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ARTICLE

The effect of caffeine on skeletal muscle anabolic signaling and hypertrophy

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Abstract: Caffeine is a widely consumed stimulant with the potential to enhance physical performance through multiple mechanisms. However, recent in vitro findings have suggested that caffeine may block skeletal muscle anabolic signaling through AMP-activated protein kinase (AMPK)-mediated inhibition of mechanistic target of rapamycin (mTOR) signaling pathway. This could negatively affect protein synthesis and the capacity for muscle growth. The primary purpose of this study was to assess the effect of caffeine on in vivo AMPK and mTOR pathway signaling, protein synthesis, and muscle growth. In cultured C2C12 muscle cells, physiological levels of caffeine failed to impact mTOR activation or myoblast proliferation or differentiation. We found that caffeine administration to mice did not significantly enhance the phosphorylation of AMPK or inhibit signaling proteins downstream of mTOR (p70S6k, S6, or 4EBP1) or protein synthesis after a bout of electrically stimulated contractions. Skeletal muscle-specific knockout of LKB1, the primary AMPK activator in skeletal muscle, on the other hand, eliminated AMPK activation by contractions and enhanced S6k, S6, and 4EBP1 activation before and after contractions. In rats, the addition of caffeine did not affect plantaris hypertrophy induced by the tenotomy of the gastrocnemius and soleus muscles. In conclusion, caffeine administration does not impair skeletal muscle load-induced mTOR signaling, protein synthesis, or muscle hypertrophy.

Key words: skeletal muscle hypertrophy, caffeine, AMPK, LKB1, mTOR, contraction.

Résumé : La caféine est un stimulant de grande consommation ayant le potentiel d'améliorer la performance physique au moyen de multiples mécanismes. Toutefois, selon des récentes observations in vitro, la caféine pourrait bloquer le signal anabolique du muscle squelettique en inhibant, au moyen de la protéine kinase activée par l'AMP (AMPK), le mécanisme de la voie de signalisation de la cible de la rapamycine (mTOR). Ce phénomène pourrait nuire à la synthèse protéique et à la capacité de croissance du muscle. Cette étude a pour objectif principal d'évaluer l'effet in vivo de la caféine sur l'AMPK et la voie de signalisation de mTOR, la synthèse protéique et la croissance musculaire. Chez des cellules musculaires C2C12 en culture, des taux physiologiques de caféine n'ont pas d'impact sur l'activation de la mTOR ou la prolifération/différentiation des myoblastes. D'après nos observations, l'administration de la caféine à des souris n'améliore pas significativement la phosphorylation de l'AMPK ou n'inhibe pas en aval la signalisation des protéines de mTOR (p70S6k, S6 ou 4EBP1) ou la synthèse protéique consécutive à des contractions obtenues par stimulation électrique. D'autre part, l'activateur primaire de l'AMPK dans le muscle squelettique sans LKB1 élimine l'activation de l'AMPK par des contractions et améliore l'activation de S6k, S6 et 4EBP1 avant et après les contractions. L'ajout de caféine chez des rats ne modifie pas l'hypertrophie du muscle plantaire induite par la ténotomie des muscles gastrocnemius et soleus. En conclusion, l'administration de caféine ne nuit pas à la signalisation de mTOR, à la synthèse protéique ou à l'hypertrophie musculaire du muscle squelettique stimulé par une charge. [Traduit par la Rédaction]

Mots-clés : hypertrophie du muscle squelettique, caféine, AMPK, LKB1, mTOR, contraction.

Introduction

Caffeine is arguably the most widely consumed drug in the world today. In the United States, average caffeine consumption amongst coffee drinkers is estimated at about 150–200 mg per day, with 10% of the population ingesting over 100 mg daily (Graham 2001). The health effects of caffeine can be positive as it drives a temporary increase in attention, alertness, and metabolic rate, and its acute use can also enhance exercise performance, primarily through effects on the central nervous system. Because of this, it is very commonly used amongst athletes as an ergogenic aid (Tarnopolsky 2008).

However, caffeine use could potentially be detrimental in some respects. For instance, caffeine treatment blocks insulin signaling through protein kinase B (Akt) and activation of the mechanistic target of rapamycin (mTOR) signaling pathway (McMahon et al. 2005; Kolnes et al. 2010; Egawa et al. 2011*b*; Miwa et al. 2012). Since mTOR is a major regulator of protein synthesis and muscle fiber size, this could be a major concern for anyone trying to elicit muscle growth (aged populations, those with injuries, athletes, etc.). In vitro studies showed that caffeine activates adenosine monophosphate-activated protein kinase (AMPK) in incubated skeletal muscles (Jensen et al. 2007; Raney and Turcotte 2008; Egawa et al. 2009, 2011*a*, 2011*b*; Lally et al. 2012; Tsuda et al. 2015) and cells (Mathew et al. 2014), which could provide an explanatory mechanism for mTOR inhibition since AMPK is known to block mTOR activation in skeletal muscle (Bolster et al. 2002; Thomson and Gordon 2005, 2006; Thomson et al. 2008).

The physiological relevance of these findings is unclear since the concentrations of caffeine used in those studies were supra-

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physiological, in the 1–5 mM range. Two to five cups of coffee can result in blood concentrations of 15–80 μM depending upon the individual, caffeine concentration, and duration of consumption (Birkett and Miners 1991). A blood caffeine level of about 0.5 mM (100 mg/L) is generally considered lethal (Winek et al. 2001). Therefore, the purpose of this study was to determine whether caffeine attenuates skeletal muscle AMPK and mTOR signaling, protein synthesis, and hypertrophy in response to contractions and (or) increased loading in rodent models at dosages consistent with physiological caffeine concentrations that would be observed in humans with moderate to high caffeine consumption.

Materials and methods

Cell culture

C2C12 myoblasts were proliferated at 37 °C and 5% CO_2 in growth media (high-glucose Dulbecco's Modified Eagle's medium (DMEM, Corning #15-013-CV) with 10% fetal bovine serum, 200 mM glutamine, and 1% penicillin/streptomycin/amphotericin B antibiotic) to ~80% confluence. Where indicated, cells were then allowed to differentiate to myotubes for 5–7 days in differentiation media (high-glucose DMEM supplemented with 2% horse serum; 200 mM glutamine, and 1% penicillin/streptomycin antibiotic).

For assessment of insulin-stimulated signaling, myotubes were prepared as described above in 6-well plates and pretreated for 1 h with serum-free media followed by treatment with variable concentrations of caffeine (0–0.3 mM in serum-free DMEM) for 75 min. Insulin (100 nM) was added to half of the wells for the final 15 min of the incubation period to induce activation of the Akt-mTOR pathway. Following treatment, cells were harvested by scraping with lysis buffer. Lysates were freeze-thawed 3 times, centrifuged at 10 000*g* for 10 min, then protein concentration was assessed and western blotting performed as described below.

For determination of the effect of caffeine on myoblast proliferation, C2C12 cells were seeded in 96-well plates and allowed to adhere to the plate for 1 h in growth media. Fresh growth media with 0–4 mM caffeine was then added to the wells ($n = 4$ wells per group). Cells were allowed to proliferate for 16 h after which fresh media with the indicated concentration of caffeine and 10 μM 5-bromo-2′-deoxyuridine (BrDU) was added for the labeling of proliferating nuclei. After an additional 4 h the cells were washed with phosphate-buffered saline (PBS), fixed with cold ethanol for 5 min, permeabilized with 0.3% Triton X-100 in PBS for 15 min, and incubated in 2N hydrochloric acid for 15 min. After washing twice with PBS, the cells were blocked with normal goat serum and incubated overnight at 4 °C with BrDU primary antibody (G3G4, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). Cells were washed twice with PBS, then incubated in secondary antibody (AlexaFluor 488 goat-antimouse IgG_1, Life Technologies #A21121, Carlsbad, CA) for 1 h in the dark. After washing 3 times with PBS, one drop of 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) in glycerol mounting medium (25% glycerol in PBS) plus 1 drop Flourmout G (Southern Biotech, Birmingham, AL) were added and the number of BrDU and DAPI positive nuclei was determined using fluorescent microscopy.

For determination of the effect of caffeine on myoblast differentiation to myotubes, C2C12 myoblasts were cultured in 12-well plates to ~80% confluence, then differentiated as described above but with 0–2.5 mM caffeine added to the differentiation media. Myotubes were then washed and fixed with 3.7% formaldehyde for 10 min at room temperature, washed twice with PBS, permiabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature, washed twice with PBS, and incubated overnight at 4 °C in antimyosin primary antibody (Developmental Studies Hybridoma Bank #MF20, Iowa City, IA). Cells were washed with PBS, then incubated with AlexaFluor 488 goat anti-mouse secondary antibody (Invitrogen #A11059) for 1 h at room temperature in the dark. Cells were washed then incubated in 1 μg/mL DAPI for 5 min in the dark and washed twice with PBS. Two drops of Fluormount G were added to each well and the percentage of cells positive for myosin heavy chain was determined by fluorescence microscopy.

Animals

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Brigham Young University. Rats and mice were housed in cages maintained in a 12-h light–dark, temperature controlled (20–25 °C) room with ad libitum food and water access.

High-frequency electrical stimulation (STIM)

We assessed the effect of caffeine on hypertrophic signaling after a bout of muscle contractions in skeletal muscle specific LKB1-knockout (KO) and wild-type (WT) littermate mice (Tanner et al. 2013; Chen et al. 2016). LKB1 is the primary upstream kinase for AMPK in skeletal muscle and, therefore, any activating effect of caffeine on AMPK would be expected to be greatly attenuated in KO muscles. At approximately 12 weeks of age, mice were stratified into groups according to weight. Mice were injected intraperitoneally with either caffeine (20 mg/kg body weight (BW)) or an equivalent volume of saline using a 26-gauge needle. Using general allometric scaling guidelines (Sharma and McNeill 2009), this dosage of caffeine is equivalent to approximately 193 mg of caffeine in a 70 kg human (about 2 cups of coffee, depending on type) and results in a serum caffeine concentration of ~40 μM (Ohta et al. 2007). Ten min after injection, the mice were anesthetized with 2%–3% isoflurane in supplemental oxygen and placed on a water-circulating heat pad set to 37 °C. The sciatic nerve was isolated from the lateral aspect of both upper hindlimbs, and the left nerve was electrically stimulated to produce a bout of 6 sets of 10 contractions (3 s per contraction, 10–12V, 100 Hz) with 10 s of rest between contractions and 1 min of rest between sets. The tibialis anterior–extensor digitorum longus and gastrocnemius–plantaris–soleus muscle groups were harvested from the animals either immediately or 8 h after the contraction bout. For the 8-h time point, the incision was closed with wound clips after the contraction bout. Mice in the 8-h group received an injection of buprenorphine (0.075 mg/kg BW) and were then allowed to awaken and resume normal activity with ad libitum access to food until 30 min prior to the designated time for tissue collection at which time some received an intraperitoneal (IP) injection of 5 mg/mL puromycin (22 mg/kg BW) using a 26-gauge needle for the assessment of protein synthesis.

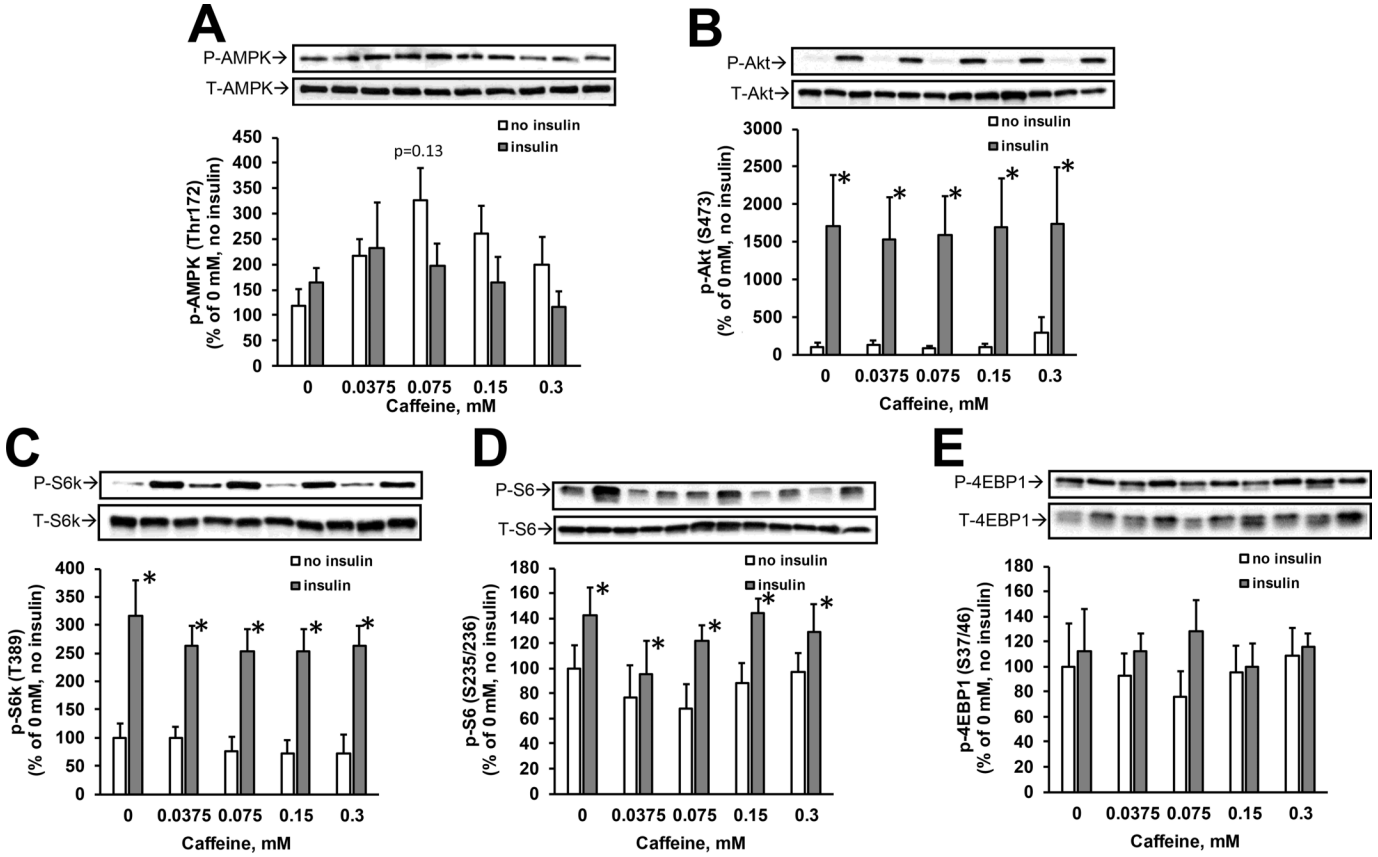
Assessment of protein synthesis

Some mice were injected with 22 mg/mL puromycin 30 min prior to the collection of muscle tissue. This antibiotic labels newly synthesized proteins and is a validated indicator of protein synthesis (Goodman and Hornberger 2012). Measurement of puromycin content in the protein lysates was determined by western blotting and immunodetection with an antipuromycin antibody (EMD Millipore, Temecula, CA #MABE343) as described below.

Synergist Tenotomy

Sprague Dawley rats (~2 months of age) were obtained from Charles River Laboratories (Wilmingtion, MA). Rats were stratified based on weight into tap water or tap water + caffeine (1 g/L) groups. This concentration has been shown to result in serum caffeine concentrations of ~32 μM in rats, or the equivalent of ~6 cups of coffee per day in humans (Costenla et al. 2010). Water and food consumption was monitored throughout the treatment period. Two days after initiation of caffeine treatment, all rats underwent unilateral tenotomy of the gastrocnemius and soleus muscles of the right hindlimb to induce hypertrophy of the synergistic plantaris muscle. Rats were anesthetized using isoflurane (2%–3%) dispersed in supplemental oxygen and injected IP with the anal-

Fig. 1. Effect of caffeine on anabolic signaling in cultured myotubes. C2C12 myoblasts were differentiated to myotubes for 7 days. They were then treated with varying concentrations of caffeine for 1 h, followed by the addition of 100 nM insulin or no insulin for an additional 15 min. Cells were lysed in homogenization buffer, then assessed by western blotting for levels phosphorylated (P) and total (T): AMPK (A), Akt (B), p70 S6 kinase (S6k; C), ribosomal protein S6 (S6; D) and eIF4E binding protein 1 (4EBP1; E). n = 5 cultures/condition. *, significant difference vs. corresponding caffeine treatment group without insulin. AMPK, adenosine monophosphate-activated protein kinase; Akt, protein kinase B.



gesic buprenorphine (0.075 mg/kg BW) using a 26-gauge needle. Ophthalmic ointment was applied to the eyes prior to surgery. The hindlimbs were shaved and scrubbed with povodone–iodine solution. Using aseptic technique, a 15–20 mm incision was made along the posterior aspect of the lower hindlimb. The gastrocnemius tendon of the right hindlimb was cut and sutured back onto the body of the muscle using 6–0 silk sutures to prevent reattachment of the tendon. The contralateral hindlimb was sham operated (incision without the tenotomy). Both incisions were closed using surgical staples, and the rats were returned to the animal quarters and fed ad libitum for the remainder of the study. At 2 weeks postsurgery plantaris, adipose, and heart tissues were harvested, quickly weighed, and then flash frozen in liquid nitrogen for protein analysis.

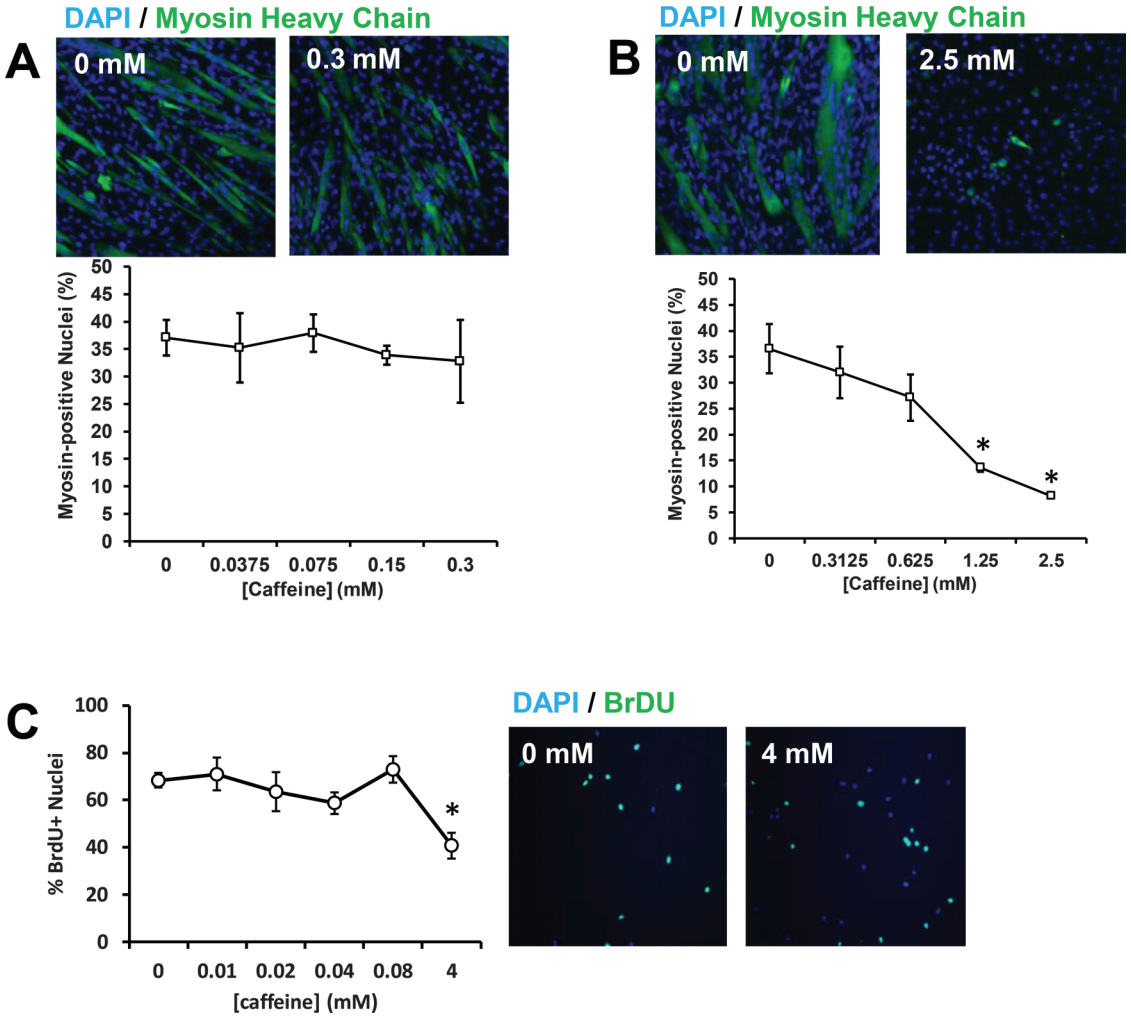
Tissue homogenization

Tissues were ground to powder using a mortar and pestle, then homogenized (Bullet Blender, Next Advance Laboratories, Averill Park, NY) in 19 μL of homogenization buffer (50 mM Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 1% Triton X-100, 50 mM β-glycerol phosphate, pH 7.5, 1 M dithiothreitol (DTT), 1 M benzamidine, 5 ug/uL soybean trypsin inhibitor, 200 mM Na_3VO_4, and 200 mM phenylmethane sulfonyl fluoride (PMSF)) per μg tissue. Homogenized tissues were then sonicated at 30% amplitude for 10 s on ice or subjected to 3 cycles of freezing at –90 °C and thawing to ensure disruption of cellular components, after which a small aliquot was removed to determine the total protein content of the muscle and frozen at –90 °C. The remaining homogenate was vortexed and clarified by centrifugation at 21 000g for 10 min. Supernatants were removed, flash frozen in liquid nitrogen, and stored at –90 °C.

Protein quantification and western blotting

Muscle homogenates and cell lysates were analyzed for protein concentration using a modified Lowry assay (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA) according to manufacturer's protocols. Equal amounts of protein were then separated by electrophoresis on (Criterion Precast Tris-HCl gels, Bio-Rad Laboratories) and transferred to polyvinylidene fluoride (PVDF) membranes. Proper transfer and equal protein loading were verified by Ponceau-S staining of the PVDF membranes after transfer. Primary antibodies (pAMPKa (Cell Signaling #2535), AMPKa (Cell Signaling #2532), LKB1 (Millipore #07-694), p-p70 S6 kinase (pS6k; Cell Signaling #9206), S6K (Cell Signaling #2708), phospho-eukaryotic initiation factor 4E binding protein-1 (p-4EBP1; Cell Signaling #2855), 4EBP1 (Cell Signaling #9644), phospho-eukaryotic elongation factor 2 (p-eEF2; Cell Signaling #2331), eEF2 (Cell Signaling #2332), phospho-Akt (pAkt; Cell Signaling #4060), Akt (Cell Signaling #9272), phospho-ribosomal protein S6 (p-S6; Cell Signaling #4858), S6 (Cell Signaling #2217)) were applied overnight at 4 °C. Membranes were exposed to autoradiographic film and resulting band intensities were determined using AlphaEase FC Software version 3.1.2 (Alpha Innotech Corporation, San Leandro, CA), or Gel-Pro software (Media Cybernetics, Rockville, MD).

Fig. 2. Effect of caffeine on myoblast proliferation and differentiation. C2C12 myoblasts were proliferated to near-confluence, then differentiated with or without lower (A) or higher, nonphysiological (B) concentrations of caffeine for 7 days, followed by staining for myosin heavy chain (green) and nuclei (blue). C2C12 myoblasts were subcultured and allowed to adhere to their wells overnight, then allowed to proliferated for in the presence or absence of varying concentrations of caffeine for X hours. Bromo-deoxyuridine (BrDU) was added for the final X minutes of the culture period. The percentage of nuclei that stained positive for BrDU was used as an index of myoblast proliferation. n = 3 cultures/conditions. *, significant difference vs. 0 mM caffeine. DAPI, 4′,6-diamidino-2-phenylindole. [Colour online.]



Statistical analysis

Statistical analysis was performed using NCSS Statistical Software (Kaysville, UT). All data are expressed as mean ± standard error (SE). Factorial ANOVA with repeated measures was used for the analysis of caffeine on resting and stimulated or sham operated and overloaded muscles. Factorial ANOVA was used for the analysis of caffeine on insulin- and non-insulin-treated cells. One-way ANOVA was used for the analysis of caffeine's effect on cell proliferation and differentiation, with significance set at $P \leq 0.05$. Fisher's least significant difference posthoc analysis was used where appropriate based on ANOVA testing.

Results

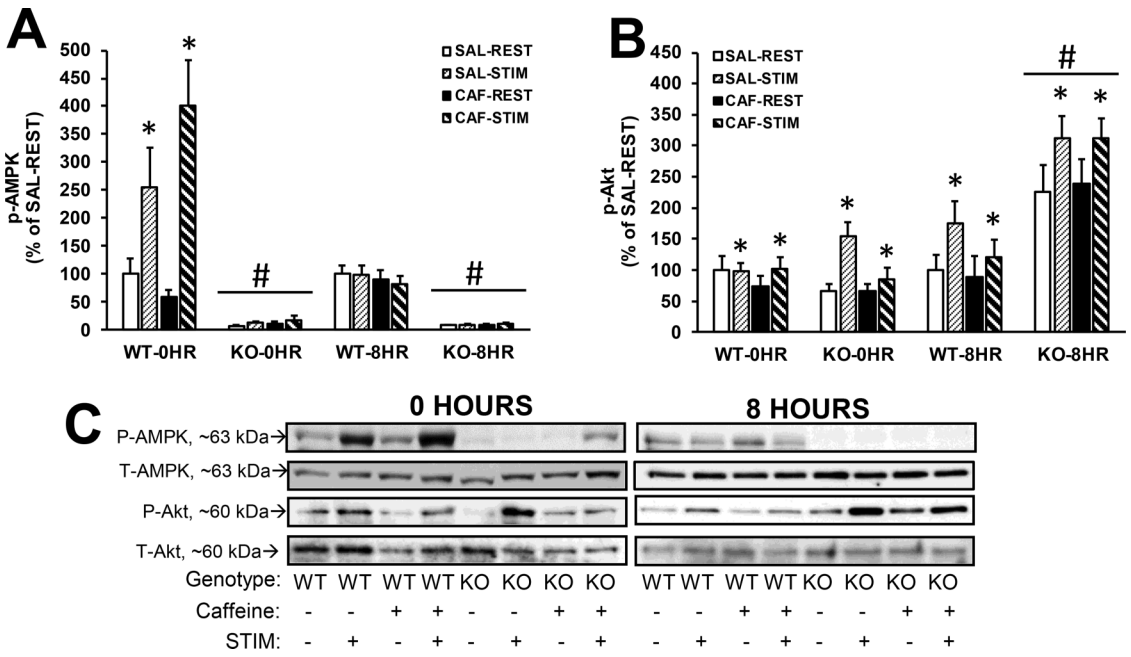
Effect of physiological caffeine concentrations on cultured skeletal muscle anabolic signaling

To determine the effect of varying levels of caffeine (0–0.3 mM) on basal and insulin-stimulated signaling in skeletal muscle cells, we measured total and phosphorylated AMPK, Akt, S6k, S6, and 4EBP-1 via western blotting. Total protein levels were not significantly changed by caffeine or insulin treatment, except for total 4EBP1, which was increased by 53% in insulin-treated cells (main effect across all caffeine treatment levels; Fig. 1E, representative blot). AMPK phosphorylation in C2C12 myotubes was not significantly affected by a range of caffeine concentrations from 0.0375 mM to 0.3 mM with or without insulin, although a trend was observed for an increase at 0.075 mM caffeine (Fig. 1A). Insulin did not affect AMPK phosphorylation (Fig. 1A). Akt phosphorylation was induced, as expected, by insulin but this was not affected by caffeine treatment at any concentration (Fig. 1B). Phosphorylation of S6k (Fig. 1C) and S6 (Fig. 1D) were likewise increased by insulin treatment, but unaffected by caffeine at any concentration, while 4EBP1 phosphorylation was not affected by insulin or caffeine (Fig. 1E).

Effect of physiological caffeine concentrations on cultured skeletal muscle differentiation and proliferation

Treatment of cultured C2C12 myoblasts with 0.0–0.625 mM caffeine did not significantly affect differentiation into myotubes, as gauged by the presence of nuclei in myosin positive cells (Figs. 2A and 2B). However, treatment with higher caffeine concentrations (1.25–2.5 mM) did significantly decrease differentiation (Fig. 2B). Similarly, proliferation of myoblasts, as determined by BrdU incorporation into myoblasts, was significantly decreased by 4 mM but not 0.01–0.08 mM caffeine (Fig. 2C).

Fig. 3. Effect of caffeine on contraction-induced AMPK and Akt phosphorylation. Tibialis anterior/extensor digitorum longus muscles were harvested immediately after (0HR) or 8 h after (8HR) unilateral sciatic nerve stimulation (STIM) in wild-type (WT) or skeletal muscle-specific LKB1 knockout (KO) mice pre-treated with an intraperitoneal injection of caffeine (CAF) or saline (SAL). Unstimulated muscles from the contralateral limb served as resting (REST) controls. (A) adenosine monophosphate-activated protein kinase (AMPK) phosphorylation. (B) Protein kinase B (Akt) phosphorylation. *, significant ($P \leq 0.05$) main effect of STIM vs REST; #, significant main effect of KO vs. WT. n = 7–8 muscles/condition. P or p, phosphorylation; T, total.



Effect of caffeine on AMPK and Akt phosphorylation in resting and contracted muscles

To determine the effect of caffeine on activation of signaling pathways upstream of mTOR, we measured total and phosphorylated AMPK and Akt levels from mouse muscles at rest and after STIM. Total protein levels were not affected by caffeine or stimulation for either AMPK or Akt, although total-Akt tended (P = 0.16) to be lower in caffeine-treated muscles at 0 h post STIM (Fig. 3C). STIM activated AMPK, as indicated by increased phosphorylation, immediately after the contraction bout. However, caffeine had no significant effect on AMPK phosphorylation in rest or STIM muscles at 0 or 8 h postcontractions (Fig. 3A). AMPK phosphorylation returned to resting levels within 8 h postcontraction with or without caffeine. As expected, AMPK phosphorylation in KO muscles was nearly undetectable and was not significantly affected by caffeine or STIM (Fig. 3A).

A main effect of STIM on Akt phosphorylation was observed at both 0 and 8 h, across both genotypes and caffeine treatment conditions, although the effect of STIM on Akt phosphorylation at 0 h in WT mice appears to be quite minimal (Fig. 3B). Akt phosphorylation was also higher in LKB1 KO versus WT muscles in both rest and STIM muscles 8 h postcontraction, regardless of caffeine treatment (main effect), suggesting systemic-mediated activation of Akt in the KO muscles (Fig. 3B).

Effect of caffeine on anabolic signaling and protein synthesis in resting and contracting muscles

To determine the effect of caffeine on activation of mTOR pathway signaling, and protein synthesis, we measured total and phosphorylated S6k, 4EBP1, and S6 levels, as well as the incorporation of puromycin into nascent proteins in mouse muscles at rest and after electrically stimulated contractions. Total protein levels were not significantly affected by stimulation or caffeine treatment in either genotype (Fig. 4D). Activation of the mTOR pathway, as indicated by the phosphorylation of its direct targets S6k (Figs. 4A and 4D) and 4EBP1 (Figs. 4B and 4D) as well as the S6k target rpS6 (Figs. 4C and 4D), was elevated in STIM muscles at 0 and 8 h postcontraction. Caffeine treatment had no significant effect on S6k or 4E-BP1 phosphorylation, but enhanced the phosphorylation of rpS6 immediately after the contraction bout in WT muscles (Fig. 4C). A main effect for increased phosphorylation in muscles from KO mice, regardless of contraction or caffeine treatment, was observed for S6k at 0 and 8 h, as well as on 4E-BP1 and S6 at 0 h postcontraction.

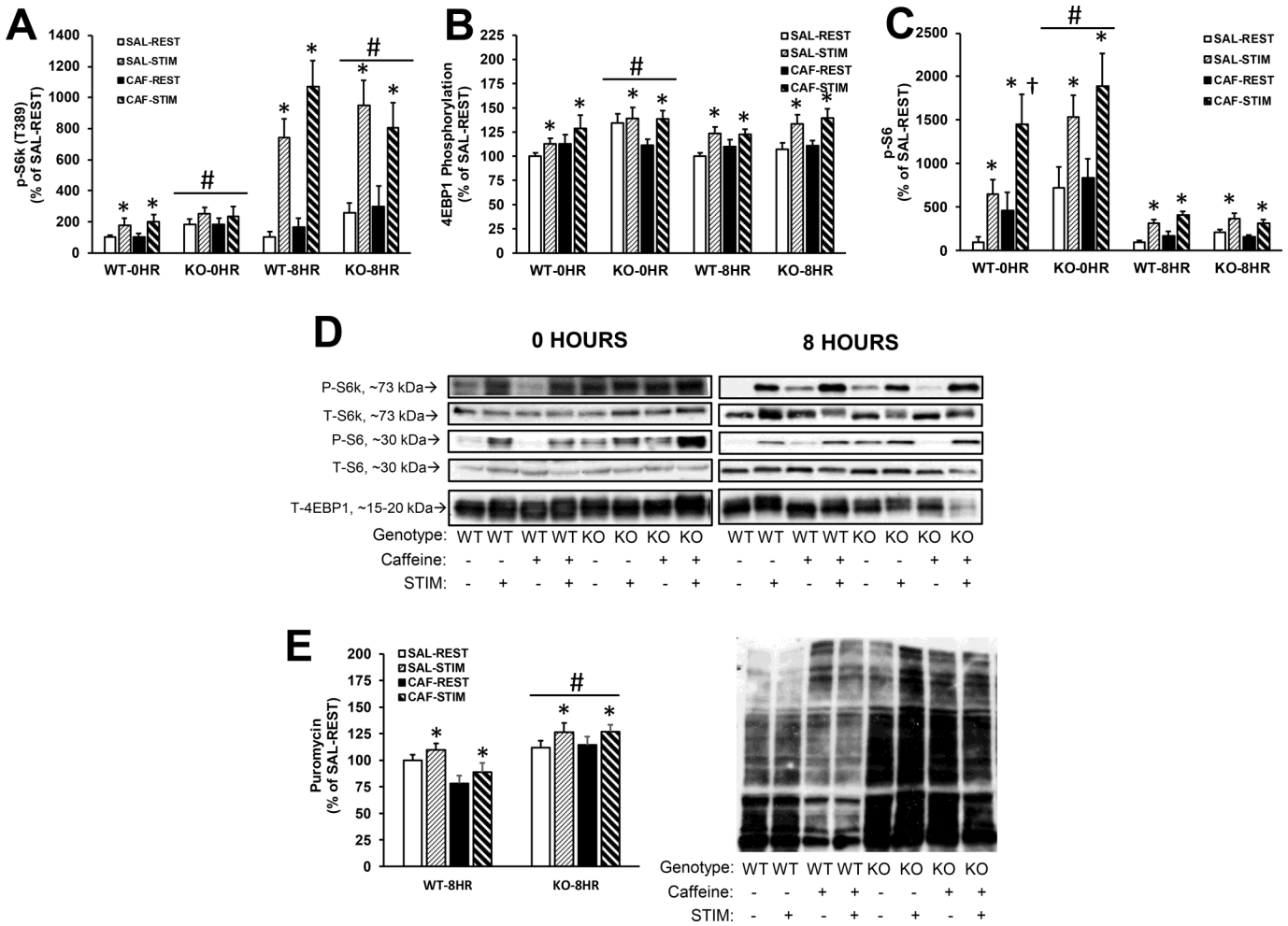
Puromycin incorporation into proteins, which is reflective of protein synthetic rate, was increased 8 h postcontraction regardless of genotype or caffeine treatment, and was also higher in KO versus WT muscles (Fig. 4E).

Effect of caffeine on overload-induced skeletal muscle hypertrophy

The effect of caffeine on skeletal muscle hypertrophy was assessed using synergist tenotomy model for muscle growth. Two weeks of plantaris overload (OVLD) induced by gastrocnemius and soleus tenotomy resulted in significant hypertrophy (~15%) of the plantaris muscle regardless of caffeine treatment (Figs. 5A, 5B). AMPK phosphorylation was not affected at this timepoint by OVLD (Fig. 5C), but Akt phosphorylation was elevated in OVLD muscles from water-treated animals. Caffeine treatment completely eliminated the OVLD-induced increase in Akt phosphorylation (Fig. 5D). 4EBP1 phosphorylation (downstream of mTOR) was increased by OVLD regardless of caffeine treatment (Fig. 5E). An attempt was made to assess S6k and S6 phosphorylation in these samples as well, but was unsuccessful in our rat tissue with our antibodies for unknown reasons.

Caffeine treatment led to a decrease in food consumption and a decreased retroperitoneal fat pad weight, and a trend for decreased body weight gain (P = 0.22) and water consumption (P = 0.12) but similar heart weight (Table 1).

Fig. 4. Effect of caffeine on contraction-induced anabolic signaling and protein synthesis. Tibialis anterior/extensor digitorum longus muscles were harvested immediately after (0HR) or 8 h after (8HR) unilateral sciatic nerve stimulation (STIM) in wild-type (WT) or skeletal muscle-specific LKB1 knockout (KO) mice pretreated with an intraperitoneal injection of caffeine (CAF) or saline (SAL). Unstimulated muscles from the contralateral limb served as resting (REST) controls. (A) p70S6k phosphorylation. (B) 4EBP1 phosphorylation (% of total 4EBP1 signal in upper, slower migrating band). (C) S6 phosphorylation. (D) Representative blots for total and phospho-proteins in panels A–C. (E) Puromycin incorporation into proteins with representative blot. *, significant ($P \leq 0.05$) main effect of STIM vs REST; †, significant vs. WT-SAL-STIM; #, significant main effect of KO vs. WT. n = 7–8 muscles/condition. P or p, phosphorylation; T, total.



Discussion

Caffeine is arguably the most widely consumed drug today and it is also routinely found in those supplements that are intended to aid in athletic performance. However, recent work showed that caffeine, at supraphysiological concentrations, blocks mTOR signaling in cultured cells (McMahon et al. 2005; Miwa et al. 2012) as well as ex vivo soleus incubation (Kolnes, et al. 2010). Furthermore, in vivo caffeine administration was reported to impair Akt phosphorylation (Egawa et al. 2011*b*), which is a major upstream regulator of the mTOR pathway. Therefore, the purpose of this study was to test the hypothesis that caffeine at physiological doses would impair load-induced skeletal muscle anabolic signaling through the mTOR pathway, protein synthesis, and hypertrophy. Our findings indicate that, contrary to our hypothesis, caffeine at physiological levels does not induce sustained abatement of anabolic signaling, protein synthesis, or skeletal muscle hypertrophy.

Although caffeine at concentrations between 0 and 0.3 mM did not have any significant effect on AMPK phosphorylation in cultured C2C12 muscle cells, a near-significant response was observed at 75 μM. Nonetheless, we observed no significant indication of in vivo AMPK activation by caffeine either basally or in overloaded rat and contracted mouse muscles. This is in contrast to reported findings where AMPK was activated by 2.5 mM caffeine in cultured C2C12 cells (Mathew et al. 2014), 1–3 mM caffeine in ex vivo incubations of skeletal muscle (Jensen et al. 2007; Raney and Turcotte 2008; Abbott et al. 2009; Egawa et al. 2009, 2011*a*), as well as in vivo by 60 mg/kg caffeine injection in extensor digitorum longus muscles from rats (Tsuda et al. 2015). In humans, blood concentrations for caffeine typically range between 15 and 80 μM after 2–5 cups of coffee (Birkett and Miners 1991; Graham 2001). The caffeine dosage in our study was 20 mg/kg BW (via IP injection) for the mice, and 147 mg/kg BW per day (in the drinking water based on average water consumption) for the rats. Using general allometric scaling guidelines (Sharma and McNeill 2009), this dosage of caffeine would be equivalent to approximately 114 mg per injection and 1660 mg per day, respectively, in a 70 kg human. This would be equivalent to about 1–2 cups of coffee, depending on type, for the injection study and 10–20 cups per day for the chronic drinking water study. Previous works indicates that these dosages result in serum caffeine concentrations of 30–40 μM (Ohta et al. 2007; Costenla et al. 2010), consistent with what would typically occur in a human with a relatively high consumption of caffeinated beverages such as coffee or energy drinks. Therefore, we can conclude that while supraphysiological levels

Fig. 5. Caffeine does not affect plantaris hypertrophy after synergist tenotomy. Rats were provided with plain tap water or tap water + caffeine for 16 days. Two days after initiation of water treatment, unilateral plantaris overload (OVLD) was accomplished via tenotomy of the gastrocnemius and soleus tendons. The contralateral limb was sham operated (SHAM). Rats were allowed to recover for two weeks prior to harvesting of SHAM and OVLD plantaris muscles. (A) Percent plantaris hypertrophy in water and caffeine-treated rats. (B) Plantaris wet weight. (C) AMPK phosphorylation. (D) Akt phosphorylation. (E) 4EBP1 phosphorylation (% of total 4EBP1 signal in upper, slower-migrating band). (F) Representative blots for panels C–E. *, significant ($P \leq 0.05$) difference between OVLD and corresponding REST muscles. n = 8–10 muscles/condition. AMPK, adenosine monophosphate-activated protein kinase; Akt, protein kinase B; P, phosphorylation; T, total.

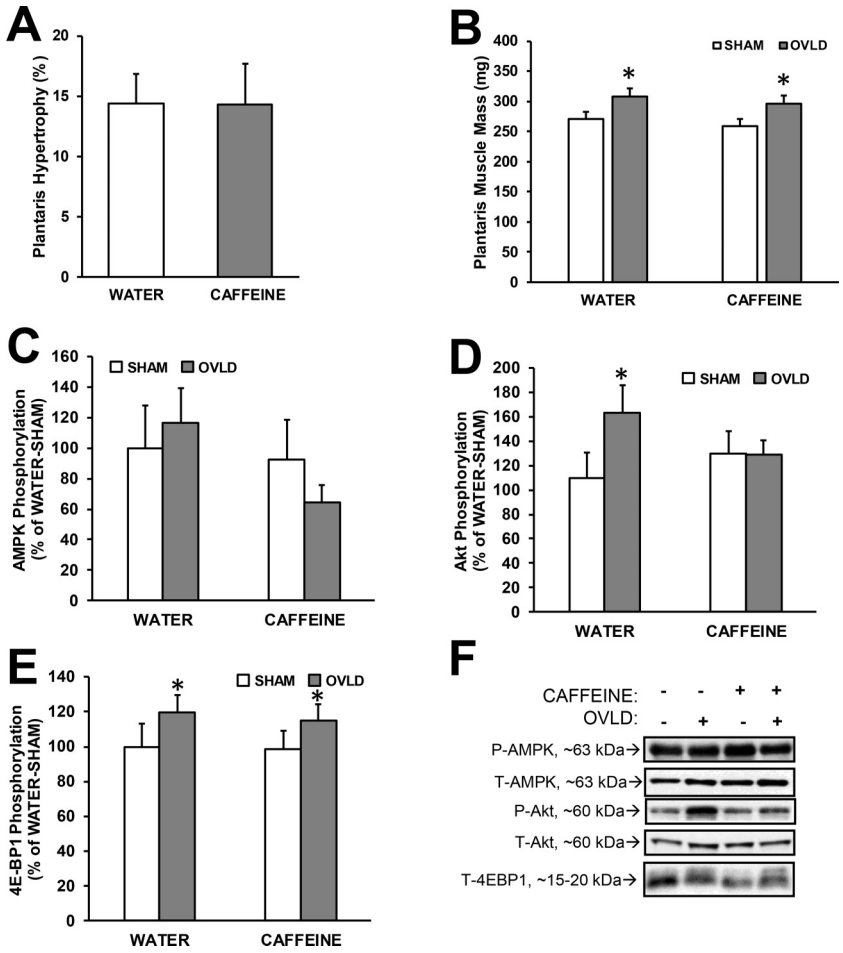


Table 1. Rat characteristics before and after 2 weeks of synergist tenotomy with or without the addition of caffeine (1 g/L) in the drinking water.

	Water (n = 10)	Caffeine (n = 10)
Initial body weight (g)	202.8±3.2	205.1±4.0
Final body weight (g)	305.5±9.3*	295.1±10.2*
BW gain (%)	50.6±3.8	43.8±3.7
Heart weight (% of BW)	0.300±0.005	0.297±0.006
Retroperitoneal fat pad weight (% of BW)	2.80±0.28	1.76±0.17†
Food consumption (g/kg BW per day)	132±3	117±4†
Water consumption (ml/kg BW per day)	165±8	147±8

Note: Values are means ± SE. BW, body weight.
*Significant difference ($P \leq 0.05$) vs. initial BW.
†Significant difference vs. water-treated group.

of caffeine do induce AMPK activation, concentrations typically found in the blood are not sufficient to do so. However, it should be kept in mind that the half-life of caffeine in mice is less than 1 h, whereas in humans it is 4–5 times longer (Ohta et al. 2007). Thus, the prolonged exposure to caffeine in humans could lead to different effects than observed in this study. Furthermore, genetic factors in humans, such as polymorphisms in the *CYP1A2* gene (major enzyme involved in caffeine metabolism), lead to wide variations in caffeine response (Yang et al. 2010) and could, potentially, drive anabolic suppression by caffeine in some individuals. It is also worth noting that insulin can inhibit AMPK activation via phosphorylation at Ser485, despite no change in phosphorylation at Thr172 on AMPK (Valentine et al. 2014). It is possible, therefore, that AMPK activity could potentially be regulated by caffeine independent of the phosphorylation site measured in this study.

At physiological concentrations, caffeine is thought to act primarily through inhibition of adenosine receptors (Graham et al. 2008). It has also been reported to block the activation of Akt by insulin in rat epitrochlearis (Egawa et al. 2011*b*) and soleus muscles (Kolnes et al. 2010) at 1–3 mM concentration. We found that while caffeine injection did not significantly affect Akt phosphorylation in resting or contracted mouse muscles, or in cultured mouse C2C12 myotubes, caffeine completely blocked the increase in Akt phosphorylation in hypertrophying plantaris muscles, but this did not significantly affect signaling through the mTOR pathway to 4EBP1 or muscle growth. This disconnect between Akt activation and downstream mTOR signaling has been observed previously using the synergist ablation model of skeletal muscle overload and may be explained by the effect of other signaling pathways' (such as extracellular signal-regulated kinases) interaction with mTOR (Miyazaki et al. 2011).

The inhibition of Akt signaling by caffeine in this context may have implications for the regulation of glucose metabolism since Akt is a major regulator of insulin-induced glucose uptake. Previous studies showed that although caffeine administration in humans did not affect skeletal muscle Akt phosphorylation, it did reduce insulin-stimulated glucose uptake and whole-body insulin sensitivity (Thong et al. 2002), suggesting that caffeine may affect glucose regulation independently or downstream of Akt. The reason for the discrepancy in the effect of caffeine on Akt activation between the 2 hypertrophy models used here is not known, but it may be that mouse muscle cells are less sensitive to caffeine in this regard than is rat muscle.

Previous work showed that caffeine at 3–5 mM levels effectively attenuated the insulin induced increases in pS6K and pAkt (Egawa et al. 2011*b*). In C2C12 cells, we found that concentrations of caffeine at 0.3 mM or lower had no significant effect on basal or insulin-induced phosphorylation of Akt, mTOR, or phosphorylation of downstream mTOR-targets and also did not affect myoblast proliferation or differentiation to myotubes. It should be recognized that the insulin concentration (100 nM) used in these experiments was supraphysiological to elicit a maximal signal, and the possibility of an effect of caffeine on submaximal insulin signaling is, therefore, not eliminated by our findings. Nonetheless, consistent with the lack of effect in cell culture at physiological caffeine concentrations, we found no evidence for attenuated mTOR-related signaling, protein synthesis, or muscle growth in caffeine-treated muscles from mice or rats using 2 models for a mechanical overload stimulus. It did not block the phosphorylation of S6k, 4E-BP1, S6, or protein synthesis (as indicated by puromycin incorporation into nascent proteins) at rest or after muscle contractions in mice, and it did not affect plantaris muscle mass, hypertrophy, or 4E-BP1 activity 2 weeks after synergist-tenotomy induced overload. Our results do not support, therefore, our hypothesis that caffeine, at typical physiological concentrations, would block muscle growth. Indeed, caffeine may even have a mild anabolic effect since it enhanced the phosphorylation of ribosomal protein S6 immediately after contractions. While not convincing on its own, future studies would be valuable in assessing whether long-term caffeine administration over repeated, distinct stimulation bouts could potentially lead to overt hypertrophy.

Two weeks of caffeine treatment did, on the other hand, reduce the size of the retroperitoneal fat pad without impacting muscle hypertrophy. This suggests that caffeine has the ability to mobilize fat stores, which is consistent with previous research where caffeine prevented adipose accretion with a high-fat diet (Conde et al. 2012). However, caffeine-treated rats in our study consumed less chow which could explain the diminished fat storage. That caffeine allowed for the maintenance of hypertrophy concurrent with significant fat loss and decreased food consumption suggests that it may actually exert a mild anabolic effect under low nutrient conditions, which would be useful in weight loss regimens.

In this study we used 2 in vivo routes for caffeine administration: orally in the chronic overload model and via one-time injection in the acute muscle stimulation model. The 2 routes of administration both have advantages and weaknesses. For the rat study, the chronic administration of the caffeine in the drinking water allowed for a more consistent (over the course of each day) and less stressful/painful administration than would be accomplished with repeated caffeine injections over the study period. However, as noted in our results, it also resulted in significantly lower food intake, and a trend for lower water intake, both of which could be confounding factors. The acute injection in the mouse study, on the other hand, allowed for a precise, immediate, and consistent dosage from mouse-to-mouse that would not be possible acutely with drinking water as the vehicle. Both treatments lend strength to the study, in that we found similar results with 2 different caffeine administration models.

Our use of the skeletal muscle specific LKB1-KO model in the present study was based on previous work showing that caffeine could activate AMPK in skeletal muscle. The AMPK α2 subunit is responsible for the majority of AMPK activity in skeletal muscle (Jorgensen et al. 2004), and we previously showed that AMPKα2 activity is greatly reduced in this mouse model (Tanner et al. 2013). Thus, we hypothesized that the KO would prevent the putative activation of AMPK by caffeine. The lack of AMPK activation by caffeine in WT muscles makes the findings in the KO muscles peripheral to the main purpose of this study, but we did find that phosphorylation of S6k, 4EBP1, and S6 were all enhanced by the lack of skeletal muscle LKB1, as was protein synthesis. This finding is novel and somewhat surprising because LKB1 KO does not impact mTOR signaling or hypertrophy after synergist ablation overload in mice as demonstrated by McGee et al. (2008) and as suggested by findings in our laboratory (unpublished results). This lack of effect may be related to findings that LKB1-KO may not completely suppress AMPKα1 activity in skeletal muscle (Jeppesen et al. 2013; Tanner et al. 2013). However, our current findings are consistent with previous work from our lab and others' labs showing that LKB1's best-characterized target, AMPK, is an important negative regulator of muscle cell size and hypertrophy (Bolster et al. 2002; Thomson and Gordon 2005; Thomson et al. 2008; Mounier et al. 2009). The comparison of these findings is further complicated by the fact that LKB1 has many other targets besides AMPK, but our current findings suggest that LKB1 activity may indeed inhibit anabolism during skeletal muscle growth.

In conclusion, we observed that caffeine at physiological concentrations did not generally impair AMPK or anabolic signaling (except for blocking overload-induced Akt phosphorylation) or skeletal muscle hypertrophy after OVLD or STIM in rats and mice respectively. Indeed, although it did not affect protein synthesis or hypertrophy, caffeine enhanced the phosphorylation of S6 immediately after muscle contractions. Though inconclusive, this suggests that if anything, caffeine may provide some anabolic benefit.

Conflict of interest

The authors report no conflicts of interest associated with this manuscript.

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