Muscle-specific overexpression of the type 1 IGF receptor results in myoblast-independent muscle hypertrophy via PI3K, and not calcineurin, signaling

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Quinn LS, Anderson BG, Plymate SR. Muscle-specific overexpression of the type 1 IGF receptor results in myoblast-independent muscle hypertrophy via PI3K, and not calcineurin, signaling. Am J Physiol Endocrinol Metab 293: E1538–E1551, 2007. First published October 16, 2007; doi:10.1152/ajpendo.00160.2007.—The insulin-like growth factors (IGF-I and IGF-II), working through the type 1 IGF receptor (IGF-1R), are key mediators of skeletal muscle fiber growth and hypertrophy. These processes are largely dependent on stimulation of proliferation and differentiation of muscle precursor cells, termed myoblasts. It has not been rigorously determined whether the IGFs can also mediate skeletal muscle hypertrophy in a myoblast-independent fashion. Similarly, although the phosphatidylinositol 3-kinase (PI3K) and calcineurin signaling pathways have been implicated in skeletal muscle hypertrophy, these pathways are also involved in skeletal myoblast differentiation. To determine whether the IGFs can stimulate skeletal muscle hypertrophy in a myoblast-independent fashion, we developed and validated a retroviral expression vector that mediated overexpression of the human IGF-1R in rat L6 skeletal myotubes (immature muscle fibers), but not in myoblasts. L6 myotubes transduced with this vector accumulated significantly higher amounts of myofibrillar proteins, in a ligand- and receptor-dependent manner, than controls and demonstrated significantly increased rates of protein synthesis. Stimulation of myotube hypertrophy was independent of myoblast contributions, inasmuch as these cultures did not exhibit increased levels of myoblast proliferation or differentiation. Experiments with PI3K and calcineurin inhibitors indicated that myoblast-independent myotube hypertrophy was mediated by PI3K, but not calcineurin, signaling. This study demonstrates that IGF can mediate skeletal muscle hypertrophy in a myoblast-independent fashion and suggests that muscle-specific overexpression of the IGF-1R or stimulation of its signaling pathways could be used to develop strategies to ameliorate muscle wasting without stimulating proliferative pathways leading to carcinogenesis or other pathological sequelae.

insulin-like growth factor; insulin-like growth factor receptor; protein synthesis; protein degradation; sarcopenia

THE INSULIN-LIKE GROWTH FACTORS (IGF-I and IGF-II), signaling via the type 1 IGF receptor (IGF-1R) are crucial mediators of skeletal muscle development, hypertrophy, and regeneration (7, 29, 33, 46, 60). IGF signaling is required for fetal skeletal muscle development (7, 29, 46, 60), a process largely dependent on the proliferation and differentiation of muscle precursor cells known as myoblasts (5, 12, 29, 51, 60). Cell culture studies have shown that the IGFs are strong stimulators of myoblast proliferation and differentiation (15, 20, 29). Overexpression of the IGF-1R via constitutive promoters in skeletal myogenic cell lines and primary skeletal myogenic cultures also resulted in greatly enhanced proliferation and differentiation responses to IGF-I (55, 61, 62). In vivo, adult muscle regeneration following injury and muscle hypertrophy in response to loading are correlated with increased IGF mRNA expression as well as enhanced proliferation and differentiation of the postnatal myoblast population, termed “satellite” cells (2, 24, 32, 37, 70, 80). Prevention of age-associated muscle wasting (“sarcopenia”) induced by muscle-specific overexpression of IGF-I is accompanied by stimulation of muscle satellite cell proliferation and differentiation (8, 54). Gamma irradiation to prevent satellite cell proliferation inhibits postnatal muscle growth and load-induced hypertrophy (58, 67–69). On the basis of these observations, many investigators support the idea that IGF-induced muscle hypertrophy is mediated largely, if not entirely, through the effects of IGF on myoblasts or satellite cells (1, 36, 39, 78). However, it is unclear whether IGF can also mediate hypertrophic effects on skeletal muscle by a myoblast-independent pathway and, if so, whether the signal transduction pathways underlying putative myoblast-independent muscle hypertrophy are similar to those mediating myoblast differentiation. The present study was undertaken to develop a system in which the effects of IGF on myoblasts could be separated from the effects of IGF on muscle fibers. Previous in vitro studies utilized a muscle-specific promoter to overexpress the IGF ligand, which nevertheless could be secreted and stimulate the proliferation and differentiation of neighboring myoblasts (52, 53). To avoid such paracrine effects, in the present study, we constructed and validated a retroviral expression vector that mediated overexpression of the receptor for IGF (the IGF-1R), specifically in cultured rat skeletal myotubes (immature muscle fibers). Thus, compared with control myogenic cultures, IGF signaling was enhanced in myotubes, but not in myoblasts. Analysis of this system provided the first rigorous test of the idea that IGF-I can have myoblast-independent actions on skeletal muscle protein dynamics and muscle hypertrophy. Additionally, this system was utilized to determine the roles of the phosphatidylinositol 3-kinase (PI3K) and calcineurin signaling pathways in myoblast-independent myotube hypertrophy.

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**MATERIALS AND METHODS**

Retroviral vector construction and transduction of cell lines. To produce a retroviral expression vector in which the IGF-1R was overexpressed from a myotube-specific promoter, a fragment of the human α-skeletal actin (HSA) promoter, originally characterized by Brennan and Hardeman (10), was utilized. The human IGF-1R cDNA (43), coding for α- and β-subunits of IGF-1R and previously demonstrated to confer ligand-dependent IGF signaling in skeletal myogenic cultures (61, 62), was ligated into a plasmid vector (modified in our laboratory to expand the cloning site) containing the HSA promoter (17). The construct was cloned into the pSIR retroviral vector (Clontech, Palo Alto, CA), a self-inactivating retroviral vector designed for use with tissue-specific promoters, which was modified in our laboratory with an improved multiple cloning site. The vector contains a 176-bp deletion in the 5'-long terminal repeat (LTR) that includes the enhancer sequences. After reverse transcription, this deletion is present in both LTRs, inactivating the provirus in infected cells. Since the 5'-LTR is inactive, the transgene is expressed from the inserted promoter, in this case, the HSA promoter fragment; virally transduced cells still express the selectable marker neomycin phosphotransferase.

For production of control and HSA-IGF1R retroviral vectors, modified pSIR (empty) and HSA-IGF1R plasmids were purified and transfected into EcoPack2-293 producer cells (Clontech) using Lipo-fectamine and Plus Reagents (Invitrogen, Carlsbad, CA). Virus-containing medium resulting from the transfected producer cells was filtered through 0.45-μm nitrocellulose filters and utilized for transduction of target cells.

**Cell culture for experimental assays.** L6-pSIR and L6-HSA-IGF1R myoblast populations were expanded in 10% FBS-DMEM and incubated with antibiotics (105 U/ml penicillin, 10 mg/ml streptomycin, and 25 μg/ml amphotericin; Sigma) and stored frozen in aliquots in 10% FBS-DMEM + 10% DMSO (Sigma) at −135°C. For production of L6-pSIR and L6-HSA-IGF1R cell lines, L6 cells were cultured in antibiotic-free 10% FBS-DMEM and incubated with virus-containing medium + 8 μg/ml hexadimethrine bromide (Polybrene, Sigma) for 24 h, and then medium was replaced with no virus for an additional 24 h. Cells were selected in DMEM with 10% FBS-DMEM + 1 mg/ml G418 (GIBCO, Grand Island, NY) for 10–14 days with subculturing to maintain subconfluence. Stable cell lines were stored frozen in aliquots as described above.

**Assays for IGF-1R expression.** IGF-1R expression was assayed by Western blots using antibodies specific to the α- and β-subunit chains of the IGF-1R (IGF-1Rα and IGF-1Rβ, respectively). Goat polyclonal D-16 anti-IGF-1Rα affinity-purified antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) react with rat and human IGF-1Rα chains. Mouse anti-IGF-1Rβ antibody (MAb 1123, Upstate Chemicon, Temecula, CA) reacts with rat and human IGF-1Rβ chains and exhibits no cross-reactivity with the insulin receptor. For analysis, pSIR and L6-HSA-IGF1R cells were cultured in 0.5% FBS-DMEM (differentiation medium) for 0, 2, 4, or 6 days, rinsed, harvested in reducing SDS sample buffer for Western blot analysis of expression of IGF-1Rα and IGF-1Rβ with use of the above-described antibodies and appropriate secondary antibodies, and quantified by densitometry using UN-SCAN-IT version 5.1 software (Silk Scientific, Orem, UT). To compare and quantitatively analyze data on different blots, a standard was run on each gel, and the densitometric signal was normalized to the standard.

IGF-1R expression was also assayed by cross-linking or binding of 125I-IGF-I (specific activity 2 × 105 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) to cell surfaces. L6-pSIR and L6-HSA-IGF1R cells were cultured in 0.5% FBS-DMEM (differentiation medium) for 0–5 days and used for each of these assays. For radioligand cross-linking, at the indicated time points, sister cultures were rinsed three times for 15 min each with DMEM containing 1% BSA to reduce binding of serum-derived IGF and then incubated for 2 h at 4°C in 0.5 ml of 1% BSA-DMEM containing 0.125 μCi of 125I-IGF-I, washed twice with Tris-buffered saline and once with PBS, and exposed to the cross-link reagent disuccinimidyl suberate (0.1 mM; Pierce, Rockford, IL) in PBS for 5 min (59, 61). Cultures were rinsed three times with 0.01 M Tris-HCl-1 mM EDTA (pH 7.5), removed from the dishes by use of plastic cell scrapers into 1 ml of Tris-EDTA, and pelleted by centrifugation. Pellets were solubilized in 50 μl of reducing SDS sample buffer, and proteins were separated on 7.5% SDS-polyacrylamide gels. Gels were fixed in a solution of 10% acetic acid-40% methanol-50% H2O, dried, and exposed to Hyperfilm MP (Amersham) in cassettes with intensifying screens at −70°C for 8 wk. Autoradiographic bands migrating at ~140 kDa, the expected mass of the dissociated IGF-1Rx cross-linked to the radioligand (59, 61), were quantified by densitometry. For radioligand binding assays, the subset of cultures to be assayed was rinsed three times for 15 min each with 1% BSA-DMEM and incubated for 2 h at 4°C in 1% BSA-DMEM containing 0.02 μCi of 125I-IGF-I. At the end of the incubation period, cultures were rinsed three times with cold DMEM and solubilized in 0.5 ml of 0.5% SDS, and radioactivity was determined using a gamma counter (Gamma 5500, Beckman).

**Assays for myofibrillar protein accretion, proliferation, and differentiation.** To assess myofibrillar protein accretion, proliferation, and differentiation, L6-pSIR and L6-HSA-IGF1R cells were established and cultured in differentiation medium (0.5% FBS-DMEM) with 0–100 ng/ml IGF-I for 0–6 days (see above). Myofibrillar protein accretion was assessed by Western blotting to determine sarcomeric myosin heavy chain (MHC) and α-skeletal actin content per well, without normalization to DNA or protein (which would confound the assay). MHC was assessed using the mouse monoclonal antibody MF-20 (Developmental Studies Hybridoma Bank, Iowa City, IA), and α-skeletal actin was assessed using 5C5 antibody (Sigma); both were visualized using enhanced chemiluminescence (ECL) and quantified by densitometry (63). To remain in the linear range of signal on the ECL film, samples were loaded at graded volumes, and the densitometric signal was normalized by volume. To compare and quantitatively analyze data on different blots, a standard was run on every gel, and the densitometric signal was also normalized to the standard. DNA per well was quantified in cultures fixed at the times indicated, stained with Hoechst 33258 dye (Sigma), and measured in a fluorescence microplate reader (35, 63). This assay was previously demonstrated to be as sensitive as, and less variable than, [3H]thyminide incorporation assays in experiments with skeletal myogenic cultures (63). Differentiation was assessed after 4 days of culture with 0–10 ng/ml IGF-I using anti-MHC fluorescence immunocytochemistry (63). Cultures were rinsed three times with cold DMEM, fixed for 30 s with cold 70% ethanol-formalin-glacial acetic acid at 20:2:1, and then rinsed three times again. MHC was assessed using MF-20 antibody (1:5 dilution) and visualized using Alexa Fluor 488-labeled chicken anti-mouse IgG (H & L) secondary antibody (Molecular Probes, Eugene, OR). Nuclei were counterstained with 0.01% ethidium bromide, and the numbers of nuclei within MHC-positive cytoplasm per
unit area were quantified by counting 16 quarter-fields (4 quarter-fields each in 4 wells) for each sample in 2 independent experiments. Anti-MHC immunofluorescence microscopy was also utilized to record the morphology of L6-pSIR and L6-HSA-IGF1R myotubes that arose in different IGF-I concentrations.

**Signal transduction pathway experiments.** L6-pSIR and L6-HSA-IGF1R cultures were established as described above and cultured in differentiation medium with 3 ng/ml IGF-I for 48 h to allow formation of myotubes. At 48 h, the P13K inhibitor LY-294002 (LY; Alexis Biochemicals, San Diego, CA) or the calcineurin inhibitor cyclosporin A (CsA; Alexis Biochemicals) was added (plus 3 ng/ml IGF-I). LY was administered at 150 μM in 1% DMSO, and CsA was administered at 5 μM in 0.1% DMSO. Respective control cultures (no inhibitor) were treated with appropriate amounts of DMSO carrier. After an additional 48 h, cultures were collected for assays of MHC accretion or differentiation (see above) or for validation of inhibitor activity. LY activity was verified by Western blotting for Akt (protein kinase B) and phosphorylated Akt using anti-Akt and anti-phosphorylated Akt (Cell Signaling Technology, Beverly, MA). CsA activity was verified by assay of nuclear factor of activated T cells (NFAT) translocation in the nucleus (31, 66). Cultures were harvested and separated into nuclear and cytoplasmic fractions using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) with addition of Halt protease inhibitor cocktail (Pierce) and 5 mM EDTA, according to the directions supplied by the manufacturer. NFAT content in the nucleus was determined by Western blotting using a monoclonal anti-NFAT2 antibody (Affinity BioReagents, Golden, CO).

**Protein synthesis and degradation assays.** L6-pSIR and L6-HSA-IGF1R cells were established as described above and cultured with 0–30 ng/ml IGF-I in 0.5% FBS. For analysis of total protein synthesis, 1 µCi/ml [3H]tyrosine ([3H]Tyr, specific activity 46.0 Ci/mmol; Amersham) was added to L6-pSIR and L6-HSA-IGF1R myotube cultures 4 days after medium change in the presence or absence of the indicated amounts of IGF-I for 24 h. At the completion of the labeling period, cultures were rinsed three times with DMEM, and protein was precipitated with cold TCA in PBS overnight, rinsed once with cold 10% TCA, solubilized in 0.5 M NaOH, and mixed with scintillation fluid, and radioactivity was determined using a scintillation counter. For analysis of protein degradation, myotubes (3 days after medium change) were labeled with 5 µCi/ml [3H]Tyr for 24 h and incubated for an additional 24 h with 2 mM unlabeled tyrosine in the presence or absence of the indicated amounts of IGF-I. TCA-nonprecipitable counts released into the medium (α) + TCA-precipitable counts in the cell layer (β) and in the medium (γ) were determined as described above, and percent protein degraded was calculated as follows: \[\frac{\alpha}{\alpha + \beta + \gamma} \times 100.\] For each treatment, the mean of three to five determinations of percent protein degraded ± SE was calculated. In one experiment, myotubes were prelabeled with [3H]Tyr for 24 h and then incubated with 2 mM unlabeled tyrosine and 10−5 M water-soluble dexamethasone (DEX; cyclodextrin encapsulated; Sigma) with or without 3 ng/ml IGF-I for an additional 24 h, and percent protein degraded was determined as described above.

**RESULTS**

**IGF-1R expression is elevated in L6-HSA-IGF1R cultures coincident with differentiation.** When cultured in low-serum medium, rat L6 myoblasts commence a well-documented program of differentiation into immature muscle fibers termed myotubes (29, 30). At the time of switch to low-serum medium (day 0), the cultures consist almost exclusively of undifferentiated myoblasts; differentiated myotubes begin to form 2 days after the switch to low-serum medium, and cultures are highly differentiated by day 4 of culture. Western blot analysis of IGF-1R expression, using antibodies to IGF-1Rα and IGF-1Rβ, in control L6-pSIR and L6-HSA-IGF1R cultures using antibodies to IGF-1Rα and IGF-1Rβ on day 0 and 2, 4, and 6 days after the medium switch was shown in Fig. 1. A band reacting with anti-IGF-1Rα and anti-IGF-1Rβ appeared at ~200 kDa, corresponding to disulfide-linked αβ dimmers, which are the reduction products of mature αβ2 IGF-1R heterotrimers and are resistant to further separation into α- and β-subunits of IGF-1R by reducing agents (28, 77). Before differentiation (day 0), expression of the IGF-1R did not differ significantly between control L6-pSIR myoblasts and L6-HSA-IGF1R myoblasts (Fig. 1, A and B). However, upon differentiation (days 2, 4, and 6), expression of the IGF-1R was significantly higher (P < 0.05) in L6-HSA-IGF1R than in control L6-pSIR cultures (Fig. 1, A and B).

Two assays of IGF-I binding were also performed using control L6-pSIR and L6-HSA-IGF1R myogenic cultures on day 0 and 2–5 days after the switch to differentiation-permissive low-serum medium (Fig. 2). Before differentiation (day 0), cell surface 125I-IGF-I binding (Fig. 2A) and 125I-IGF-I cross-linking to IGF-1Rα (Fig. 2B) did not differ significantly between the two sublines. 125I-IGF-I binding was significantly (P < 0.001) higher in the L6-HSA-IGF1R than in control L6-pSIR cultures on days 2–5 (Fig. 2A). 125I-IGF-I cross-linking to IGF-1Rα was significantly (P < 0.05, t-tests) increased on days 2 and 3 in L6-HSA-IGF1R cultures compared with controls on days 2 and 3 (Fig. 2B). 125I-IGF-I cross-linking was elevated, but not significantly different, in L6-HSA cultures on days 4 and 5 because of increased variation, possibly caused by the serum-free incubations necessary for the assays.

As expected from the nature of the molecular construct utilizing a promoter (HSA) that is active only after muscle differentiation, these findings indicate that IGF-1R is overexpressed in differentiated myotubes that arise after culture in low-serum medium, but not in undifferentiated myoblasts (day 0). Moreover, these assays indicated that the fusion polypeptide coded by the transgene appeared to be processed normally and was able to bind ligand. Some of the assays revealed a trend (not significant) of slightly higher IGF-1R expression in L6-HSA-IGF1R cultures on day 0; although undifferentiated myoblasts predominated at this time, the small percentage of differentiated cells in such conditions may account for this trend. Greater differences between the two sublines were revealed by radioligand cross-linking (Fig. 2B) and the Western blot assays (Fig. 1) than by the radioligand binding assay (Fig. 2A). The differences among these assays is due to the higher specificity of the radioligand cross-linking assay and Western blots to the IGF-1R, whereas the radioligand binding assay includes a background of binding to other cell surface proteins, such as IGF-binding proteins.

L6-HSA-IGF1R myotubes display a ligand-dependent hypertrophic morphology. When allowed to differentiate in culture without exogenous IGF-I, control L6-pSIR and L6-HSA-IGF1R myogenic cultures exhibited extensive myotube formation, but little difference in morphology (Fig. 3). With the addition of as little as 1 ng/ml IGF-I, L6-pSIR and L6-HSA-IGF1R myotubes exhibited a hypertrophic morphology characterized by broad myotubes and clustered nuclei, similar to that described for L6 myogenic cultures that overexpressed the IGF-I ligand (52). The hypertrophic morphology was greatly enhanced in L6-HSA-IGF1R myotubes cultured with 1 ng/ml
IGF-I compared with L6-pSIR control myotubes exposed to the same concentration of IGF-I (Fig. 3).

Myofibrillar protein accretion is elevated in L6-HSA-IGF1R myotubes. Morphological analysis (Fig. 3) suggested that L6-HSA-IGF1R myotubes exhibited increased ligand-dependent hypertrophy. To quantify this effect, accretion of muscle-specific MHC and α-skeletal actin was assessed in day 4 myotube cultures by Western blotting (Fig. 4, A and B). MHC content per well was stimulated by increasing doses of IGF-I (0–100 ng/ml) in L6-pSIR and L6-HSA-IGF1R cultures. Moreover, MHC content was significantly (P < 0.01) higher in L6-HSA-IGF1R cultures at all concentrations of IGF-I (Fig. 4A). A similar result was observed for α-skeletal actin accretion (Fig. 4B). Accretion of both myofibrillar proteins was significantly higher in L6-HSA-IGF1R than L6-pSIR cultures, even when no exogenous IGF-I was added to the cultures, an effect attributable to autocrine production of low levels of IGF-I and IGF-II by L6 myogenic cells (30).

To confirm that the hypertrophic effect was indeed dependent on the overexpressed IGF-1R, an anti-human IGF-1R neutralizing antibody, A12, was utilized (11, 79). The A12 antibody blocks IGF-I- and IGF-II-dependent IGF-1R phosphorylation and signaling but does not block the insulin receptor (11). When cultured in the presence of 10 ng/ml IGF-I, addition of the A12 antibody reduced MHC accretion in L6-HSA-IGF1R cultures to levels similar to those of L6-pSIR cultures (Fig. 4C).

The addition of 10–100 ng/ml IGF-I significantly stimulated DNA accretion in both sets of cultures (Fig. 4D). However, in contrast to the effects of IGF-I on myofibrillar protein accretion, the stimulation of DNA accretion did not differ significantly between L6-pSIR and L6-HSA-IGF1R cultures. Moreover, DNA per well was not significantly different between the two sublines at any concentration of IGF-I. These results indicate that the overexpressed IGF-1R was not active in myoblasts and, thus, did not cause increased IGF-mediated myoblast proliferation in L6-HSA-IGF1R cultures. Thus the increased accretion of myofibrillar proteins observed in L6-HSA-IGF1R cultures compared with L6-pSIR control cultures was not due to the effects of IGF on myoblast proliferation or overall nuclear number.

Myoblast differentiation is not altered in L6-HSA-IGF1R cultures. Previous studies using skeletal myogenic cell lines and primary myoblasts indicated that constitutive overexpression of the IGF-1R stimulated myoblast differentiation, as well as myoblast proliferation, in a ligand-dependent manner (61, 62). Differential rates of differentiation in the two sublines, quantitative immunocytochemical analysis of differentiation was performed. Since myotubes are multinucleated, arising by fusion of differentiated myocytes, counts of the numbers of nuclei within MHC-positive cytoplasm (regardless of myotube nuclearity or percentage of differentiated cells) reflect the absolute numbers of differentiated myocytes that arose in these cultures. Figure 5 shows the numbers of such nuclei on days 2 and 4 in L6-pSIR and L6-HSA-IGF1R cultures in the presence of 0, 1, and 10
ng/ml IGF-I. The addition of IGF-I significantly stimulated differentiation in both sets of cultures. However, the numbers of nuclei in MHC-positive cytoplasm did not differ between L6-pSIR and L6-HSA-IGF1R myogenic cultures at any concentration of IGF-I at either time point. This indicates that the transgene construct did not increase the rate of differentiation in L6-HSA-IGF1R cultures. Additionally, the numbers of nuclei in MHC-positive cytoplasm increased only slightly between days 2 and 4, indicating that the differentiation process is substantially completed by 2 days after the switch to low-serum medium.

Because of extreme multilayering of cells and nuclei at concentrations of IGF-I exceeding 10 ng/ml, differentiation could not be assessed accurately at these higher IGF-I concentrations. However, at the concentrations of IGF-I (0–10 ng/ml) that were assessed for differentiation, myofibrillar protein accretion was two to three times higher in L6-HSA-IGF1R cultures (Fig. 4, A and B). At these concentrations, there were no differences in DNA content (Fig. 4D) or differentiation (Fig. 5B) between the two sublines. Taken together, these findings indicate that the increases in myofibrillar protein accretion in L6-HSA-IGF1R cultures shown in Fig. 3, at least at 0–10 ng/ml, were the result of a hypertrophy process that was not dependent on increases in the rate of myoblast differentiation.

**Time course of hypertrophy in L6-pSIR and L6-HSA-IGF1R myogenic cultures.** To determine the time course of the hypertrophic process, L6-pSIR and L6-HSA-IGF1R cultures were treated with 10 ng/ml IGF-I, and sister cultures were collected for analysis of MHC and DNA content sequentially on days 2–6 of culture (Fig. 6). Both sets of cells accumulated increasing amounts of MHC as culture progressed (Fig. 6A). However, at each time point, L6-HSA-IGF1R cultures contained significantly higher amounts of MHC than L6-pSIR cultures, with the relative difference between the two sublines increasing with time in culture. In contrast, because of the differentiation-permissive low-serum conditions, there were no increases in DNA content in either set of cultures from day 2 to day 6 (Fig. 6B). Furthermore, there was no significant difference in DNA content between L6-pSIR and L6-HSA-IGF1R cultures at any time point. Similar results for MHC and DNA were obtained when cells were cultured with 0 and 1 ng/ml IGF-I (not shown). Taken together with the findings reported above (Fig. 5) indicating that the differentiation process was substantially completed by day 2, these observations suggest that the progressive increase in MHC accretion reflects a myoblast-independent hypertrophic process that occurs after the differentiation phase in these cultures. The progressively greater difference in MHC content between the two sets of cultures is consistent with dependence of this hypertrophy process on increased signaling mediated by the overexpressed IGF-1R.

Myoblast-independent hypertrophy is dependent on PI3K, but not calcineurin, signaling. The findings presented above indicate that an IGF-stimulated, myoblast-independent process of skeletal myotube hypertrophy can be separated from the differentiation process in this culture system. This system was next used to analyze the involvement of two signal transduction pathways, PI3K signaling and calcineurin signaling, which have been implicated in muscle hypertrophy and muscle differentiation (16, 23, 25, 31, 40, 52, 53, 66, 71, 74).

To determine the roles of these pathways, L6-pSIR and L6-HSA-IGF1R cells were cultured in low-serum medium with 3 ng/ml IGF-I for 2 days to allow formation of myotubes and then cultured in the presence of absence of the PI3K inhibitor LY or the calcineurin inhibitor CsA + 3 ng/ml IGF-I. MHC content and differentiation were determined 48 h later, on day 4 of culture. Additionally, to ensure that the inhibitors were effective in both sets of cultures, Akt phosphorylation and translocation of NFAT in the nucleus, downstream signaling targets of PI3K and calcineurin, respectively, were assessed.

Figure 7 shows the effects of administration of LY or CsA on day 2 on differentiation in L6-pSIR and L6-HSA-IGF1R cultures, assessed immunocytochemically on day 4. Administration of the inhibitors caused small (~20%) decreases (not significant) in the numbers of differentiated nuclei. Moreover, the numbers of differentiated nuclei did not differ between the L6-pSIR and L6-HSA-IGF1R sublines in any condition. Therefore, when administered according to this protocol (days 2–4), any effects of the inhibitors on myofibrillar protein accretion are not due to differential effects on differentiation or survival of differentiated cells.
LY inhibited Akt phosphorylation ~75% in L6-pSIR and L6-HSA-IGF1R cultures (Fig. 8A), indicating that LY inhibited PI3K signaling equally in both sublines. Similarly, LY inhibited MHC accumulation in both sets of cultures by ~75% (Fig. 8B). Since LY decreased the numbers of differentiated nuclei by only 20% (Fig. 7), these findings indicate that PI3K signaling mediates myoblast-independent accumulation of myofibrillar proteins in this system.

CsA inhibited NFAT translocation to the nucleus by ~50% in L6-pSIR and L6-HSA-IGF1R cultures (Fig. 8C), indicating that CsA inhibited calcineurin signaling equally in both sublines. However, CsA had no significant effect on MHC accretion in either subline (Fig. 8D). This observation indicates that calcineurin signaling does not play a role in myoblast-independent muscle hypertrophy.

Protein dynamics are altered in L6-HSA-IGF1R myotubes. Since the accumulation of higher levels of myofibrillar proteins in L6-HSA-IGF1R cultures in this system was not due to increased rates of myoblast proliferation or differentiation, differences in protein dynamics (protein synthesis and degradation rates) were assessed. Protein dynamics were determined by [3H]Tyr labeling in differentiated L6-pSIR and L6-HSA-IGF1R cultures treated with 0–30 ng/ml IGF-I (Fig. 9). At all concentrations of IGF-I, protein synthesis rates were significantly higher (P < 0.05) in L6-HSA-IGF1R than in L6-pSIR cultures (Fig. 9A). In contrast, protein degradation rates were not significantly different between the two sublines at any concentration of IGF-I (Fig. 9B). Moreover, increasing concentrations of IGF-I did not decrease protein degradation rates in either subline. These data suggest that, in these culture conditions, the increased accumulation of myofibrillar proteins in L6-HSA-IGF1R myotubes is due to elevated protein synthetic rates, but not to decreased rates of proteolysis.

Since IGF-mediated PI3K signaling has been implicated in the inhibition of muscle protein degradation in other skeletal muscle model systems (44, 71, 75), the rates of protein degradation in L6-pSIR and L6-HSA-IGF1R cultures exposed to DEX, a potent proteolysis-inducing glucocorticoid (44), were determined (Fig. 9C). In the absence of exogenous IGF-I, DEX increased proteolysis by a factor of 4 in L6-pSIR myotubes (P < 0.001) but had no effect on proteolysis in L6-HSA-IGF1R myotubes. These observations suggest that low autocrine levels of IGFs released by L6 myotubes (30) resulted in enhanced antiproteolytic signaling from overexpressed IGF-1Rs in the L6-HSA-IGF1R subline sufficient to inhibit the proteolysis-inducing effect of 10⁻³ M DEX, whereas similar autocrine levels of IGF release by L6-pSIR cells were not sufficient to inhibit DEX-induced proteolysis. When cultured with as little as 3 ng/ml IGF-I, both sublines were resistant to the proteolysis-inducing effects of 10⁻³ M DEX (Fig. 9C). These observations indicate that the overexpressed IGF-1R can increase IGF signaling in the presence of low levels of ligand, resulting in inhibition of the effects of a potent proteolysis-inducing agent.

DISCUSSION

A large body of literature indicates that IGF-I and IGF-II, working through the IGF-1R, are essential for skeletal muscle growth and muscle fiber hypertrophy (29). Targeted mutation of IGF-I, IGF-II, or the IGF-1R results in severe muscle dysgenesis (7, 29, 46, 60). Declining IGF levels in aged muscle tissue are thought to be responsible for the age-related deficit in muscle regenerative responses (13, 32, 54). Conversely, muscle hypertrophy in response to exercise, stretch, or increased loading is correlated with increased expression of IGF-I mRNA in muscle tissue (2, 24, 80). Muscle-specific overexpression of IGF-I or infusion of IGF-I into skeletal muscle tissue results in muscle hypertrophy (3, 8, 15, 54). Maintenance of muscle IGF-I expression using transgenic technology or viral vectors prevents age-associated muscle wasting (sarcopenia) in mouse models (8, 54). Muscle growth, hypertrophy, and repair processes are strongly correlated with proliferation and differentiation of dividing muscle precursor cells (5), termed

**Fig. 3.** Hypertrophic morphology of L6-HSA-IGF1R myotubes. Representative immunofluorescence images of L6-pSIR and L6-HSA-IGF1R cultures after 4 days in 0.5% FBS ± 1 ng/ml IGF-I. Cytoplasm of differentiated myotubes was visualized by sarcomeric myosin heavy chain (MHC) staining, whereas all nuclei (differentiated and undifferentiated) were visualized by ethidium bromide staining. All images are shown at the same magnification.
myoblasts (developmentally) and satellite cells (postnatally). The IGFs stimulate myoblast proliferation and differentiation in myogenic cell lines and primary myogenic cultures (29, 30). Indeed, two cell culture studies have shown that IGF signaling is required for muscle differentiation (20, 30). Studies using gamma irradiation to inhibit satellite cell proliferation suggested that postnatal muscle fiber hypertrophy is partly or wholly satellite cell dependent (58, 68). Taken together, these observations have led to the consensus that the effects of IGF on muscle hypertrophy are mediated largely, if not entirely, through stimulation of myoblast or satellite cell proliferation and differentiation (1, 36, 39, 78). Thus it has not been clear...
whether IGF can also mediate hypertrophic effects on skeletal muscle by a myoblast-independent pathway.

The present study demonstrates that, at least in a cell culture model, IGF can cause myotube hypertrophy without stimulating myoblast proliferation or differentiation. Cultured rat L6 skeletal myoblasts that overexpressed the IGF-1R accumulated significantly higher amounts of the myofibrillar proteins MHC and α/β-actin in a ligand- and receptor-dependent manner, accompanied by significantly increased rates of protein synthesis. The stimulation of muscle hypertrophy was independent of myoblast contributions, inasmuch as these cultures did not exhibit increased levels of myoblast proliferation or differentiation. This study constitutes the first rigorous test of the idea that IGF-I can have myoblast-independent actions on skeletal muscle protein dynamics and muscle hypertrophy.

Cell culture and transgenic mouse models with targeted deletions of each of the components of the IGF signaling pathway indicate that the effects of IGF-I and IGF-II on skeletal muscle growth are mediated by the IGF-1R (29, 33, 46). In previous studies, overexpression of the IGF-1R using constitutive promoters in skeletal myogenic cell lines and primary skeletal myogenic cultures resulted in greatly enhanced proliferation and differentiation responses to IGF-I and sensitivity to very low concentrations of IGF (55, 61, 62). However, new or aberrant responses to IGF were not observed (55, 61, 62). In the present study, the effects of IGF on myoblasts were separated from the effects of IGF on myotubes by overexpression of the IGF-1R from a muscle-specific promoter, the HSA, which is not active in myoblasts (10, 15, 17). The HSA promoter confers strong, muscle-specific expression of target genes, since α-actin comprises a large proportion of muscle protein (10). Moreover, skeletal myoblasts do not express muscle-specific contractile proteins until differentiation into myocytes or myotubes (5, 29). Finally, our strategy to overexpress the IGF-1R, rather than one of the IGF ligands, was designed to confer cell-autonomous actions of the transgene. To confirm that this was the case, expression of IGF-1Rα and IGF-1Rβ, cell surface 125I-IGF-I binding, and 125I-IGF-I cross-linking to IGF-1Rα in L6-pSIR (control) and L6-HSA-IGF1R myogenic cultures were compared before and after...
differentiation. Quantitative assays of the numbers of receptors expressed by each cell type at each stage of myogenesis were not technically feasible, inasmuch as other binding sites for IGF-I, such as IGF-binding proteins, are present on the cell surfaces. However, all these assays indicated that expression of the IGF-1R was equivalent in the two myogenic sublines at the myoblast stage but, coincident with differentiation, increased significantly in L6-HSA-IGF1R cultures relative to L6-pSIR controls. More importantly, functional assays supported this contention. The effects of IGF-I on the myoblast-specific processes of proliferation and differentiation were identical in the L6-pSIR and L6-HSA-IGF1R sublines, strongly indicating that IGF responsiveness was not enhanced in L6-HSA-IGF1R myogenic cultures at the myoblast stage. In contrast, the accumulation of contractile proteins MHC and \(\text{HSA} \), an indication of hypertrophy, was greatly enhanced in HSA-IGF1R cultures. Additionally, the difference in accumulation of contractile protein was progressively increased with time in culture, at points well beyond the peak of differentiation. Moreover, differences in contractile protein accumulation between the two sublines were abrogated by anti-human IGF-1R neutralizing antibodies, indicating that the overexpressed human IGF-1R was indeed responsible for these hypertrophic responses to IGF-I. These findings demonstrate that IGF-mediated skeletal muscle hypertrophy can occur in a myoblast-independent fashion.

Since muscle fiber nuclei do not synthesize DNA, multinucleated muscle fibers are formed by the proliferation, differentiation, and subsequent fusion of skeletal myoblasts throughout embryonic and fetal development (5, 51, 76). In mammals,
there is little increase in muscle fiber number during normal postnatal growth (5). However, muscle fibers undergo continued hypertrophy until adult fiber size is achieved (5). Before 1961, when the application of transmission electron microscopy to skeletal muscle tissue revealed a population of postnatal myoblasts, termed satellite cells because of their position surrounding muscle fibers (49), there was no apparent source of new postnatal muscle nuclei (51). Thus it was assumed that all postnatal hypertrophy of skeletal muscle fibers was due to protein dynamics, such that the rate of protein synthesis exceeded the rate of protein degradation (72). Since that period, newer data have indicated that, in mammalian muscle development, the majority of adult muscle nuclei arise postnatally from satellite cell contributions (5, 12, 51). The addition of new muscle nuclei from satellite cell proliferation, differentiation, and fusion into preexisting muscle fibers results in increased protein synthetic capacity in developing muscle tissue (5, 12, 51). Gamma irradiation to inhibit satellite cell proliferation inhibits much, but not all, postnatal muscle fiber hypertrophy during normal growth (68).

After the growth period, satellite cells are quiescent but can be mitotically reactivated in adult skeletal muscle by injury, stretch, or increased loading (5, 37). Activated satellite cells can reenter the cell cycle, undergo proliferation and differentiation, and contribute to hypertrophy of existing muscle fibers or form new muscle fibers (5, 37). The resulting hypertrophic or repair processes are correlated with increased expression of IGF-I mRNA in muscle tissue (32, 37). Indeed, after load- or IGF-induced muscle hypertrophy, muscle protein and DNA contents have been reported to increase in parallel (2, 3, 12, 70). However, similar studies indicated that IGF- or load-induced muscle hypertrophy was not, or only partially, dependent on satellite cell activities (9, 47). Thus the most common interpretation of the mechanism of muscle fiber hypertrophy and that of its main mediator, IGF, has changed completely since 1961. Many authors support the idea that IGF-induced muscle hypertrophy is mediated entirely through the effects of IGF on satellite cells (1, 36, 39, 78). The present study demonstrates the existence of an additional IGF-stimulated pathway whereby IGF can mediate muscle fiber hypertrophy without additional satellite cell input.

Because of the multinucleation of muscle fibers and the complexities of the relationships between myoblasts and muscle nuclei, confusion still exists in the literature concerning the definition of terms such as muscle hyperplasia, which can refer to nuclear hyperplasia (more numerous muscle nuclei or a higher nucleus-to-cytoplasm ratio) or fiber hyperplasia (more numerous muscle fibers) (6, 27, 50). As well, muscle hypertrophy can refer to gross increases in muscle weight, cross-sectional area, or protein content or, specifically, to increases in mean muscle fiber cross-sectional area (2, 3, 70). In the latter case, some authors assume that this process is myoblast independent (14, 72), whereas others assume that it is entirely myoblast dependent (1, 36, 39, 78). In fact, evidence for both has been published (4, 41, 47, 58, 64, 67, 69).

The immunocytochemical assay for differentiation utilized here is not commonly used in cell culture studies of skeletal myogenesis but has been validated (20, 63). Many studies of skeletal myogenesis utilize assays of contractile protein accretion (23) or M-type creatine kinase activity (16, 30) similar to the MHC and α-actin assays also utilized in the present study. However, when utilized without an immunocytochemical assay of differentiation, muscle-specific protein assays cannot distinguish the roles of differentiation and hypertrophy, which this study was designed to address. Our assay quantified the absolute numbers of myocytes that arose in the cultures, a specific assay of the decision of an individual myoblast to undergo differentiation (20). This did not differ between L6-pSIR and L6-HSA-IGF1R cultures at the IGF-I concentrations we were able to test because of multilayering of cultures at high IGF-I concentrations. Thus we could conclude that differences in myofibrillar protein accruement, at least at lower concentrations of IGF-I, reflected a hypertrophy process that...
was independent of the process of differentiation. Similarly, our assay of proliferation by measuring DNA per well (35), rather than the more commonly used labeled thymidine or bromodeoxyuridine incorporation assays (35, 61), reflects not only proliferation but also the survival of myogenic cells (20), which could impact total myofibrillar protein accretion as well. As with differentiation, DNA per well did not differ between L6-pSIR and L6-HSA-IGF1R cultures at any concentration of IGF-1.

The overlap between the myoblast proliferation and differentiation processes and the muscle fiber hypertrophy process has similarly confounded the literature concerning the signal transduction processes underlying IGF-induced skeletal muscle hypertrophy. Two distinct intracellular signaling pathways, mediated by PI3K signaling (66) or activation of the calcium-sensitive trimeric calcineurin complex (25, 53, 74), have been implicated in IGF-induced muscle fiber hypertrophy. Since the PI3K and calcineurin pathways are also implicated in muscle differentiation (16, 23, 31, 40), it remains unclear whether hypertrophy mediated by either or both of these pathways was induced indirectly by stimulation of myoblast differentiation or directly by action on muscle fibers themselves. Various in vitro and in vivo studies have indicated that PI3K, but not calcineurin, signaling mediates skeletal muscle hypertrophy (66, 56, 71), whereas others have indicated the reverse (25, 74). In some of these studies, an attempt was made to direct IGF action specifically to myotubes by overexpression of IGF-I from a muscle-specific promoter (similar to that utilized here) that is active only in differentiated cells (52, 53). However, since myogenic differentiation occurs somewhat asynchronously (63), IGF-I secreted from differentiated myotubes could nevertheless have acted in a paracrine fashion to stimulate differentiation of myoblasts present in the cultures at the same time. Thus such studies could not distinguish the roles of these signal transduction pathways in hypertrophy from their roles in differentiation (52, 53). In another study in which inhibitors of PI3K signaling were added at progressively later time points, it was deduced that the IGF-induced PI3K pathway was necessary for myoblast differentiation, but not for maturation or hypertrophy (52). A different study, using similar temporal methods, indicated that PI3K signaling mediated muscle hypertrophy independently from IGF effects on myoblasts and that IGF actually inhibited calcineurin signaling (66). A third in vitro study indicated that calcineurin activity was necessary only for the earliest stages of myoblast differentiation (31). In vivo studies yielded equally conflicting data on the involvement of the calcineurin pathway in skeletal muscle hypertrophy (25, 56, 74). It was not possible in any of these studies to determine how much of the muscle hypertrophy in question was due to myoblast-dependent vs. myoblast-independent mechanisms.

The present study indicated that myoblast-independent muscle hypertrophy could be inhibited by blocking PI3K signaling in conditions in which this treatment had little effect on differentiation. In contrast, inhibition of calcineurin signaling, validated by measuring NFAT translocation to the nucleus, had no effect on contractile protein accretion. These findings indicate that, at least in this cell culture system, myoblast-independent skeletal muscle hypertrophy is mediated by PI3K, but not by calcineurin, signaling. Since PI3K and calcineurin mediate myoblast differentiation, this study suggests that the signal transduction pathway for myoblast-independent muscle hypertrophy partially overlaps, but is distinct from, the pathways leading to myoblast differentiation.

The vast majority of in vitro and in vivo studies have indicated that IGF-I stimulates muscle protein synthesis (19, 26, 38, 65). This effect is consistent with the well-established role of IGF in adding new synthetic capacity to muscle fibers by incorporation of new muscle nuclei via stimulation of myoblast or satellite cell proliferation and differentiation (5, 12). The results of our study indicate that IGF can also stimulate muscle protein synthesis in a myoblast-independent manner. Our study did not address whether the increases in MHC and α-actin accretion were due to specific increases in myofibrillar protein accretion or to global increases in the rate of protein synthesis, or whether this was regulated at the transcriptional or translational level. Rather the intent of the study was simply to establish the existence of, and a model for, IGF-dependent myoblast-independent hypertrophy, so that such mechanisms could be investigated in future studies. Similarly, the involvement of translational regulators such as mammalian target of rapamycin and S6 kinase type 1 (44, 66) in IGF-dependent myoblast-independent hypertrophy, so that such mechanisms could be investigated in future studies. Similarly, the involvement of translational regulators such as mammalian target of rapamycin and S6 kinase type 1 (44, 66) in IGF-dependent myoblast-independent hypertrophy, so that such mechanisms could be investigated in future studies. Similarly, the involvement of translational regulators such as mammalian target of rapamycin and S6 kinase type 1 (44, 66) in IGF-dependent myoblast-independent hypertrophy, so that such mechanisms could be investigated in future studies. Similarly, the involvement of translational regulators such as mammalian target of rapamycin and S6 kinase type 1 (44, 66) in IGF-dependent myoblast-independent hypertrophy, so that such mechanisms could be investigated in future studies. Similarly, the involvement of translational regulators such as mammalian target of rapamycin and S6 kinase type 1 (44, 66) in IGF-dependent myoblast-independent hypertrophy, so that such mechanisms could be investigated in future studies.
(38), or proinflammatory cytokines (22). In contrast, IGF-I inhibited muscle protein breakdown in response to severe burns (26) and glucocorticoid administration (44, 73). Recent studies have suggested the existence of at least two signal pathways leading to muscle proteolysis, only one of which is inhibited by IGF and Akt signaling (22).

Circulating and muscle IGF-I levels decline with age (8, 13, 42), whereas muscle-specific IGF-I overexpression inhibited sarcopenia in aging mice (8, 54). However, greatly enhanced satellite cell proliferation was observed in these studies, suggesting that the primary mechanism of IGF action in those studies was to stimulate satellite cell proliferation and differentiation, essentially replacing, rather than preserving, degenerating muscle fibers or fiber nuclei (8, 54). The prudence of administration of IGF-I to aging subjects is controversial (34, 81), since IGF-I is a strong mitogen for virtually all cell types and, consequently, is associated with development of several age-associated types of cancer (prostate, breast, colorectal, and lung). Overexpression of the IGF-1R in skeletal myoblasts resulted in a transformed phenotype in appropriate conditions and in the presence of high levels of IGF-I (55, 61, 62). Several studies have indicated that IGF-1R mRNA expression and/or receptor density are decreased in aging skeletal muscle fibers (45, 48) or that aging muscles fail to upregulate IGF-1R mRNA after overload (57). The findings reported in this study suggest that enhancing IGF signaling or IGF-1R expression specifically in aging muscle fibers may constitute a strategy to inhibit sarcopenia without inducing proliferation of any cell type, including satellite cells. Our findings suggest that muscle-specific IGF-1R overexpression could effect enhanced IGF signaling in the presence of low levels of IGF ligand similar to those in aging subjects.

In summary, this study describes establishment of a retroviral expression vector and cell culture system that, by muscle fiber-specific overexpression of the IGF-1R (rather than the IGF ligand), can be utilized to separate the effects of IGF on myogenic precursor cells from those on differentiated muscle cells. Analysis of this system provided the first rigorous test of the idea that IGF-I can have myoblast-independent actions on skeletal muscle protein dynamics and muscle hypertrophy. Additionally, this system was utilized to determine that PI3K signaling mediates myoblast-independent myotube hypertrophy. Further work is necessary to extend these studies to the in vivo situation. In the long term, muscle-specific overexpression of the IGF-1R or stimulation of its signaling pathways could be used to develop strategies to ameliorate muscle wasting or age-associated sarcopenia without stimulating proliferative or differentiation pathways leading to carcinogenesis or other pathological sequelae. -->

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