Application of the [γ-32P] ATP kinase assay to study anabolic signaling in human skeletal muscle

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1Health & Exercise Sciences Research Group University of Stirling, Stirling, United Kingdom; 2Department of Orthopaedic Surgery, University of California San Diego, La Jolla, California; 3Biomedical Sciences Graduate Program, University of California San Diego, La Jolla, California; 4INSERM U845, Université Paris Descartes, Paris, France; 5Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, United Kingdom; and 6School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, Edgbaston, United Kingdom

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McGlory C, White A, Treins C, Drust B, Close GL, MacLaren DP, Campbell IT, Philp A, Schenk S, Morton JP, Hamilton DL. Application of the [γ-32P] ATP kinase assay to study anabolic signaling in human skeletal muscle. J Appl Physiol 116: 504–513, 2014. First published January 16, 2014; doi:10.1152/japplphysiol.01072.2013.—AMPK (AMP-dependant protein kinase)-mTORC1 (mechanistic target of rapamycin in complex 1)-p70S6K1 (ribosomal protein S6 kinase 1 of 70 kDa) signaling plays a crucial role in muscle protein synthesis (MPS). Understanding this pathway has been advanced by the application of the Western blot (WB) technique. However, because many components of the mTORC1 pathway undergo numerous, multisite posttranslational modifications, solely studying the phosphorylation changes of mTORC1 and its substrates may not adequately represent the true metabolic signaling processes. The aim of this study was to develop and apply a quantitative in vitro [γ-32P] ATP kinase assay (KA) for p70S6K1 to assess kinase activity in human skeletal muscle to resistance exercise (RE) and protein feeding. In an initial series of experiments the assay was validated in tissue culture and in p70S6K1-knockout tissues. Following these experiments, the methodology was applied to assess p70S6K1 signaling responses to a physiologically relevant stimulus. Six men performed unilateral RE followed by the consumption of 20 g of protein. Muscle biopsies were obtained at pre-RE, and 1 and 3 h post-RE. In response to RE and protein consumption, p70S6K1 g of protein. Muscle biopsies were obtained at pre-RE, and 1 and 3 h post-RE, whereas phosphorylated ACC ser79 was un-elevated at 3 h post-RE from pre-RE. These data highlight the utility of the KA to study skeletal muscle plasticity.

nTORC1; p70S6K1; AMPK; resistance exercise

THE AMPK (AMP-DEPENDANT PROTEIN KINASE)-mTORC1 (mechanistic target of rapamycin in complex 1)-p70S6K1 (ribosomal protein S6 kinase 1 of 70 kDa) cascade is a key regulatory signaling axis controlling a plethora of human metabolic events such as skeletal muscle protein synthesis (MPS) (11), glucose disposal (18, 29), and fatty acid metabolism (26). Our understanding of how the AMPK-mTORC1-p70S6K1 pathway responds to physiological perturbation such as exercise (11) and nutrition (9) has been advanced by the application of the phosphorylation-specific Western blot (WB) technique. This technique assesses the phosphorylation of a kinase or a kinase target on serine, threonine, and tyrosine residues, and infers the activity of a kinase on the basis of the magnitude of phosphorylation as determined by densitometry. The WB technique is highly advantageous because it offers the capacity to measure phosphorylation changes in many targets in a cost-effective way. However, in some cases, the WB technique possesses a limited dynamic range that can lead to type II statistical errors (18). Furthermore, differences in methodological approaches to the WB are known to lead to different statistical outcomes for the same data sets (14). Another consideration is that p70S6K1 has a constitutively low baseline phosphorylation. As such, when changes in p70S6K1 phosphorylation to anabolic stimulation are represented as a fold or percentage change, this low baseline phosphorylation results in an inflated response that is not representative of a physiological change in activity (21, 27). Hence, our understanding of how various stimuli such as exercise and nutrition affect p70S6K1 signaling is in part confined to both the limitations and assumptions of the WB technique.

In a recent commentary, Murphy and Lamb (24) describe a fully quantitative approach to WB. These authors show that by using calibration curves for each gel, a quantitative assessment of changes in protein expression can be made. However, conducting calibration curves for the analysis of posttranslational modifications (PTM) such as phosphorylation would be contingent upon 100% of the recombinant protein modified specifically at the specific PTM residue. Furthermore, the use of such calibration curves on every gel would prove costly when analyzing numerous samples, thus undermining the financial viability of the WB technique. As such, the use of the WB to assess changes in the phosphorylation of a kinase as a proxy of kinase activity remains a challenge.

The in vitro [γ-32P] ATP kinase assay (KA) is the gold standard for assessing kinase activity (16). This methodology involves immunoprecipitating the kinase of interest from homogenized tissue. The activity of the kinase is then assessed in vitro against a kinase-specific or kinase family-specific substrate. Gamma (γ)-32P ATP is subsequently used to measure the incorporation of phosphate into the substrate via liquid scintillation counting, thus enabling a quantitative assessment of activity. The dual layer of specificity and quantitative nature of the KA may obviate some of the methodological shortcom-
ings associated with using the WB (14) and its use to assess AMPK activity in response to exercise is now a feature in the human exercise sciences (10, 37, 40). A semiquantitative p70S6K1 KA does exist for use in rodent tissue (21), and a quantitative p70S6K1 KA has previously been used in cell culture studies (33). However, no study has described a fully quantitative KA methodology for the assessment of p70S6K1 activity in human skeletal muscle.

Therefore, the primary aim of this methodological study was to develop and validate a fully quantitative p70S6K1 KA methodology to assess p70S6K1 activity in human skeletal muscle in response to resistance exercise (RE) and protein feeding. Because muscle tissue availability is often a major limitation to routine analytical procedures, a secondary aim was to simultaneously assess AMPK activity and another regulator of mTOR (mechanistic target of rapamycin), protein kinase B (PKB), from the same muscle biopsy sample as p70S6K1. In this regard, we also aimed to validate a serial immunoprecipitation (IP) protocol to enable the dual assessment of p70S6K1 and PKB, from the same muscle homogenate. It is hoped that these methodological developments will enhance our capacity to accurately delineate the molecular mechanisms that regulate human skeletal muscle plasticity.

**METHODS**

**Materials**

Unless otherwise stated, all materials were from Fisher Scientific (Loughborough, UK). All antibodies, unless otherwise stated, were used at a concentration of 1:1,000, and were from New England Biolabs (Herts, UK). Selected primary antibodies were mTOR (Santa Cruz Biotechnology), total p70S6K1 (#2708), PKB thr308 (PRAS40)thr246 (#2997) and total PRAS40 (#2691), 4EBP1 thr37/46 (#2965), total PKB (#4691), proline-rich Akt/PKB substrate 40 kDa (Loughborough, UK). All antibodies, unless otherwise stated, were a gift from Professor Graham Hardie (Division of Cell Signaling and Immunology, University of Dundee).

p70S6K1−/− tissues. All animal experiments on p70S6K1−/− and littermate controls (wild type; WT) were approved by and conducted in accordance with the Direction Départementale des Services Vétérinaires, Préfecture de Police, Paris, France (authorization 75–1313). Mice (p70S6K1−/−) were generated as previously described (26a). The mice were housed in plastic cages and maintained at 22°C with a 12-h dark/12-h light cycle and had free access to food. Starved mice were WT; p70S6K1−/− mice had food withdrawn overnight and were then refed standard chow for 4 h. Animals were killed by cervical dislocation, and tibialis anterior muscles were rapidly dissected, blotted dry, and snap-frozen in liquid N₂.

**Mouse ex vivo and in vivo insulin stimulations.** All mouse experiments were approved by and conducted in accordance with the Animal Care Program at the University of California, San Diego, for the ex vivo insulin stimulations; and the Animal Care Program at the University of California, Davis, for the in vivo insulin stimulations. Ex vivo insulin stimulations were carried out as follows: 6 male C57/B16 mice were fasted for 4 h and anesthetized (150 mg/kg nembutal) via ip injection. Paired extensor digitorum longus muscles were excised and incubated at 35°C for 30 min in oxygenated (95% O₂, 5% CO₂) flasks of Krebs-Henseleit buffer (KHB) containing 0.1% BSA, 2 mM sodium pyruvate, and 6 mM mannitol. One muscle per pair was incubated in KHB without insulin, and the contralateral muscle was incubated in KHB with insulin [60 μU/ml (0.36 nM); Humulin R, Eli Lilly]. After 30 min, muscles were blotted on ice-cold filter paper, trimmed, freeze-clamped, and then stored at −80°C (n = 6). In vivo insulin stimulations were carried out as follows: 2 female C57/B16 mice were fasted for 4 h and anesthetized with 2% isofluran vaporized in 100% O₂. One mouse was ip injected with 100 mU/kg of insulin (Humulin R, Eli Lilly). After 30 min the muscles from the lower limb were dissected and snap-frozen in liquid N₂. The control mouse went through the same procedure except that it was injected with 0.9% saline.

**Human Experimental Study**

**Participants.** Six healthy, moderately trained men [mean ± SD: age, 23 ± 2 yr; body mass, 76 ± 5 kg; height, 179 ± 5 cm; unilateral 1 repetition maximum (1 RM) leg press, 128 ± 8 kg; 1 RM leg extension, 54 ± 3 kg] were recruited to participate in this study. All participants engaged in resistance training approximately two times per week and played team sports recreationally. Prior to the commencement of the experiment each participant provided written informed consent after all procedures and risks were fully explained in lay terms. Participants also were required to satisfy a routine physical activity readiness questionnaire. The study procedures were approved by the Research Institute for Sport and Exercise Sciences Ethics Committee, Liverpool John Moores University, and conformed to the standards as outlined in the most recent version of the Declaration of Helsinki.

**Study design.** Seven days after confirmation of unilateral 1 RM for leg press and leg extension, six healthy, moderately trained men reported to the laboratory at ~7:00 a.m. in a 10-h postabsorptive state. Each participant’s height and body mass were recorded, after which they rested (~30 min) in a semisupine position on a bed, and a resting biopsy was obtained. Immediately after the biopsy participants were transported by wheelchair to the resistance-training laboratory where they performed a bout of unilateral RE. Immediately following the bout of unilateral RE, participants were required to consume 20 g of pure egg white powder in a 500-ml solution. Participants were then transported back to the resting laboratory and rested again in a sucrose, and 0.1% (vol/vol) 2-mercaptoethanol and Complete protease inhibitor cocktail (Roche)] and then stored at ~80°C. HEK293 cell lysates overexpressing either the c1 or the c2 subunit of AMPK were a gift from Professor Graham Hardie (Division of Cell Signaling and Immunology, University of Dundee).

C₂C₁₂ myoblasts were grown to confluence on T75 plates in growth media [GM] 20% fetal bovine serum (FBS) (Dundee Cell Products, Dundee, UK), 1% penicillin/streptomycin (Invitrogen, Paisley, UK) in high-glucose DMEM (Invitrogen)]. Confluent myoblasts were then transferred to differentiation media [DM] 2% donor horse serum (Dundee Cell Products), 1% penicillin/streptomycin (Invitrogen) in high-glucose DMEM (Invitrogen)]. Prior to the addition of inhibitors cells were serum- and amino acid-starved in PBS with 5 mM glucose (Invitrogen) for 3 h. Starved cells were then pretreated with an inhibitor [100 nM rapamycin (Sigma Aldrich), 10 μM LY294002 (Cell Signaling) or vehicle control (0.1% DMSO)] for 1 h prior to serum and amino acid stimulation by the addition of the stimulation media supplemented with or without inhibitors. After 30 min of stimulation cells were lysed on ice in 1 ml of radio immunoprecipitation assay (RIPA) buffer [50 mmol/l Tris-HCl pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EDTA, 1% (vol/vol) Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27 mmol/l.
on the exercising limb at pre-RE, 1 h post-RE, and 3 h post-RE using a form of vigorous exercise. These controls were implemented in an experimental trial, participants also were asked to refrain from any activity for 3 days prior to the initial single 1 RM testing session, and to repeat a similar protocol at the same intensity with their dominant limb. Recovery time between exercises and sets was 3 min and 2 min, respectively. Participants were provided with verbal cues to ensure correct exercise technique. Each repetition consisted of a 1-s concentric action, 0-s pause, then a 1-s eccentric action as previously reported (4). Study controls. Participants were required to record dietary intake for 3 days prior to the initial single 1 RM testing session, and to repeat this pattern of consumption for the 3 days preceding the day of the experimental trial. For 3 days prior to both 1 RM testing and the experimental trial, participants also were asked to refrain from any form of vigorous exercise. These controls were implemented in an attempt to prevent any nutritional or exercise-induced changes in protein activity that might adversely affect the results of the study.

**Skeletal muscle biopsies.** Skeletal muscle biopsies were obtained on the exercising limb at pre-RE, 1 h post-RE, and 3 h post-RE using a Bard Monopry Disposable Core Biopsy Instrument (12 gauge × 10 cm length; Bard Biopsy Systems, Tempe, AZ). For each biopsy, the lateral portion of the vastus lateralis was cleaned before an incision into the skin and fascia was made under local anesthetic (MD92672; 0.5% marcareine without adrenaline). A sample of muscle (~30 mg) was extracted, rinsed with ice-cold saline, blotted dry, and any visible fat or connective tissue was removed. Muscle samples were then snap-frozen in liquid nitrogen and stored at −80°C for further analysis.

**Muscle tissue processing.** Approximately 30 mg of human skeletal muscle tissue (~5 mg of mouse skeletal muscle tissue) was homogenized by scissor mincing on ice in RIPA buffer [50 mM Tris-HCl pH 7.5, 50 mM NaCl, 50 mM NaF, 1 mM sodium vanadate, 1 mM EDTA, 1% (vol/vol) Triton X-100, 5 mM sodium pyrophosphate, 0.27 mM sodium succrose, and 0.1% (vol/vol) 2-mercaptoethanol and Complete protease inhibitor cocktail (Roche)] followed by shaking at 1,000 rpm on a shaking platform for 60 min at 4°C. Debris was removed by centrifugation at 4°C for 15 min at 13,000 g. The supernatant was then removed, and protein concentration was determined using the bichinonic acid protein assay according to the manufacturer’s instructions (Sigma Aldrich, UK).

**Western blotting.** For WB, 300 μg of supernatant was made up in Lamelli sample buffer, and 5–15 μg of total protein was loaded per well with the same amount of protein loaded in all wells for each gel, and run at 150 V for 1 h 15 min. Proteins were then transferred onto Whatman Immunobilon Nitrocellulose membranes (Fisher Scientific, Loughborough, UK) at 30 V overnight on ice. Membranes were blocked in 3% BSA-Tris-buffered saline (containing vol/vol 0.1% Tween 20) for 1 h at room temperature, followed by incubation in primary antibodies at 4°C overnight. Membranes underwent three 5-min washes in TBST followed by incubation in the appropriate secondary antibodies for 1 h at room temperature. Membranes were again washed three times for 5 min followed by incubation in enhanced chemiluminescence reagent (BioRad, Herts, UK). A BioRad ChemiDoc (Herts, UK) was used to visualize and quantify protein expression. All phospho proteins were normalized to the corresponding total proteins after stripping the phospho antibody for 30 min at 50°C in stripping buffer (65 mM Tris-HCl, 2% SDS, vol/vol, 0.8% mercaptethanol and Complete protease inhibitor cocktail). The antibody for the corresponding total protein. All phospho proteins were normalized to the expression of the corresponding total protein with the exception of phosphorylated Raptoreo792, which was normalized to the expression of GAPDH.

**[γ32P] ATP kinase assays.** All KA were carried out by IP either for 2 h at 4°C or overnight at 4°C in homogenization buffer [AMPK 50 mM Tris-HCl pH 7.25, 150 mM NaCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 5 μM soybean trypsin inhibitor, 1% (vol/vol) Triton X-100 and p70S6K1/panPKB (50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 1% (vol/vol) Triton X-100, 50 mM NaF, 5 mM NaPPi, 0.27 M sucrose, 0.1% β-mercaptoethanol, 1 mM Na3(OV)4, and 1 Complete (Roche) protease inhibitor tablet per 10 ml)]. Protein G sepharose (2.5 μl per IP) was used to precipitate the immune complexes. Immune complexes were washed twice in assay-specific high-salt washes (homogenization buffers as above with 0.5 M NaCl added) followed by one wash in assay-specific assay buffer (see below). Prior to carrying out the activity assay the immune-bead-complex was suspended in a total of 10 μl of assay buffer for p70S6K1 and panPKB assays, and 20 μl of assay buffer for AMPK assays. All assays were carried out in a 50-μl reaction. Assays were started every 20 s by the addition of a hot assay mix, which consisted of assay buffer [PKB/p70S6K1 (50 mM Tris-HCl pH 7.4, 0.03% Brij35, and 0.1% β-mercaptoethanol), AMPK (50 mM HEPES pH 7.4, 1 mM DTT, and 0.02% Brij35), ATP-MgCl2 (100 μM ATP + 10 mM MgCl2 for p70S6K1/panPKB, and 200 μM ATP + 50 μM MgCl2 for AMPK), 32γ-ATP [specific activities as follows; panAMPK (0.25 × 106 cpn/mmol), panPKB (0.5 × 106 cpn/mmol), p70S6K1 (1 × 106 cpn/mmol)], and finally synthetic peptide substrates [Crotidine for panPKB (GRPRSTTSSFAEG at 30 μM), S6tide for p70S6K1 (KRRKLASLRT at 30 μM), and AMARA for AMPK (AMARRAASAALARRR at 200 μM)]. Assays were stopped at 20-s intervals by spotting onto squares of p81 chromatography paper (Whatman; GE Healthcare, UK) and immersing in 75 mM phosphoric acid. Papers (p81) were washed three times for 5 min in 75 mM phosphoric acid and once in acetone. They were then dried and immersed in Gold Star Quanta scintillation fluid (Meridian Biotechnologies, Chesterfield, UK) and counted in a Packard 2200CA TriCarb scintillation counter (United Technologies). Assay results were quantified in nmol·min−1·mg−1 (U/mg). Blanks for background subtractions were carried out with immunoprecipitated kinases with no peptide included in the assay reaction. For the AMPK antibody validation assays the AMPK α1 antibody (5 μg) was used to immunoprecipitate AMPK α1 complexes from 100 μg of lysate in duplicate, whereas AMPK α2 antibody (5 μg) was used to immunoprecipitate AMPK α2 complexes from 100 μg of lysate. These lysates were from HEK cells overexpressing either AMPK α1 or AMPK α2, and were a kind gift from Prof. Grahame Hardie (University of Dundee). Assays were carried out for 15 min. For p70S6K1 antibody validation assay, 2 μg of p70S6K1 antibody was used to immunoprecipitate p70S6K1 from 250 μg of muscle lysate from WT starved/refed and p70S6K1−/− refed mice. Activities assays for panPKB and p70S6K1 were carried out on cell lysates by IP from 200 μg of cell lysate. The IP step was performed with 2 μg each of PKBα/β antibodies (DSTT, Dundee University) or 2 μg of p70S6K1 antibody (H-9; Santa Cruz Biotechnology, Heidelberg, Germany), respectively. Antibodies were used with 2.5 μl of protein G sepharose per IP to immunoprecipitate for 2 h at 4°C. p70S6K1 and panPKB were assayed for 45 min and 20 min, respectively.

**Time-dependent saturation assays.** Three human skeletal muscle biopsy samples were pooled and homogenized. Homogenate was aliquoted to 2.4 mg for panPKB assays, 6 mg for p70S6K1 assays, and 0.6 mg for AMPK assays. Antibodies of PKBα/β/γ (72 μg each) were used to immunoprecipitate panPKB, 48 μg of p70S6K1 antibody was used to immunoprecipitate p70S6K1, and 60 μg each of AMPK α1 and α2 were used to immunoprecipitate panAMPK. Following IP, each of these immune complexes were aliquoted into 12 aliquots for activity assays; 9 of the aliquots were used for activity assays for the time course of 7.5, 15, and 30 min for AMPK; 15, 30, and 60 min for panPKB and p70S6K1. The three remaining aliquots were used for no-peptide controls to generate assay-specific blanks. Each assay represented an IP from 50 μg of lysate for panAMPK, 200 μg of lysate for panPKB, and 500 μg of lysate for p70S6K1.

For the serial IP validation, lower limb muscles from a 4-h fasted (Con) and an insulin-stimulated mouse [Ins (4-hr fasted + 100 mU insulin/kg for 30 min)] were homogenized and aliquoted into 6 × 200-μg aliquots each. IPs were set up to immunoprecipitate panPKB (3.2 μg of each PKB antibody) from three Con and three Ins aliquots, whereas the other aliquots had p70S6K1 immunoprecipitated (4 μg of p70S6K1 antibody) prior to immunoprecipitating with panPKB as before. Activity assays for panPKB were carried out as before following IP.

For p70S6K1/panPKB KA in human tissue, 500 μg of lysate was aliquoted, and p70S6K1 was immunoprecipitated with 4 μg of p70S6K1 and 2.5 μl of protein G sepharose (GE Healthcare) for 2 h at 4°C. The p70S6K1 KA was carried out for 45 min. Two hundred micrometers of the post-IP supernatant was then used for PKB IP. Two micrograms each of PKBα/β/γ antibodies (DSTT, Dundee University) were used with 2.5 μl of protein G sepharose to immunoprecipitate PKB at 4°C for 2 h. KA for panPKB were carried out as previously described for a 30-min assay. Following homogenization, 50 μg of lysate was aliquoted for AMPK activity assays. AMPK activity assays were carried out by IP with complexes in AMPK IP buffer (homogenization buffer as above). Immunoprecipitates were then washed, and AMPK activity was determined against AMARA peptide as previously described in a 20-min assay.

Statistical Analysis

Data were analyzed using GraphPad Prism Software version 6.0 (GraphPad, San Diego, CA). Differences in kinase signaling activity and phosphorylation (i.e., p70S6K1thr389, PKBthr308, AMPK activity) were analyzed using a one-way ANOVA and, when appropriate, a Tukey’s post hoc analysis. Post hoc sample size calculations were conducted using GPower 3.0.8 software on the basis of an estimated effect size of 0.53, a 1-β error probability of 0.8, and a significance level < 0.05. All data unless otherwise stated are presented as means ± SE, and P < 0.05 indicates statistical significance.

RESULTS

Antibody/Assay Validation

panAMPK. Total (or pan) AMPK activity is measured by immunoprecipitating both catalytic subunits of AMPK (AMPK α1 and AMPK α2). We commissioned our own AMPK α1 and AMPK α2 antibodies (GL Biochem, China) against the following antigens: α1, CTSPPD5FLDDHHLTR; and α2, CM-DDSAMHHPGLKPH (38). To confirm that our AMPK antibodies were specific for AMPK α1 and AMPK α2 and therefore capable of immunoprecipitating total AMPK when the antibodies are combined, we carried out a validation experiment (Fig. 1A). Cell lysates overexpressing either AMPK α1 or AMPK α2 underwent an IP with either the AMPK α1 or AMPK α2 antibody. AMPK α1 immunoprecipitated substantial activity from the AMPK α1 overexpressing cell lysates—approximately 10-fold more activity than the AMPK α2 antibody immunoprecipitated. The reverse experiment demonstrated a similar result, in that AMPK α2 immunoprecipitated approximately 10-fold more activity from the AMPK α2 overexpressing cell lysates than did the AMPK α1 antibody. These data demonstrate the specificity of our AMPK α1 and α2 antibodies. To further prove that these antibodies are immunoprecipitating active endogenous AMPK complexes, we carried out a positive control experiment by treating C2C12 myotubes with 100 μM 2,4-dinitrophenol [a known AMPK activator (39)] for 30 min, and followed this with panAMPK activity assays. This treatment resulted in an approximately fourfold increase in panAMPK activity (Fig. 1B), concurrent with a substantial increase in phosphorylation of AMPK at Thr172 (Fig. 1B, inset).

panPKB. Total (or pan) PKB activity can be assessed by utilizing recombinant glycogen synthase-3 (GSK3) as a substrate and then running a standard WB with a phosphorylated GSK3 antibody to determine phosphate incorporation (3). However, this approach again relies upon densitometry analysis and makes comparisons across large sample sets difficult. Therefore, we utilized a filter binding assay that also allowed for quantitative scintillation counting. We used antibodies and a peptide substrate (6) that have been previously well characterized (6, 22). However, to confirm that we were detecting panPKB activity with the immune complex we carried out a positive control experiment (Fig. 1C). We serum-stimulated C2C12 myotubes that had been treated with or without the PI3K inhibitor LY294002 (35). Serum stimulation led to an approximate fivefold increase in panPKB activity, whereas the inhibition of PI3K with LY294002 significantly inhibited panPKB activity. The changes in activity were reflected by changes in phosphorylation (Fig. 1C, inset).

p70S6K1. Traditionally, p70S6K1 activity assays are carried out with recombinant S6 as a substrate (21) wherein the radioactively labeled substrate is run on a gel before being exposed to radiography film. This assay is more difficult to accurately quantitate with large sample numbers due to the necessity to expose all samples to SDS-PAGE. Furthermore, this method still requires the use of densitometry analysis that can be subjective, leading to variable outputs depending upon the method of quantification (14). However, several laboratories have utilized a scintillation assay to quantitatively assess p70S6K1 activity (7, 33). To utilize a quantitative p70S6K1 activity assay that can be applied more easily to large sample numbers we employed a similar assay protocol with a peptide substrate analog of S6 corresponding to amino acids 230–238 on human 40S ribosomal protein S6 (KRRRLASLR) (12). This approach allowed for the use of filter paper capture of the labeled peptide that can then be quantitatively analyzed via scintillation counting. To confirm that this method did not alter the output of the assay we carried out a validation experiment in C2C12 myoblasts (Fig. 1D). We used serum and amino acid stimulation as a positive control with rapamycin (specifically inhibits mTORC1 activity) as a control to confirm that serum and amino acid-induced activation of kinase activity was in fact p70S6K1-specific. We showed that serum and amino acid stimulation induces an approximately 10-fold increase in activity, whereas rapamycin completely blocks this activation (Fig. 1D) and the phosphorylation of p70S6K1thr389 (Fig. 1D, inset). These data demonstrate the mTORC1 dependence of the kinase activity we measured. To further validate that no other contaminating kinases could be contributing activity in our assay we also ran the assay from starved/refed WT mice and refed p70S6K1−/− mice. We found approximately 19-fold more activity in refed mouse muscle vs. starved mouse muscle, and we could not detect any activity in the p70S6K1−/− mice. These data highlight the specificity of our assay to p70S6K1. Prior to moving the assay into human tissue we first needed to define the amount of antibody required to saturate p70S6K1 in human skeletal muscle. This would ensure that all the p70S6K1 in the lysate was immunoprecipitated, thus improving consistency across sample sets. We used increasing amounts of...
antibody to immunoprecipitate p70S6K1 from 500 μg of protein lysate extracted from pooled human muscle biopsy material from at least three volunteers. We found that despite increasing amounts of IgG-heavy chain (from the p70S6K1 antibody), the amount of p70S6K1 that was immunoprecipitated from 500 μg of protein lysate was saturated by 2 μg antibody. We therefore used 4 μg of p70S6K1 antibody for every 500 μg of protein lysate to ensure that our antibody was always in excess.

**Time-Dependent Saturation Curves**

To select the most appropriate duration for each assay in human biopsy samples we carried out a time-dependent saturation curve for each assay from a pool of human muscle biopsies (Fig. 2). We carried out the AMPK assays for 7.5, 15, and 30 min, whereas PKB and p70S6K1 assays were carried out for 15, 30, and 60 min. These assays revealed linearity across the time course for each assay, indicating that assays carried out for anywhere between 7.5 and 30 min for panAMPK, and 15–60 min for panPKB and p70S6K1, would be within the linear range for time.

**Validation of the Serial IP**

To economize on tissue with human muscle samples, panPKB and p70S6K1 activity assays were carried out via serial IP with p70S6K1 immunoprecipitated first. To confirm that this serial IP process did not affect PKB activity we performed a validation of this procedure in response to maximal insulin.
stimulation (Fig. 3). Serially immunoprecipitating panPKB after p70S6K1 had no significant effect upon panPKB activity compared with a standard IP (Fig. 3).

**Application of the [$\gamma$,S-32P] ATP KA in a Physiological Context in Human Skeletal Muscle**

We next determined whether we could measure the activity of panAMPK, panPKB, and p70S6K1 from the same human skeletal muscle sample following a well-defined anabolic stimulus in humans (23). In our study we identified a significant increase in p70S6K1 activity from pre-RE at 1 and 3 h post-RE ($P < 0.05$; Fig. 4C). However, there was no significant change in panPKB activity at any time point (Fig. 4B). Finally, panAMPK activity was significantly repressed ($P < 0.05$; Fig. 4A) at 3 h post-RE compared with pre-RE. To confirm that we were able to detect physiologically relevant changes in panPKB activity, we assessed the activation of panPKB in response to a physiologically relevant ($0.36 \text{ nM}$) insulin stimulus in ex vivo mouse skeletal muscle (Fig. 4B, inset). Indeed, we detected a significant increase in panPKB activity in response to 50 min of insulin stimulation, thus confirming that this assay is capable of detecting changes in panPKB activity in a physiological context.

**Western Blotting**

Following the assessment of kinase activity as markers of anabolic responses in humans we next measured the phosphorylation of proteins that are typically used as surrogate readouts of anabolic signaling activity. The responses of kinases as determined by WB are shown in Fig. 5 (AMPK readouts), Fig. 6 (PKB readouts), and Fig. 7 (mTORC1 readouts). In response to RE and nutrition, there were no significant changes in phosphorylated mTOR$^{\text{ser}2448}$ (Fig. 7A), ACC$^{\text{ser}79}$ (Fig. 5A), Raptor$^{\text{ser}792}$ (Fig. 5B), p70S6K1$^{\text{thr}389}$ (Fig. 7B), PKB$^{\text{thr}308}$ (Fig. 6A), and PRAS40$^{\text{thr}246}$ (Fig. 6B). However, phosphorylated 4EBP1$^{\text{thr}37/46}$ was significantly elevated at 3 h post-RE compared with pre-RE ($P < 0.05$). All data are expressed as means ± SE.

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**Fig. 2.** Saturation time course of activity assays carried out from pooled human skeletal muscle protein lysate. $R^2$ values are as follows: AMPK = 0.969; panPKB = 0.982; and p70S6K1 = 0.856. All data are expressed as means ± SD.

**Fig. 3.** Serial immunoprecipitation (IP) validation. IPs were set up to immunoprecipitate panPKB alone or p70S6K1 immunoprecipitated prior to immunoprecipitating with panPKB. *Significantly different from both control (Con) conditions. All data are expressed as means ± SE.

**Fig. 4.** Application of three kinase assays in human skeletal muscle in response to a physiological anabolic stimulus of resistance exercise combined with feeding 20 g of protein ($n = 6$). A: panAMPK activity was determined from 50 μg of lysate in a 20-min reaction against the synthetic substrate AMARA. B: panPKB activity serially immunoprecipitated after p70S6K1 IP. Inset: panPKB activity response to a physiological insulin stimulation of 0.36 nM for 50 min in ex vivo mouse skeletal muscle ($n = 6$). panPKB activity was determined from 200 μg of lysate in a 30-min reaction against the synthetic peptide substrate Crosstide. C: p70S6K1 activity was determined from 500 μg of lysate in a 45-min reaction against the synthetic peptide substrate S6K1tide. Pre-RE indicates biopsy taken prior to resistance exercise and feeding, 1 h post-RE indicates the biopsy taken 1 h following combined resistance exercise and feeding, 3 h post-RE indicates biopsy taken 3 h following combined resistance exercise and feeding. *Significantly different from Con or Pre-RE ($P < 0.05$). All data are expressed as means ± SE.
pared with pre-RE (P < 0.05; Fig. 7C). Representative WB images appear as insets above each graph.

DISCUSSION

The main aim of the present methodological study was to develop and validate a quantitative p70S6K1 KA for use in human skeletal muscle biopsy samples. Second, we aimed to examine the physiological context of alterations in p70S6K1 activity by examining parallel alterations in PKB and AMPK activity in response to acute RE and protein feeding (23). For the first time we demonstrated that combined RE and protein feeding significantly increases p70S6K1 activity by approximately twofold, as determined by the KA with a similar, approximate twofold but nonsignificant change in p70S6K1thr389 phosphorylation. In addition, we observed a suppression of AMPK activity that was not apparent when assessing ACCser79 phosphorylation, a known readout of AMPK activity (36). Furthermore, we demonstrate the capacity to achieve a dual measure of panPKB and p70S6K1 activity from the same sample via a serial IP protocol. This study therefore highlights the potential application of the KA described in this investigation to study the molecular signaling responses of skeletal muscle to RE and nutrition.

Although we observed a significant increase in p70S6K1 activity to RE and protein feeding, we detected no significant changes in the phosphorylation of p70S6K1thr389. This finding is unexpected, given previous reports of significant, approximate twofold (5) and 12-fold (2) increases in phosphorylated p706K61thr389 to an acute bout of RE and protein feeding. Although the lack of detectable change in phosphorylated p70S6K1thr389 in our investigation appears to be related to low statistical power. Indeed, a post hoc sample size calculation from the present study determined that a participant sample of 12 would have been necessary to detect a statistically significant difference in phosphorylated p70S6K1thr389 between pre-RE and 1 h post-RE and protein ingestion. However, by utilizing the KA, we were able to detect a modest increase in p70S6K1 activity from pre-RE at 1 and 3 h post-RE and feeding. Thus these data highlight not only the precision but also the utility of this p70S6K1 KA to assess p70S6K1 activity to anabolic stimulation.

Due to issues associated with ethical practice and participant compliance in human research, muscle tissue availability is often a limiting factor. In this investigation we provided a validated, serial IP protocol for the dual assessment of p70S6K1 and panPKB activity from a single muscle homogenate. We showed that this serial IP protocol has no effect on panPKB activity, hence economizing on muscle tissue requirements. When applying this protocol to study panPKB responses of human skeletal muscle to RE and feeding, we...
showed no change in panPKB activity at any time point, a finding that corroborates previous reports (25, 28, 32). However, it is important to note that the panPKB KA described in this methodological investigation failed to provide information regarding PKB isoform-specific effects that could be useful in understanding cell growth and metabolism (30). The development of such a methodology is therefore a topic for future work.

The increase in p70S6K1 activity in our investigation was associated with a decrease in AMPK activity. These data are similar to findings showing that RE (1) or feeding (13) also repress AMPK\textsuperscript{thr172} phosphorylation, but these findings are incongruent with previous work that demonstrated RE increases AMPK\textsubscript{\alpha2} activity 1 h post-RE (10). However, in that study, RE was not followed by feeding, and one possibility is that the protein feeding in our study may have overridden RE-induced increases in AMPK activity, perhaps via restoration of the AMP:ATP ratio (15). Alternatively, it is known that p70S6K1 can inhibit AMPK via phosphorylation at Ser491 in mouse hypothalamic cells (8), although this latter hypothesis has yet to be observed in human skeletal muscle. A reduction in AMPK activity also is known to relieve inhibition on mTOR-p70S6K1 signaling (17), which could partially explain the sustained increase in p70S6K1 activation at 3 h post-RE and feeding in our investigation. Interestingly, the significant reduction in AMPK activity in our study was not mirrored by a reduction in ACC\textsuperscript{ser79} phosphorylation ($P < 0.05$). We chose to assess the phosphorylation of ACC\textsuperscript{ser79} as a readout of AMPK activity because phosphorylated AMPK\textsuperscript{thr172} possesses a low dynamic range that renders phosphorylated AMPK\textsuperscript{thr172} on this residue a poor surrogate of true AMPK activity (18). Therefore, the decrease in AMPK activity paralleled with a nonsignificant change in ACC\textsuperscript{ser79} phosphorylation further emphasizes the potential application of the KA to assess RE and nutrition-induced changes in signaling.

Both RE and protein ingestion are known to increase MPS via mTOR-p70S6K1 signaling (9, 11, 20). However, in response to RE and protein ingestion, we detected no significant change in the phosphorylation status of Raptor\textsuperscript{ser792}, PRAS40\textsuperscript{thr246}, or mTOR\textsuperscript{ser2448}. This finding was surprising because there was a significant increase in the phosphorylation of the mTOR substrate 4EBP1\textsuperscript{thr37/46} at 3 h post-RE and protein feeding. Others have also shown no change in mTOR\textsuperscript{ser2448} phosphorylation to a 48-g whey bolus at both 1 and 3 h postfeeding despite increases in phosphorylated p70S6K1\textsuperscript{thr389} and 4EBP1\textsuperscript{thr37/46} (3). Furthermore, it is also known that mutation of the ser2448 residue on mTOR fails to significantly affect p70S6K1 activity in cell-based systems (31). It therefore appears that mTOR\textsuperscript{ser2448} phosphorylation does not offer the most accurate readout of mTORC1 activity. Hence, studies that aim to infer changes in mTORC1 activity to anabolic stimulation using the WB technique may be better served by assessing changes in the phosphorylation of the mTOR substrates 4EBP1 and p70S6K1 rather than mTOR\textsuperscript{ser2448} phosphorylation itself.

In summary, this study provides a novel, fully quantitative methodology to assess p70S6K1-specific activity in human skeletal muscle. In addition, we provide a validated serial IP protocol that enables the dual assessment of PKB and p70S6K1 activity from a single skeletal muscle biopsy sample. Given that the number and yield of human muscle biopsies present...
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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