Nutrition & Metabolism

BRIEF REPORT Open Access

The polyphenol metabolite urolithin A suppresses myostatin expression and augments glucose uptake in human skeletal muscle cells

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Abstract

Purpose Polyphenolic plant extracts have demonstrated anti-inflammatory and anti-catabolic effects in vitro, however their meaningful translation into humans remains elusive. Urolithin A (UA), a gut-derived metabolite of ellagitannins, has shown promise for improving muscle function and metabolic health in rodent models. This study aimed to explore the impact of UA on insulin and anabolic sensitivity in human skeletal muscle cells.

Methods Primary human myogenic cultures were derived from skeletal muscle biopsies of eight healthy adults. After differentiation, myotubes were treated with 0.002, 1 and 50 µM UA or vehicle for 24 h. Cell viability was assessed using a resazurin assay. Basal and insulin-stimulated glucose uptake was measured using tritiated deoxy-D-glucose, whilst amino acid-stimulated protein synthesis was estimated using the surface sensing of translation (SuNSET) technique. Expression of myostatin and glucose transporters was quantified via real-time PCR.

Results UA treatment at ≤ 50 µM did not compromise cell viability. Treatment with 50 µM UA enhanced both basal- and insulin-stimulated glucose uptake by 21% ($P < 0.05$) and 24% ($P < 0.01$), respectively, compared to vehicle and was accompanied by a 1.8-fold upregulation of *GLUT4* expression ($P < 0.01$). 50 µM UA reduced myostatin (*MSTN*) expression by 14% ($P < 0.01$) but did not alter amino acid-stimulated global cell protein synthesis.

Conclusion This study provides evidence of UA's metabolic benefits in primary human myotubes, notably improving basal- and insulin-stimulated glucose uptake and supressing *MSTN* expression. These findings suggest UA could be an effective nutraceutical for mitigating insulin resistance and warrants further investigation.

Keywords Insulin sensitivity, Anabolic sensitivity, Skeletal muscle, Primary human myotubes, Urolithin A

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Introduction

According to estimates from The World Health Organization (WHO), non-communicable diseases (NCD) account for 74% of all deaths globally; cardiovascular diseases, cancers, chronic respiratory diseases and diabetes collectively comprising over 80% of all premature NCD deaths [1]. Raised blood pressure overweight/obesity, hyperglycaemia and hyperlipidaemia are the major physiological risk factors for these most common NCDs. Commonly underlying such diseases is a characteristic, subclinical, systemic inflammation, which is associated with a deleterious metabolic cascade involving cellular oxidative stress, atherosclerotic processes and insulin resistance [2]. The identification and exploration of so-called nutraceuticals – naturally-occurring, bioactive compounds with purported health benefits beyond the provision of energy substrates – is growing industry and field of research interested in the prevention of NCD.

Of particular interest, plants produce a class of phytochemicals called polyphenols, some of which are purported to confer health benefits, particularly with regard to NCD and the pathophysiological process of ageing [3]. Certain polyphenolic plant extracts have demonstrated anti-inflammatory and anti-catabolic effects on skeletal muscle cells in both in vitro and in vivo in rodent models [4–7], however their meaningful translation into humans remains elusive [8]. Amongst the most studied polyphenolic compounds are ellagitannins (ETs) and ellagic acid (EA), which are present in fruits, nuts and seeds, such as pomegranates and walnuts. ETs and EA are metabolised by the microbiota of the gut, with ETs first being hydrolysed into EAs in the upper gastrointestinal tract by tannases [9]. Whilst ETs and EA can be readily obtained from the diet, they exhibit poor pharmacokinetic profiles, low water solubility and intestinal permeability, and a short plasma half-life due to rapid elimination by first-pass metabolism [10–14]. EA is further metabolised by microflora of the large intestine to form a group of readily absorbed and biologically active compounds termed urolithins; the most intensively investigated being Urolithin A (UA) [15]. However, only ~ 40% of individuals demonstrate significant ability to convert ETs and EAs from dietary sources, such as pomegranate juice, into circulating UA, demonstrating the requirement to consider direct supplementation [16]. Indeed, in humans, acute supplementation with 500 mg UA was found to provide > 6-fold greater exposure to circulating UA than pomegranate juice [16].

In skeletal muscle culture, c. elegans and rodent models of ageing, treatment with UA has been shown to increase muscle function, angiogenesis, respiratory and exercise capacity, which have been attributed to enhanced mitophagy and mitochondrial functioning [17–19]. Additionally, studies have shown beneficial effects of UA on glucose tolerance and insulin sensitivity in rodent models of obesity and ageing [20–22]. In human trials, UA supplementation (500 or 1000 mg·day^{-1}) demonstrated a favourable safety profile in older adults, with significant biological availability and upregulated skeletal muscle mitochondrial gene expression [23, 24]. Myostatin, which principally serves to negatively regulate skeletal muscle growth, has been implicated in the metabolic dysregulation associated with pathophysiological ageing in humans [25] and positively associated with insulin resistance [26]; pertinently, its suppression has been repeatedly demonstrated through the actions of numerous polyphenols [8]. To date, however, the effects of UA on insulin and anabolic sensitivity in human skeletal muscle have not been investigated. This gap is important to address, since metabolic dysfunction in skeletal muscle contributes to the aetiology of sarcopenia and thus may explain the mechanisms by which UA supplementation in humans contributes to its beneficial effects in healthy middle-aged and older adults [23, 24, 27, 28]. Therefore, the aim of the present study was to establish whether acute treatment of primary human myotubes with UA would confer positive changes in insulin and anabolic sensitivity.

Methods

Primary cell culture and urolithin A treatments

Primary human myogenic cultures were established from skeletal muscle biopsy samples of eight healthy adults (4 male, 4 female) of varying age and BMI (mean [SD] age: 47 [± 26]; BMI: 27.1 [± 5.8] kg·m^{-2}). Following approval by the University of Nottingham Faculty of Medicine & Health Sciences Research Ethics Committee (reference No 143–1811) in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki), written informed consent was obtained from all participants. Following this, they undertook a medical screening (comprising of medical history, health questionnaires, 12-lead electrocardiogram, blood pressure, weight and height measurements, and blood sample for routine chemistry and haematology) prior to obtaining the muscle sample. Myogenic cultures were proliferated and subsequently differentiated into myotubes, as described previously [25, 29]. Cultures were passaged no more than twice to best preserve skeletal muscle and donor phenotype. After 6–8 days of differentiation, myotubes were incubated for 24 h with 0.002, 1 and 50 μM UA (lyophilised UA provided by Amazentis SA, Lausanne, CH) or a matched vehicle treatment (0.1% DMSO). Viability of myotubes after UA treatment was assessed via resazurin assay, using UA concentrations from 0.002 to 100 μM.

Insulin and anabolic sensitivity

Basal and insulin-stimulated (100 nM) glucose uptake was assessed in myotubes using nominally radio-labelled

(tritiated) deoxy-D-glucose (DOG) (2-[1,2–3 H (N)]; PerkinElmer, MA, USA), as previously described [30], with glucose uptake normalised against total cell protein content and converted to absolute glucose uptake rate ($pmol \cdot mg^{-1} \cdot min^{-1}$).

Changes in amino acid stimulated global myotube protein synthesis were estimated using the surface sensing of translation (SuNSET) technique [31], which measures the incorporation of the amino-nucleoside antibiotic puromycin into newly synthesised muscle proteins. Cells were stimulated for 3 h with 2 mM L-leucine and 5 mM D-glucose and for the final 30 min incubated with 1µM puromycin dihydrochloride, as previously described [25]. Briefly, equal amounts of total protein (determined via Pierce bicinchoninic acid assay) were separated on 12% SDS-PAGE gels, transferred onto PVDF membranes, incubated with anti-puromycin antibody (clone 12D10; Millipore, US) and visualized using chemiluminescence. Following immunodetection, membranes were stained with 0.1% Coomassie and digitally imaged. Total lane intensity was measured by densitometry and relative protein synthetic activity was estimated as the ratio of puromycin-labelled protein intensity to total protein (Coomassie) intensity.

mRNA expression

Myotubes were harvested using Tri-Reagent™ (ThermoFisher Scientific), prior to RNA extraction and cDNA synthesis using Invitrogen SuperScript™ in accordance with manufacturer's guidelines. Gene expression was assessed via real time PCR (qPCR) with TaqMan probes (custom designed or commercially available pre-designed assay kits; Supplementary Table 1) on a StepOnePlus™ system (Applied Biosystems, CA, US). For the analysis of relative mRNA expression, genes of interest were normalised against the geometric mean of Hydroxymethylbilane Synthase (*HMBS*) and Ribosomal Protein Lateral Stalk Subunit P0 (*RPLP0*) expression, which demonstrated high stability within and between experiments and donors under these experimental conditions.

Statistical analysis and terminology to describe Biological and Technical Replication

All statistical analysis was performed in GraphPad Prism (Version 9.2). Descriptive data are presented as mean ± SD, whilst experimental data are presented as mean ± SEM. Assumptions of normality were tested via Shapiro-Wilk test, whereafter analysis of variance (ANOVA) with repeated measures, was performed. Significant ($P < 0.05$) post-hoc comparisons are presented as mean difference [95% CI], where relevant. All reported N values on each figure are the number of independent donor experiments, whereas the number of in vitro treatment replicates performed within each donor are reported in the legend, where relevant.

Results

Effect of urolithin A on the cell viability of human myogenic culture

Following differentiation, myotubes were either untreated (vehicle control), or treated with UA concentrations between 0.002 and 100 µM for a period of 24 h. No statistically significant loss of cell viability was observed with 24 h treatment with UA concentrations ≤ 50 µM, whereas at a concentration of 100 µM, there was a ~ 40% significant decline in cell viability (Fig. 1A).

Effects of urolithin A on insulin and anabolic sensitivity

Mixed Effects ANOVA revealed significant treatment effects of both UA ($P = 0.043$; $\eta^2_P = 0.410$) and insulin ($P = 0.008$; $\eta^2_P = 0.781$) on glucose uptake, but not an interaction effect between the two (Fig. 1B). Post-hoc comparisons revealed statistically significant increases in basal (Mean Difference [95% CI]: 0.21 [0.01, 0.41]-fold greater; $P < 0.05$) and insulin-stimulated (Mean Difference: 0.24 [0.04, 0.43]-fold greater; $P < 0.01$) glucose uptake after treatment with 50 µM UA only, compared to Vehicle. In determining whether UA affected anabolic sensitivity, we observed no effect of UA treatment on amino acid-stimulated global cell protein synthesis (Fig. 1C).

Effects of urolithin A on the mRNA expression of myostatin, glucose transporters and autophagy

Repeated measures one-way ANOVA found a significant effect of UA treatment on the relative mRNA expression of *MSTN* ($P = 0.031$; $\eta^2_P = 0.382$), with significant suppression (14%) after treatment with 50 µM UA compared to Vehicle (Mean Difference [95% CI]: -0.210 [-0.031, -0.390] a.u.; $P < 0.01$; Fig. 2A). A statistically significant effect of UA treatment on *GLUT4* ($P = 0.016$; $\eta^2_P = 0.427$) (Fig. 2B), but not *GLUT1* (Fig. 2C), gene expression was also found, with *GLUT4* expression being 1.8-fold greater with 50 µM UA vs. Vehicle (Mean Difference [95% CI]: 0.788 [0.080, 1.496] a.u.; $P < 0.01$). Data on expression of genes involved in cell autophagy, mitophagy and mitochondrial function are included in Fig. 3. One-way ANOVA revealed a significant effect of UA treatment on the relative mRNA expression of *MAP1LC3B* only ($P = 0.028$; $\eta^2_P = 0.506$), which was driven by upregulation (20%) with 50 µM UA compared to Vehicle (Fig. 3A).

Discussion

To our awareness, the present study is the first to directly assess the effects of UA on indices of insulin and anabolic sensitivity in primary human myotubes. Expanding upon previous findings in non-human myogenic

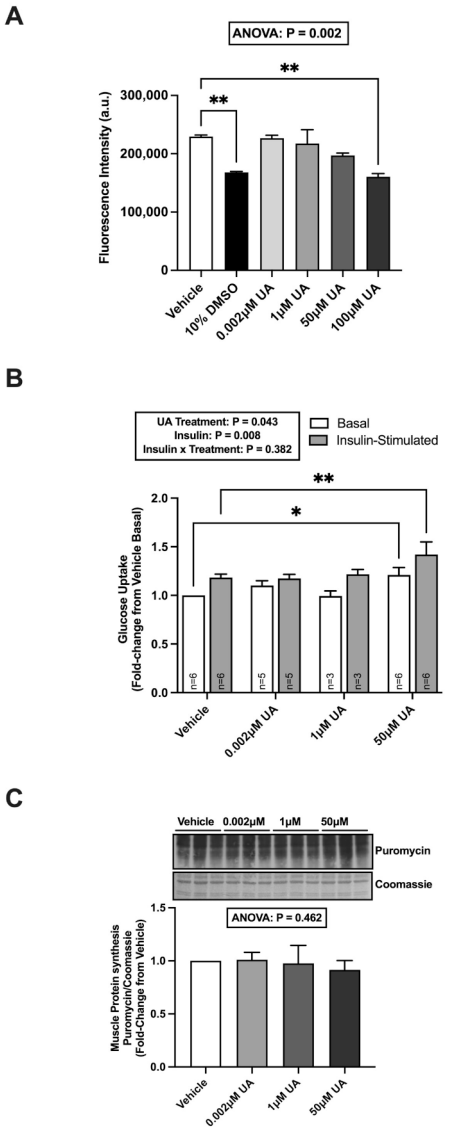

Fig. 1 Effect of 24 h UA exposure on cell viability, glucose uptake and amino-acid stimulated protein synthesis. (**A**) Fully differentiated human myotubes were incubated with either vehicle (0.1% DMSO), a positive control (10% DMSO), or varying concentrations of UA for 24 h. Viability was assayed fluorometrically vis Resazurin assay. Data are presented as Mean ± SEM of fluorescence intensity background ($n = 3$ replicate wells per treatment). (**B**) Basal (white bars) and insulin-stimulated (100 nM; grey bars) glucose uptake was measured using [^{3}H] 2-DOG, following serum and glucose starvation. Glucose uptake was measured in pmol·mg^{-1} protein·min^{-1}, however, to account for absolute differences in glucose uptake rate between myotubes from independent donor repeats. (**C**) Amino-acid stimulated protein synthesis was measured using the incorporation of puromycin (1μM) into newly synthesised muscle proteins following serum starvation and subsequent glucose (5mM) and leucine (2 mM) incubation. Proteins were separated via SDS-PAGE and probed with an anti-puromycin antibody to label newly synthesised peptides. Data for (**B**) and (**C**) are presented as Mean ± SEM of fold-change from Vehicle Basal for each donor repeat. Individual data points for each donor are the mean of 4–6 replicate wells for each treatment. Significant differences between treatments, as indicated: *$P < 0.05$, **$P < 0.01$

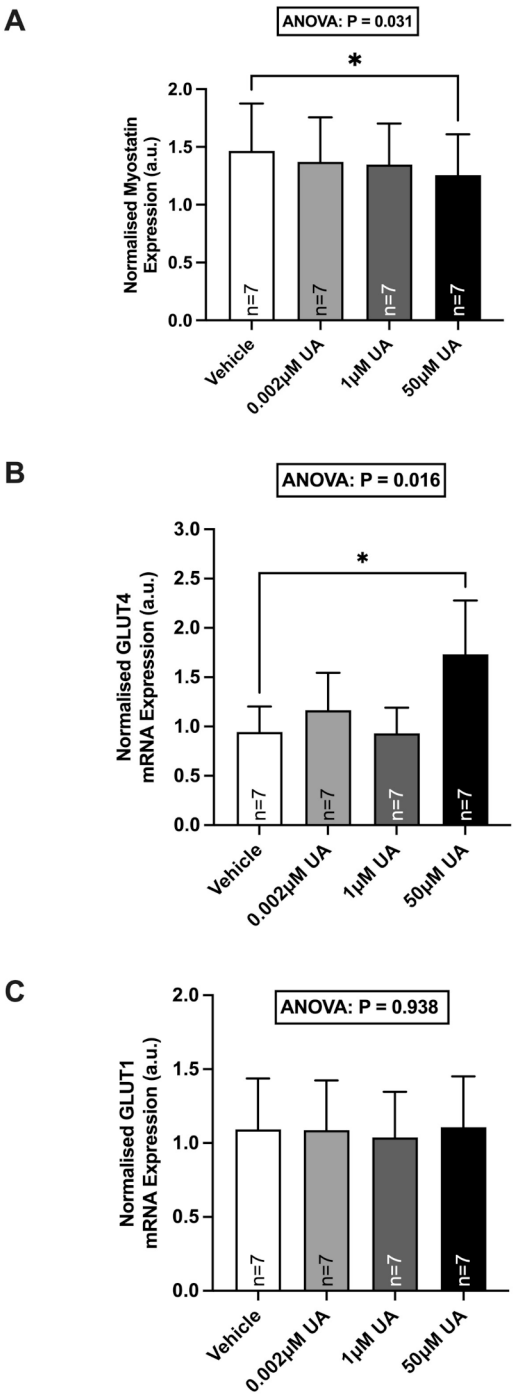

Fig. 2 Effect of 24 h UA exposure on *MSTN* **(A)**, *GLUT4* **(B)** and *GLUT1* **(C)** mRNA expression. Repeated measures one-way ANOVA revealed a significant effect of UA on *MSTN* (**A**) and *GLUT4* (**B**), but no effect on *GLUT1* (**C**) mRNA expression. Data presented as Mean ± SEM of gene of interest expression, relative to the geometric mean of *HMBS* and *RPLP0*. Significant differences between treatments, as indicated: *$P < 0.05$

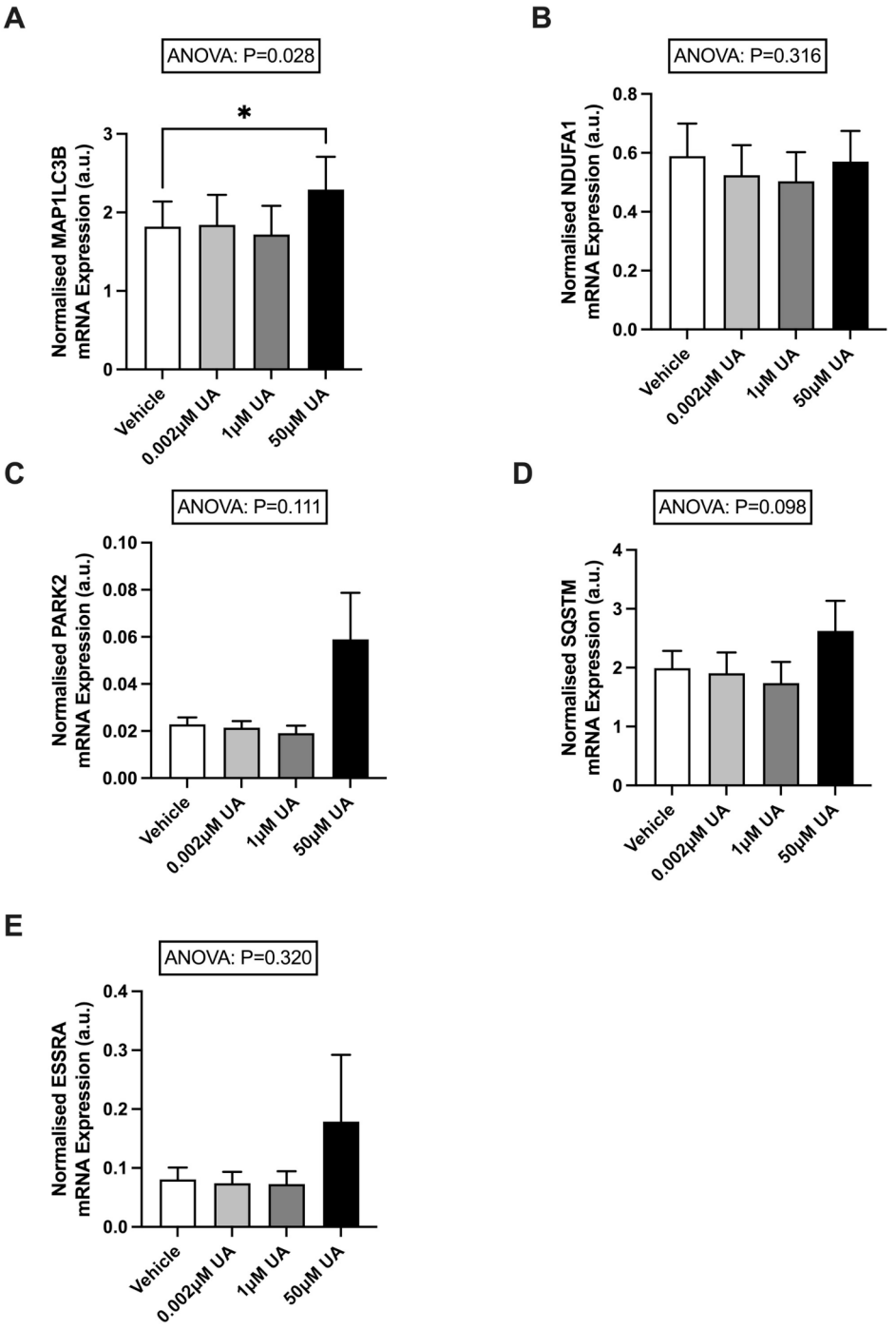

Fig. 3 Effect of 24 h UA exposure on *MAP1LC3B* **(A)**, *NDUFA1* **(B)**, *PARK2* **(C)**, *SQSTM* **(D)** and *ESSRA* **(E)** mRNA expression. Repeated measures one-way ANOVA revealed a significant effect of UA on *MAP1LC3B* mRNA expression only. Data presented as Mean ± SEM; $n = 7$ independent donors. Significant differences between treatments, as indicated: $^{*}P < 0.05$

cultures and animal models, we found that acute (24 h) treatment of primary human myotubes with 50 μM UA markedly improved both basal- and insulin-stimulated glucose uptake, which was associated with significant upregulation of *GLUT4* mRNA expression. Whilst we did not observe an acute improvement in global amino-acid stimulated cell protein synthesis, we did find a significant UA-induced reduction (14%) in the mRNA expression of *MSTN*, which is a potent negative regulator of skeletal

muscle mass and development, which could prove beneficial in the context of chronic UA supplementation.

In the present study, treatment of myotubes with 50 μM UA elicited a 21% increase in basal glucose uptake, which was preserved but not further enhanced in the insulin-stimulated state. This suggests that UA has comparable potential to confer beneficial effects in skeletal muscle glucose uptake in both the post-absorptive and post-prandial state. Mechanistically, the increased glucose uptake observed with UA may be attributable to upregulation of *GLUT4*; treatment with 50 μM UA in our study induced an almost 2-fold increase in *GLUT4* mRNA expression, which may explain the observed increases in glucose handling in both the basal and insulin-stimulated states. In accordance, recent observations in rat-derived L6 myogenic cultures have shown enhanced basal glucose uptake via increased recruitment of GLUT4 to the plasma membrane and activation of PI3K/Akt- and AMPK-dependent pathways in response to treatment with 100 and 200 μM but not 50 μM UA [32]. While GLUT4 abundance in primary human myotubes is low, it is still significantly recruited to the plasma membrane upon insulin stimulation [33] and is translocated in C2C12 cells upon stimulation with low doses of EA [34]. This observation therefore warrants further investigation under in vivo conditions.

In the context of the established propensity for UA to promote mitochondrial function and biogenesis in skeletal muscle [17, 23], the increased capacity for myotubes to uptake glucose in the present study may contribute to an increased capacity for oxidative phosphorylation, which is perturbed in insulin resistant individuals [35, 36]. Supporting this hypothesis, it has previously been demonstrated that chronic UA administration upregulates the activity of mitochondrial respiratory complexes I and II in the skeletal muscle of muscular dystrophic or HFD-fed mice [17, 19]. In the postprandial state, as much as 50% of the glucose that is taken up by skeletal muscle undergoes oxidation, demonstrating the importance of this mechanism in glucose homeostasis [37]. To that effect, glucose is first metabolised to pyruvate via glycolysis and can subsequently enter the mitochondria for further metabolism within the TCA cycle. Thus, it is plausible that the observed enhancement of glucose uptake serves the UA-mediated increased capacity of the mitochondria, which expands upon the previously demonstrated UA-induced upregulation of basal oxygen consumption in C2C12 myotubes [19] and whole-body oxygen consumption in mice [21]. The significant upregulation of *MAPLC3B* (a key gene in cell autophagy) observed in the current study further support this notion. Interestingly, UA can activate AMPK [38], a common regulator of both autophagy and *GLUT4* expression [39, 40], which may provide the mechanism by which UA exerts its dual effects on glucose uptake and mitochondrial function.

Another novel finding of this study was that UA treatment reduced *MSTN* mRNA expression by ~ 14%. Given myostatin's involvement in the inhibition of muscle growth and regeneration [41–43] and the elevated *MSTN* mRNA expression reported in older adults with obesity [25], even modest sustained suppression of myostatin may present a promising avenue for nutraceutical targeted mitigation of sarcopenic obesity. Indeed, myostatin is considered a highly druggable target and the present study demonstrates a potential novel nutritional approach for its modulation that negates prior issues of poor ET/EA bioavailability.

Despite the suppression of *MSTN* mRNA expression, we didn't observe any changes in amino acid-stimulated myotube protein synthesis, as indicated by the incorporation of the tyrosyl-tRNA analogue Puromycin into newly synthesised peptides. This is consistent with a study of C2C12 myotubes by Rodriguez and colleagues who reported that 24 h treatment with 15 μM UA did not affect myotube protein synthesis, and suggests an uncoupling of insulin and anabolic resistance in the UA-mediated suppression of myostatin in human skeletal muscle cells [44]. Therefore, longer duration of treatment with UA may be required to investigate the effects of sustained myostatin suppression on protein synthesis.

Limitations

In these experiments, myotubes were treated with so-called 'parent UA'. It must be considered that parent UA is not the dominant form within the circulation. Rather, its phase II metabolites (predominantly UA-glucuronide and UA-sulfate) abound in far greater concentrations [23]. There is some evidence that UA conjugates have shown lower biological potency in vitro, compared to parent UA, on the bioactive anti-proliferation effect [13]. Future research must seek to better understand the role of conjugation in relation to UA effects on skeletal muscle biology. Furthermore, reflecting the time and resource intensive nature of primary human myogenic tissue culture, these experiments employed modest numbers of independent repeats, limiting extrapolation to the wider population. Nevertheless, the presence of significant effects in cultures from a relatively heterogenous sample of donors reinforces the robustness of these results and lays the ground for future studies in humans. Finally, the molecular events studied in the present study were limited to mRNA measurements, which may not always predict changes in protein levels [45]. However, correlations between mRNA and protein levels have been observed in human cell lines [46] and this relationship can be enhanced by applying gene-specific, cell type independent RNA-to-protein conversion factors [47].

Conclusions

This study provides the first direct evidence of metabolically beneficial effects of Urolithin A in primary human skeletal muscle cells. It was demonstrated that acute (24 h) UA treatment improves both basal and insulin-stimulated glucose uptake in human skeletal muscle cells, and that these effects may be mediated by the upregulation of *GLUT4* expression. Additionally, it was revealed that UA exerts a suppressive effect on the expression of *MSTN* in human skeletal muscle cells; while this did not translate to enhanced muscle protein synthesis in the acute setting, it could feasibly be beneficial in the context of chronic supplementation. Together these findings suggest plausible translation into human studies of obesity and ageing, where these effects, should be investigated further.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12986-025-00909-0.

Supplementary Material 1

Author contributions

Conceptualization: KT, SWJ, LGK and AW designed the studies. Funding Acquisition: The MRC PhD studentship grant was awarded to KT, SWJ and LGK. Investigation: AW, LGK, AJB, DD, AMF, SWJ and KT conducted research. Data Curation and Formal Analysis: AW, KT, DD, AMF analysed data and performed statistical analysis. Roles/Writing: AW, LGK, SWJ and KT wrote the original manuscript. Review & Editing: AJB, DD, AMF, all reviewed and approved the manuscript prior to submission.

Funding

This work was supported by an MRC PhD studentship grant awarded to KT, SWJ and LGK.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

Conflict of interest

DD and AMF are employees of Amazentis SA, Lausanne, Switzerland. LGK was an employee of Nestlé Health Science, Vevey, Switzerland, during the project, is on the scientific advisory boards of Vital Proteins and NUUN, has participated on advisory boards of Liquid I.V and has received personal fees from RNWY and Nestlé Health Science, and is a board member of Siftlink. The other authors report no potential conflicts of interest.

Received: 5 November 2024 / Accepted: 6 February 2025
Published online: 17 February 2025

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