

Arginine protects muscle cells from wasting *in vitro* in an mTORC1-dependent and NO-independent manner

Daniel J. Ham, Marissa K. Caldow, Gordon S. Lynch, and René Koopman

Basic and Clinical Myology Laboratory, Department of Physiology, The University of Melbourne, Victoria 3010; Australia

Authors last name: Ham, Caldow, Lynch, Koopman

Short title: Arginine reduces muscle cell wasting

Word count: 4740

Number of figures: 6

Reprint requests & correspondence to:

René Koopman, PhD

Department of Physiology

The University of Melbourne

Victoria 3010 Australia

Ph: +61 3 8344 0243

Fax: +61 3 8344 5818

Email: rkoopman@unimelb.edu.au

ABSTRACT

Amino acids are potent regulators of muscle protein synthesis and breakdown and have received considerable attention for the treatment of muscle wasting conditions. Arginine is critically involved in numerous physiological functions including providing substrate for the production of creatine, urea and nitric oxide (NO) and in the synthesis of new proteins. However, little is known about the direct effects of arginine on skeletal muscle protein synthesis during catabolic conditions. The aims of this study were to determine whether exogenous arginine could protect skeletal muscle cells from wasting directly and whether this effect was dependent on production of NO and/or activation of the rapamycin-sensitive mTORC1 signalling pathway. To explore these aims, we deprived mature C2C12 myotubes from nutrients and growth factors by incubating them in HEPES buffered saline with arginine or equimolar concentrations of alanine (control). Our results show that arginine: increased the ratio of phosphorylated to total mTOR (146%), S6 (40%) and 4EBP1 (69%); increased protein synthesis (69%) during the first hour of treatment; and increased myotube diameter by ~15%. Experiments using the NO synthase inhibitor L-NAME showed a NO-independent protection from muscle wasting. On the other hand, the mTORC1 inhibitor rapamycin prevented increases in phosphorylated S6, protein synthesis and myotube diameter. The activation of mTORC1 and protein synthesis by arginine was not associated with changes in the phosphorylation status of Akt, but rather increased expression of the amino acid-sensitive type III PI3-kinase Vps34 signalling protein. These data support a direct role for arginine in the regulation of mTORC1 in skeletal muscle.

INTRODUCTION

Skeletal muscle wasting, the loss or atrophy of skeletal muscle (Evans 1995), is a serious complication of a wide range of diseases and conditions such as aging, disuse, muscular dystrophy, chronic heart failure, sepsis, and cancer (Lynch et al. 2007). Muscle wasting occurs when there is a chronic imbalance between rates of synthesis and breakdown, such that muscle protein degradation exceeds protein synthesis (Koopman and van Loon 2009). Amino acids are potent regulators of muscle protein synthesis and breakdown and have consequently received considerable attention for the treatment of muscle wasting with ageing, diabetes and cancer cachexia (Koopman et al. 2014).

The mechanistic target of rapamycin complex 1 (mTORC1) is a master regulator of cell size, integrating signals from nutrients, growth factors, energy status and stress (Koopman et al. 2014). During nutrient abundance, mTORC1 drives protein synthesis and growth, and mediates growth arrest and protein breakdown under starvation conditions. Growth factors stimulate mTORC1 by a well-defined mechanism involving the phosphorylation of Akt which leads to the phosphorylation and subsequent inhibition of two key suppressors of mTORC1 activation, TSC2 and PRAS40 (Efeyan et al. 2012). Upon activation, mTORC1 phosphorylates and activates two parallel signalling pathways involved in the control of translation. S6 kinase 1 (S6K1) phosphorylation leads to activation of the ribosomal protein S6, while phosphorylation of the eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP1) releases its inhibition of the translation initiation factor eIF-4E, allowing initiation of translation and the synthesis of new proteins. Interestingly, growth factors cannot efficiently activate mTORC1 without the presence of amino acids, which activate mTORC1 in a variety of growth factor independent mechanisms, described in detail elsewhere (Jewell et al. 2013; Efeyan et al. 2012). Although essential amino acids, in particular leucine, have been strongly implicated in the activation of mTORC1 both *in vitro* (Atherton et al. 2010) and *in vivo* (Koopman et al. 2005), other amino acids can also modulate anabolic signalling and skeletal muscle mass.

The semi-essential amino acid arginine is critically involved in numerous physiological functions including providing substrate for the production of creatine, urea and nitric oxide (NO) and in the synthesis of new proteins (Wu and Morris 1998). Supplementation with arginine or its precursor citrulline in mice restores anabolic signalling and prevents muscle wasting in a number of catabolic conditions (Moinard and Cynober 2007). The regulation of skeletal muscle protein metabolism and mass by arginine *in vivo* is multifaceted. Arginine

stimulates the release of growth factors such as insulin and growth hormone, which may occur in both a NO-dependent and NO-independent manner (Collier et al. 2005; Jun and Wennmalm 1994). NO is a key signalling molecule within skeletal muscle and plays a role in satellite cell activation (Anderson 2000), myoblast fusion (Long et al. 2006) and overload-induced skeletal muscle hypertrophy (Sellman et al. 2006; Smith et al. 2002). Through its vasodilatory function, NO may play a role in nutrient delivery to the muscle (Goto et al. 2007) and can impact nutrient transport into the cell (Merry et al. 2010). It is currently unclear if skeletal muscle mTORC1 is directly sensitive to arginine, but recent work in cultured primary intestinal epithelial cells (IPEC) found that arginine activates mTORC1, stimulates protein synthesis and promotes survival in starved cells (Bauchart-Thevret et al. 2010).

Thus, arginine represents a potential nutritional intervention for muscle wasting conditions. However, little is known about the direct effects of arginine on skeletal muscle protein synthesis during catabolic conditions. To examine the role of exogenous arginine on muscle wasting, independent of potential effects on blood flow or growth factor release, mature C2C12 myotubes were growth factor and nutrient deprived by incubation in HEPES buffered saline supplemented with arginine or equimolar concentrations of alanine. We hypothesised that arginine preserves anabolic signalling and protein synthesis and reduce muscle wasting in an mTORC1 and NO-dependent manner.

METHODS

Cell culture

Murine C2C12 myoblasts (Cryosite distribution, NSW, Australia) were plated in 6 or 12 well plates and cultured in DMEM (Life Technologies, Australia) containing 10% (v/v) fetal calf serum (Life Technologies) and antimycotic antibiotic solution (100 unit/ml penicillin/streptomycin, Life Technologies) at 37°C in an atmosphere of 5% CO₂. Upon confluency, the media was changed to DMEM containing 2% (v/v) horse serum (Life Technologies) for 5 days to promote formation of mature multinucleated myotubes (Atherton et al. 2010). To induce wasting, cells were washed once in HEPES buffered saline (HBS, 20 mM HEPES/Na pH 7.4, 140 mM NaCl, 2.5 mM MgSO₄, 5 mM KCl and 1 mM CaCl₂), then incubated in HBS with L-arginine or equimolar concentrations of L-alanine (control) for 0.5, 1, 2 or 4 h. For serum free experiments, cells were washed once in arginine and serum free DMEM (Life Technologies, Australia) and then incubated in arginine and serum free media supplemented with either 0, 0.4 or 2.5 mM arginine for 48 h. L-arginine and L-alanine were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Based on an initial dose-ranging experiment (Fig 2), 2.5 mM was chosen for all future experiments. L-NG-Nitroarginine Methyl Ester (L-NAME, 10 mM, Sigma-Aldrich) and rapamycin (100 nM, Sigma-Aldrich) were used to inhibit NO production (Long et al. 2006) and mTORC1 activation (Herningtyas et al. 2008), respectively. A thirty minute pre-treatment period was used for both inhibitors.

Immunohistochemistry

Cells were washed 2 × 5 min in phosphate buffered saline (PBS) and then fixed with 3.7% formaldehyde for 15 min. Cells were then washed in PBS (3 × 5 min), permeabilised with 0.3% TritonX100, washed in PBS (3 × 5 min) and then incubated in anti- α -myosin (1:50, Sigma-Aldrich) in PBS at room temperature for 1 h. Cells were then washed (3 × 5 min) and incubated in goat-anti-rabbit Alexa555 secondary antibody (1:400, Life Technologies) and DAPI (1:1000) for 30 min in PBS. Cells were washed in PBS (3 × 5 min) and then imaged on a Zeiss Axiovert 40 CFL inverted microscope at 20X magnification. Four images were taken in each well from pre-defined locations within each quadrant. Myotube diameter was measured using Axiovision software (Zeiss, USA). A total of ~50-80 myotubes were measured per well and the average diameter of each well was used for statistical analysis.

Protein synthesis

Myotubes were grown in 6-well plates and treated as described above. To determine the rate of protein synthesis we utilised SUnSET methodology, as described (Goodman et al. 2011). Briefly, puromycin (Sigma-Aldrich) was administered to the media at a final concentration of 1 μ M exactly 30 min before cells were collected in ice cold homogenising buffer as described below. Anti-puromycin was purchased from Millipore (Kilsyth, Victoria, Australia).

Protein extraction and western blotting

Cell lysates were homogenised in ice-cold extraction buffer (10 mM Tris.HCL (pH 7.4), 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X, 10% glycerol, 0.1% SDS, 20mM $\text{Na}_4\text{P}_2\text{O}_7$, 2 mM Na_3VO_4 , 1 mM NaF, 0.5% sodium deoxycholate and 1 mM PMSF) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Homogenates were centrifuged at 10000g for 10 min at 4°C to remove cell debris. Supernatant protein concentrations were determined using the Bradford-Lowry protein assay method, as per the manufacturer's instructions (Bio-Rad laboratories, NSW, Australia). Samples were standardised to a protein content of 2 mg/ml in homogenising buffer. Equal amounts of protein (30 μ g/lane) in 4 x Laemmli buffer were run on SDS-PAGE gels and proteins were transferred to 0.45 mm PVDF via Trans-Blot® Turbo™ transfer system (Bio-Rad). After Ponceau S staining and de-staining, membranes were blocked for 1 h at room temperature (RT) in 5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) in Tris-buffered saline-Tween 20 (TBST). Membranes were incubated overnight at 4°C in primary antibodies. All primary antibodies (pAkt (S473), Akt, Vps34, LC3B, pmTOR (S2448), mTOR, pS6 (S235/236), S6, p4EBP1 (T37/46) and 4EBP1) were diluted 1:1000 in 5% BSA/TBST and purchased from Cell Signalling Technologies (Beverly, MA, USA). The following day membranes were washed (5 \times 5 min in TBST) and then incubated for 1 h at RT in horseradish peroxidase-conjugated secondary antibodies (mouse anti-rabbit or goat anti-mouse immunoglobulins, GE Healthcare Life Sciences, Australia) diluted in 5% BSA/TBST. After washes (5 \times 5 min in TBST), membranes were treated with enhanced chemiluminescence (Super Signal West Femto; Thermo Scientific). Membranes were imaged using ChemiDoc™ imaging system (Bio-Rad) and blots were quantified using ImageLab 4.0 software (Bio-Rad), and normalized to total protein as determined by BLOT-FastStain™ as per manufacturer's instructions (G-Biosciences, St Louis, MO).

Statistical analyses

All values are expressed as mean \pm SEM. Phosphorylated proteins were normalised to total protein of the protein of interest (p/t), while all other proteins were normalised to total protein (BLOT-FastStain™). All data was then normalised to the appropriate control group for ease of visualisation. Data were tested for normality and homogeneity of variance using a Shapiro-Wilk and Levene's test, respectively. For dose response and timecourse experiments, two-way ANOVAs (time/dose, treatment) were used to compare between groups, while one-way ANOVAs were used for all other comparisons. Tukey's post hoc test was used to determine significant differences between individual groups. $P < 0.05$ was considered significant. Unless otherwise stated, data were normalised to control values.

RESULTS

Growth factor and nutrient deprivation reduces mTORC1 signalling and protein synthesis and induces muscle wasting

Incubation of mature C2C12 myotubes in HBS rapidly reduced anabolic signalling, such that protein synthesis was reduced by ~70% during the first 30 min, and by 80% after four hours (Fig. 1B). After incubation in HBS for 1 h, phosphorylated levels of Akt (-29%, $P<0.05$), mTOR (-69%, $P<0.01$), S6 (-69%, $P<0.01$) and 4EBP1 (-44%, $P<0.01$) were all significantly reduced (Fig. 1C). After an incubation time of 5 h, this marked reduction in anabolic signalling and protein synthesis was associated with a reduction in myotube diameter of 29% ($P<0.001$, Fig. 1A).

Arginine reduces muscle wasting in a dose dependent manner

The addition of arginine to the HBS solution attenuated wasting resulting from growth factor and nutrient deprivation in a dose-dependent manner, reaching statistical significance at a concentration of 1 mM (Fig. 2). As compared to incubation in HBS alone, 1 mM of arginine increased myotube diameter by 12.0% ($P<0.01$, Fig. 2B). Higher arginine concentrations of 2.5 mM and 5 mM increased myotube diameter by 13.4% ($P<0.01$) and 13.0% ($P<0.01$), respectively, but were not statistically different from a concentration of 1 mM. Importantly, equimolar concentrations of alanine did not increase myotube diameter as compared to HBS alone. All further experiments were performed using 2.5 mM arginine or alanine. The effect of arginine on C2C12 myotube diameter was also explored using a serum free model of muscle wasting. Supplementation with arginine (2.5 mM) did not attenuate serum free-induced muscle wasting, however, in the absence of arginine (i.e. incubation in serum and arginine free media) myotube diameter was further reduced by ~15% (Fig. 3).

Arginine increases anabolic signalling and protein synthesis

Arginine did not increase the phosphorylation of Akt ($P>0.05$, Fig. 4B), a growth factor sensitive signalling protein upstream of mTORC1. In addition, arginine did not alter the ratio of lipidated LC3B (LC3B-II) to non-lipidated LC3B (LC3B-I) protein ($P>0.05$, Fig. 4E), indicating no change in autophagosome number. However, arginine increased the ratio of phosphorylated to total mTOR by 146% ($P<0.01$, Fig. 4C) after 1 h incubation in HBS. The activation of mTORC1 was associated with increased protein content of the amino acid sensitive type III PI3-kinase Vps34 (90%, $P<0.01$, Fig. 4D) and increased protein synthesis

($P < 0.05$, 38%, Fig. 4A) after 1 h incubation in HBS. By 4 h, arginine-stimulated increases in mTOR, Vps34 and protein synthesis had returned to baseline and were not different to alanine.

The protective effect of arginine is NO-independent and mTORC1 dependent

To determine the involvement of NO in the protective effect of arginine, myotubes were incubated with arginine and the general NOS inhibitor L-NAME. NOS inhibition did not impact the protective effect of arginine on myotube diameter (Fig. 5), demonstrating a NO-independent mechanism of mTORC1 activation and protection from wasting. To establish the relative involvement of mTORC1 activation in the attenuation of muscle wasting and reduced protein synthesis by arginine, we incubated myotubes with or without the mTORC1 inhibitor rapamycin. We then measured the effect of rapamycin on myotube diameter, protein synthesis and the expression of two key downstream targets of mTORC1, S6 and 4EBP1. Arginine increased phosphorylated, total and the ratio of phosphorylated to total S6 protein expression (Fig. 6A) and the ratio of phosphorylated to total 4EBP1 (Fig. 6B) at the 1 h time point. Rapamycin inhibited all increases in S6 protein expression, but did not reduce the increase in the ratio of phosphorylated to total 4EBP1. Rapamycin also completely prevented the arginine-induced increase in protein synthesis at the 1 h time point (Fig. 6C). Furthermore, rapamycin completely prevented the protective effect of arginine on myotube diameter (Fig. 6D). Together, these findings suggest that arginine activates mTORC1, potentially through Vps34, which leads to increases in protein synthesis and an attenuation of growth factor and nutrient deprivation-induced muscle wasting *in vitro*.

DISCUSSION

The aim of this study was to determine whether exogenous arginine could protect skeletal muscle cells from wasting directly and whether this effect was dependent on production of NO and/or activation of the rapamycin sensitive mTORC1 signalling pathway. To explore this aim, we nutrient deprived mature C2C12 myotubes by incubating them in HBS with arginine or equimolar concentrations of alanine (control). Our results show that arginine attenuates: 1) muscle wasting; 2) the reduction in protein synthesis and; 3) activation of mTORC1 and its downstream targets, S6 and 4EBP1. Experiments using the NOS inhibitor L-NAME showed a NO-independent mechanism of protection from muscle wasting, while incubation with the mTORC1 inhibitor rapamycin demonstrated that activation of mTORC1 was required for these effects. Furthermore, the activation of mTORC1 by arginine was not associated with changes in the phosphorylation status of Akt, but was associated with increased expression of the amino acid-sensitive signalling protein Vps34 (Nobukuni et al. 2005). Importantly, initial dose response experiments indicate the maximal response (1-2.5 mM) occurs above the normal physiological concentration range (0.1-0.3 mM) (Evans et al. 2004; Tangphao et al. 1999), suggesting that increasing arginine availability may be beneficial during muscle wasting conditions.

Growth factor and nutrient deprivation rapidly reduces mTORC1 signalling and protein synthesis and induces muscle wasting

mTORC1 integrates signals from nutrients, growth factors, energy status and stress to drive growth during nutrient abundance and to mediate growth arrest under starvation conditions. Our growth factor and nutrient deprivation model resulted in a rapid decrease in mTOR phosphorylation, and its downstream targets S6 and 4EBP1 (Fig. 1C). Consequently, protein synthesis was reduced by more than 75% (Fig. 1B) within 1 h of nutrient and growth factor deprivation and myotube diameter was reduced by 29% after 5 h incubation in HBS (Fig 1A). These results are consistent with previous data showing a rapid reduction in S6 and 4EBP1 activation during amino acid starvation (Hara et al. 1998), and demonstrate the effectiveness of the model in reducing protein synthesis and inducing muscle wasting.

Arginine reduces muscle wasting in a dose-dependent manner

We observed a dose-dependent attenuation of growth factor and nutrient deprivation-induced muscle wasting with arginine administration. Furthermore, removal of arginine from the media increased growth factor withdrawal-induced muscle wasting. This is the first study to

show a direct role for arginine in the protection of skeletal muscle from wasting *in vitro*. Our observations suggest that concentrations of arginine ≥ 1 mM are required to elicit protection from HBS-induced skeletal muscle wasting, but the arginine concentration in normal DMEM (~0.4 mM) is sufficient to provide protection from SF-induced muscle wasting since supplemental arginine dose not significantly increase myotube diameter. These findings are consistent with previous work showing that 1 mM of supplemental arginine promotes C2C12 myoblast fusion in a NO-dependent manner (Long et al. 2006). Normal baseline serum and plasma arginine concentrations are ~0.1 mM (Evans et al. 2004; Tangphao et al. 1999), but are increased to around 0.3 mM after ingestion of 10 g arginine or to >5 mM after intravenous infusion of 30 g arginine (Tangphao et al. 1999). Importantly, equimolar concentrations of the non-essential amino acid alanine did not provide any protection from muscle wasting, indicating an amino acid specific effect of arginine.

Arginine increases anabolic signalling and protein synthesis

In our model of growth factor and nutrient deprivation-induced muscle wasting, we observed increased phosphorylation of mTOR and its downstream targets S6 and 4EBP1 with arginine, compared to alanine-treated cells. Following a 4 h period of serum withdrawal and a 1 h incubation in HBS (Atherton et al. 2010), showed that out the 9 amino acids tested (leucine, isoleucine, valine, lysine, methionine, phenylalanine, tryptophan and threonine) only leucine was able to simultaneously activate mTOR, S6 and 4EBP1. Both leucine and arginine are known to reduce mRNA levels of the key ubiquitin-proteolysis pathway genes atrogin-1 and MuRF1 during serum withdrawal in an mTORC1 dependent manner (Herningtyas et al. 2008). However, protein synthesis and the prevention of muscle wasting were not measured in this study. In the current study, the arginine-stimulated improvement in protein synthesis was only observed during the first hour of incubation in HBS, after which protein synthesis was not significantly different to control levels. Although we did not detect an effect of arginine on the ratio of LC3B-II:LC3B-I, a marker of autophagosome number, it is likely that the observed activation of mTORC1 resulted in changes to other downstream targets involved in the regulation of muscle size, such as the ubiquitin-proteasome muscle breakdown pathway. Indeed, previous data in model of C2C12 muscle cell wasting show that arginine reduces atrogin-1 expression (Herningtyas et al. 2008).

The protective effect of arginine is NO independent

Aside from its role in providing substrate for protein synthesis, arginine is also a precursor for NO, a key signalling molecule involved in numerous cellular functions. Previous studies have identified an important role for NO in the regulation of skeletal muscle mass. NO is involved in satellite cell activation and fusion (Anderson 2000) and endogenous production of NO is important for sarcomere addition in stretched muscle (Koh and Tidball 1999) and overload-induced hypertrophy (Sellman et al. 2006; Smith et al. 2002) *in vivo*. There is also evidence that arginine availability for the production of NO by NOS may become limiting during myoblast fusion, since supplemental arginine enhances C2C12 differentiation, an effect prevented by NOS inhibition (Long et al. 2006). In this study, we hypothesised that NO would play a central role in the observed effect of arginine on muscle wasting. However, incubation with the general NOS-inhibitor, L-NAME, did not attenuate the arginine-induced increase in myotube diameter (Fig. 5). These findings suggest that the attenuation of growth factor and nutrient deprivation-induced wasting with arginine treatment is not reliant on NOS activity.

The protective effect of arginine is mTORC1 dependent

mTOR exists as part of two protein complexes, mTOR complex 1 (mTORC1) which consists of mTOR, raptor and G protein-subunit-like protein (G-L) and mTOR complex 2 (mTORC 2) which consists of mTOR, rictor and G-L (Corradetti and Guan 2006). mTORC1 is rapamycin-sensitive, activated by amino acids and considered the master regulator of protein synthesis (Kim et al. 2008). mTORC2, on the other hand, is rapamycin-insensitive and can activate Akt, a key regulator of cell survival upstream of mTORC1 (Sarbasov et al. 2006). In order to determine the extent to which activation of mTORC1 by arginine contributes to the preservation of myotube diameter, we co-incubated arginine-treated cells with the specific mTORC1 inhibitor rapamycin. The arginine-induced increases in p-S6 (Fig. 5A) and protein synthesis (Fig. 5C) were completely blocked by rapamycin. Furthermore, rapamycin completely prevented the arginine-stimulated improvement in myotube diameter. Together these results implicate activation of the rapamycin-sensitive mTORC1 as the critical step for the attenuation of growth factor and nutrient deprivation-induced muscle wasting by arginine. This is in line with previous work in intestinal epithelial cells (IPEC-J2) showing that arginine-induced increases in protein synthesis were entirely mTORC1 dependent (Bauchart-Thevret et al. 2010).

Despite the complete inhibition of arginine-induced S6 phosphorylation by rapamycin, the arginine-induced increase in p-4EBP1 was not reduced by rapamycin. The mechanisms responsible for this differential regulation of 4EBP1 and S6 phosphorylation by arginine in the presence of rapamycin in the current study are unclear. However, differential regulation of S6K and 4EBP1 phosphorylation by rapamycin has been described previously (Choo et al. 2008). Interestingly, this rapamycin-resistant 4EBP1 phosphorylation still requires mTORC1 activity and can be blocked by mTOR catalytic inhibitors. Although beyond the scope of this work, the rapamycin-resistant phosphorylation of 4EBP1 by arginine observed in the current study suggests that arginine may play a role in the regulation of mTORC1 catalytic activity. While it is clear that activation of mTORC1 by arginine is required to improve protein synthesis and myotube diameter, the specific upstream signalling cascade responsible for its action remains to be established. Although not definitive, the activation of mTORC1 by arginine was associated with an increase in the expression of the amino acid-sensitive signalling protein Vps34 (Nobukuni et al. 2005), suggesting a potential role for Vps34 in the observed arginine-induced increase in muscle protein synthesis.

In summary, our results using mature C2C12 myotubes are the first to demonstrate a direct role for arginine in the protection of skeletal muscle cells from cachectic stimuli, *in vitro*. Arginine reduced muscle wasting in a dose-dependent manner, with the maximal response occurring at a concentration of 1 mM. After 1 h of nutrient and growth factor deprivation, activation of mTOR, S6 and 4EBP1 as well as protein synthesis rates, were significantly higher in arginine-treated cells than control (alanine-treated) cells. Using pharmacological inhibition, we showed that the arginine-induced increase in protein synthesis, S6 activation and attenuation of muscle wasting was entirely mediated through activation of the rapamycin-sensitive mTORC1. Finally, the upstream signalling pathways responsible for the arginine-induced attenuation of muscle wasting do not involve activation of Akt and are NO-independent but may involve Vps34, since increased expression coincided with increases in protein synthesis and activation of mTORC1. How the activation of mTORC1 by arginine impacts other downstream pathways central to muscle wasting, such as the lysosomal and proteolytic muscle breakdown pathways warrant further investigation.

ACKNOWLEDGEMENTS: This work was funded by a grant from the Ajinomoto Amino Acid Research Program (3ARP: 09/07).

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

REFERENCES

- Anderson JE (2000) A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells. *Mol Biol Cell* 11 (5):1859-1874
- Atherton PJ, Smith K, Etheridge T, Rankin D, Rennie MJ (2010) Distinct anabolic signalling responses to amino acids in C2C12 skeletal muscle cells. *Amino Acids* 38 (5):1533-1539. doi:10.1007/s00726-009-0377-x
- Bauchart-Thevret C, Cui L, Wu G, Burrin DG (2010) Arginine-induced stimulation of protein synthesis and survival in IPEC-J2 cells is mediated by mTOR but not nitric oxide. *Am J Physiol Endocrinol Metab* 299 (6):E899-909. doi:10.1152/ajpendo.00068.2010
- Choo AY, Yoon SO, Kim SG, Roux PP, Blenis J (2008) Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc Natl Acad Sci U S A* 105 (45):17414-17419. doi:10.1073/pnas.0809136105
- Collier SR, Casey DP, Kanaley JA (2005) Growth hormone responses to varying doses of oral arginine. *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society* 15 (2):136-139. doi:10.1016/j.ghir.2004.12.004
- Corradetti MN, Guan KL (2006) Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? *Oncogene* 25 (48):6347-6360. doi:10.1038/sj.onc.1209885
- Efeyan A, Zoncu R, Sabatini DM (2012) Amino acids and mTORC1: from lysosomes to disease. *Trends in molecular medicine* 18 (9):524-533. doi:10.1016/j.molmed.2012.05.007
- Evans RW, Fernstrom JD, Thompson J, Morris SM, Jr., Kuller LH (2004) Biochemical responses of healthy subjects during dietary supplementation with L-arginine. *The Journal of nutritional biochemistry* 15 (9):534-539. doi:10.1016/j.jnutbio.2004.03.005
- Evans WJ (1995) What is sarcopenia? *J Gerontol A Biol Sci Med Sci* 50 Spec No:5-8
- Goodman CA, Mabrey DM, Frey JW, Miu MH, Schmidt EK, Pierre P, Hornberger TA (2011) Novel insights into the regulation of skeletal muscle protein synthesis as revealed by a new nonradioactive in vivo technique. *Faseb J* 25 (3):1028-1039. doi:10.1096/fj.10-168799
- Goto C, Nishioka K, Umemura T, Jitsuiki D, Sakaguchi A, Kawamura M, Chayama K, Yoshizumi M, Higashi Y (2007) Acute moderate-intensity exercise induces vasodilation through an increase in nitric oxide bioavailability in humans. *Am J Hypertens* 20 (8):825-830. doi:10.1016/j.amjhyper.2007.02.014
- Hara K, Yonezawa K, Weng QP, Kozlowski MT, Belham C, Avruch J (1998) Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem* 273 (23):14484-14494
- Herningtyas EH, Okimura Y, Handayaningsih AE, Yamamoto D, Maki T, Iida K, Takahashi Y, Kaji H, Chihara K (2008) Branched-chain amino acids and arginine suppress MaFbx/atrogen-1 mRNA expression via mTOR pathway in C2C12 cell line. *Biochim Biophys Acta* 1780 (10):1115-1120. doi:10.1016/j.bbagen.2008.06.004
- Jewell JL, Russell RC, Guan KL (2013) Amino acid signalling upstream of mTOR. *Nature reviews Molecular cell biology* 14 (3):133-139. doi:10.1038/nrm3522
- Jun T, Wennmalm A (1994) NO-dependent and -independent elevation of plasma levels of insulin and glucose in rats by L-arginine. *British journal of pharmacology* 113 (2):345-348
- Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL (2008) Regulation of TORC1 by Rag GTPases in nutrient response. *Nat Cell Biol* 10 (8):935-945. doi:10.1038/ncb1753
- Koh TJ, Tidball JG (1999) Nitric oxide synthase inhibitors reduce sarcomere addition in rat skeletal muscle. *J Physiol* 519 Pt 1:189-196
- Koopman R, Ly CH, Ryall JG (2014) A metabolic link to skeletal muscle wasting and regeneration. *Frontiers in physiology* 5:32. doi:10.3389/fphys.2014.00032
- Koopman R, van Loon LJ (2009) Aging, exercise, and muscle protein metabolism. *J Appl Physiol* (1985) 106 (6):2040-2048. doi:10.1152/jappphysiol.91551.2008
- Koopman R, Wagenmakers AJ, Manders RJ, Zorenc AH, Senden JM, Gorselink M, Keizer HA, van Loon LJ (2005) Combined ingestion of protein and free leucine with carbohydrate increases

- postexercise muscle protein synthesis in vivo in male subjects. *Am J Physiol Endocrinol Metab* 288 (4):E645-653. doi:10.1152/ajpendo.00413.2004
- Long JH, Lira VA, Soltow QA, Betters JL, Sellman JE, Criswell DS (2006) Arginine supplementation induces myoblast fusion via augmentation of nitric oxide production. *Journal of muscle research and cell motility* 27 (8):577-584. doi:10.1007/s10974-006-9078-1
- Lynch GS, Schertzer JD, Ryall JG (2007) Therapeutic approaches for muscle wasting disorders. *Pharmacol Ther* 113 (3):461-487
- Merry TL, Steinberg GR, Lynch GS, McConell GK (2010) Skeletal muscle glucose uptake during contraction is regulated by nitric oxide and ROS independently of AMPK. *Am J Physiol Endocrinol Metab* 298 (3):E577-585. doi:10.1152/ajpendo.00239.2009
- Moinard C, Cynober L (2007) Citrulline: a new player in the control of nitrogen homeostasis. *J Nutr* 137 (6 Suppl 2):1621S-1625S. doi:137/6/1621S [pii]
- Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P, Byfield MP, Backer JM, Natt F, Bos JL, Zwartkruis FJ, Thomas G (2005) Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc Natl Acad Sci U S A* 102 (40):14238-14243. doi:10.1073/pnas.0506925102
- Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM (2006) Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* 22 (2):159-168. doi:10.1016/j.molcel.2006.03.029
- Sellman JE, DeRuisseau KC, Betters JL, Lira VA, Soltow QA, Selsby JT, Criswell DS (2006) In vivo inhibition of nitric oxide synthase impairs upregulation of contractile protein mRNA in overloaded plantaris muscle. *J Appl Physiol* (1985) 100 (1):258-265. doi:10.1152/jappphysiol.00936.2005
- Smith LW, Smith JD, Criswell DS (2002) Involvement of nitric oxide synthase in skeletal muscle adaptation to chronic overload. *J Appl Physiol* (1985) 92 (5):2005-2011. doi:10.1152/jappphysiol.00950.2001
- Tangphao O, Grossmann M, Chalon S, Hoffman BB, Blaschke TF (1999) Pharmacokinetics of intravenous and oral L-arginine in normal volunteers. *British journal of clinical pharmacology* 47 (3):261-266
- Wu G, Morris SM, Jr. (1998) Arginine metabolism: nitric oxide and beyond. *Biochem J* 336 (Pt 1):1-17

FIGURE LEGENDS

Fig. 1: Nutrient and growth factor deprivation rapidly reduces protein synthesis and induces muscle wasting. C2C12 myotubes were incubated in normal differentiation media (DM) or were growth factor and nutrient deprived (HBS). Myotube diameter (**A**, n=6), protein synthesis as assessed using puromycin (**B**, n=4-6) and mTORC1 associated signalling (**C**, n=4-5) are presented as means±SEM, normalised to DM control. Representative western blots are presented. Significant differences ($P<0.05$) between groups are displayed.

Fig. 2: Arginine preserves myotube diameter in a dose-dependent manner. C2C12 myotubes were incubated in normal differentiation media (DM) or were growth factor and nutrient deprived (HBS) and co-treated with 5 different concentrations of arginine or alanine (control) for 5 h to identify the optimal arginine concentration. Representative images of myotubes treated with DM, HBS, alanine (ALA, 2.5 mM) and arginine (ARG, 2.5 mM) stained for myosin (red), with nuclei visualised using DAPI (blue) (**A**). Myotube diameter is presented as means±SEM, normalised to DM control for alanine and arginine concentrations of 0.2, 0.5, 1, 2.5 and 5 mM (**B**, n=6 per treatment, per concentration). Significant differences ($P<0.05$) between groups are displayed.

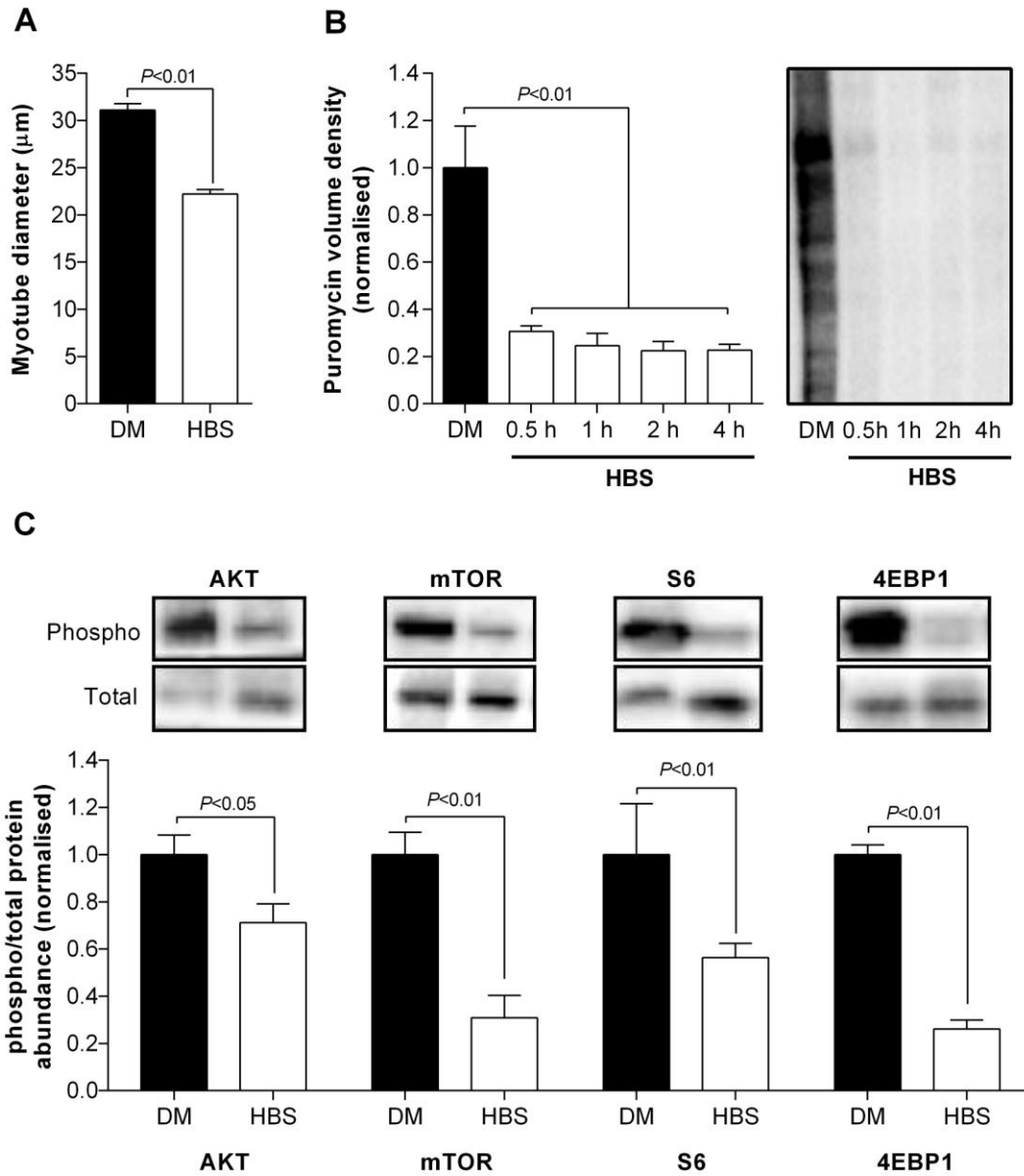
Fig. 3: Serum free-induced muscle wasting is increased in arginine free media. C2C12 myotubes were incubated in normal differentiation media (DM) or were growth factor deprived (SF) for 48 h in media containing 3 different concentrations of arginine. Representative images of myotubes treated with DM, serum free media (SF, 0.4mM arginine), arginine free media (ARG⁻) or serum free media supplemented with 2.5mM arginine (ARG⁺) stained for myosin (red), with nuclei visualised using DAPI (blue) (**A**). Myotube diameter is presented as means±SEM, normalised to DM control (**B**, n=4 per treatment). Significant differences ($P<0.05$) between groups are displayed.

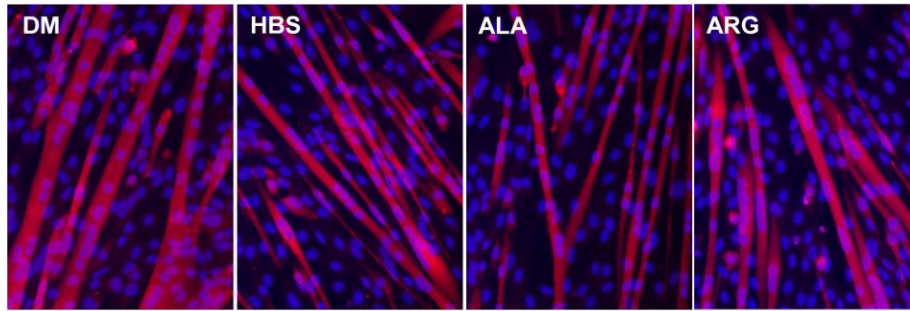
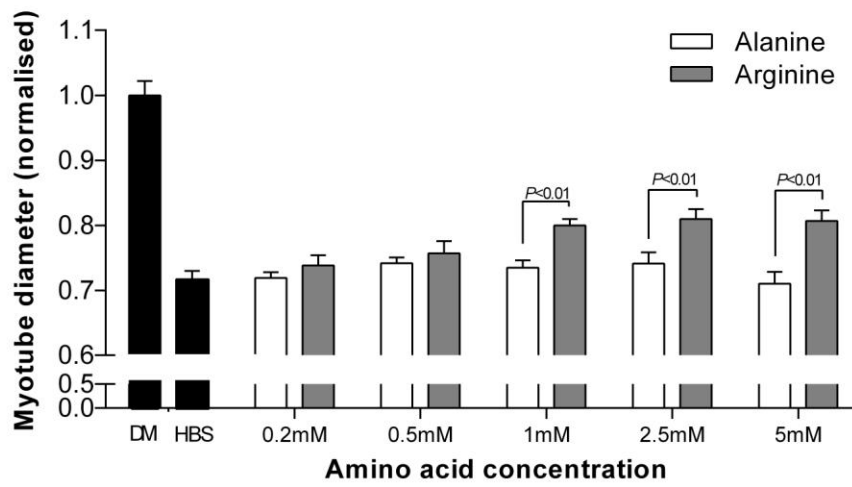
Fig. 4: Arginine increases anabolic signalling and protein synthesis. C2C12 myotubes were growth factor and nutrient deprived (HBS) and treated with alanine or arginine for up to 4 h. Protein synthesis and representative puromycin western blots (**A**), phosphorylation status (phospho/total protein) of Akt (**B**) and mTOR (**C**); and protein expression of Vps34 (**D**), and LC3BII/LC3BI ratio (**E**) were measured during wasting conditions. Data are presented as

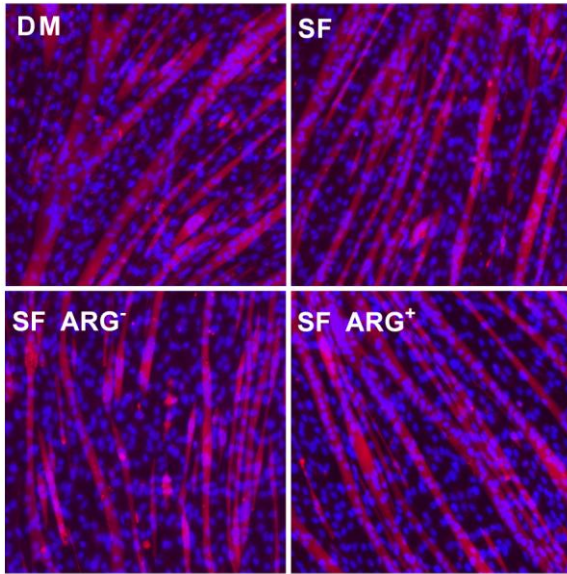
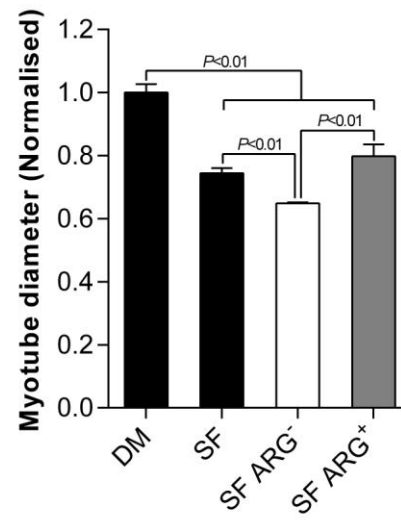
means±SEM (n=4-5), normalised to alanine. Significant differences ($P<0.05$) and trends ($P<0.1$) between groups are displayed.

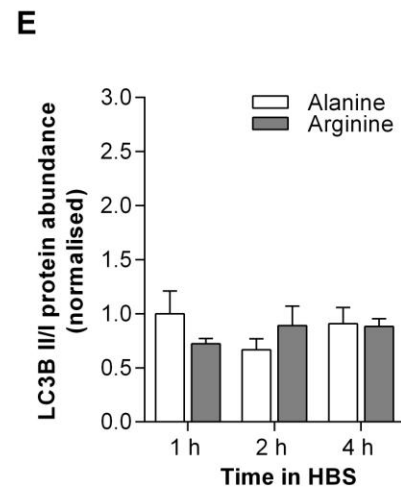
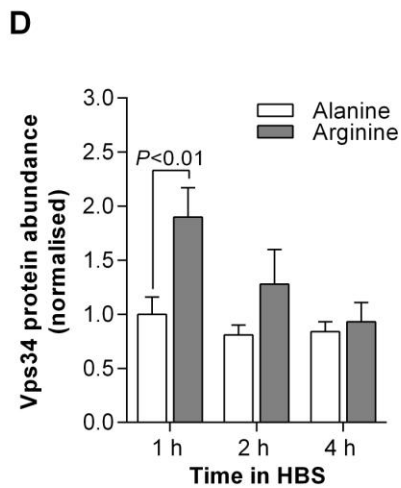
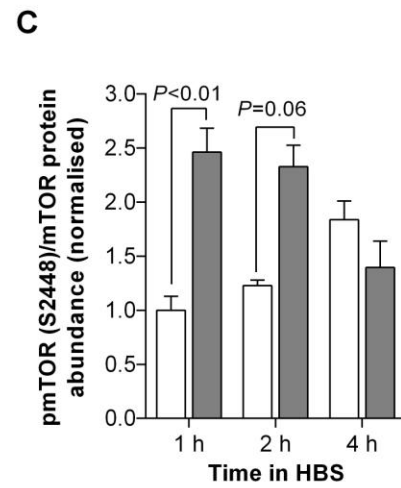
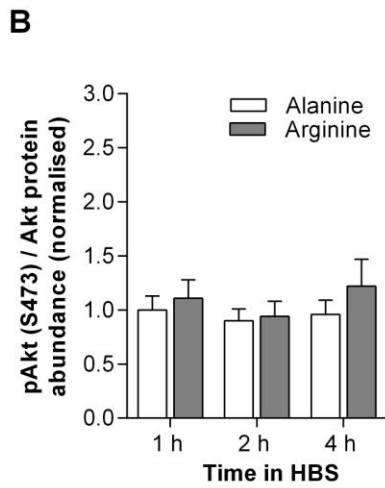
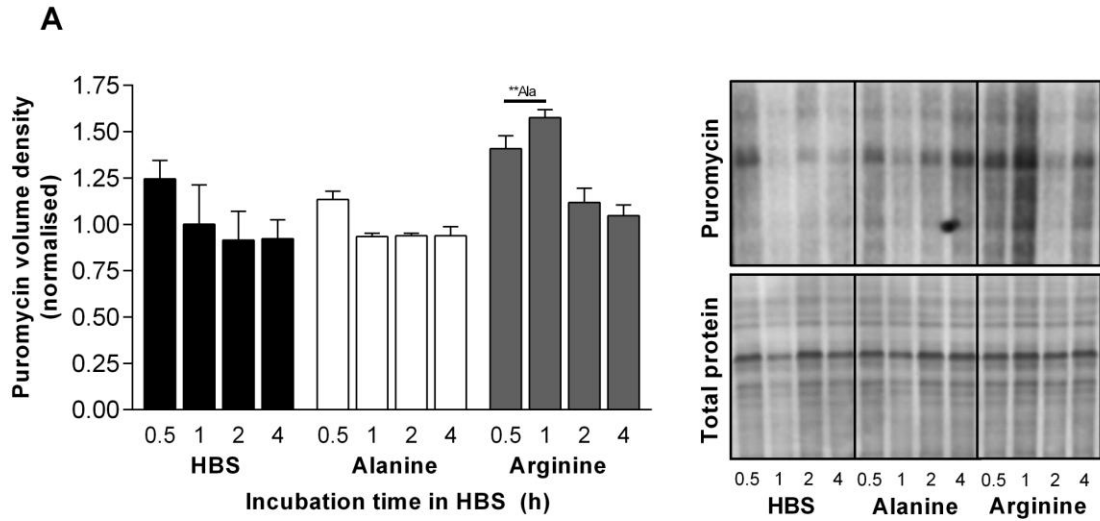
Fig. 5: Arginine attenuates myotube atrophy in a NO-independent manner. C2C12 myotubes were incubated in normal differentiation media (DM) or were growth factor and nutrient deprived (HBS) and co-treated with 2.5 mM arginine or alanine (control) for 5 h with or without the general NOS inhibitor L-NAME (10mM). Representative images of myotubes treated with alanine + L-NAME (ALA+L-NAME) or arginine + L-NAME (ARG+L-NAME) stained for myosin (red), with nuclei visualised using DAPI (blue) (**A**). Myotube diameter is presented as means±SEM, normalised to DM control (**B**, n=6 per treatment). Significant differences ($P<0.05$) between groups are displayed.

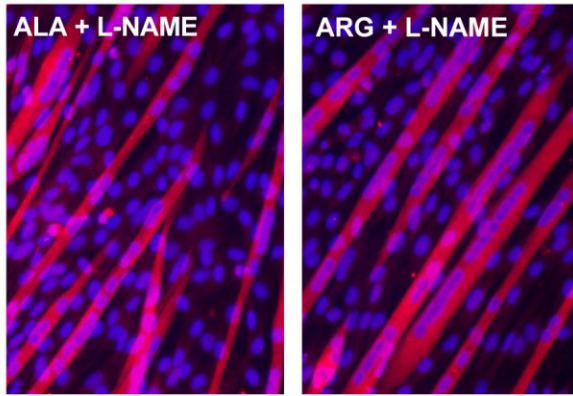
Fig. 6: Arginine attenuates myotube atrophy in an mTORC1 dependent manner. C2C12 myotubes were incubated in HBS with alanine or arginine and co-treated with the mTORC1 inhibitor rapamycin (100 nM) for 1 h. Representative western blots and normalised protein expression are presented for pS6, tS6 and p/tS6 (**A**); phosphorylation status of 4EBP1(**B**); and protein synthesis as assessed by puromycin (**C**, n=5-6). Myotube diameter is presented as means±SEM, normalised to alanine (**D**, n=6-8 per treatment). Significant differences ($P<0.05$) between groups are displayed.



A**B**

A**B**



A**B**