Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways

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Skeletal muscle is composed of multinucleated fibres, formed after the differentiation and fusion of myoblast precursors[1]. Skeletal muscle atrophy and hypertrophy refer to changes in the diameter of these pre-existing muscle fibres. The prevention of atrophy would provide an obvious clinical benefit; insulin-like growth factor 1 (IGF-1) is a promising anti-atrophy agent[2–5] because of its ability to promote hypertrophy. However, the signalling pathways by which IGF-1 promotes hypertrophy remain unclear, with roles suggested for both the calcineurin/NFAT (nuclear factor of activated T cells) pathway[6,7] and the PtdIns-3-OH kinase (PI(3)K)/Akt pathway[8]. Here we employ a battery of approaches to examine these pathways during the hypertrophic response of cultured myotubes to IGF-1. We report that Akt promotes hypertrophy by activating downstream signalling pathways previously implicated in activating protein synthesis: the pathways downstream of mammalian target of rapamycin (mTOR) and the pathway activated by phosphorylating and thereby inhibiting glycogen synthase kinase 3 (GSK3). In contrast, in addition to demonstrating that calcineurin does not mediate IGF-1-induced hypertrophy, we show that IGF-1 unexpectedly acts via Akt to antagonize calcineurin signalling during myotube hypertrophy.

The idea that calcineurin has a role in skeletal muscle hypertrophy was based largely on findings that either cyclosporin A (CsA; a calcineurin inhibitor) or dominant-negative forms of calcineurin could block IGF-1-induced hypertrophy in muscle cultures[6,7]. However, these findings might have resulted from an inhibition of myoblast differentiation and fusion, as opposed to direct inhibition of muscle hypertrophy, because the calcinueurin blockers were administered to undifferentiated myoblasts, before fusion[6,7], and because calcineurin has since been shown to be required for myoblast differentiation[9]. The possibility that the PI(3)K/Akt pathway accounts for the hypertrophic actions of IGF-1 has not been explored adequately, although IGF-1 has been shown to activate this pathway[10].

To distinguish between effects on myoblast differentiation and those on the trophic state of existing myofibres, we exploited the C2C12 myoblast differentiation system. C2C12 myoblasts proliferate until they reach confluence; then, in serum-poor medium, the myoblasts fuse into multi-nucleated myotubes. Differentiated myotubes are predominant by 2 days after fusion (D2 myotubes) and survive for another 3 days. D2 myotubes can be treated with 10 ng ml^{-1} IGF-1, resulting in hypertrophy, as measured by a comparison of myotube diameters, or by an increase in protein content per myotube[8]. In contrast, if pre-differentiation myoblasts are treated with IGF-1, the result is an increase in proliferation[11,12]. Thus, myoblasts and myotubes respond differently to growth-factor stimuli, demonstrating the need to distinguish between pre-differentiation and post-differentiation effects. IGF-1-induced myotube hypertrophy, as measured by an increase in diameter and an increase in total protein, is normally accompanied by an increase in nuclei within the myofibres. However, hypertrophy can be uncoupled from DNA replication, as myotube diameter and total protein still increase as a result of stimulation with IGF-1 in the presence of cytosine arabinoside (Ara-C), an inhibitor of DNA synthesis (see below).

As a first step towards examining the roles of the calcineurin/NFAT and PI(3)K/Akt pathways during IGF-induced myotube hypertrophy, we added IGF-1 to differentiated myotubes and determined whether these pathways were activated. Calcineurin is a protein phosphatase that is activated by increases in intracellular calcium levels, which in turn dephosphorylates the NFAT transcription factors; this dephosphorylation results in the translocation of NFATs to the nucleus and subsequent gene transcription[13]. NFAT dephosphorylation and nuclear translocation are therefore markers of calcineurin activation (Fig. 1, right). For markers of activation of the PI(3)K/Akt pathway, the phosphorylation of Akt itself or of downstream targets such as p70S6 kinase (p70S6K), PHAS-1/4E-BP1 or GSK3 were used (Fig. 1).

Doses of IGF-1 sufficient to cause hypertrophy (see below) did not cause a downshift in dephosphorylation or a nuclear translocation of NFAT, but instead caused a hyperphosphorylation of NFAT (Fig. 2a), indicating that IGF-1 might be inhibiting calcineurin activity. As a control to show that the calcineurin pathway could be activated in myotubes, treatment with a calcium ionophore (a standard method of activating calcineurin[14]) resulted in a dephosphorylation-induced downshift in NFAT gel mobility (Fig. 2a, top) and nuclear translocation (Fig. 2a, bottom) of the NFAT isoform previously implicated in myotube hypertrophy, NFAT-C1 (ref. 6). The calcineurin inhibitor CsA blocked the calcium-ionophore-induced dephosphorylation and translocation of NFATC-1 (Fig. 2a, lane 4). Further supporting the notion that IGF-1 might antagonize calcineurin signalling, simultaneous treatment with IGF-1 and calcium ionophore blunted the ability of the calcium ionophore to activate NFAT-C1 (Fig. 2a, lanes 7–10).

Although IGF-1 could not activate the calcineurin pathway in myotubes, it induced the phosphorylation of many of the defined downstream targets of the PI(3)K/Akt/mTOR pathway (Fig. 2b, lane 3). Consistent with the notion that this IGF-1-induced activation of the PI(3)K/Akt pathway was independent of the calcineurin pathway was the observation that none of these markers of Akt activation was inhibited by using CsA as a calcineurin blocker (Fig. 2b, lane 4), and none could be induced with calcium ionophore as a calcineurin activator (Fig. 2b, lane 2). Interestingly, the only shared signalling molecule induced by activation both with IGF-1

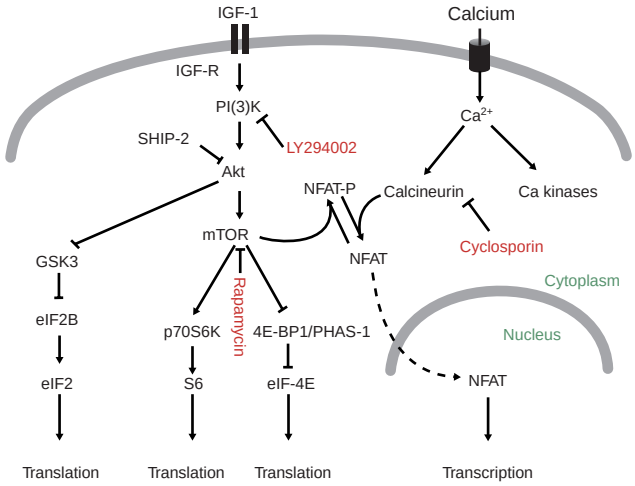

Figure 1 **Schematic overview of a signalling network downstream of the growth factor IGF-1, emphasizing the primary role of the PI(3)K/Akt/mTOR cascade, linking receptor tyrosine kinase derived signals to growth regulatory mechanisms.** The data also suggest possible cross-talk between the interacting kinases Akt and mTOR, and the NFAT-C1 transcription factor.

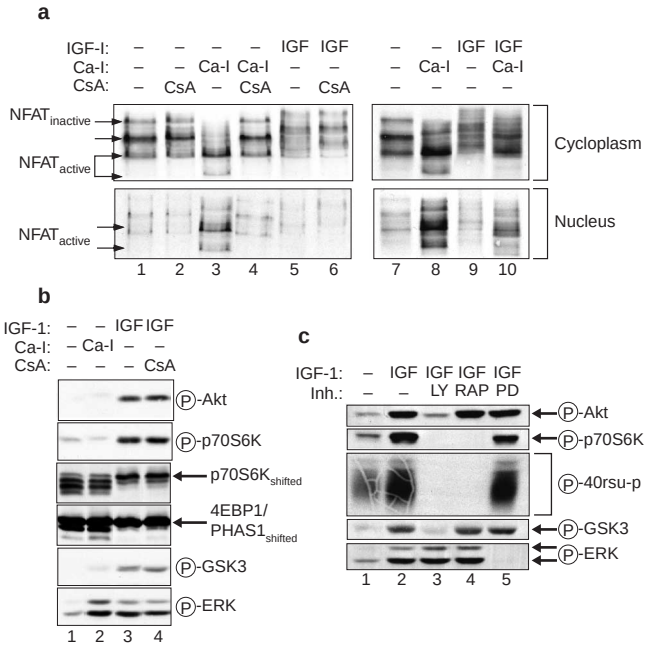

Figure 2 **Signalling pathways activated by IGF-1 or the calcium ionophore A23187 (Ca-I) in C2C12 differentiated myotubes. a**, Opposing effects of Ca-I and IGF-1 on the activation of the transcription factor NFAT-C1 in differentiated myotubes; immunoblot analysis. Ca-I (1.0 μM, 15 min) induces a dephosphorylation (compare lanes 1 and 3, top panel) and translocation (lane 3, bottom panel) of NFAT-C1 from the cytoplasm into the nuclear fraction. Treatment with CsA (5 μM) before stimulation with Ca-I shows that dephosphorylation and translocation requires calcineurin (lane 4). IGF-1 stimulation (10 ng ml^{-1}) leads to a hyperphosphorylation of NFAT-C1 (lane 5). In an independent experiment, treatment of the myotubes simultaneously with IGF-1 and Ca-I inhibits the dephosphorylation and translocation of NFAT-C1 induced by Ca-I (compare lanes 8 and 10). **b**, Stimulation with IGF-1 activates the PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways independently of calcineurin blockade; immunoblot analysis. Stimulation of myotubes with IGF-1 (10 ng ml^{-1}, 15 min) increased the phosphorylation of Akt, p70S6K, GSK3, PHAS-1/4E-BP1 and Erk1/2 (lane 3) compared with control (lane 1); treatment with Ca-I (1.0 μM, 15 min), an activator of calcineurin, had no effect on these kinases (lane 2). CsA (5 μM, 15 min), a calcineurin inhibitor, did not inhibit IGF-1 (lane 4). **c**, Treatment of myotubes with kinase inhibitors for PI(3)K (10 μM LY294002 (LY)) and mTOR (2 ng ml^{-1} rapamycin (RAP)) before stimulation with IGF-1 (10 ng ml^{-1}, 15 min); immunoblot analysis. Akt and GSK3 phosphorylation depend on PI(3)K activity and are independent of mTOR. p70S6K phosphorylation and kinase activity, assayed on the 40S ribosomal subunit (40rsu-p), is blocked by the inhibitors LY294002 and rapamycin. Rapamycin did not block GSK3 phosphorylation. An inhibitor of Mek1/2, PD98059 (PD), showed that the IGF-1-induced phosphorylation of Akt, GSK3 and p70S6K does not require MAP-kinase kinase/Erk kinase activity.

and with calcineurin was extracellular signal-regulated kinase (ERK) (Fig. 2b, bottom); previous evidence indicates that in myotubes the ERK pathway acts counter to the PI(3)K/Akt pathway and inhibits the hypertrophic response[8].

Pharmacological inhibitors of the PI(3)K/Akt/mTOR pathway were subsequently used in phenotypic assays of hypertrophy. To confirm the specificity of these inhibitors, we characterized them with regard to the biochemical activations induced by IGF-1. The PI(3)K inhibitor LY294002 (Fig. 1) blocked all measured activations in the pathway beginning with Akt, but did not affect ERK activation (Fig. 2c, lane 3). The more downstream inhibitor rapamycin (Fig 1), which acts on mTOR, did not affect upstream Akt activation nor a branch of this pathway represented by GSK-3, but did block the phosphorylation of two known mediators of protein synthesis downstream of mTOR, p70S6K and PHAS-1/4E-BP1 (Fig. 2c, lane 4).

The above correlative findings supported the possibility that IGF-1 promotes muscle hypertrophy through the PI(3)K/Akt pathway in a calcineurin-independent manner. To obtain more direct evidence, we next used the pharmacological inhibitors of the calcineurin and PI(3)K/Akt pathways in assays of IGF-1-induced myotube hypertrophy. Further, we introduced gene-expression vectors into the myotubes that induced the expression of either activators or dominant-negative blockers of these pathways (see Methods). Hypertrophy was quantified by measuring myotube diameter; increases in diameter were correlated with total protein increases in IGF-1-treated cultures (data not shown), but unfortunately we could not use the independent measure of change in total protein in some of the following experiments because only a fraction of the fibres expressed the transfected gene of interest.

Addition of the calcineurin inhibitor CsA at a pharmacologically efficacious dose (as shown in Fig. 2a) to pre-differentiation myoblasts resulted in significantly fewer myotubes, which is consistent with a requirement for calcineurin during differentiation (Fig. 3a). To establish a requirement for calcineurin in hypertrophy, as opposed to differentiation, CsA was added to already-differentiated D2 myotubes; in this setting, CsA did not prevent IGF-induced hypertrophy — instead, there was a potentiation of hypertrophy by CsA in both control and IGF-treated myotubes (Fig. 3b). Treatment of differentiated myotubes with a calcium ionophore that biochemically activates calcineurin (Fig. 2a), or expression in myotubes of a doxycycline-regulatable gene expression vector, permitting the doxycycline-inducible expression of a constitutively active form of calcineurin only after myotube formation (see Fig. 3d, inset), led to thinner myotubes (Fig. 3c, d), indicating that neither the pharmacological nor the genetic activation of the calcineurin pathway is sufficient to cause hypertrophy in differentiated myotubes.

We next blocked the PI(3)K/Akt pathway pharmacologically, to see whether, in addition to being correlated with hypertrophy (Fig. 2b, c), the PI(3)K/Akt pathway was required for hypertrophy. Inhibition of PI(3)K with LY294002 led to a mild atrophy of control myotubes and completely blocked IGF-induced hypertrophy (Fig. 3e). Among the downstream effectors of the PI(3)K/Akt pathway, the mTOR kinase is an important regulator of protein synthesis

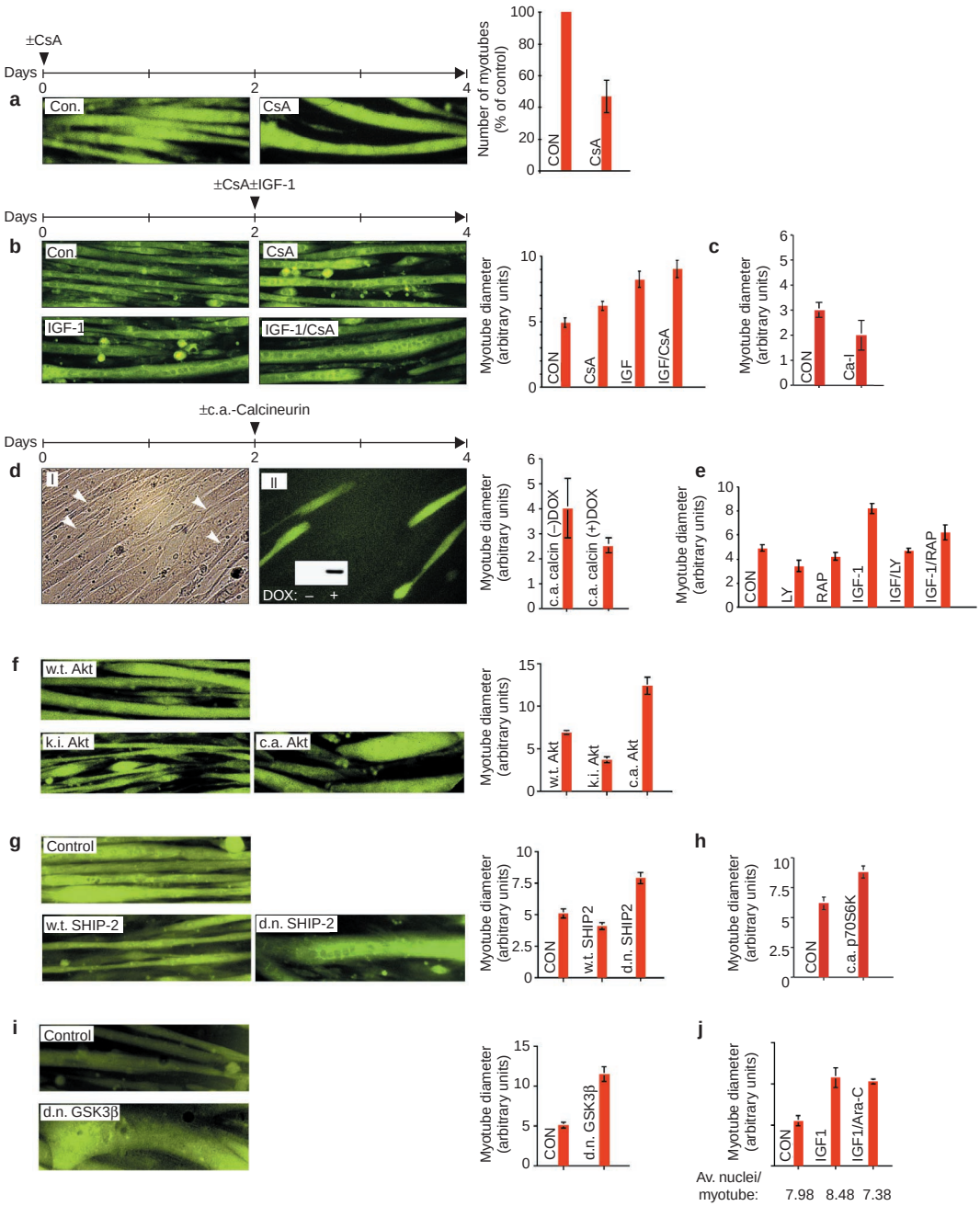

Figure 3. **IGF-1 induces skeletal myotube hypertrophy via the PI(3)K/Akt/mTOR kinase cascade, independently of calcineurin activity.** **a**–**d**, Calcineurin inhibition blocks myotube differentiation but is not required for hypertrophy. **a**, C2C12 myoblasts, at confluence on day 0, then differentiated in 5 μM CsA, analysed for morphological effects at day 4 after fusion. Untreated myotubes are shown (Con.). **b**, Myotubes treated at day 2 after fusion with either or both of CsA (5 μM) or IGF-1 (10 ng ml^{-1}), analysed at day 4 of differentiation. IGF-1 caused hypertrophy; CsA did not block hypertrophy. **c**, Myotubes treated with the calcium ionophore A23187 (Ca-I) at 1 μM. The ionophore did not stimulate myotube hypertrophy. **d**, C2C12 cells genetically-engineered for inducible expression of constitutively active calcineurin (c.a. calcin), induced with 2 μg ml^{-1} doxycycline (DOX) at day 2 after differentiation. Calcineurin did not induce hypertrophy. **e**, Pharmacological inhibitors of PI(3)K and mTOR kinase on IGF-1-induced skeletal myotube hypertrophy. Myotubes were treated at day 2 after fusion with the PI(3)K inhibitor LY294002 (LY; 10 μM), the mTOR inhibitor rapamycin (RAP, 2 ng ml^{-1}), IGF-1 (10 ng ml^{-1}) and with a combination of IGF-1 and each inhibitor, and analysed for morphological effects at day 4. Blockade of the PI(3)K/Akt/mTOR pathway inhibited hypertrophy. **f**–**l**, Comparison of the phenotypes of C2C12 myotubes expressing Akt mutants, SHIP-2 mutants, constitutively active p70S6K (c.a. p70S6K) and dominant-negative GSK3β (d.n. GSK3β). **f**, Expression of wild-type Akt (w.t. Akt), c.a. Akt and kinase-inactive Akt (k.i. Akt) shows that c.a. Akt increased myotube size, whereas k.i. Akt diminished it. **g**, Comparison of EGFP (control), wild-type SHIP-2 (w.t. SHIP-2) and dominant-negative SHIP-2 (d.n. SHIP-2): SHIP-2 decreased myotube size; d.n. SHIP-2 increased myotube size. **h**, Constitutively active p70S6K (c.a. p70S6K) increased myotube size. **i**, Expression of dominant-negative GSK3β (d.n. GSK3β) resulted in a markedly increased myotube size. EGFP is shown as control. **j**, Effect of Ara-C, an inhibitor of DNA replication, on IGF-1-mediated hypertrophy. C2C12 myotubes were treated at day 2 after induction of differentiation with IGF-1 (10 ng ml^{-1}) or a combination of Ara-C (10 μM) and IGF-1 (10 ng ml^{-1}), and analysed the next day. Untreated myotubes are shown (CON). Ara-C did not block IGF-1-mediated hypertrophy. Quantification of myotube diameters is shown. The average number of nuclei per myotube is presented for each condition in the bottom row.

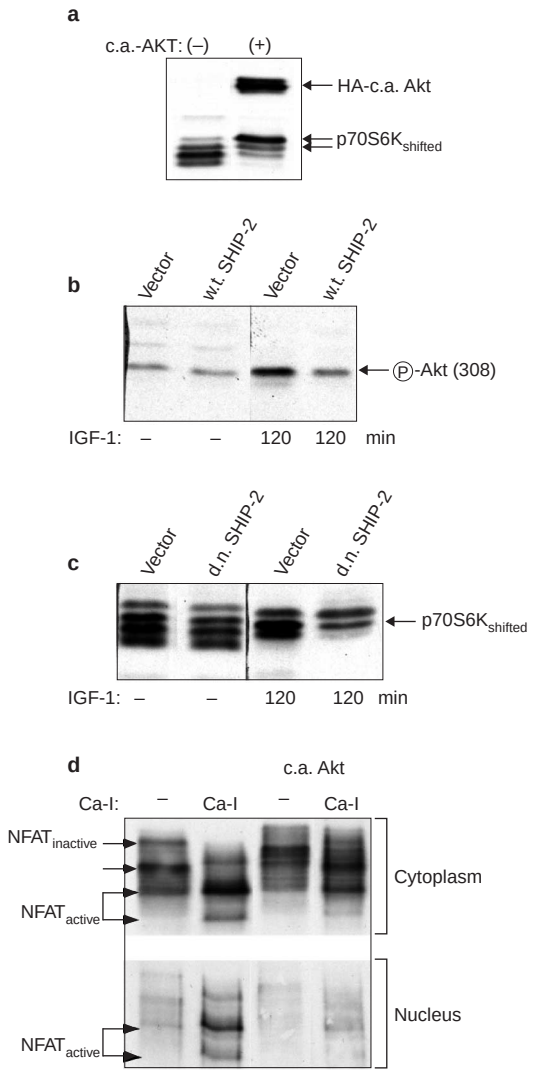

Figure 4. **Direct or indirect Akt activation results in p70S6 kinase activation.** **a**, Phosphorylation of p70S6K is induced by constitutively active Akt (c.a. Akt), shown as a control for Akt activity in the cells transfected in Fig. 3f. Immunoblot analysis demonstrates the expression of the haemagglutinin-tagged c.a. Akt (HA-c.a. Akt), compared with the control cell line. **b**, Expression of wild-type SHIP-2 decreases IGF-induced Akt activity, as measured by immunoblotting with a phospho-specific antibody for Akt, which detects activated Akt. **c**, Expression of dominant-negative SHIP-2 (d.n. SHIP-2) increases p70S6 activity, as determined by a shift in the ratio of p70S6 kinase phosphorylated bands, demonstrating a shift to the higher-molecular-mass, active form of p70S6 kinase in the presence of d.n. SHIP-2. **d**, Expression of c.a. Akt in serum-starved differentiated myotubes increases the phosphorylation of NFAT-C1 and diminishes the ability of calcineurin to dephosphorylate NFAT-C1. Immunoblot analysis shows that stimulation of C2C12 with the calcium ionophore A23187 (Ca-I, 1.0 μM, 15 min) causes dephosphorylation and nuclear translocation of NFAT-C1 (control, lanes 1 and 2). Expression of c.a. Akt (lanes 3 and 4) leads to a hyperphosphorylation of NFAT-C1 (lane 3) and diminishes the Ca-I-induced dephosphorylation and translocation of NFAT-C1 (compare lanes 2 and 4).

(Fig. 1); pharmacological blockade with the mTOR inhibitor rapamycin was less effective than PI(3)K blockade but significantly blunted IGF-induced hypertrophy (Fig. 3e); the more marked effects seen with upstream inhibition of the PI(3)K/Akt/mTOR pathway suggested that multiple branches of this pathway might contribute to hypertrophy (see below) and that mTOR is not required for all of these. As a specificity control for these manipulations, an inhibitor of the ERK pathway known as PD98059 did indeed block ERK activation without affecting steps in the PI(3)K/Akt/mTOR pathway (Fig. 2c); the PD98059 inhibitor did not block phenotypic hypertrophy (data not shown).

To confirm the role of the PI(3)K/Akt pathway independently, and to determine whether it might be sufficient to cause hypertrophy, we exploited a genetic approach. The first genetic construct permitted the expression of a kinase-inactive Akt that has been shown to block endogenous Akt activity when overexpressed[15]; the second construct permitted the expression of a constitutively active Akt[8]. These two constructs behaved as would be predicted if the Akt pathway were sufficient for hypertrophy: the kinase-inactive Akt resulted in thinner myotubes, whereas the constitutively active Akt caused phenotypic hypertrophy (Fig. 3f). The third and fourth constructs permitted the indirect regulation of the PI(3)K/Akt pathway by way of the inositol phosphatase SHIP-2 (Fig. 1). Because Akt activity depends on levels of phosphatidylinositol-3,4,5-trisphosphate PtdIns(3,4,5)P_3 (refs 16–18), overexpression of the inositol phosphatase SHIP-2 should attenuate the Akt pathway by decreasing PtdIns(3,4,5)P_3 levels (as has been shown for SHIP1 (refs 16, 18)), whereas overexpression of a dominant-negative mutant of SHIP-2 should promote the Akt pathway by increasing PtdIns(3,4,5)P_3 levels (as shown for SHIP1 (ref. 19)). Overexpression of wild-type SHIP-2 resulted in atrophic myotubes (Fig. 3g), whereas expression of the dominant-negative mutant of SHIP-2 induced hypertrophy (Fig. 3g). Thus, indirect regulation of Akt, by manipulation of SHIP-2 activity, resulted in phenotypes consistent with the direct manipulation of Akt. We confirmed that the gene expression constructs were affecting intracellular signalling as expected: overexpression of constitutively active Akt caused activation of p70S6K (Fig. 4a); overexpression of wild-type SHIP-2 accelerated inactivation of Akt (Fig. 4b); overexpression of the dominant-negative form of SHIP-2 resulted in IGF-induced hyperphosphorylation of p70S6K (Fig. 4c).

The above genetic approach confirmed the unexpected and surprising finding noted above, that IGF-1 inhibits the calcineurin/NFAT pathway by promoting NFAT-C1 hyperphosphorylation, and further demonstrated that IGF-1 acts in this manner via the Akt pathway: we found that the constitutively active Akt resulted in the hyperphosphorylation of NFAT-C1 (Fig. 4d, lane 3) and blocked its activation and nuclear translocation (Fig. 4d, lane 4).

We next examined the PI(3)K/Akt/mTOR pathway genetically, by making use of a constitutively active form of p70S6K. This construct caused hypertrophy (Fig. 3h) but was less potent than the constitutively active Akt construct (Fig. 3f), which is consistent with the idea that p70S6K might be only one of several subpathways (including, for example, the 4E-BP1/PHAS-1 subpathway) downstream of Akt and mTOR. The smaller effects seen by more downstream inhibition or activation of the PI(3)K/Akt/mTOR pathway suggests that Akt targets in addition to mTOR are important in hypertrophy. One obvious such target is GSK3 (Fig. 1), whose activity is inhibited after phosphorylation by Akt; because GSK3 normally acts to inhibit the translation initiation factor eIF2B, blockade of GSK3 by Akt might promote translation initiation and protein synthesis, and thus might contribute to hypertrophy. To examine this possibility directly, we expressed a dominant-negative form of GSK3β in myotubes and found that it caused profound myotube hypertrophy (Fig. 3i). Together with the above findings that IGF-1 results in phosphorylation of GSK3 via a mechanism that is blocked by the PI(3)K inhibitor LY294002 (Fig. 2c) but not by rapamycin (Fig. 2c), the results with the dominant-negative GSK3 strongly argue that this represents a downstream target of the IGF/PI(3)K/Akt pathway that has a crucial role in regulating muscle hypertrophy in an mTOR-independent manner (Fig. 1).

In this study we explored the signalling pathways by which IGF-1 mediates muscle hypertrophy *in vitro*. We found that PI(3)K/Akt pathway activation occurs during IGF-induced hypertrophy, and showed further that this pathway is necessary for IGF-1-mediated

hypertrophy by demonstrating that its inhibition prevents hypertrophy. We further report that Akt pathway activation is sufficient to cause hypertrophy. Akt activates multiple downstream signalling pathways, and our findings define at least two major downstream targets of Akt in the hypertrophic response (Fig. 1). We first circumstantially implicated the Akt/mTOR and Akt/GSK3 subpathways in the hypertrophic response by showing that they were biochemically regulated by IGF-1 and Akt. We then demonstrated required roles for both the mTOR and GSK3 subpathways during hypertrophy by exploiting either pharmacological or genetic means. However, we cannot rule out the possibility that other Akt targets might also be involved.

While our findings seem to confirm a crucial role for Akt-dependent pathways in the IGF-1-induced hypertrophy response, our efforts seem to have ruled out a required role for the calcineurin/NFAT pathway after differentiation. In mature myotubes this pathway is not activated by IGF-1, inhibition of calcineurin by CsA does not blunt IGF-induced hypertrophy, and constitutive activation of calcineurin does not cause hypertrophy. In contrast, we find that calcineurin activity is necessary for myotube differentiation, which is consistent with a recent study[9]. In reconciling these findings with two previous reports indicating that calcineurin was crucial for IGF-1-mediated hypertrophy[6,7], it should be noted that those studies added CsA as a calcineurin inhibitor before the differentiation of myoblasts into myotubes, which is consistent with the possibility that the lack of hypertrophy seen in those studies was a consequence of an inhibition of differentiation. It is certainly possible that at different points in the development process, muscle fibre size can be regulated both by the fusion of additional satellite cells into a myofibre and by enhanced protein synthesis within a myofibre. We show here that the addition of Ara-C, an inhibitor of DNA replication, does not inhibit IGF-1-mediated hypertrophy; myotube hypertrophy can therefore be uncoupled from DNA replication, because myotube diameter and total protein still increase as a result of stimulation with IGF-1 (Fig. 3j). In terms of the role of the calcineurin/NFAT pathway in hypertrophy, our data suggest that calcineurin might even oppose the hypertrophic response in the mature myotube, because IGF-1 and Akt antagonize the calcineurin-mediated dephosphorylation and translocation of NFAT-C1.

Our findings are consistent with previous findings in *Drosophila*, in which overexpression of the insulin receptor substrate IRS-1 or Akt or p70S6K were sufficient to cause hypertrophy of the cells in which they were expressed[20–23]. The role of the Akt pathway in mammalian myotubes might be analogous to that in *Drosophila*, in which the Akt pathway promotes hypertrophy by way of pro-synthetic pathways and can be separated from proliferation effects. Furthermore, the findings reported by Bodine *et al.* in this issue[24] extend our *in vitro* findings and confirm their physiological relevance, by defining a crucial role for Akt signalling in a variety of muscle hypertrophy responses *in vivo*. Although further work is needed to understand how the Akt pathway contributes to hypertrophy, the immediate therapeutic ramifications of these findings indicate that pharmacological inhibitors of either SHIP-2 or GSK3, as well as pharmacological activators of Akt, mTOR or p70S6K, could provide therapeutic benefit for muscle atrophy. □

Methods

Cell culture and myotube analysis.

C2C12 myoblasts were cultured and transfected as described[8,25]. Flow cytometry and cell sorting were performed on a Cytomation MoFlo (Fort Collins, Colorado) high-speed cell sorter (FACS)[8]. Myoblasts were fused into myotubes at confluence, by shifting the medium to DMEM + 2% horse serum. Myotube diameter was quantified as follows: 10 fields were chosen randomly, and approximately 10 myotubes were measured per field. The average diameter per myotube was calculated as the mean of ten measurements taken along the length of the myotube. For the constitutively active Akt, kinase-inactive Akt, wild-type Akt, wild-type SHIP-2, dominant-negative SHIP-2, constitutively active p70S6K and dominant-negative GSK3β constructs, transfected myotubes were determined by the co-expression of enhanced green fluorescent protein (EGFP; see below for construct details); thus, only transfected myotubes were assessed for hypertrophy. For the tetracycline-inducible calcineurin, myotubes expressing calcineurin were identified by EGFP expression, and comparisons were made between EGFP-positive and EGFP-negative myotubes (see below for construct details). Photographs shown in Fig. 3 were all taken at a magnification of ×200.

Growth factors, pharmacological agents and antibodies.

Before the preparation of cell lysate, serum-starved cells were treated with R3-IGF-1 (IGF-1: 10 ng ml^{-1}; Sigma), A23187 (0.1–10 μM in dimethylsulphoxide (DMSO); Calbiochem), LY294002 (10 μM in DMSO; Calbiochem), rapamycin (20 ng ml^{-1} in DMSO; Calbiochem), CsA (5 μM in ethanol; Calbiochem), PD98059 (10 μM in DMSO; Calbiochem) or cytosine-β-D-arabinofuranoside (Ara-C, 10 μM in water; Sigma). Protein analysis of total cell lysates was conducted as described[8]. The antibodies used were anti-phospho-Erk1/2 (Thr 202/Tyr 204; NEB); anti-phospho-Akt (Ser 473; NEB); anti-phospho-GSK3 (Ser 9/21; NEB); anti-phospho-p70S6K (Thr 389; NEB); anti-phospho-PHAS-1/4E-BP1 (Thr 65; NEB); anti-Erk1/2 (UBI), anti-Akt (NEB), anti-p70S6K (C-18; Santa Cruz), anti-PHAS-1/4E-BP1 (Zymed) and anti-NFAT-C1 (7A6; Santa Cruz).

Protein analysis.

Immunoprecipitations and immunoblotting were performed as described[8]. Proteins were detected with the chemiluminescence detection system (Renaissance; NEN). To measure the enzymatic activity of p70S6K an immunocomplex kinase assay was performed as described[26].

Molecular biology and selection of inducible-expression constructs.

Constitutively active calcineurin (carboxy-terminal deletion mutant encoding amino acid residues 1–398 of calcineurin) tagged with the Flag epitope ([EYKEEEK]$_2$) at the carboxy terminus was generated by the polymerase chain reaction from mouse skeletal muscle complementary DNA (Marathon-Ready; Clontech) and was subsequently subcloned into a tetracycline-inducible internal ribosomal entry site (IRES) bicistronic expression vector (pTRE–Flag–c.a.-calcineurin–IRES–EGFP). The reverse tetracycline-controlled transcriptional activator (rtTA) was fused at its C terminus to enhanced blue fluorescence protein (EBFP; Clontech) and subcloned into an expression vector containing the muscle creatine kinase (MCK) promoter[8]. The tetracycline-responsive vector encoding constitutively active calcineurin and EGFP on the same transcript (as a constitutively active calcineurin–IRES–EGFP cassette) was stably transfected into an MCK-rtTAEBFP cell line. Myoblasts harvested after FACS analysis were treated 48 h after the induction of myogenic differentiation with 2 μg ml^{-1} doxycycline (from a 10 mg ml^{-1} stock solution in water; SIGMA). At day 4 of differentiation, cell lysates were prepared as described[8] and calcineurin was immunoprecipitated with an anti-Flag antibody (Sigma) followed by immunoblot analysis with anti-Flag. Constitutively active Akt was as described previously[8]; it was expressed in a vector containing the MCK promoter and an IRES–EGFP cassette. The kinase-inactive Akt was a gift from the Tsichlis laboratory and was cloned into the same MCK–IRES–EGFP vector. The constitutively active form of p70S6K was a gift from John Blenis's laboratory and was also cloned into the same MCK–IRES–EGFP vector; the kinase activity of the constitutively active p70S6K was determined by transiently transfecting the construct, a wtp70S6K construct and a negative-control vector construct into COS cells, starving those cells and determining the kinase activity as described[26] (data not shown). Human SHIP-2 was cloned from an Origene library. The dominant-negative mutant of SHIP-2 contained a D690A mutation. SHIP-2 was tagged with the haemagglutinin epitope and cloned into the MCK–IRES–EGFP vector[8].

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