECC-induced anabolic signalling. We found that

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mechano-sensitive signalling pathways related to P38, ERK1/2, and Akt were not altered by the cachectic environment in wasting muscle. While cachexia did not attenuate the ECC induction of mTORC1 signalling, the capacity for protein synthesis remained suppressed compared with healthy controls. Interestingly, we found that reducing muscle STAT3/NFκB signalling improved basal and ECC-induced protein synthesis during severe cachexia. These studies demonstrate that mechano-sensitive signalling pathways are maintained in skeletal muscle, but STAT3/NFκB signalling serves to attenuate basal and ECC-induced protein synthesis. Further work is necessary to determine whether intermittent anti-inflammatory therapies combined with exercise training may be useful to alleviate suppressed muscle protein synthesis during cancer cachexia.

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Conflict of interest

Justin P. Hardee, Brittany R. Counts, Song Gao, Brandon N. VanderVeen, Dennis K. Fix, Ho-Jin Koh,, and James A. Carson declare that they have no conflict of interest.

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