Mechanical stimuli regulate rapamycin-sensitive signalling by a phosphoinositide 3-kinase-, protein kinase B- and growth factor-independent mechanism

Troy A. HORNBERGER*, Rudy STUPPARD†, Kevin E. CONLEY†, Mark J. FEDELE*, Marta L. FIOROTTO‡, Eva R. CHIN§ and Karyn A. ESSER*¹

*School of Kinesiology, University of Illinois at Chicago, 901 W. Roosevelt, Chicago, IL 60608, U.S.A., †Department of Radiology, University of Washington, Seattle, WA 98195, U.S.A., ‡Department of Paediatrics, Baylor College of Medicine, USDA/ARS Children's Nutrition Research Center, Houston, TX 77030, U.S.A., and §Pfizer Global Research and Development, Groton, CT 06340, U.S.A.

In response to growth factors, mTOR (mammalian target of rapamycin) has been identified as a central component of the signalling pathways that control the translational machinery and cell growth. Signalling through mTOR has also been shown to be necessary for the mechanical load-induced growth of cardiac and skeletal muscles. Although the mechanisms involved for mechanically induced activation of mTOR are not known, it has been suggested that activation of PI3K (phosphoinositide 3-kinase) and protein kinase B (Akt), via the release of locally acting growth factors, underlies this process. In the present study, we show that mechanically stimulating (passive stretch) the skeletal muscle *ex vivo* results in the activation of mTOR-dependent signalling events. The activation of mTOR-dependent signalling events was necessary for an increase in translational efficiency, demonstrating the physiological significance of this pathway. Using pharmacological inhibitors, we show that activation of

mTOR-dependent signalling occurs through a PI3K-independent pathway. Consistent with these results, mechanically induced signalling through mTOR was not disrupted in muscles from Akt1^{−/−} mice. In addition, *ex vivo* co-incubation experiments, along with *in vitro* conditioned-media experiments, demonstrate that a mechanically induced release of locally acting autocrine/paracrine growth factors was not sufficient for the activation of the mTOR pathway. Taken together, our results demonstrate that mechanical stimuli can activate the mTOR pathway independent of PI3K/Akt1 and locally acting growth factors. Thus mechanical stimuli and growth factors provide distinct inputs through which mTOR co-ordinates an increase in the translational efficiency.

Key words: hypertrophy, mammalian target of rapamycin (mTOR), mechanotransduction, protein synthesis, rapamycin, ribosomal S6 kinase (p70^{S6k}).

INTRODUCTION

Increased rates of protein synthesis and decreased protein degradation are hallmarks of skeletal-muscle growth, and mechanical stimuli play a major role in their regulation. For example, increasing the tension on skeletal muscles can activate protein synthesis and this results in enhanced translational efficiency (rate per ribosome) as opposed to translational capacity (number of ribosomes), with translation initiation being the principal stage of regulation [1–5].

Translation initiation is regulated by a co-ordinated series of biochemical events and most of these events involve inputs from signalling molecules that alter the phosphorylation state of the initiation factors [6,7]. For example, initiation can be regulated by altering the phosphorylation state of eIF2B (eukaryotic initiation factor 2B) and GSK-3 β (glycogen synthase kinase 3 β) and has been commonly linked to this reaction [8]. Alternatively, formation of the eIF4F complex regulates cap-dependent translation and signals from p38 (stress-activated protein kinase), protein kinase B (Akt) and mTOR (mammalian target of rapamycin) have all been implicated in this process [7,9,10]. The translation initiation of mRNAs that contain a 5'-TOP (5'-tract of polypyrimidines) are also mTOR-dependent and p70^{S6k} (ribo-

somal S6 kinase) has been linked to the translational regulation of these mRNAs [11,12]. The co-ordinated regulation of initiation factors by multiple signalling pathways allows for the integration of several inputs to protein synthesis, including signals from growth factors and nutrient availability [13].

Most of the signalling pathways involved in the regulation of translation initiation have been shown to lie downstream of PI3K (phosphoinositide 3-kinase) [13,14]. In skeletal muscles, inhibition of PI3K with wortmannin or LY294002 completely blocks both growth factor-induced signalling through GSK-3 β and p70^{S6k} and increases in protein synthesis [15–19]. Thus PI3K appears to be a central integrator for the signalling pathways that regulate protein synthesis in response to signals from growth factors. In co-ordination with PI3K-dependent inputs, mTOR has also been shown to integrate growth factor-based signals. For example, growth factor-induced p70^{S6k} activity and protein synthesis are PI3K- and mTOR-dependent, and mTOR is considered to lie downstream of PI3K in this pathway (PI3K $\rightarrow$ mTOR) [18–22].

In addition to integrating stimuli from growth factors and nutrients, mTOR-dependent signalling events have also been shown to be necessary for the mechanically induced growth of skeletal muscles. In fact, it has been suggested that mechanical stimuli regulate mTOR-dependent signalling via an Akt-dependent

Abbreviations used: CNT, control; EDL muscle, extensor digitorum longus muscle; eEF2, eukaryotic elongation factor 2; eIF, eukaryotic initiation factor; GSK-3 β , glycogen synthase kinase-3 β ; IGF, insulin-like growth factor; mTOR, mammalian target of rapamycin; p38, stress-activated protein kinase; p70^{S6k}, ribosomal S6 kinase; PA, phosphatidic acid; PCr, phosphocreatine; PI3K, phosphoinositide 3-kinase; STR, stretch; TCr, total creatine; TOP, tract of polypyrimidines.

¹ To whom correspondence should be addressed (e-mail mlc25@uic.edu).

mechanism [23]. Since PI3K activity is considered to be indispensable for Akt activity [24], it has been proposed that mechanical stimuli are similar to growth factors in that they regulate mTOR-dependent signalling events and protein synthesis through a PI3K $\rightarrow$ Akt $\rightarrow$ mTOR pathway; however, evidence for this pathway is lacking. Furthermore, mechanical stimuli have been suggested to induce the release of locally acting factors such as IGF-1 (insulin-like growth factor), which in turn can activate the PI3K $\rightarrow$ Akt $\rightarrow$ mTOR cascade and protein synthesis. However, evidence for this mechanism is also lacking [25]. Therefore the primary focus of the present study was to determine whether signalling through PI3K and mTOR is necessary for a mechanically induced increase in protein synthesis and to assess whether mechanical stimuli activate mTOR via a growth factor-induced PI3K $\rightarrow$ Akt $\rightarrow$ mTOR pathway.

MATERIALS AND METHODS

Materials

Peroxidase-conjugated anti-rabbit antibody was purchased from Vector Laboratories (Burlingame, CA, U.S.A.). Rabbit polyclonal anti-phospho-Thr⁵⁶-eEF2 antibody (eEF, eukaryotic elongation factor) was generously provided by Dr Angus C. Narin (Rockefeller University). All other antibodies were purchased from Cellular Signaling (Lake Placid, NY, U.S.A.). PVDF membranes were purchased from Millipore (Bedford, MA, U.S.A.), enhanced chemiluminescence (ECL[®]) detection reagent from Amersham Biosciences (Buckinghamshire, U.K.), DC protein assay kit from Bio-Rad Laboratories (Hercules, CA, U.S.A.), Dulbecco's modified Eagle's medium from Invitrogen (Carlsbad, CA, U.S.A.), Ly294002 from Calbiochem (San Diego, CA, U.S.A.) and wortmannin and rapamycin from Sigma (St. Louis, MO, U.S.A.). Force measurements and stretch movements were produced on a 300-BLR force transducer/dynamometer (Aurora Scientific, Aurora, ON, Canada). Data from the force transducer was integrated with a PCI-MIO A/D board (National Instruments, Austin, TX, U.S.A.) and interfaced with Labview software for data analysis (National Instruments). Muscle field stimulations were delivered through a Grass stimulator (Grass Telefactor, West Warwick, RI, U.S.A.).

Animal care and use

All the experimental procedures were approved by the University of Illinois at Chicago Animal Care and Use Committee. Male C57BL/10 mice (Jackson Laboratories, Bar Harbor, MA, U.S.A.; Harlan, Indianapolis, IN, U.S.A.), 2–4 months old, were randomly assigned to experimental groups. All animals were allowed free access to food and water. Animals were anaesthetized with sodium pentobarbital (40 mg/kg) and EDL muscle (extensor digitorum longus muscle) of the hind limb was exposed. Sutures (4-0 silk) were tied at the proximal and distal myotendinous junctions of the EDL. A lever arm for attachment to a force transducer/dynamometer was attached to the suture at the distal end of the EDL and a lever arm for attachment to a micromanipulator was attached to the suture at the proximal end of the EDL. The EDL was excised and immediately placed in an organ culture bath.

Akt1 transgenic animals

The Akt1 null mutation was generated through homologous recombination by deleting exons 8–13 of the *akt1* gene [26]. The genetic background of the mice used for these studies were

>99% C57BL/6. At the time of weaning (28 days), the mice were identified by an ear hole punch and a 1 cm snip of the tail was taken for the isolation of DNA for genotyping. Tails were dissolved overnight at 55 °C in 0.5 ml of a buffer containing 100 mM NaCl, 50 mM Tris (pH 8.0), 100 mM EDTA (pH 8.0), 1% SDS and 1 mg/ml proteinase K. After incubation, 0.2 ml of 5 M NaCl was added to the tubes, which were shaken and then centrifuged at 8000 *g* for 10 min. The supernatant was transferred to a new tube, and the DNA was isolated by propan-2-ol precipitation, followed by ethanol (70%, v/v) washing of the DNA pellet. The DNA pellet was air-dried and then resuspended in 0.4 ml of 10 mM Tris (pH 7.4). The presence or deletion of the *akt1* gene was determined by PCR. The sequences for the primers to detect *akt1* were the following: (i) forward 5'-ACCAGGGG-AGGATGTTTCTACTG and (ii) reverse 5'-ACGACTATGGTG-CAGCAATGC, which yield a 1200 bp product. The following were the sequences for the primers used to detect the *akt1* deletion: forward, 5'-CTACTATGCCATGAAGATCCTC; reverse, 5'-TGC-TACTTCCATTTGTCACGTCC, which yield a 700 bp fragment. The genotype was then determined by resolving the PCR products on a 1.2% (w/v) agarose/TBE (45 mM Tris/borate/1 mM EDTA) gel containing 3 μ g/ml ethidium bromide and visualized under UV light.

Organ culture (*ex vivo* mechanical stimulator)

Design of the *ex vivo* organ culture system was based on the method of Reeds et al. [27] and consisted of a refined myograph system (Kent Scientific, Torrington, CT, U.S.A.) with the DMEM (Dulbecco's modified Eagle's medium; high-glucose) cell culture medium maintained at 37 °C under continuous 95% O₂ and 5% CO₂ gassing (Figure 1A). With the lever arms attached to the force transducer and micromanipulator, the EDL was adjusted to optimal length *L*_o. The optimal length was determined in pilot experiments by stimulating the muscle with a 0.5 ms, 10 V pulse and the twitch tension was measured. The length of the muscle was adjusted until a peak in twitch tension was observed, and the passive tension at this length was recorded (8.25 $\pm$ 1.25 mN, *n* = 4). In all subsequent experiments, muscle length was adjusted until a passive tension of 8.25 mN was obtained, and this length was assumed to be approximately *L*_o. Fresh medium was added to the bath at 30 min intervals.

Passive stretch paradigm

After a 30 min preincubation at *L*_o, muscles were subjected to stretch or static conditions. The stretch method consisted of a 15% stretch using a 50 ms ramp, with a 100 ms holding pattern. Muscles were stretched once every 3 s for up to 90 min (Figures 1B and 1C). The average peak passive tension produced by a 15% stretch was 203 $\pm$ 23 mN (*n* = 13), and static muscles were held at *L*_o.

Co-incubations

In some experiments, static muscles were co-incubated with stretched muscles. In these experiments, the right EDL muscle was connected to the lever arms of the mechanical stimulator as described above, and adjusted until a tension of 8.25 mN was obtained (*L*_o). The EDL muscle from the left leg was connected to lever arms of a motionless apparatus, which allowed for the muscle to be fixed at a chosen length. The length of the left EDL was adjusted to match that of the right EDL on the mechanical stimulator. The left EDL was then co-incubated with the right EDL by setting the muscles parallel to one another in the culture

Figure 1 Mechanical stimulation promotes an increase in the rate of protein synthesis via enhanced translational efficiency

(A–C) Schematic representation of the *ex vivo* mechanical stimulator used to apply intermittent stretch on skeletal muscles. (A) Muscles were attached to lever arms that extend from a micromanipulator and force transducer/dynamometer and then placed in a culture medium bath that was gassed with 95% O₂/5% CO₂. The force transducer was integrated to a computer that controlled muscle length (stretch) and provided a continuous display of muscle tension. (B) The muscle was stretched to 115% of the optimal length L₀ using a 50 ms ramp and 100 ms holding pattern. (C) The stretch cycle was repeated once every 3 s for up to 90 min. (D) After a 30 min preincubation at optimal length L₀, rates of protein synthesis were measured at 30 min intervals in muscles held static at L₀ [control (CNT); spotted bars] or subjected to stretch (STR; solid bars). (E) In the presence of 2.5 mM phenylalanine (20 µCi/ml [³H]phenylalanine), the specific activities of the muscle-free pool and the medium were within 5% of equilibrium, an effect that was not altered by 90 min of STR. (F) The concentration of total RNA was not altered by 90 min of STR. Results are means ± S.E.M., n = 3–6/group. *Significantly different from CNT (P ≤ 0.05).

bath (≤ 1.5 mm of space between the two muscles). The right EDL was then subjected to stretch or static conditions on the mechanical stimulator, whereas the left EDL was maintained in a static state.

Conditioned-media experiments in cell culture

C2C12 skeletal myotube cultures were grown by plating C2C12 myoblasts on type I collagen-coated Bioflex membranes (Flexcell, Hillsborough NC, U.S.A.) or 6-well plastic dishes (Corning, Corning, NY, U.S.A.). Myoblasts were maintained in DMEM, supplemented with antibiotics [100 µg/ml streptomycin and 100 units/ml penicillin (Sigma)] and 10% (v/v) foetal bovine serum. After confluence, cells were switched to DMEM, supplemented with antibiotics and 10% (w/v) horse serum to promote differentiation. Cells were maintained in this medium for 24 h and then switched back to the 10% foetal bovine serum medium for an additional 5–6 days, resulting in the formation of distinct multinucleated myotubes.

C2C12 myotubes grown on Bioflex membranes were switched to serum-free DMEM (serum-starved) for 90 min, followed by the addition of a fresh serum-free medium immediately before initiating 10 min of 15% intermittent (1 Hz) multi-axial stretch using a triangular-waveform [Flexercell (FX-3000) device; Flexcell] or static conditions. The conditioned medium from static or stretched myotubes was immediately removed and placed (for 10 min) on a new set of serum-starved myotubes that had been grown on plastic dishes. Myotubes were collected and subjected to Western-blot analysis as described below.

High-energy phosphates

Muscles were removed from the organ culture and immediately frozen with liquid-nitrogen-cooled tongs. Frozen muscles were pulverized in a mortar cooled with liquid nitrogen and then extracted in unlabelled perchlorate as described previously [28]. The perchlorate extracts were then assayed to determine the concentrations of ATP, phosphocreatine (PCr) and total creatine (TCr)

by HPLC as described previously [29]. Metabolite concentrations were expressed per volume of cell water by assuming 0.7 ml of intracellular water per g of muscle mass, as found for skeletal muscles [30].

Protein synthesis

Protein synthesis rates [nmol of phenylalanine incorporated $\cdot$ (mg of protein) $^{-1} \cdot$ h $^{-1}$] were measured by switching the culture medium to DMEM, supplemented with 2.5 mM phenylalanine and 20 μ Ci/ml [3 H]phenylalanine (Amersham Biosciences). The resulting concentration of phenylalanine represents a flooding dose that ensures rapid equilibration with the cellular-free amino acid precursor pool, and undergoes insignificant change over the duration of the labelling period [31]. Muscles were incubated for 30 min and then washed four times with ice-cold PBS (pH 7.5), followed by homogenization in 10% (w/v) trichloroacetic acid. A portion of the culture medium was saved for determining the specific activity of the medium (c.p.m./nmol of phenylalanine). Trichloroacetic acid homogenates were incubated on ice for 30 min, followed by centrifugation at 4500 *g* for 5 min. Trichloroacetic acid-soluble material was removed and the trichloroacetic acid-insoluble material was washed by resuspension of the pellet in 10% trichloroacetic acid, followed by centrifugation at 4500 *g*. After four consecutive washes, the trichloroacetic acid-insoluble material was dissolved in 0.15 M NaOH at 55 °C with frequent vortex-mixing for 1 h. Aliquots of the sample were analysed to determine the specific activity (c.p.m./mg of protein) of trichloroacetic acid-insoluble material by scintillation counting and DC protein assay (Bio-Rad Laboratories). The rate of protein synthesis was calculated using the following equation:

$$\text{Rate} = [(A/B)/\text{mg of protein}] \times \text{incubation time}$$

where A represents the specific activity of the trichloroacetic acid-insoluble material and B the specific activity of the medium; incubation time is in h.

In a separate trial, equilibration of the phenylalanine tracer medium with the tissue-free phenylalanine pool was determined. Muscles were subjected to 90 min of static or stretch conditions, washed with ice-cold PBS as described above and then frozen in liquid nitrogen. Frozen muscles were homogenized for 60 s in ice-cold deionized water using a Polytron. The homogenate was immediately acidified to 0.2 M HCl₄ and centrifuged at 12000 *g* at 2 °C for 20 min. The supernatant containing the muscle-free phenylalanine was decanted and neutralized with 4.0 M KOH. An aliquot of the medium collected from each muscle bath was similarly processed. The neutralized supernatants were vacuum-desiccated, resuspended in 1 M acetic acid and the amino acids were purified by ion-exchange chromatography (Dowex AG-50W-W8). Phenylalanine in the neutralized homogenate and supernatants of the medium was isolated by anion-exchange HPLC as described previously [32]. Amino acids were post-column-derivatized with *o*-ophthalaldehyde reagent and detected fluorimetrically. The phenylalanine concentration of each peak was determined by comparison with a known standard. The eluted fraction containing the phenylalanine peak was collected and the associated radioactivity was determined by scintillation counting.

Total RNA

Total RNA was extracted by homogenizing muscles in TRIzol[®] reagent (Invitrogen). After performing a series of centrifugation and separation steps according to the manufacturer's instructions,

a pellet of RNA was obtained and suspended in diethyl pyrocarbonate-treated water. Total RNA was quantified by measuring the absorbance A_{260} and was expressed relative to muscle weight.

Western blots

At various time points (5, 15, 30, 60 and 90 min), EDL muscles were removed from the organ culture bath and frozen in liquid nitrogen. Frozen muscles were homogenized by a Polytron in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P40, 1 mM β -glycerolphosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin and 1 mM PMSF. The protein concentration was determined by the DC protein assay (Bio-Rad Laboratories). From the homogenate, samples containing 10–60 μ g of protein were dissolved in Laemmli buffer and subjected to SDS/PAGE (7.5% gel). After electrophoretic separation, proteins were transferred to a PVDF membrane, blocked in 5% (w/v) blotto [5% powdered milk in TBST (Tris-buffered saline/1% Tween 20)] for 3 h, followed by overnight incubation at 4 °C with a primary antibody. After overnight incubation, the membranes were washed for 30 min with TBST and then probed with an anti-rabbit antibody for 45 min at room temperature (21 °C). After 30 min of washing with TBST, the blots were developed using ECL[®]. Once the appropriate image was captured, membranes were stained with Coomassie Blue to verify equal loading in all lanes. Densitometric measurements were performed using the FluorSmax Imager with QuantityOne software (Bio-Rad Laboratories).

Pharmacological inhibitors and insulin stimulation

Throughout the incubation period, the vehicle (0.2% DMSO), 500 nM wortmannin in DMSO, 20 μ M Ly294002 in DMSO or 150 nM rapamycin in DMSO was present in the medium. The concentrations employed for each of these inhibitors were selected from pilot studies in which the minimal effective dose was determined. Protein synthesis measurements were performed during the final 30 min of the 2 h incubation as described above. For the evaluation of signalling responses, samples were collected at the end of the 2 h incubation (90 min $\pm$ stretch). Insulin-stimulated samples were generated by adding 100 nM insulin during the final 30 min of the 2 h incubation.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Statistical significance was determined using ANOVA, followed by Student's Newman–Kuels post hoc analysis. Differences between groups were considered significant if $P \leq 0.05$.

RESULTS

Muscle viability

To assess the viability of the EDL muscle in the organ culture system, several criteria, including stability in [ATP], [PCr]/[TCr] ratio as well as twitch tension and protein synthesis, were evaluated. The results indicate that after a 30 min preincubation, [ATP] and the [PCr]/[TCr] ratio remained stable for at least 90 min and was not altered by intermittent passive stretch (Table 1). In addition, no alterations in twitch tension or rates of protein synthesis were observed in muscles held static at L_0 (Table 1 and Figure 1D).

Table 1 Muscle viability

Measurements of [ATP], the ratio of PCr to TCr ([PCr]/[TCr]) and twitch tension in muscles held static at optimal length after a 30 min preincubation (Control 0 min) or an additional 90 min of static (Control 90 min) or stretch conditions (Stretch 90 min). Results represent the means $\pm$ S.E.M. ($n = 3$ /group); NA, not measured.

	ATP (mM)	[PCr]/[TCr]	Twitch tension (mN)
Control 0 min	6.93 $\pm$ 1.58	0.63 $\pm$ 0.12	51.5 $\pm$ 8.3
Control 90 min	6.82 $\pm$ 0.54	0.71 $\pm$ 0.04	50.4 $\pm$ 1.9
Stretch 90 min	6.67 $\pm$ 0.42	0.74 $\pm$ 0.07	NA

Mechanical stimulation promotes an increase in the rate of protein synthesis via enhanced translational efficiency

Subjecting the EDL muscle to intermittent passive stretch produced a progressive increase in the rate of protein synthesis (130% of control by 60–90 min, $P \leq 0.05$; Figure 1D). To confirm the validity of the protein synthesis rates estimated with the specific activity of the medium phenylalanine as a surrogate for the phenylalanine-tRNA precursor pool, the specific activity of the phenylalanine tissue-free pool was measured and compared with the corresponding values for the medium. The specific activity of the tissue-free pool has been shown to be a valid measure of the specific activity of the precursor pool when a flooding dose of phenylalanine is administered [33]. It was observed that the values of the specific activity for the medium and the tissue-free pool were within 5% of each other, an effect that was not altered by the stretch. Thus the rates of protein synthesis estimated from the specific activity of the medium are valid (Figure 1E).

To determine whether the increase in protein synthesis was due to enhanced translational efficiency (as calculated by synthesis rate/RNA) or enhanced translational capacity (as inferred by increases in total RNA), total RNA was measured. Since 80–90% of the total RNA is composed of rRNA, any alteration in translational capacity or ribosomal content will probably be reflected in the measurements of total RNA. The results demonstrated that after 90 min of intermittent stretch, the total RNA/mg of muscle weight was not significantly altered (Figure 1F). When the values obtained for μg of RNA/mg of muscle weight were used to calculate translational efficiency, the values for static and stretched muscles were 16.1 ± 1.0 and 26.6 ± 1.9 (protein synthesis rate/ng of RNA) respectively ($n = 12$ –15/group, $P \leq 0.05$). These results indicate that the mechanically induced increase in protein synthesis was due to enhanced translational efficiency.

Mechanically induced signalling events

Intermittent stretch produced signalling (phosphorylation) through several molecules that have been implicated in the regulation of translational efficiency [6,7]. The mechanically induced signalling events occurred in a time-dependent manner, which could be separated into four general categories, namely (1) eEF2, tuberlin Thr¹⁴⁶² and Akt Thr³⁰⁸ phosphorylation did not reveal any alterations, (2) phosphorylation of Akt on Ser⁴⁷³ revealed a delayed (15 min) and transient (returned to baseline at 60 min) increase, (3) p38 and p70^{S6k}-421/424 phosphorylation showed an immediate (within 5 min) and sustained increase and (4) p70^{S6k}-389 and GSK-3 β phosphorylation showed a delayed (for at least 30 min) and sustained increase (Figure 2). Of these responses, the alterations in p70^{S6k}-389 and GSK-3 β phosphorylation were of particular interest, since both were highly correlated with the concomitant changes in protein synthesis ($R^2 = 0.90$ and 0.99 respectively, $n = 5$ –6/group).

Figure 2 Time course of mechanically induced signalling events

After a 30 min preincubation at optimal length L_0 , muscles were subjected to 5, 15, 30, 60 or 90 min of static (Stretch-) or stretch (Stretch+) conditions. Western-blot analysis of (A) phosphorylated eEF2 (P-eEF2), (B) phosphorylated tuberlin on Thr¹⁴⁶² (P-Tuberlin 1462), (C) phosphorylated Akt on Thr³⁰⁸ (P-Akt 308), (D) phosphorylated Akt on Ser⁴⁷³ (P-Akt 473), (E) phosphorylated p38 (P-p38), (F) phosphorylated p70^{S6k} on threonine/serine 421/424 (P-p70 421/424), (G) phosphorylated p70^{S6k} on Thr³⁸⁹ (P-p70 389) and (H) phosphorylated GSK-3 β (P-GSK-3 β). Average values for each group are presented at the top of the lane and expressed as a ratio of the value obtained in control (Stretch-, 5 min) samples, $n = 5$ –6/group. * $P \leq 0.05$, significantly different from time-matched static samples.

Mechanically induced protein synthesis is PI3K- and mTOR-dependent

Increases in protein synthesis after growth factor stimulation have been shown to be PI3K-dependent [15,16]. To determine whether PI3K is also required for mechanically induced protein synthesis, muscles were incubated in the presence of two chemically distinct inhibitors of PI3K, namely Ly294002 (20 μM) and wortmannin (500 nM). Both inhibitors completely blocked the mechanically induced increase in protein synthesis, indicating that PI3K activity is necessary for an increase in protein synthesis (Figure 3).

Signalling through mTOR has also been shown to be involved in the regulation of protein synthesis by growth factors and nutrients [20,21]. To assess the role of mTOR in the mechanically induced increase in protein synthesis, muscles were incubated in the presence of 150 nM rapamycin. Rapamycin completely blocked the mechanically induced increase in protein synthesis, indicating that signalling through mTOR was also necessary for the increase in protein synthesis (Figure 3).

Figure 3 Mechanically induced protein synthesis is PI3K- and mTOR-dependent

Muscles were held at optimal length L_0 for a 30 min preincubation, followed by an additional 90 min of static [Control (CNT) spotted bars] or STR (solid bars) conditions in a medium containing a vehicle (0.2% DMSO), 150 nM rapamycin (RAP), 20 μ M Ly294002 (LY) or 500 nM wortmannin (Wort) throughout the entire incubation period. Protein synthesis rates were measured during the final 30 min. Results are expressed as means $\pm$ S.E.M., $n = 3-11$ /group. * $P \leq 0.05$, significantly different from vehicle control.

Mechanically induced signalling through mTOR occurs via a PI3K-independent pathway

Similar to stretch, incubating the EDL muscle with insulin promoted an increase in phosphorylation of GSK-3 β and p70^{S6k}-389. The insulin-induced increase in GSK-3 β phosphorylation was blocked by wortmannin but not rapamycin, whereas the insulin-induced increase in p70^{S6k}-389 phosphorylation was blocked by

both wortmannin and rapamycin. To confirm the specificity of the kinase inhibitors, insulin-induced Akt-473 phosphorylation was examined. As expected, the increase in Akt phosphorylation was blocked by wortmannin but not by rapamycin. No significant effect of insulin on p70^{S6k}-421/424 or p38 phosphorylation were observed (Figure 4A).

Unlike insulin stimulation, mechanically induced phosphorylation of GSK-3 β or p70^{S6k}-389 was not affected by wortmannin (Figure 4B). Wortmannin also had no effect on the mechanically induced increase in p70^{S6k}-421/424 or p38 phosphorylation (Figure 4B). Consistent with these results, a mechanically induced alteration in Akt-473 phosphorylation was not observed at the time point evaluated (90 min). Similar results were obtained with 20 μ M Ly294002 (results not shown).

To evaluate the contribution of mTOR to the mechanically induced signalling events, muscles were incubated with rapamycin. Rapamycin blocked the mechanically induced increase in GSK-3 β and p70^{S6k}-389 phosphorylation, but had no effect on p70^{S6k}-421/424 or p38 phosphorylation (Figure 4B). Taken together, results of these experiments demonstrate that mechanical stimulation promotes signalling through mTOR via a PI3K-independent pathway.

Akt1 is not necessary for mechanically induced signalling through mTOR or an increase in protein synthesis

One of the primary signalling molecules downstream of PI3K is Akt. Akt is a serine/threonine kinase that has been implicated in the control of several signalling events that regulate protein synthesis and overall skeletal-muscle mass [34]. In skeletal muscles, three isoforms of Akt (Akt1, Akt2 and Akt3) are expressed

Figure 4 Mechanically induced signalling through mTOR occurs via a PI3K-independent pathway

Muscles were held at optimal length L_0 for a 30 min preincubation, followed by an additional 90 min of static or stretch conditions in a medium containing a vehicle (0.2% DMSO), 150 nM rapamycin (RAP) or 500 nM wortmannin (WORT) present throughout the entire incubation period. (A) During the final 30 min, static muscles were stimulated with a 0.1% ethanol (control, Insulin-) or 100 nM insulin in 0.1% ethanol (Insulin+) and subjected to Western-blot analysis for phosphorylated p38 (P-p38), phosphorylated Akt on Ser⁴⁷³ (P-Akt 473), phosphorylated p70^{S6k} on threonine/serine 421/424 (P-p70 421/424), phosphorylated p70^{S6k} on Thr³⁸⁹ (P-p70 389) and phosphorylated GSK-3 β (P-GSK-3 β ; $n = 3-4$ /group). (B) Muscles subjected to 90 min of static (control, STR-) or STR (STR+) conditions were Western-blotted as described above ($n = 6-9$ /group). Average values for each group are presented at the top of the lane and expressed as a ratio of the value obtained in vehicle-treated control samples. * $P \leq 0.05$, significantly different from treatment (i.e. vehicle, RAP, WORT) control; † $P \leq 0.05$, significantly less than vehicle control.

Figure 5 Akt1 is not necessary for mechanically induced signalling through mTOR or an increase in protein synthesis

(A) Western-blot analysis of muscles from wild-type (WT) and Akt1^{-/-} mice for Akt1, total Akt (all isoforms) and total phosphorylated Akt on Ser⁴⁷³ (P-Akt 473), $n = 4-8$ /group. (B-D) WT and Akt1^{-/-} muscles were held at optimal length for a 30 min preincubation, followed by 90 min of static (STR-) or STR (STR+) conditions. (B, C) Western-blot analysis of phosphorylated mTOR on Ser²⁴⁴⁸ (P-mTOR), phosphorylated p38 (P-p38) or phosphorylated p70^{S6k} on Thr³⁸⁹ (P-p70 389), $n = 4-8$ /group. For the Western blots, the average value of each group is presented at the top of the lane and expressed as a ratio of the value obtained in WT muscles (A, B) or STR muscles (C). (D) Protein synthesis rates in Akt1^{-/-} muscles were measured during the final 30 min of STR- or STR+ conditions. Results are expressed as means $\pm$ S.E.M., $n = 5$ /group. * $P \leq 0.05$, significantly different from STR-; † $P \leq 0.05$, significantly different from WT.

[35]. Of the three isoforms, Akt1 is the most active and is the only isoform that appears to respond to mechanical stimuli [36]. To assess the role of Akt in the mechanically induced increase in protein synthesis, EDL muscles of Akt1 null mice were employed. Muscles from Akt1 null mice express no Akt1 protein, have a 53 % reduction in total Akt (all isoforms) and a 56 % reduction in total Akt phosphorylated on Ser⁴⁷³ (Figure 5A).

The regulation of mTOR is an important component of Akt signalling and it has been reported that Akt can regulate mTOR via phosphorylation of Ser²⁴⁴⁸ [37]. Consistent with a role for Akt in regulating the phosphorylation of Ser²⁴⁴⁸ on mTOR, Akt1^{-/-} muscles revealed a significant decrease in the phosphorylation of this site when compared with wild-type muscles (Figure 5B). However, mechanical stimulation did not alter the Ser²⁴⁴⁸ phosphorylation of mTOR in both wild-type and Akt1^{-/-} muscles,

suggesting that Akt-dependent alterations in mTOR phosphorylation were not required for the activation of mTOR-dependent signalling events (Figure 5B). Additional evidence for this conclusion was obtained from experiments, which indicated that muscles from Akt1^{-/-} mice revealed a normal mechanically induced increase in both rapamycin-dependent (p70^{S6k}-389) and rapamycin-independent (p38) signalling events (Figure 5C). Furthermore, muscles from Akt1^{-/-} mice also displayed a normal mechanically induced increase in protein synthesis (Figure 5D). Together, these results demonstrate that Akt1 is not required for mechanically induced signalling through mTOR or an increase in protein synthesis.

Mechanically induced release of locally acting factors is not sufficient for the induction of mTOR-dependent signalling events

To determine if the mechanically induced signalling through mTOR was elicited by the release of locally acting factors, static muscles were co-incubated with muscles subjected to intermittent stretch. Results from these experiments indicate that a mechanically induced release of locally acting factors was not sufficient for the activation of either the rapamycin-dependent (GSK-3 β and p70^{S6k}-389) or rapamycin-independent (p70^{S6k}-421/424 or p38) signalling events (Figures 6A and 6B).

The extracellular matrix that surrounds myofibres in whole muscle might limit the release of locally acting factors into the surrounding medium. This limitation could be used to explain the negative results that were observed in the co-incubation experiments. Therefore additional experiments were conducted with muscle cells grown in cell culture. The muscles cells grown in culture are readily exposed to the surrounding medium and, therefore, limitations in the release of locally acting factors through the extracellular matrix should not be a confounding variable. Results from these experiments indicated that an intermittent stretch in cell culture is also capable of activating signalling to the rapamycin-sensitive p70^{S6k}-389 phosphorylation site (Figure 6C). Furthermore, conditioning of the medium by the release of locally acting growth factors was not sufficient for the induction of p70^{S6k}-389 phosphorylation. Although these results cannot fully exclude a role for locally acting growth factors, they do provide additional evidence to support the conclusion that a mechanically induced release of locally acting factors is not sufficient for the activation of rapamycin-dependent signalling events (Figure 6D).

DISCUSSION

Mechanical stimuli have been shown to be important for the regulation of skeletal-muscle mass and protein synthesis, yet the molecular mechanisms involved in the mechanotransduction events are poorly understood. Previous studies have shown that mechanical stimulation of muscle tissue/cells results in the release of locally acting factors (e.g. fibroblast growth factor and IGF) [38,39]. Since these factors can affect the signalling pathways that regulate protein synthesis, it has been suggested that mechanical stimuli regulate protein synthesis primarily by the release of locally acting growth factors [25]. This hypothesis implicates the well-defined wortmannin- and rapamycin-sensitive pathways in the regulation of protein synthesis [8,21].

The mechanisms by which growth factors, such as insulin and IGF, stimulate protein synthesis in skeletal muscles have been well characterized. This includes activation of Akt, which occurs through a wortmannin-sensitive (PI3K-dependent) mechanism [14]. In turn, activated Akt phosphorylates GSK-3 β , resulting in an inhibition of GSK-3 β activity [34,40]. The decrease in

Figure 6 Mechanically induced release of locally acting factors is not sufficient for the induction of mTOR-dependent signalling events

Western-blot analyses of phosphorylated p38 (P-p38), phosphorylated p70^{S6k} on threonine/serine 421/424 (P-p70 421/424), phosphorylated p70^{S6k} on Thr³⁸⁹ (P-p70 389) and phosphorylated GSK-3β (P-GSK-3β) from experiments in which EDL muscles were attached to the mechanical stimulator or a motionless device. The muscles on both devices were adjusted to optimal length and co-incubated in the culture bath. (A) Muscles on the mechanical stimulator were held at optimal length for a 30 min preincubation, followed by an additional 90 min of either static (CNT) or STR conditions. (B) Muscles on the motionless device were held at optimal length and co-incubated (Co-Inc) with CNT or STR muscles on the mechanical stimulator. (C) Western-blot analysis of P-p70 389 from C2C12 myotubes subjected to 10 min of CNT or STR conditions or (D) static C2C12 myotubes were incubated for 10 min with the conditioned medium (Cond-Med) from CNT or STR myotubes as described in the Materials and methods section. Average values for each group are presented at the top of the lane and expressed as a ratio of the value obtained under the CNT conditions, $n = 5-8/\text{group}$. * $P \leq 0.05$, significantly different from CNT.

GSK-3β activity is linked to a decrease in phosphorylation of the ε subunit of eIF2B at Ser⁵³⁵, an event that is linked to enhanced eIF2B activity and protein synthesis [41,42]. Insulin also induces an increase in p70^{S6k} phosphorylation on Thr³⁸⁹, a site critical for the regulation of kinase activity, and this occurs through a wortmannin- and rapamycin-sensitive (mTOR-dependent) pathway [43–45]. Activated p70^{S6k} is known to phosphorylate the S6 subunit of the 40 S ribosome, an event that has been implicated in the translational control of the mRNAs that contain a 5'-TOP structure [11,12]. Thus, after insulin/IGF1 stimulation, both GSK-3β and p70^{S6k} are supposed to contribute to the wortmannin- and rapamycin-sensitive pathways that regulate protein synthesis.

Similar to insulin, the mechanically induced increase in protein synthesis was found to be both rapamycin- and wortmannin-sensitive, and it highly correlated with the changes in GSK-3β and p70^{S6k}-389 phosphorylation. These results are consistent with a model in which mechanical stimuli induce the release of locally acting growth factors, which in turn activate protein synthesis through a PI3K/Akt → mTOR pathway. However, unlike insulin

Figure 7 Schematic representation of mechanically induced signalling events that regulate protein synthesis

An intermittent stretch promotes an increase in protein synthesis. Signalling through both PI3K (---) and mTOR, but not Akt1, is necessary for the activation of protein synthesis. The increase in protein synthesis was associated with mechanically induced, time-dependent signalling through p38, Akt, p70^{S6k} and GSK-3β. The signalling to p38 and p70^{S6k} (421/424) was PI3K- and mTOR-independent (—), whereas signalling to p70^{S6k} (389) and GSK-3β were PI3K-independent but mTOR-dependent (.....). A mechanically induced release of locally acting factors was not sufficient for the induction of these signalling events, suggesting the involvement of a mechanoreceptor-related mechanism rather than an autocrine/paracrine receptor-related mechanism.

stimulation, the increase in GSK-3β and p70^{S6k}-389 phosphorylation in the present study occurred through a rapamycin-dependent but wortmannin- and Akt1-independent pathway. These surprising results indicate that mechanical stimuli are different from insulin-like growth factors in that mTOR-dependent signalling events are regulated via a PI3K/Akt1-independent mechanism. Furthermore, these results indicate that if mechanical stimuli regulate protein synthesis by the release of locally acting factors, then these factors must activate mTOR through a PI3K/Akt1-independent mechanism. However, in both the co-incubation and conditioned-media experiments, the release of locally acting factors was not sufficient for the activation of mTOR-dependent signalling events, thus suggesting that mechanotransduction (e.g. mechanoreceptor) rather than ligand binding of autocrine/paracrine growth factors as the cause for the induction of the mTOR-dependent signalling events. A schematic representation that summarizes these findings is shown in Figure 7.

Since PI3K activity is generally considered to be indispensable for mTOR signalling [22,46], the PI3K/Akt1-independent activation of mTOR signalling observed in the present study was unexpected and therefore raises questions about the mechanisms involved. One possibility is that the stretch activates the recently described PA (phosphatidic acid) → mTOR pathway [22,47]. This is an attractive possibility, since mechanical stimuli have previously been shown to induce the activation of the PA-generating enzyme phospholipase D [48]. However, PA-induced activation of p70^{S6k} activity as well as p70^{S6k}-389 phosphorylation has been shown to be wortmannin-sensitive [47,49]. The wortmannin-sensitive nature of the PA → mTOR → p70^{S6k} pathway is not

consistent with wortmannin-independent activation of the mTOR $\rightarrow$ p70^{S6k} observed after mechanical stimulation, thus suggesting that the PA $\rightarrow$ mTOR pathway is not involved.

Although the results indicate that the mechanically induced mTOR-dependent signalling events are activated by a PI3K/Akt1-independent mechanism and are necessary for an increase in protein synthesis, these events are not sufficient in the absence of PI3K activity. This result suggests that either a basal level of PI3K activity and/or mechanically induced PI3K activity is necessary for mTOR-dependent events to promote an increase in protein synthesis. Whereas PI3K activity was not directly measured, the transient increase in Akt Ser⁴⁷³ phosphorylation (15 and 30 min), a commonly used downstream marker of PI3K activity, suggests that PI3K was activated by stretch. If PI3K activity was enhanced, an increase in Akt Thr³⁰⁸ phosphorylation would also be expected. However, stretch did not alter Akt Thr³⁰⁸ phosphorylation, suggesting that PI3K was not activated by stretch. Thus, at this time, it is not clear whether or not stretch activates PI3K. In either case, muscles from Akt1 null mice displayed a normal mechanically induced increase in protein synthesis, a result consistent with a model in which basal levels of PI3K activity (which would be inhibited by wortmannin), rather than mechanically induced PI3K/Akt1 activation, is necessary for an increase in protein synthesis [50].

A role for Akt2 and Akt3 activation cannot be ruled out as possible mechanisms for the mechanically induced increase in protein synthesis and mTOR-dependent signalling events. However, in skeletal muscles, Akt1 is the most active isoform and appears to be the only isoform that responds to contractile activity [36]. Furthermore, if Akt2 or Akt3 is responsible for the activation of mTOR-dependent signalling, these isoforms of Akt must be functioning through a wortmannin (PI3K)-independent mechanism. Since PI3K activity is considered to be indispensable for Akt activation, this possibility seems unlikely [24]. Additionally, two pathways that have been proposed for linking Akt to mTOR (phosphorylations of mTOR and tuberlin) were not activated by mechanical stimulation [37,51]. Taken together, a role for the Akt2 and Akt3 isoforms in the mechanically induced increase in protein synthesis and mTOR-dependent signalling events seems unlikely.

In conclusion, mechanical stimuli are similar to growth factors in that they require signalling through both PI3K and mTOR to promote an increase in protein synthesis. However, unlike growth factors, mechanical stimuli activate mTOR-dependent signalling events through a PI3K/Akt1-independent mechanism and the release of locally acting factors is not sufficient for the induction of this pathway. Since PI3K is indispensable for growth factor-based signalling through mTOR, we propose that mechanical stimuli and growth factors provide distinct inputs through which mTOR co-ordinates an increase in the translational efficiency. In the light of recent observations [23], which have shown that mechanically induced growth of cardiac and skeletal muscles is dependent on signalling through mTOR these results will be an important step towards understanding the unique mechanisms by which mechanical stimuli regulate mTOR and overall muscle growth.

We thank N. Hay for providing the Akt1 null mice. This work was supported by NIH grants AR45617 (to K.A.E.), AR36281 (to K.E.C.), AR45184 (to M.L.F.) and T32 07692 (to M.J.F.), NASA Predoctoral Fellowship NGT-50406 (to T.A.H.) and funding from Pfizer (to K.A.E.).

REFERENCES

- Goldspink, D. F. (1977) The influence of immobilization and stretch on protein turnover of rat skeletal muscle. *J. Physiol. (Cambridge, U.K.)* **264**, 267–282
- Hornberger, T. A., Hunter, R. B., Kandarian, S. C. and Esser, K. A. (2001) Regulation of translation factors during hindlimb unloading and denervation of skeletal muscle in rats. *Am. J. Physiol. Cell Physiol.* **281**, C179–C187
- Gregory, P., Gagnon, J., Essig, D. A., Reid, S. K., Prior, G. and Zak, R. (1990) Differential regulation of actin and myosin isoenzyme synthesis in functionally overloaded skeletal muscle. *Biochem. J.* **265**, 525–532
- Wong, T. S. and Booth, F. W. (1990) Protein metabolism in rat tibialis anterior muscle after stimulated chronic eccentric exercise. *J. Appl. Physiol.* **69**, 1718–1724
- Augert, G., Monier, S. and Le Marchand-Brustel, Y. (1986) Effect of exercise on protein turnover in muscles of lean and obese mice. *Diabetologia* **29**, 248–253
- Nader, G. A., Hornberger, T. A. and Esser, K. A. (2002) Translational control: implications for skeletal muscle hypertrophy. *Clin. Orthop.* **S178–S187**
- Sonenberg, N., Hershey, J. W. and Mathews, M. B. (2000) *Translation Control of Gene Expression*, Cold Spring Harbor Laboratory Press, Plainview, NY
- Jefferson, L. S., Fabian, J. R. and Kimball, S. R. (1999) Glycogen synthase kinase-3 is the predominant insulin-regulated eukaryotic initiation factor 2B kinase in skeletal muscle. *Int. J. Biochem. Cell Biol.* **31**, 191–200
- Herbert, T. P., Kilhams, G. R., Batty, I. H. and Proud, C. G. (2000) Distinct signalling pathways mediate insulin and phorbol ester-stimulated eukaryotic initiation factor 4F assembly and protein synthesis in HEK 293 cells. *J. Biol. Chem.* **275**, 11249–11256
- Liu, G., Zhang, Y., Bode, A. M., Ma, W. Y. and Dong, Z. (2002) Phosphorylation of 4E-BP1 is mediated by the p38/MSK1 pathway in response to UVB irradiation. *J. Biol. Chem.* **277**, 8810–8816
- Jefferies, H. B., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B. and Thomas, G. (1997) Rapamycin suppresses 5'-TOP mRNA translation through inhibition of p70S6k. *EMBO J.* **16**, 3693–3704
- Kawasome, H., Papst, P., Webb, S., Keller, G. M., Johnson, G. L., Gelfand, E. W. and Terada, N. (1998) Targeted disruption of p70(S6k) defines its role in protein synthesis and rapamycin sensitivity. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5033–5038
- Martin, K. A. and Blenis, J. (2002) Coordinate regulation of translation by the PI 3-kinase and mTOR pathways. *Adv. Cancer Res.* **86**, 1–39
- Kimball, S. R., Farrell, P. A. and Jefferson, L. S. (2002) Invited review: role of insulin in translational control of protein synthesis in skeletal muscle by amino acids or exercise. *J. Appl. Physiol.* **93**, 1168–1180
- Dardevet, D., Sornet, C., Balage, M. and Grizard, J. (2000) Stimulation of *in vitro* rat muscle protein synthesis by leucine decreases with age. *J. Nutr.* **130**, 2630–2635
- Dardevet, D., Sornet, C., Vary, T. and Grizard, J. (1996) Phosphatidylinositol 3-kinase and p70 S6 kinase participate in the regulation of protein turnover in skeletal muscle by insulin and insulin-like growth factor I. *Endocrinology* **137**, 4087–4094
- Kimball, S. R., Horetsky, R. L. and Jefferson, L. S. (1998) Signal transduction pathways involved in the regulation of protein synthesis by insulin in L6 myoblasts. *Am. J. Physiol.* **274**, C221–C228
- Peyrollier, K., Hajdich, E., Blair, A. S., Hyde, R. and Hundal, H. S. (2000) L-leucine availability regulates phosphatidylinositol 3-kinase, p70 S6 kinase and glycogen synthase kinase-3 activity in L6 muscle cells: evidence for the involvement of the mammalian target of rapamycin (mTOR) pathway in the L-leucine-induced up-regulation of system A amino acid transport. *Biochem. J.* **350**, 361–368
- Armstrong, J. L., Bonavaud, S. M., Toole, B. J. and Yeaman, S. J. (2001) Regulation of glycogen synthesis by amino acids in cultured human muscle cells. *J. Biol. Chem.* **276**, 952–956
- Anthony, J. C., Yoshizawa, F., Anthony, T. G., Vary, T. C., Jefferson, L. S. and Kimball, S. R. (2000) Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *J. Nutr.* **130**, 2413–2419
- Grzelkowska, K., Dardevet, D., Balage, M. and Grizard, J. (1999) Involvement of the rapamycin-sensitive pathway in the insulin regulation of muscle protein synthesis in streptozotocin-diabetic rats. *J. Endocrinol.* **160**, 137–145
- Chen, J. and Fang, Y. (2002) A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. *Biochem. Pharmacol.* **64**, 1071–1077
- Bodine, S. C., Stitt, T. N., Gonzalez, M., Kline, W. O., Stover, G. L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J. C., Glass, D. J. et al. (2001) Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy *in vivo*. *Nat. Cell Biol.* **3**, 1014–1019
- Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F. and Hawkins, P. T. (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* **277**, 567–570
- Goldspink, G. (1999) Changes in muscle mass and phenotype and the expression of autocrine and systemic growth factors by muscle in response to stretch and overload. *J. Anat.* **194**, 323–334
- Chen, W. S., Xu, P.-Z., Gottlob, K., Chen, M.-L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K. et al. (2001) Growth retardation and increased apoptosis in mice with homozygous disruption of the *akt1* gene. *Genes Dev.* **15**, 2203–2208

- 27 Reeds, P. J., Palmer, R. M. and Smith, R. H. (1980) Protein and collagen synthesis in rat diaphragm muscle incubated *in vitro*: the effect of alterations in tension produced by electrical or mechanical means. *Int. J. Biochem.* **11**, 7–14
- 28 Crow, M. T. and Kushmerick, M. J. (1982) Chemical energetics of slow- and fast-twitch muscles of the mouse. *J. Gen. Physiol.* **79**, 147–166
- 29 Wiseman, R. W., Moerland, T. S., Chase, P. B., Stuppard, R. and Kushmerick, M. J. (1992) High-performance liquid chromatographic assays for free and phosphorylated derivatives of the creatine analogues β -guanidopropionic acid and 1-carboxy-methyl-2-iminoimidazolidine (cyclocreatine). *Anal. Biochem.* **204**, 383–389
- 30 Sjogaard, G. and Saltin, B. (1982) Extra- and intracellular water spaces in muscles of man at rest and with dynamic exercise. *Am. J. Physiol.* **243**, R271–R280
- 31 Garlick, P. J., McNurlan, M. A. and Preedy, V. R. (1980) A rapid and convenient technique for measuring the rate of protein synthesis in tissues by injection of [3 H]phenylalanine. *Biochem. J.* **192**, 719–723
- 32 Fiorotto, M. L., Davis, T. A. and Reeds, P. J. (2000) Regulation of myofibrillar protein turnover during maturation in normal and undernourished rat pups. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **278**, R845–R854
- 33 Davis, T. A., Fiorotto, M. L., Nguyen, H. V. and Burrin, D. G. (1999) Aminoacyl-tRNA and tissue free amino acid pools are equilibrated after a flooding dose of phenylalanine. *Am. J. Physiol.* **277**, E103–E109
- 34 van Weeren, P. C., de Bruyn, K. M., de Vries-Smits, A. M., van Lint, J. and Burgering, B. M. (1998) Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. *J. Biol. Chem.* **273**, 13150–13156
- 35 Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K. et al. (2001) Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev.* **15**, 2203–2208
- 36 Turinsky, J. and Damrau-Abney, A. (1999) Akt kinases and 2-deoxyglucose uptake in rat skeletal muscles *in vivo*: study with insulin and exercise. *Am. J. Physiol.* **276**, R277–R282
- 37 Nave, B. T., Ouwens, M., Withers, D. J., Alessi, D. R. and Shepherd, P. R. (1999) Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem. J.* **344**, 427–431
- 38 Clarke, M. S. and Feedback, D. L. (1996) Mechanical load induces sarcoplasmic wounding and FGF release in differentiated human skeletal muscle cultures. *FASEB J.* **10**, 502–509
- 39 Perrone, C. E., Fenwick-Smith, D. and Vandenburgh, H. H. (1995) Collagen and stretch modulate autocrine secretion of insulin-like growth factor-1 and insulin-like growth factor binding proteins from differentiated skeletal muscle cells. *J. Biol. Chem.* **270**, 2099–2106
- 40 Sakamoto, K., Hirshman, M. F., Aschenbach, W. G. and Goodyear, L. J. (2002) Contraction regulation of Akt in rat skeletal muscle. *J. Biol. Chem.* **277**, 11910–11917
- 41 Wang, X., Janmaat, M., Beugnet, A., Paulin, F. E. and Proud, C. G. (2002) Evidence that the dephosphorylation of Ser(535) in the ϵ -subunit of eukaryotic initiation factor (eIF) 2B is insufficient for the activation of eIF2B by insulin. *Biochem. J.* **367**, 475–481
- 42 Quevedo, C., Alcazar, A. and Salinas, M. (2000) Two different signal transduction pathways are implicated in the regulation of initiation factor 2B activity in insulin-like growth factor-1-stimulated neuronal cells. *J. Biol. Chem.* **275**, 19192–19197
- 43 Dennis, P. B., Pullen, N., Kozma, S. C. and Thomas, G. (1996) The principal rapamycin-sensitive p70(s6k) phosphorylation sites, T-229 and T-389, are differentially regulated by rapamycin-insensitive kinase kinases. *Mol. Cell. Biol.* **16**, 6242–6251
- 44 Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H. and Sabatini, D. M. (1998) RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 1432–1437
- 45 Isotani, S., Hara, K., Tokunaga, C., Inoue, H., Avruch, J. and Yonezawa, K. (1999) Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase α *in vitro*. *J. Biol. Chem.* **274**, 34493–34498
- 46 Gingras, A. C., Raught, B. and Sonenberg, N. (2001) Regulation of translation initiation by FRAP/mTOR. *Genes Dev.* **15**, 807–826
- 47 Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A. and Chen, J. (2001) Phosphatidic acid-mediated mitogenic activation of mTOR signaling. *Science* **294**, 1942–1945
- 48 Vandenburgh, H. H., Shansky, J., Solerssi, R. and Chromiak, J. (1995) Mechanical stimulation of skeletal muscle increases prostaglandin F $_2\alpha$ production, cyclooxygenase activity, and cell growth by a pertussis toxin sensitive mechanism. *J. Cell Physiol.* **163**, 285–294
- 49 Lim, H. K., Choi, Y. A., Park, W., Lee, T., Ryu, S. H., Kim, S. Y., Kim, J. R., Kim, J. H. and Baek, S. H. (2003) Phosphatidic acid regulates systemic inflammatory responses by modulating the Akt-mammalian target of rapamycin-p70 S6 kinase 1 pathway. *J. Biol. Chem.* **278**, 45117–45127
- 50 Tang, X., Wang, L., Proud, C. G. and Downes, C. P. (2003) Muscarinic receptor-mediated activation of p70 S6 kinase 1 (S6K1) in 1321N1 astrocytoma cells: permissive role of phosphoinositide 3-kinase. *Biochem. J.* **374**, 137–143
- 51 Dan, H. C., Sun, M., Yang, L., Feldman, R. I., Sui, X. M., Ou, C. C., Nellist, M., Yeung, R. S., Halley, D. J., Nicosia, S. V. et al. (2002) Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberlin. *J. Biol. Chem.* **277**, 35364–35370

Received 18 February 2004/13 March 2004; accepted 19 March 2004

Published as BJ Immediate Publication 19 March 2004, DOI 10.1042/BJ20040274