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Journal of Nutritional Biochemistry 110 (2022) 109150

Journal of Nutritional Biochemistry

RESEARCH PAPER

(-)-Epicatechin and its colonic metabolite hippuric acid protect against dexamethasone-induced atrophy in skeletal muscle cells

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Received 12 October 2021; received in revised form 13 May 2022; accepted 10 August 2022

ABSTRACT

Cocoa flavanols have been shown to improve muscle function and may offer a novel approach to protect against muscle atrophy. Hippuric acid (HA) is a colonic metabolite of (-)-epicatechin (EPI), the primary bioactive compound of cocoa, and may be responsible for the associations between cocoa supplementation and muscle metabolic alterations. Accordingly, we investigated the effects of EPI and HA upon skeletal muscle morphology and metabolism within an *in vitro* model of muscle atrophy. Under atrophy-like conditions (24h 100μM dexamethasone (DEX)), C2C12 myotube diameter was significantly greater following co-incubation with either 25μM HA (11.19±0.39μm) or 25μM EPI (11.01±0.21μm) compared to the vehicle control (VC; 7.61±0.16μm, both $P < .001$). In basal and leucine-stimulated states, there was a significant reduction in myotube protein synthesis (MPS) rates following DEX treatment in VC ($P = .024$). Interestingly, co-incubation with EPI or HA abrogated the DEX-induced reductions in MPS rates, whereas no significant differences *versus* control treated myotubes (CTL) were noted. Furthermore, co-incubation with EPI or HA partially attenuated the increase in proteolysis seen in DEX-treated cells, preserving LC3 α/β II:I and caspase-3 protein expression in atrophy-like conditions. The protein content of PGC1α, ACC, and TFAM (regulators of mitochondrial function) were significantly lower in DEX-treated *versus*. CTL cells (all $P < .050$). However, co-incubation with EPI or HA was unable to prevent these DEX-induced alterations. For the first time we demonstrate that EPI and HA exert anti-atrophic effects on C2C12 myotubes, providing novel insight into the association between flavanol supplementation and favourable effects on muscle health.

KEYWORDS: Muscle atrophy; dexamethasone; mitochondria; flavanols; epicatechin; hippuric acid.

1. Introduction

Skeletal muscle atrophy accompanies many physiological processes such as ageing, immobility and disease [1]. It is also a prominent consequence of corticosteroid treatments for chronic inflammatory and autoimmune conditions [2]. Muscle atrophy is underpinned by alterations to muscle protein turnover, resulting from a decline in basal and postprandial muscle protein synthesis rates (MPS) and/or a concomitant increase in muscle protein breakdown (MPB) [3–5]. Impaired mitochondrial metabolism has also been associated with muscle atrophy [6] and has been implicated in mediating the atrophic pathology [7].

Abbreviations: 4EBP1, eukaryotic initiation factor 4E binding protein 1; ACC, acetyl-CoA carboxylase; AMPKα, 5′ AMP-activated protein kinase; Akt, protein kinase B; CTL, 24h control (no atrophic stimulus); DEX, 24h 100μM dexamethasone treatment; EPI, 24h; 25μM epicatechin supplementation, HA; 24h, 25μM hippuric acid supplementation; LC3 α/β, microtubule-associated proteins 1A/1B light chain 3B; LEU, 90mins of 5mM leucine stimulation; MAFbx, muscle atrophy f-box; mTORC1, total mechanistic target of rapamycin; MURF-1, muscle ring finger protein 1; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RPS6, ribosomal protein S6; S6K1, ribosomal protein S6 kinase beta-1; TFAM, mitochondrial transcription factor A; VC, ethanol matched vehicle control.

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https://doi.org/10.1016/j.jnutbio.2022.109150

Polyphenolic compounds are regularly consumed in the human diet [8]. Flavanols are a type of flavonoids, a class of polyphenolic secondary metabolites found in plants. Cocoa is rich in flavanols [9] and has been shown to improve muscle function [10], exercise capacity [11] and recovery from exercise-induced damage [12]. (-)-Epicatechin (EPI) is the most abundant flavanol in cocoa [13], representing ~35% of total polyphenols in cocoa powder [14]. As a result, many of the health-promoting benefits of cocoa supplementation have been attributed to EPI [15]. *In vitro* experimental models suggest that EPI exerts positive effects on skeletal muscle metabolism by enhancing mitochondrial function [16]. There is also evidence to suggest that cocoa promotes myogenesis and increases C2C12 myotube width [17]. Furthermore, in C2C12 myotubes subjected to clinorotation (an *in vitro* model of atrophy), EPI treatment was associated with the downregulation of the key atrogene, muscle ring finger protein 1 (MURF1) [18]. Since oxidative stress, mitochondrial dysfunction, and an imbalance between protein anabolism and catabolism occurs in muscle atrophy [4,7,19,20], it is plausible that cocoa flavanols could abrogate muscle atrophy through maintaining mitochondrial function and supporting net muscle protein balance through the activities of its main bioactive compound, EPI. However, the specific role and mechanisms of cocoa flavanols in protecting against muscle atrophy remain to be determined.

EPI has poor oral bioavailability and, as a consequence, is only present in systemic circulation at extremely low concentrations and typically in a sulphated, methylated or glucuronidated forms (20). Unabsorbed EPI is metabolized by colonic microbiota to form smaller structurally related metabolites (20). Although research determining the role of EPI metabolites on peripheral metabolism is limited, it is possible that these smaller bioactive compounds may ultimately be responsible for the *in vivo* short-to-long term metabolic effects associated with native flavanol supplementation. The 3/1-carbon ring fission metabolite (3/1C RFM), hippuric acid (HA) is a colonic metabolite derived from EPI and proanthocyanidins (flavanol oligomers of EPI and catechin) following cocoa supplementation. In the 12–48h following cocoa ingestion, HA reaches a high circulating systemic concentration (in the mM range) [21] and may possess potent metabolic properties relevant to muscle atrophy. *In vitro*, HA has been suggested to stimulate glucose metabolism and preserve mitochondrial function following insult in C2C12 myotubes [22], which is in agreement with evidence demonstrating similar effects following supplementation with a native flavanol (e.g., [16,23]). Since mitochondrial dysfunction and insulin resistance are implicated in the aetiology of muscle atrophy, further research is warranted to assess the impact of EPI metabolites, in particular HA, as a strategy to offset muscle atrophy.

Accordingly, we investigated the effects of EPI and HA on skeletal muscle morphology, muscle protein turnover and markers of mitochondrial function within an *in vitro* model of skeletal muscle atrophy. Our hypotheses were twofold; [1] under atrophy-inducing conditions, concomitant treatment with EPI or HA would prevent declines in myotube diameter through the preservation of MPS rates, a reduction in catabolic signalling and the preservation of markers of mitochondrial function.

2. Materials and methods

2.1. C2C12 cell culture

Mouse skeletal muscle C2C12 myoblasts were maintained in growth medium consisting of: Dulbecco's Modified Eagle's Medium (DMEM; #11966025 Gibco) containing 10% *(v/v)* fetal bovine serum (FBS; #F9665 Invitrogen), 5 mM glucose (#G7021 Sigma), 1 mM sodium pyruvate (#S8636 Sigma), 1mM GlutaMax (#35050-038 Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (#15070-063 Invitrogen). Once ~70–80% confluence was obtained, cells were seeded to 6-well or 24-well plates at a density of 2×10^5 or 5×10^4 cells/well, respectively. Upon reaching ~90–95% confluence, medium was changed to differentiation medium consisting of: DMEM containing 2% *(v/v)* horse serum (#16050-122 Gibco), 5 mM glucose, 1 mM sodium pyruvate, 1 mM GlutaMax, 100 U/mL penicillin and 100 μg/mL streptomycin. Differentiation medium was replaced daily for 5 days until elongated myotubes had formed, as confirmed via visual inspection using an inverted light microscope. On day 6 of differentiation, myotubes were washed into fresh serum-free DMEM containing 5 mM glucose, 1 mM sodium pyruvate, 1 mM GlutaMax, 100 U/mL penicillin, and 100 μg/mL streptomycin for 1h prior to commencing the 24h treatment protocol as detailed below. Cell passage numbers 10–13 were used for experimentation.

2.2. Treatment protocol

The full treatment protocol is detailed in Figure 1. Briefly, to identify anti-atrophic effects of EPI and HA, C2C12 myotubes were subdivided into six groups for the 24h treatment protocol; (1) VC-CTL, with cells incubated in serum free medium (as above), (2) VC-DEX, with cells incubated in 100 μM dexamethasone (#D4902 Sigma), (3) EPI-CTL, with cells incubated with 25 μM EPI (#E1753 Sigma), (4) EPI + DEX, with cells co-incubated in 25 μM EPI and 100 μM DEX. (5) HA-CTL, with cells incubated in 25 μM HA (#112003 Sigma) and, (6) HA + DEX, with cells co-incubated with 25 μM HA and 100 μM DEX. All treatment conditions were dissolved in ethanol and matched for ethanol concentrations (0.2% of cell media). To enhance the translatability to *in vivo* physiology, a 25 μM supplementation dose was selected due to it's upper-limit representation of both circulating natural flavanol (i.e. EPI) and subsequent phenolic acid (i.e. HA) concentrations *in vivo* [21] and successful implementation in previous *in vitro* research [22].

2.3. Biochemical analysis

Immunofluorescence analysis. The immunofluorescence protocol has been outlined previously [24]. Briefly, immediately following the 24h treatment period (Fig. 1), culture medium was removed and myotubes were fixed for 30min in 2% (v/v) formaldehyde in Dulbecco's phosphate-buffered saline (dPBS). Myotubes then underwent 10min of permeabilization in 100% methanol, before being washed 3X in dPBS and blocked for 30min in 5% (v/v) goat serum in dPBS. Subsequently, myotubes were incubated for 1h with a primary anti-desmin antibody (#D8281 Sigma: 1:100 1% (v/v) bovine serum albumin (BSA): dPBS) before undergoing a wash step (as above) and incubation in the dark for 1h with a secondary goat anti-rabbit IgG H+L Alexa Fluor 488 antibody (#P-2771MP Fisher: 1:200 in dPBS). Wells were then washed once in dPBS before a 5min incubation with DAPI (#4083 CST: 1:5000 in dPBS) in the dark. Finally, cells were washed once with dPBS, before 10 μL of mountant was added to each well and a coverslip added.

MPS analysis. In the final hour of the 24h treatment protocol (described above), C2C12 myotubes were nutrient deprived in amino acid and serum free low-glucose DMEM medium (#19052222 US Biological) made according to the manufactures instructions and supplemented with 1 mM sodium pyruvate, 1 mM GlutaMax, 100 U/mL penicillin and 100 μg/mL streptomycin. Following the period of nutrient deprivation, each treatment condition underwent a 90min period of stimulation with 5 mM L-leucine (LEU; #L8912 Sigma) or a ddH_2O volume matched control to provide both a basal and LEU-stimulated measure of MPS. To obtain a proxy measurement of MPS, the Surface Sensing of Translation (SUnSET) technique developed by Goodman et al. [25] was utilized and puromycin (1 μM; #P8833 Sigma) added to cell media in the final 30min of the acute stimulation period. Subsequently, myotubes were washed and lysed with RIPA lysis buffer (#20118 Merk-Millipore) and supplemented with one cOmplete mini protease inhibitor (#4693159001 Roche) tablet and one phosSTOP (#4906845001 Roche) tablet. MPS rates were determined from cell lysates during western blot analysis utilizing an anti-puromycin antibody (#MABE343 Merk-Millipore) as detailed below.

Protein extraction and expression analysis. Protein expression was measured via western blot analysis performed on total cell lysates following centrifugation (4,500rpm, 10min, 4°C) in accordance to previously published protocols [26,27]. Gels were loaded according to the protein concentration assessed by the DC protein assay (Bio-Rad), before western blot aliquots of 2μg/1μL were prepared in 4x laemmli sample buffer and ddH_2O. To ensure membrane integrity, samples were then left to denature overnight at room temperature before subsequent analysis. Equal amounts of total protein (18-30μg) were loaded onto either in-house 10–15% or Criterion TGX Precast Midi protein gels (Bio-rad, Hertfordshire, UK) and separated by SDS-PAGE using a constant voltage of 100V for 10min and 150V for a subsequent 1h. Separated proteins were then transferred to a Protran nitrocellulose or polyvinylidene difluoride (PVDF) membrane at 100V for 1h. The membranes were then incubated overnight (4°C) with appropriate and validated primary antibodies; anti-puromycin, muscle ring finger protein 1 (MuRF-1), muscle atrophy f-box (MAFbx), caspase-3, microtubule-associated proteins 1A/1B light chain 3B (LC3 α/β), total protein kinase B (Akt), p-Akt^{S473}, p-Akt^{T308}, total mechanistic target of rapamycin complex 1 (mTORC1), p-mTORC1^{S2448}, total ribosomal protein S6 kinase beta-1 (S6K1), p-S6K1^{T389}, total ribosomal protein S6 (RPS6), p-RPS6$^{S240/244}$, total eukaryotic initiation factor 4E binding protein 1 (4EBP1), p-4EBP1$^{T37/46}$, total OXPHOS rodent antibody cocktail, total 5′ AMP-activated protein kinase (AMPKα), p-

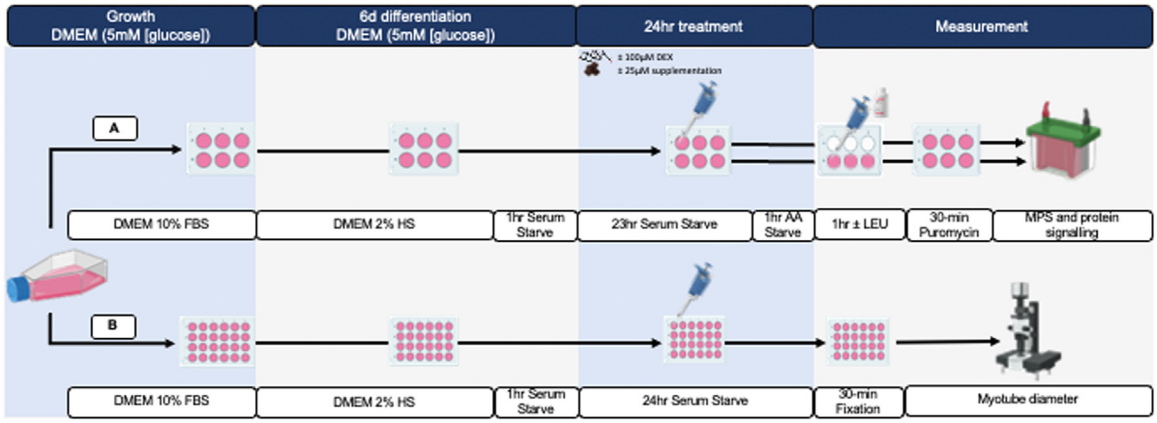

Fig. 1. Experimental protocol for the primary outcome measures of myotube protein synthesis (MPS; A) and myotube diameter (B). DEX, dexamethasone, DMEM; dulbecco's modified eagle medium, FBS; fetal bovine serum, HS; horse serum, LEU; leucine.

AMPKα^{T172}, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), citrate synthase, total acetyl-CoA carboxylase (ACC), p-ACC^{S79} and mitochondrial transcription factor A (TFAM). Samples were then washed 3 × 5min in TBST before undergoing a 60min incubation with a previously validated horseradish peroxidase (HRP)-linked anti-rabbit, anti-mouse, or anti-rat IgG. Subsequently, Immobilon western chemiluminescent HRP substrate (Millipore) was used quantify protein content, visualised using a:BOX Chemi XT4 imager with GeneSys capture software (Syngene UK, Cambridge, UK). Band quantification was achieved using a Chemi Genius Bioimaging Gel Doc System (Syngene, Cambridge, UK). Values were corrected to both a gel control and a loading control (ponceau). Following these corrections, where appropriate, the phosphorylation of proteins, as a proxy of their activation was expressed relative to the total amount of each protein. Data are presented as fold changes from the VC-CTL treatment condition.

2.4. Quantification and statistical analysis

Quantification of myotube diameter. Immunofluorescence stained C2C12 myotubes were imaged using a epifluorescence/brightfield microscope (Leica DMI6000). To improve reproducibility, for each passage triplicate wells were utilized for each treatment condition. For the quantification of myotube diameter, a total of 10 images were acquired per well at a magnification of 20x and the analysis of myotube diameter was conducted using Image J (v1.51 NIH). A myotube was classified as a desmin positive object, containing three or more DAPI positive nuclei. The myotube diameter of each individual myotube was determined as the average of five measurements acquired along its full length. For each image, the centremost five myotubes were utilized, with each passage ($n = 5$) having a total of 750 measurements across 150 myotubes analyzed per treatment condition.

Outcome measures. The primary outcome measures were myotube diameter and MPS rates in response to EPI or HA treatment in atrophy-like conditions. Alterations in intracellular signaling in response to EPI or HA treatment in atrophy-like conditions acted as a secondary outcome measure.

Statistical analysis. Data are presented as mean ± SEM. Data analyses were performed using SPSS version 25 (IBM Corp.) and statistical assumptions were checked prior to analysis. Myotube diameter were analyzed using a 2-factor mixed-model ANOVA, with a single between group factor (supplementation; VC *vs.* EPI *vs.* HA) and single within-group factor (atrophy treatment; CTL *vs.* DEX). Measures of MPS and protein expression were analyzed using a 3-factor mixed-model ANOVA, with a single between-groups factor (supplementation; VC *vs.* EPI *vs.* HA) and two within-group factors (atrophy treatment; CTL *vs.* DEX and stimulation; basal *vs.* LEU-stimulated). Missing data were not imputed into analyses and *n* numbers are reported in each figure legend. Where appropriate, Bonferroni's *post-hoc* analysis was applied whenever a significant main or interaction effect was identified. The level of significance was considered $P \leq .050$.

3. Results

3.1. Myotube diameter

The ability of EPI and its colonic metabolite (HA) to prevent DEX-induced atrophy was assessed. As shown in Figure 2B, the DEX-induced decline in myotube diameter was attenuated following treatment with HA and EPI (Fig. 2C), with significantly greater myotube diameter in EPI-DEX (11.01 ± 0.21 μm; $P < .001$) and HA-DEX (11.19 ± 0.39 μm; $P < .001$) *versus* VC-DEX (7.61 ± 0.16 μm). However, in the CTL treated myotubes, myotube diameter was significantly lower in both EPI-CTL (11.85 ± 0.20 μm) and HA-CTL (12.12 ± 0.22 μm) *versus.* VC-CTL (13.03 ± 0.19 μm, $P = .005$ and $P = .027$, respectively).

3.2. Muscle protein turnover

MPS. The current study utilized the SUnSET methodology [25] to determine the ability of EPI and HA to prevent DEX-induced differences in MPS rates. The expression of puromycin bound proteins was significantly lower in VC-DEX (fold change: 0.62 ± 0.07) *versus* VC-CTL (fold change: 1.00 ± 0.18, $P = .024$). However, the DEX-induced decline in MPS was mitigated following EPI and HA treatment with no significant differences in the expression of puromycin bound proteins between HA-CTL (fold change: 1.19 ± 0.31) and HA-DEX (fold change: 1.34 ± 0.40, $P = .205$) or EPI-CTL (fold change: 0.70 ± 0.10) and EPI-DEX (fold change: 0.89 ± 0.16, $P = .148$, Fig. 3A). Furthermore, puromycin bound protein was significantly higher in LEU-stimulated state *versus* basal state (main effect: $P = .007$, Fig. 3B) with no significant difference across all six treatment conditions.

Anabolic protein signalling pathways. Protein expression alterations in response to the 24h treatment protocol were assessed by western blotting. The phosphorylation of mTORC1^{S2448} (main effect: $P = .013$, Fig. 4B), AKT^{S473} (main effect: $P < .001$, Fig. 4F), S6K1^{T389} (main effect: $P < .001$, Figure 4H), RPS6$^{s240/244}$ (main effect $P < .001$, Fig. 4J) and AKT^{T308} (main effect: $P < .001$, Fig. 4K) were significantly lower in DEX-treated cells *versus.* CTL. The protein content of 4EBP1 (Fig. 4D) was also significantly lower following DEX treatment in EPI ($P = .004$), but not in VC ($P = .230$) or HA ($P = .653$). There was no further significant differences noted between treatment conditions (i.e., HA, EPI and VC) in the protein expression of key anabolic signalling intermediates (all $P > .050$). In response to LEU-stimulation, DEX treatment did not impair leucine stimulated anabolism across any measure of anabolic protein expression, with no significant reductions in the protein expression or phosphorylation of targets in DEX *versus* CTL treated myotubes between the basal and LEU-stimulated conditions (all $P > .050$, Fig. 4A-K). In fact, S6K1 protein content (Fig. 4C), mTORC1^{S2448} phosphorylation (Fig. 4G), and RPS6$^{s240/244}$ phosphorylation (Fig. 4J) were higher following LEU-stimulation compared to basal in DEX treated myotubes (all $P < .050$), but not CTL-treated myotubes (all $P > .050$).

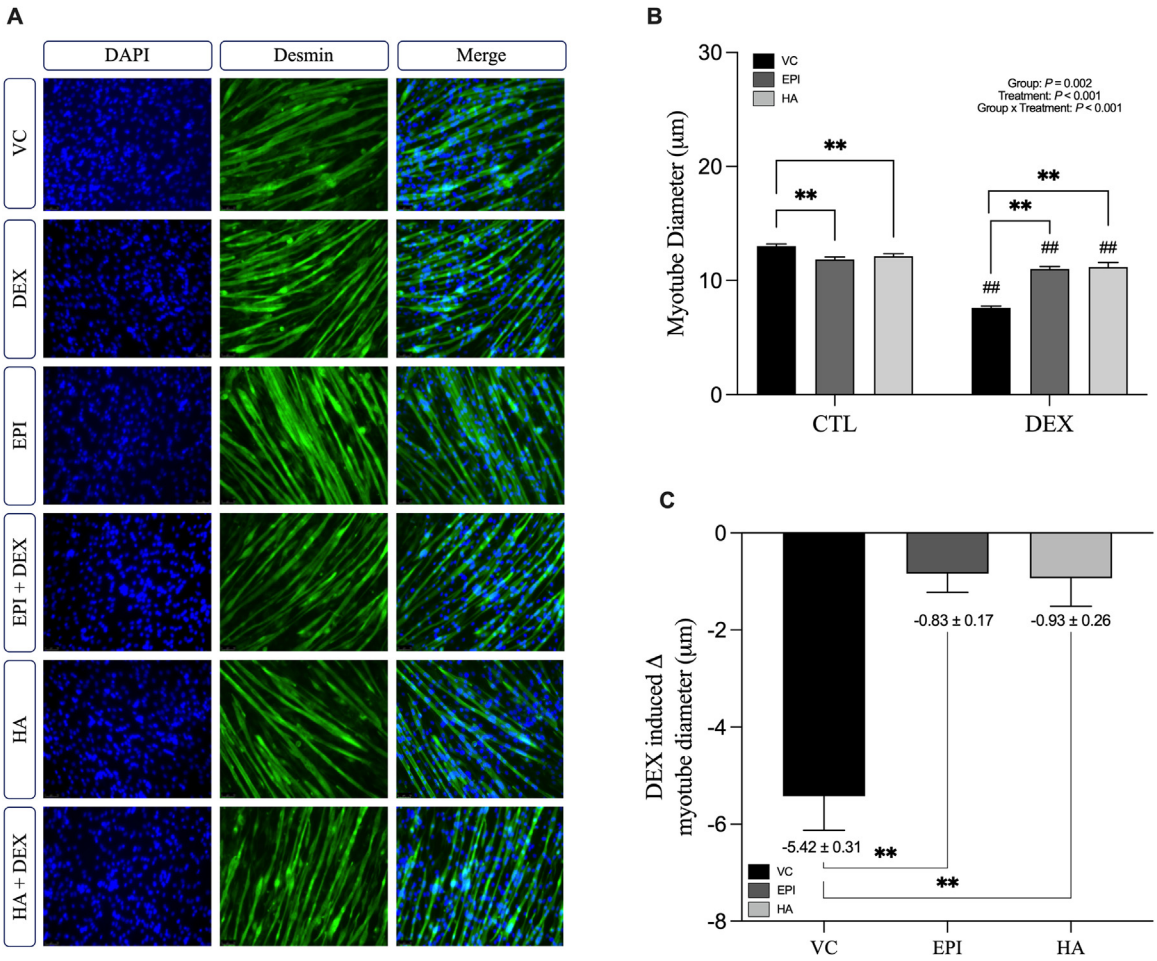

Fig. 2. Myotube diameter of C2C12 myotubes. Measures of myotube diameter following the 24h treatment protocol (A, B). Figure C displays myotube diameter of CTL treated cells expressed relative to DEX-treated cells. Data is presented as mean ± SEM. Data were analyzed using a two-factor mixed-model ANOVA (A, B) or a single factor independent ANOVA (C). In all measures $n = 5$. ** Represents a significant difference between supplementation groups at the P<0.01 level, ## DEX treated significantly different from CTL treated at the $P < .01$ level. CTL; 24h control (no atrophic stimulus), DEX; 24h, 100μM dexamethasone treatment, EPI; 24h, 25μM epicatechin supplementation, HA; 24h, 25μM hippuric acid supplementation, VC; ethanol matched vehicle control.

Catabolic signalling pathways. No significant differences were noted between supplemented conditions (i.e., EPI, HA or VC) in measures of MURF-1 (Fig. 4L) or MAFbx (Fig. 4M) protein expression. However, the protein expression of MURF-1 and MAFbx were significantly greater in response to DEX treatment *versus* CTL treated myotubes (main effect: $P = .001$ and $P = .004$, respectively). The ratio of LC3 α/β II:I (Fig. 4N) was significantly higher in VC-DEX *versus* VC-CTL ($P < .001$). However, the DEX-induced alteration in LC3 α/β II:I was mitigated following treatment with HA and EPI, with no significant differences noted between EPI-DEX *versus* EPI-CTL ($P = .181$) or HA-DEX *versus* HA-CTL ($P = .278$). Likewise, the DEX-induced decline in the protein expression of caspase-3 (Fig. 4O) in VC-DEX *versus* VC-CTL ($P = .018$) was not observed following treatment with EPI or HA, with no significant differences noted in measures of caspase-3 protein expression between EPI-DEX *versus* EPI-CTL ($P = .163$) or HA-DEX *versus* HA-CTL ($P = .654$).

Mitochondrial protein expression. In CTL treated myotubes, total OXPHOS content (Fig. 5A) was significantly lower in HA-treated cells compared to VC (HA-CTL *vs.* VC-CTL: $P = .041$). Similarly, total OXPHOS content was significantly lower in HA-DEX compared to both VC-DEX and EPI-DEX ($P = .003$ for both). Compared to the basal state, LEU-stimulation significantly decreased total OXPHOS protein content (Fig. 5A) in both EPI ($P = .041$) and HA ($P = .017$) supplemented conditions, but not in VC ($P = .364$). Indeed, total OXPHOS content was significantly lower following LEU-stimulation in HA *versus*VC ($P = .002$). Furthermore, when analyzed individually, OXPHOS complex I (CI) protein content was significantly lower under basal conditions in VC ($P = .04$), HA ($P = .004$), HA-DEX ($P = .003$) compared to VC-DEX. Similarly, following LEU-stimulation, CI protein content was significantly lower in VC ($P = .05$), HA ($P < .0001$), HA-DEX ($P < .0001$) compared to VC-DEX. Additionally, CI protein content was significantly lower in HA ($P = .02$) and HA-DEX ($P = .01$), compared to EPI after LEU-stimulation. In CTL treated myotubes complex II (CII) protein content was also significantly lower in HA ($P = .04$) and HA-DEX ($P = .03$) compared to VC-DEX. Furthermore, in LEU-stimulated myotubes CII protein content was significantly lower in VC ($P = .05$), EPI ($P = .004$), HA ($P = .0003$) and HA-DEX ($P < .0001$) compared to VC-DEX. Additionally, CII protein content was also significantly lower in HA-DEX compared to EPI-DEX post LEU-stimulation ($P = .02$). In LEU-stimulated myotubes complexes III (CIII) and V (CV) protein content was significantly lower in HA ($P = .04$ for both) and HA-DEX ($P = .02$, $P = .04$ respectively) compared to VC-DEX. No significant difference was identified between basal vs LEU-stimulated conditions.

In CTL treated myotubes, TFAM protein content was significantly lower following treatment with EPI (EPI-CTL *vs.* VC-CTL;

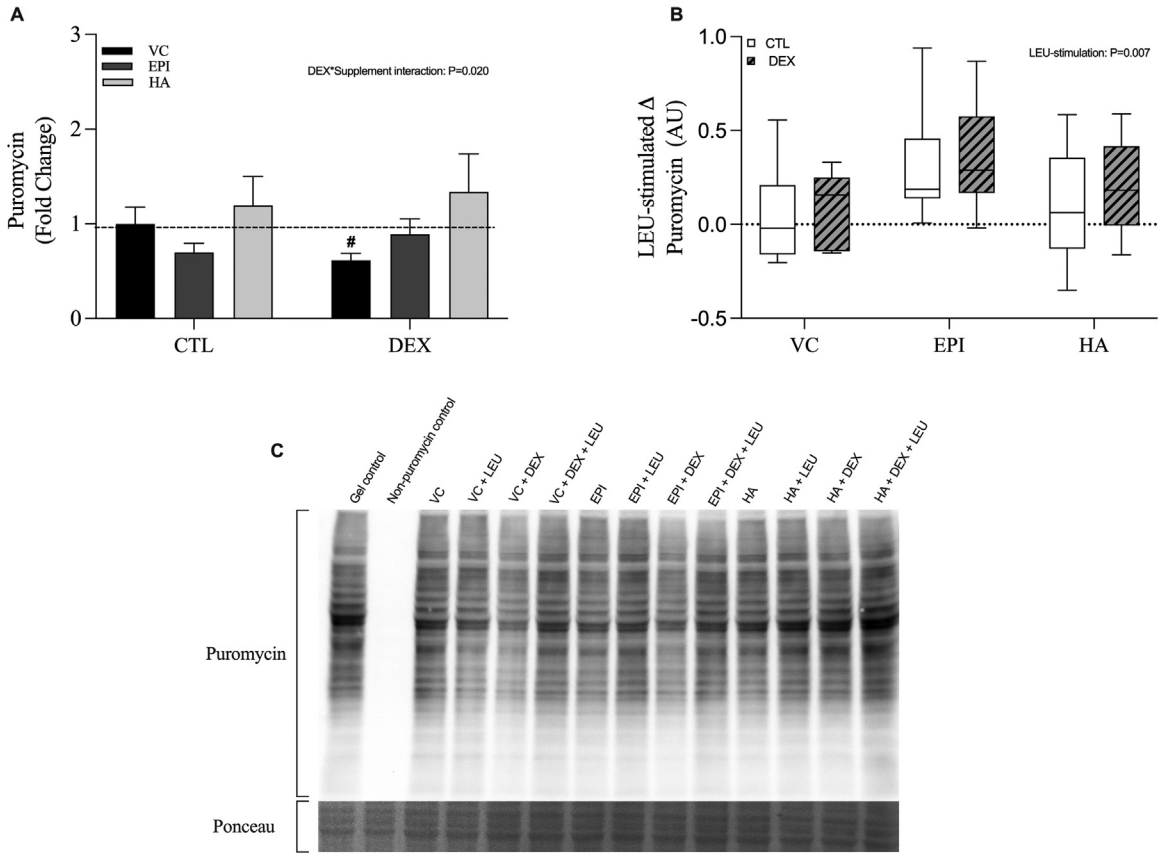

Fig. 3. Myotube protein synthesis (MPS) rates of C2C12 myotubes. The incorporation of puromycin labelled proteins following the 24h treatment protocol as a proxy of MPS rates (A,C). Figure B displays puromycin bound protein of LEU-stimulated cells expressed relative to corresponding basal (i.e. no LEU) values. In figure A, data is presented as mean ± SEM. In figure B, the boxes represent the 25th–75th percentile, the error bars represent min-max values and the lines represent medians. Data was analyzed using a three-factor mixed-model ANOVA. In all measures n=6. $^{\#}$ DEX treated significantly different from CTL treated at the $P < .05$ level. CTL; 24h control (no atrophic stimulus), DEX; 24h, 100μM dexamethasone treatment, EPI; 24h, 25μM epicatechin supplementation, HA; 24h, 25μM hippuric acid supplementation, LEU; 90min of 5mM leucine stimulation, VC; Ethanol matched vehicle control.

$P = .038$) or HA (HA-CTL *vs.* VC-CTL; $P = .002$) compared to VC (Fig. 5D). Following DEX treatment, TFAM protein content was lower than CTL (main effect: $P < .001$), but there were no significant group (i.e. HA, EPI or VC) differences noted in DEX-treated myotubes (all $P > .050$). The final between group difference was noted in T-AMPKα, where T-AMPKα was significantly lower in HA-treated cells *versus* VC ($P = .043$; Fig. 5E). There were no between group (i.e., HA, EPI or VC) differences noted in measures of PGC1α (Fig. 5B), AMPKα^{T172} phosphorylation (Fig. 5F) T-ACC (Fig. 5G), or ACC^{S79} phosphorylation (Fig. 5 H; all $P > .050$). However, compared to CTL, DEX-treated myotubes had a significantly lower protein expression of PGC1α (main effect: $P = .012$, Fig. 5B) and T-ACC (main effect: $P < .001$, Fig. 5G). Conversely, the protein expression of citrate synthase was significantly higher in DEX *versus* CTL treated myotubes (main effect: $P = .009$, Fig. 5C).

4. Discussion

Skeletal muscle atrophy results in declines of physiological functioning and metabolic health, highlighting a need for effective strategies to combat its progression. Here, we report that myotube diameter was significantly lower in DEX-treated *versus* CTL C2C12 myotubes, and concomitant treatment with EPI or HA abrogated these DEX-induced alterations in myotube diameter. Mechanistically, DEX-induced reductions in myotube diameter were accompanied by declines in basal MPS *versus* CTL treated cells. However, these DEX-induced declines in basal MPS were mitigated following a co-incubation with EPI or HA treatment, whereas there were no significant differences from CTL treated myotubes. Finally, markers of mitochondrial function were altered in the atrophy-like conditions, with the protein expression of PGC1α, ACC and TFAM were significantly lower following 24h DEX treatment compared to CTL. However, concomitant treatment with EPI or HA did not prevent these DEX-induced alterations in markers of mitochondrial function.

Polyphenolic compounds have been identified as effective anti-atrophic agents following DEX treatment [28–31]. Here we investigated the ability of the native flavanol EPI to preserve myotube diameter in atrophy-like conditions. Due to the reduced bioavailability of EPI *in vivo* [32] and the importance of colonic metabolites in the manifestation of phenotypic changes following flavanol supplementation [22,32], we also investigated the ability of the 3/1C RFM metabolite HA to protect against DEX-induced atrophy. In line with previous literature [33], we report that 24h DEX treatment was successful at inducing a ~41% decline in myotube diameter *versus* CTL. Interestingly, in CTL treated myotubes there was a marginal, albeit significant decline in myotube diameter following treatment with EPI or HA, compared to VC. However, in atrophy-like conditions (i.e. co-incubation with DEX), EPI or HA treatment abrogated DEX-induced declines in myotube diameter compared to VC. The marginal discrepancy between the CTL and DEX states suggest that the ability of flavanols to favourably influence protein metabolism may be context dependent and is suggestive that in these C2C12 myotubes flavanol metabolites act to offset atrophy

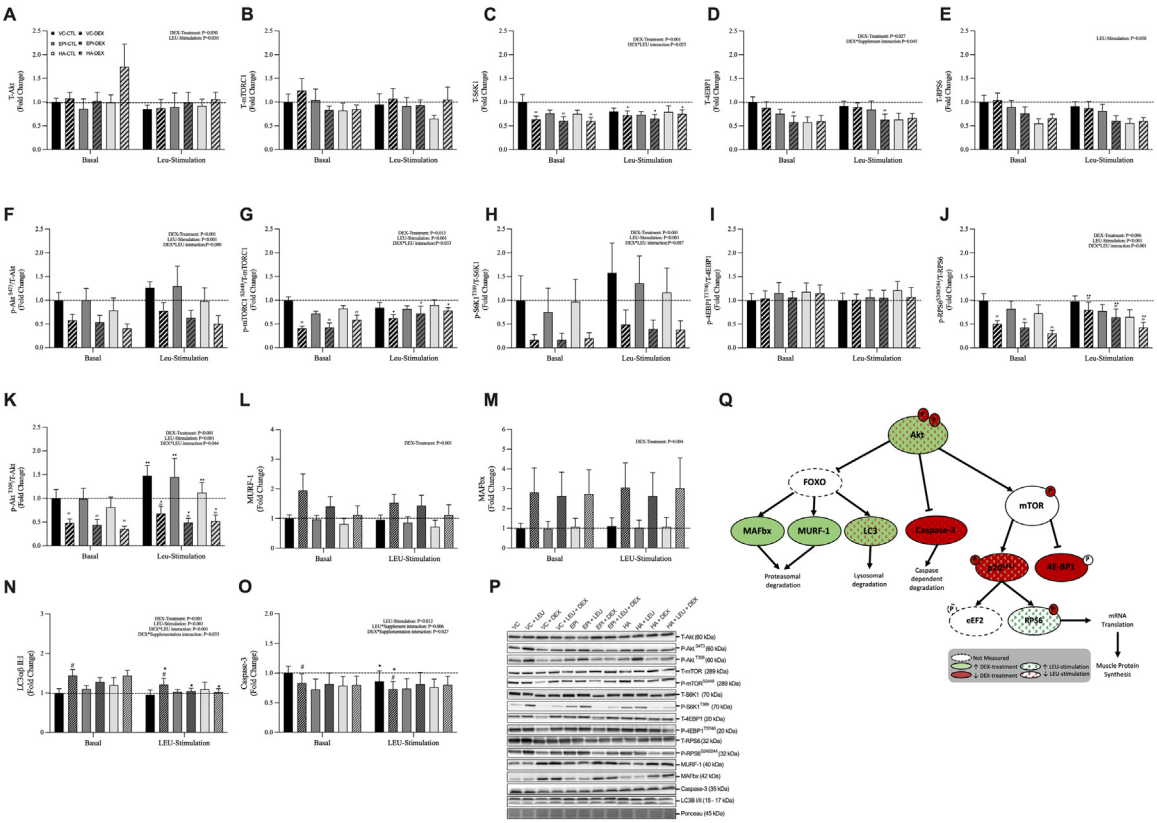

Fig. 4. Anabolic and catabolic protein expression in response to the 24h treatment protocol. (A) total Akt ($n = 5$), (B) total mTORC1($n = 5$), (C) total S6K1($n = 6$), (D) total 4EBP1($n = 6$), (E) total RPS6 ($n = 6$), (F) p-AKT^{S473}/T-Akt ($n = 5$), (G) p-mTORC1^{S2448}/t-mTORC1 ($n = 5$), (H) p-S6K1^{T389}/T-S6K1 ($n = 6$), (I) p-4EBP1$^{T37/46}$/T-4EBP1 ($n = 6$), (J) p-RPS6$^{S240/244}$/T-RPS6 ($n = 6$), (K) p-Akt^{T308}/T-Akt ($n = 5$), (L) MURF-1 ($n = 6$), (M) MAFbx ($n = 6$), (N) LC3 α/β II:I ($n = 6$), (O) Caspase-3 (n=6), (P) western blot representative image of anabolic and catabolic signaling proteins, (Q) schematic overview of measured targets in the anabolic and catabolic signaling pathways following 24h treatment. Data are presented as means ± SEMs and were analyzed using a 3-factor mixed-model ANOVA. * and ** LEU-stimulated significantly different from basal at the $P < .05$ level and $P < .01$ level, respectively. # and ## DEX treated significantly different from CTL treated at the $P < .05$ level and $P < .01$ level, respectively. 4EBP1; eukaryotic initiation factor 4E binding protein 1, Akt; total protein kinase B, CTL; 24h control (no atrophic stimulus), DEX; 24h, 100μM dexamethasone treatment, EPI; 24h, 25μM epicatechin supplementation,
HA; 24h, 25μM hippuric acid supplementation, LC3 α/β; Microtubule-associated proteins 1A/1B light chain 3B, LEU; 90min of 5mM leucine stimulation, MAFbx; muscle atrophy f-box, mTORC1; total mechanistic target of rapamycin, MURF-1; muscle ring finger protein 1, RPS6; ribosomal protein S6, S6K1; ribosomal protein S6 kinase beta-1, VC; Ethanol matched vehicle control.

through pathways divergent to those promoting hypertrophy in non-catabolic conditions (i.e. through alterations in catabolic signaling as discussed below). Since HA is present in circulation from 12–48h following cocoa ingestion at a relatively high concentration [21], these novel results indicate that HA may account for a fraction of the observed bioactivities following flavanol supplementation, especially in chronic supplementation protocols where cocoa is provided every 12–24h (i.e.[10]). Therefore, our findings extend on previous investigations [5,6] that highlight the importance of the splanchnic metabolism of cocoa following oral ingestion and offer novel insights as to how cocoa supplementation could support skeletal muscle health in conditions of atrophy, despite the poor bioavailability of its main bioactive compound, EPI.

A decline in postabsorptive, postprandial and exercise-induced MPS rates, combined with normal and/or dysregulated MPB, underscores the skeletal muscle atrophy that accompanies aging [34], inactivity [35,36] and diseased states [37]. We hypothesized that EPI and its metabolite HA would similarly maintain myotube diameter in response to DEX treatment, through the preservation of basal and LEU-stimulated MPS rates. We report that the 24h DEX treatment induced a ~34% decline in basal MPS, which was accompanied with significant declines in the phosphorylation of AKT^{S473}, AKT^{T308}, mTORC1^{S2448}, S6K1^{T389} and RPS6$^{s240/244}$ in response to DEX treatment *versus* CTL treated myotubes. Concomitant treatment with EPI or HA was able to abrogate the DEX-induced reductions in MPS rates to a similar extent. However, the protective effects of EPI and HA against the DEX-induced decline in MPS was not reflected in the phosphorylation of key anabolic signaling intermediates. This finding was perhaps surprising since previous literature reported significant increases in markers of myogenesis following EPI treatment [17]. Nevertheless, our novel finding that HA was able to preserve MPS rates to a similar extent as the natural flavanol EPI furthers our understanding to the importance of EPI metabolites present in circulation for relatively long durations (~24h) following supplementation in the regulation of myotube metabolism. Furthermore, these results may explain discrepancies between acute and chronic *in vivo* supplementation interventions. Acute interventions have measured MPS rates over a 4–6h period post cocoa ingestion (i.e. [38]). Due to the differences in absorption rates of gut metabolites, these acute interventions likely only measure the effectiveness of the fast-absorbing structurally related epicatechin metabolites (SHREMs) on skeletal muscle metabolism [32]. However, it is likely, due to the differences in the chemical structure between metabolites, that each metabolite will have divergent effects on skeletal muscle metabolism and may explain why chronic studies (which account for all metabo-

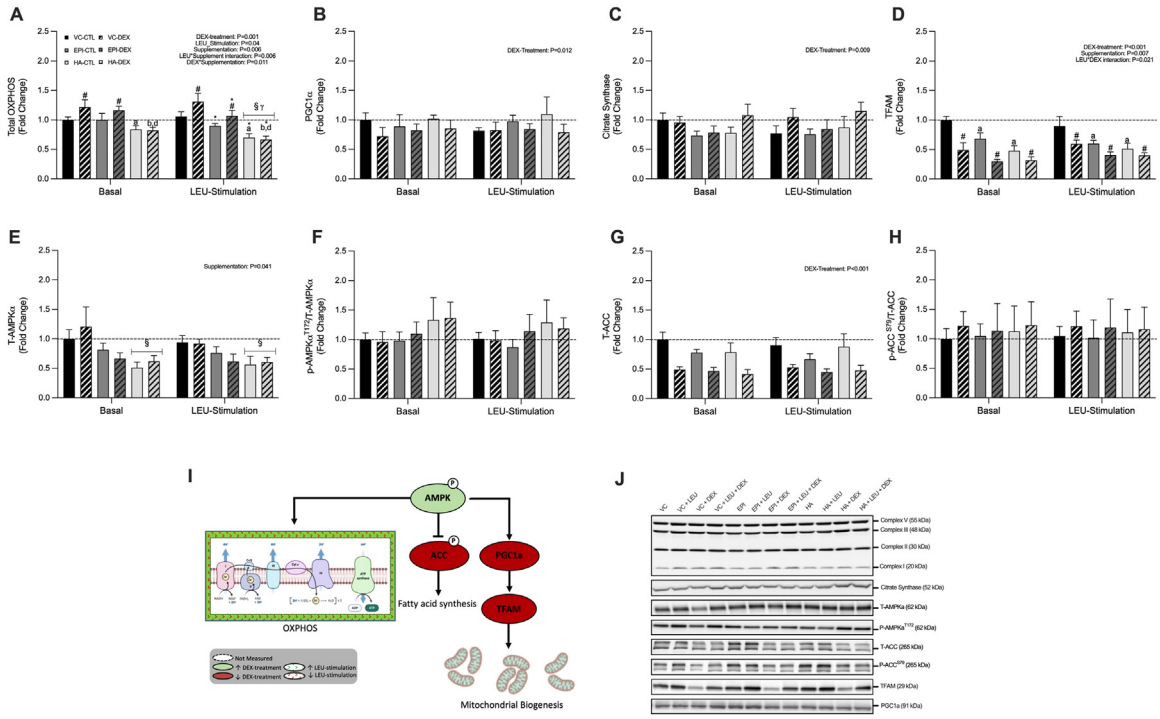

Fig. 5. Oxidative metabolism protein expression in response to 24hr treatment. (A) total OXPHOS ($n = 6$), (B) PGC1α ($n = 5$), (C) citrate synthase ($n = 6$), (D) TFAM ($n = 6$), (E) total AMPKα ($n = 6$), (F) p-AMPKα^{T172}/T-AMPKα ($n = 6$), (G) total ACC ($n = 6$), (H) p-ACC^{S79}/T-ACC ($n = 6$), (I) schematic overview of alterations in measured oxidative metabolism markers following 24h treatme nt, (G) western blot representative image of oxidative metabolism markers. Data are presented as means ± SEMs and were analyzed using a 3-factor mixed-model ANOVA.* LEU-stimulated significantly different from basal at the $P < .05$ level. # DEX-treated significantly different from CTL-treated at the $P < .05$ level. § supplementation group significantly different from VC group at the $P < .05$ level. $^{\gamma}$ supplementation group significantly different from EPI group at the $P < .05$ level. [a] significantly different from VC-CTL at the $P < .05$ level. [b] significantly different from VC-DEX. [d] significantly different from EPI-DEX at the $P < .05$ level. ACC; acetyl-CoA carboxylase, AMPKα; 5′ AMP-activated protein kinase, CTL; 24hr control (no atrophic stimulus), DEX; 24hr, 100μM dexamethasone treatment, EPI; 24hr, 25μM epicatechin supplementation, HA; 24hr, 25μM hippuric acid supplementation, LEU; 90min of 5mM leucine stimulation, PGC1α; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, TFAM; Mitochondrial transcription factor A, VC; Ethanol matched vehicle control.

lites) have shown improvements in muscle function [10,11], yet acute interventions do not show any alterations in muscle protein metabolism despite preferential changes to muscle vasculature [38]. As such, the present findings highlight the need for further research to determine the different roles of flavanol metabolites on *in vivo* skeletal muscle protein metabolism in order to optimize supplementation protocols to support adaptation.

Given evidence of the central role of impaired muscle anabolic responsiveness in conditions of atrophy [3–5,27], we also examined the ability of EPI and HA treatment to offset DEX-induced alterations in LEU-stimulated MPS rates to provide some translation to evidence from *in vivo* human models. We have recently demonstrated the utility of this approach, detecting a reduced anabolic responsiveness to an acute LEU-treatment (5mM) in C2C12 myotubes treated in old *versus* young serum [39]. Herein, we were unable to detect any DEX-induced alterations in LEU-stimulated MPS rates or the protein expression of key anabolic signaling markers *versus* CTL treated myotubes. Similarly, previous research has demonstrated that 1h of LEU treatment (5mM) was sufficient to overcome DEX-induced decrements in the protein expression of key markers of anabolic signalling [40]. Therefore, it is plausible that DEX treatment does not induce declines in anabolic responsiveness, instead exerting an atrophic effect primarily by dampening basal MPS. The fact that myotube atrophy occurred independently of any 'anabolic resistance' in the present study, could be viewed as a limitation when utilizing this model to examine the effectiveness of treatments to offset muscle atrophy *in vivo*. That said, declines in basal MPS play an important role in the manifestation of muscle atrophy in human disease [5,41,42], highlighting the utility of this model as a means to investigate potential countermeasures to muscle atrophy.

Muscle atrophy under catabolic conditions is also regulated by an increase in muscle protein breakdown, where rates exceed those of MPS promoting a negative net protein balance [43,44]. Within skeletal muscle, two main degradation systems are suggested to be activated under atrophy-inducing conditions; [1] the ubiquitin proteasome system and, [2] the autophagy lysosome system [43]. In line with previous literature, which noted significant increases in MPB following DEX treatment [45], we hypothesized that 24h DEX treatment would significantly increase the protein expression of key markers of catabolic signalling and concomitant treatment with EPI or HA would prevent such alterations. Here, we report significant differences in protein expression of key markers in the ubiquitin proteasome (i.e. MAFbx and MURF1 higher in DEX) and autophagy lysosome (i.e. higher LC3 α/β II:I and lower caspase-3 in DEX) systems in DEX-treated compared to CTL-treated myotubes. Similar to previous research in DEX and lipopolysaccharide treated C2C12 myotubes, concomitant treatment with EPI or HA was unable to prevent alterations in MAFbx or MURF1 protein expression [18,46]. However, the concomitant treatment did abrogate the DEX-induced alterations in LC3 α/β II:I and caspase-3 protein expression. This is important as the autophagy lysosome system is active under catabolic conditions to degrade and recycle proteins [47]. Furthermore, under conditions of oxidative stress, autophagy can trigger the selective degradation and removal of mitochondria, through mitophagy (for a review see [48]). Previously, EPI has been demonstrated to exhibit anti-oxidative properties stimulating the protein expression of multiple anti-oxidant en-

zymes (i.e. SOD and catalase [49]). In combination with the aforementioned data, it is plausible that flavanols may act to preserve myotube diameter partially through this oxidative stress-autophagy mechanism, although research is warranted to examine this further.

Mitochondria are critical to contractile function [50,51] and are proposed to play an important role in muscle protein turnover, which has high ATP requirements [52]. In atrophy-like conditions, previous research reports significant declines in mitochondrial protein synthesis [27,53] as well as alterations in mitochondrial metabolism [54–57]. Polyphenolic compounds have been demonstrated to enhance mitochondrial function [16] and protect against mitochondrial insult [22]. Therefore, we hypothesized that EPI or HA treatment would preserve myotube diameter, partially through the maintenance of mitochondrial function in atrophy-like conditions (i.e. DEX treatment). In line with previous research, we report alterations to markers of mitochondrial function in atrophy-like conditions. Specifically, we report a significant reduction in the protein expression of PGC1α, ACC and TFAM in response to 24h DEX treatment *vs.* CTL treated myotubes. In contrast to previous literature [16,22], we did not detect any preservation of these markers in myotubes co-incubated with EPI or HA. Similarly, under CTL conditions, the marginally lower myotube diameter following HA and EPI *versus* VC (as discussed above) coincided with lower total OXPHOS (HA only) and TFAM (EPI and HA) expression *vs*. VC. These results were somewhat surprising in the context of previous literature and may be due to differences in culture conditions between studies. For example, previous literature has utilized immortalized cell lines cultured in media containing higher glucose concentrations (e.g. [16,17,31]), which promotes a heavy reliance upon anaerobic glycolysis [58] and mitochondrial dysfunction [59–62]. In contrast, lower media glucose concentrations (such as 5mM as used in this study) promotes a more oxidative phenotype in immortalized cell lines [63]. Therefore, we speculate that the contrasting findings between the current and previous studies in response to DEX treatment may be secondary to differences in basal metabolism during growth and differentiation. To limit type I errors, the current study focused on the measurement of key markers of mitochondrial function. Therefore, we were unable to measure the protein expression of markers involved in Ca^{2+} handling or oxidative stress, both which have been purported as secondary mechanisms of mitochondrial function improvements in basal conditions following treatment with polyphenolic compounds [49,64]. Therefore, in combination with the aforementioned alterations in autophagic signaling it is possible that flavanol metabolites could improve myotube metabolism via these mechanisms, although this remains to be elucidated.

Despite the importance and novelty of the current findings, the authors note that a limitation of the current study was the absence of measurement of additional EPI metabolites (i.e., SHREMs or additional RFMs) due to the lack of commercial availability of these compounds. Further research utilizing an *ex vivo* approach with multiple serum samples obtained over the 12h post cocoa supplementation may now be required to extend on the current findings. That said, this study provides important and novel insights into the role of the 3/1C RFM metabolite HA in preserving myotube diameter and MPS rates during atrophy-like conditions in C2C12 myotubes, and findings may have relevance in the context of muscle atrophy observed following corticosteroid treatment.

Conclusions

In summary, our data demonstrate that EPI was able to exert anti-atrophic effects on C2C12 myotubes and the colonic metabolite HA was able to preserve myotube diameter and MPS rates to a similar extent to its natural flavanol (i.e., EPI). Alterations in basal MPS and myotube diameter following co-incubation of EPI/HA, occurred independently to alterations in markers of mitochondrial function, with a possible attenuation of MPB markers instead contributing to preserve myotube diameter. Overall, our data provides important insight on the role of flavanol gut metabolites on skeletal muscle. Furthermore, the current study highlights the significance of accounting for slow-absorbing colonic metabolites (e.g., HA) in experimental protocols, providing evidence against the use of acute *in vivo* interventions to determine the effectiveness of cocoa supplementation to alter muscle metabolism. Future research needs to be conducted in an *in vivo* model of atrophy to confirm the ecological validity of chronic cocoa supplementation.

Declarations of competing interest

None

Author contributions

S.J.E, S.C, T.N, S.W.J, C.R and L.B conceived and designed the research. S.J.E conducted all in vitro experiments. S.J.E and S.A analysed the data. S.J.E and L.B wrote the manuscript. S.J.E, S.C, T.N, S.A, R.N.M, P.T.M, S.W.J, C.R and L.B contributed to the interpretation of the results, edited and approved of the final manuscript.

Funding

This work was supported by a studentship to S.J.E from the BBSRC Midlands Integrative Biosciences Training Partnership.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2022.109150.

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