GSK-3β negatively regulates skeletal myotube hypertrophy

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Vyas, Dharmesh R., Espen E. Spangenburg, Tsghe W. Abraha, Thomas E. Childs, and Frank W. Booth. GSK-3β negatively regulates skeletal myotube hypertrophy. Am J Physiol Cell Physiol 283: C545–C551, 2002. First published April 3, 2002; 10.1152/ajpcell.00049.2002.—To determine whether changes in glycogen synthase kinase-3β (GSK-3β) phosphorylation contribute to muscle hypertrophy, we delineated the effects of GSK-3β activity on C2C12 myotube size. We also examined possible insulin-like growth factor I (IGF-I) signaling of NFAT (nuclear factors of activated T cells)-inducible gene activity and possible modulation of NFAT activation by GSK-3β. Application of IGF-I (250 ng/ml) or LiCl (10 mM) alone (i.e., both inhibit GSK-3β activity) increased the area of C2C12 myotubes by 80 and 85%, respectively. The application of IGF-I (250 ng/ml) elevated GSK-3β phosphorylation and reduced GSK-3β kinase activity by ~800% and ~25%, respectively. LY-294002 (100 μM) and wortmannin (150 μM), specific inhibitors of phosphatidylinositol 3’-kinase, attenuated IGF-I-induced GSK-3β phosphorylation by 67 and 92%, respectively. IGF-I suppressed the kinase activity of GSK-3β. IGF-I (250 ng/ml), but not LiCl (10 mM), induced an increase in NFAT-activated luciferase reporter activity. Cotransfection of a constitutively active GSK-3β (cGSK-3β) inhibited the induction by IGF-I of NFAT-inducible reporter activity. LiCl, which inhibits GSK-3β, removed the block by cGSK-3β on IGF-I-inducible NFAT-responsive reporter gene activity. These data suggest that the IGF-I-induced increase in skeletal myotube size is signaled, in part, through the inhibition of GSK-3β.

IN RESPONSE TO AN INCREASE in functional demand, striated muscle undergoes a phenotypic adaptation characterized by a compensatory increase in mass with an alteration in contractile and metabolic properties of the cell (3). Studies on the molecular mechanisms underlying growth factor-induced skeletal muscle hypertrophy have elucidated various prohypertrophic signaling molecules. However, little is known regarding potential negative regulators of the adaptational circuitry in response to insulin-like growth factor I (IGF-I)-stimulated muscle hypertrophy.

IGF-I is capable of activating many signal transduction pathways; however, recent data suggest that specific activation by IGF-I of the phosphatidylinositol 3’-kinase (PI3’-kinase) pathway significantly contributes to muscle hypertrophy (2). IGF-I binds to the IGF-I receptor with high affinity, and this binding of IGF-I to its specific receptor ultimately leads to activation of the 85-kDa regulatory subunit of PI3’-kinase. Activation of PI3’-kinase induces the activation of protein kinase B (PKB or Akt) by way of phosphorylation of specific serine/threonine residues. Bodine et al. (2) suggested that activation of Akt is a major contributor of muscle hypertrophy process. Furthermore, a downstream target of Akt is a signaling protein termed glycogen synthase kinase-3β (GSK-3β). Akt reduces GSK-3β kinase activity through specific phosphorylation of Ser9 (7). Decreases in activity (2) and increases in phosphorylation (2; unpublished observations) of GSK-3β occur in overloaded skeletal muscle in animals. Although the importance of the inhibition of GSK-3β is not completely understood, it appears that the reduction in GSK-3β kinase activity affects the activity of various transcription factors and global protein synthesis (11). Obviously, if GSK-3β can impact both the regulation of transcription factor activation and protein synthesis, then it is very possible that GSK-3β could play an important role in skeletal muscle hypertrophy.

Recent data have implicated a role for GSK-3β as a negative regulator of cardiac muscle hypertrophy (1, 6, 10). For example, inactivation of GSK-3β (phosphorylation of Ser9) appears to be requisite for transcriptional activation of the atrial natriuretic factor (ANF) gene, an established marker of cardiac hypertrophy (10). Furthermore, the regulation of cardiac hypertrophy-responsive gene expression is mediated via the modulation by GSK-3β of the NFAT (nuclear factors of activated T cells) and GATA family of transcription factors (6). In addition, Rommel et al. (12) found that inactivation of GSK-3β significantly contributed to skeletal muscle hypertrophy.

Recent evidence points to an important role of GSK-3β in the IGF-I-stimulated skeletal muscle hypertrophy through the PI3’-kinase/Akt signaling cascade (12). In the current study, we undertook a more detailed examination of the role of GSK-3β as a nega-
tive regulator of skeletal muscle hypertrophy. We hypothesized that inhibiting GSK-3β activity through IGF-I exposure would increase the activity of an NFAT-inducible reporter gene.

METHODS

Materials. Recombinant human IGF-I was purchased from Austral Biologics (San Ramon, CA). The PI3′-kinase inhibitors LY-294002 and wortmannin were from Calbiochem (San Diego, CA) and Sigma (St. Louis, MO), respectively. All antibodies were purchased from Cell Signaling Technology (Beverly, MA) unless otherwise indicated: phospho-GSK-3β (Ser9) rabbit polyclonal antibody raised against human phospho-GSK-3β Ser9 peptide (1:1,000 dilution); GSK-3β mouse monoclonal antibody raised against amino acids 1–160 of rat GSK-3β (1:2,500 dilution; BD Transduction Laboratories, Lexington, KY); phospho-Akt (Ser473) rabbit polyclonal antibody raised against mouse phospho-Ser473 Akt peptide (1:1,000 dilution); and Akt rabbit polyclonal antibody raised against amino acids 466–479 of mouse Akt (1:300 dilution). pGSK-3β(M authentic) cDNA was a gift from James R. Woodgett (University of Toronto, Canada) (15).

Cell culture. All cell culture experiments were performed by using C2C12 skeletal muscle myoblasts (ATCC), maintained at 37°C and 10% CO2 in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Rockville, MD) supplemented with 1% penicillin-streptomycin antibiotic. Myoblasts were maintained at a subconfluent seeding density in growth medium; DMEM was supplemented with 20% fetal bovine serum (FBS). Differentiation into myotubes was induced by transferring myoblasts into differentiation medium (DM) consisting of DMEM with 2% horse serum. IGF-I and/or LiCl. Cultures were maintained in supplemented or nonsupplemented DM for various indicated time points between 1 min and 5 days.

Myotube staining and area calculation. Myotubes were stained after 5 days in DM by using a modified Wright stain with minor modifications to the manufacturer’s recommendations (Sigma). Briefly, after aspiration of the DM, the culture plates were gently washed twice with ice-cold 1× PBS. Next, the cells were fixed in 10% methanol for 15 min and stained with Wright solution for 2 min. The myotubes were then visualized by using a microscope (Olympus BH-2) and a SPOT Insight imaging color camera (Diagnostic Instruments). The image system was calibrated by using a microometer at each magnification. Myotube area was quantified according to the previously described techniques of Semsarian et al. (13). Medium was changed every 24 h to ensure maintenance of optimal concentrations of IGF-I and LiCl. Cultures were maintained in supplemented or nonsupplemented DM for various indicated time points between 1 min and 5 days.

For cell culture experiments, myoblasts were grown on 100-mm plates and stained with the use of 400 μl of lysis buffer [50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 2 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 10 mM Na4P2O7, 100 mM NaF, 25 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM diethiothreitol (DTT), 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml tosyl-l-phenylalanine chloromethyl ketone, 10 μg/ml Nα-p-tosyl-l-lysine chloromethyl ketone, 0.1 μM okadaic acid, 1 mM benzamidine, and 2 mM Pefabloc SC Plus]. The scraped cells were lysed by rotation for 30 min at 4°C. Protein concentration was determined by standard Bradford assay (Bio-Rad), and equal amounts (25 μg) were fractionated by SDS-PAGE on 10% gel, electrophoretically transferred to a nitrocellulose membrane in transfer buffer containing 25 mM Tris·HCl (pH 8.3), 192 mM glycine, and 20% (vol/vol) methanol. Membranes were stained with Ponceau S (Sigma) to verify equal loading between lanes and subsequently blocked in 5% nonfat dry milk/0.05% (vol/vol) Tween 20 for 1 h at room temperature. The filter was then incubated in primary antibody for 12 h at 4°C and washed six times (5 min each) with TBS-T (25 mM Tris-base, pH 8.3, 150 mM NaCl, and 0.05% Tween 20). Next, the filter was incubated (21°C, 1 h) in 1:8,000 dilution of a 1 mg/ml donkey antirabbit IgG horseradish peroxidase-conjugated secondary antibody (Amersham) and washed with TBS-T. Immunocomplexes were treated with the enhanced chemiluminescence reagent (ECL; NEN Life Science Products) and visualized by autoradiography.

GSK-3β immune complex kinase assay. GSK-3β activity was measured by using 100 μg of total cellular protein prepared as described in Immunoblotting analysis. The extract was diluted to 400 μl with fresh cell lysis buffer and immunoprecipitated with 1 μg of the monoclonal anti-GSK-3β antibody by rotation for 2 h at 4°C. The immune complexes were isolated by the addition of 25 μl of a 50% slurry of protein G-Sepharose and incubation for 1.5 h. Im-

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munoprecipitates were washed four times in lysis buffer and twice in kinase reaction buffer (8 mM MOPS, pH 7.4, 0.2 mM EDTA, 10 mM magnesium acetate, 1 mM Na4VO4, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml aprotonin, 0.1 mM okadaic acid, and 2 mM Pefabloc SC Plus). Kinase assays were performed in 40 µl of total reaction buffer containing 62.5 µM phospho-glycogen synthase peptide-2 (Upstate Biotechnology), 20 mM MgCl2, 125 µM ATP, and 10 µCi [γ-32P]ATP. The reaction mixture was allowed to proceed for 30 min at 30°C with shaking, and 25 µl of the supernatant were spotted onto Whatmann P81 phosphocellulose paper. The filters were washed five times for 5 min each in 0.75% phosphoric acid. Next, the filters were briefly rinsed in acetone, dried at room temperature, and subjected to liquid scintillation counting. A sample including all assay components except the immunoprecipitate was included in each experiment as a background control.

**RESULTS**

**Inhibitors of active GSK-3β produce hypertrophy of C2C12 myotubes.** When LiCl, an inhibitor of GSK-3β activity, was included in the culture medium, the area of C2C12 myotubes was 85% greater (Fig. 1). Inclusion of IGF-I (250 ng/ml), instead of LiCl, in the incubation medium resulted in a similar increase in myocyte area (80%). The IGF-I-induced increase in myocyte area confirmed the finding of Rommel et al. (12).

**IGF-I produces a transient phosphorylation of GSK-3β in C2C12 myotubes.** GSK-3β phosphorylation was increased 732, 908, 914, and 1,312% after exposure to IGF-I (250 ng/ml) for 15 min, 30 min, 1 h, and 12 h, respectively, in C2C12 myotubes (Fig. 2A). However, after 24 h of IGF-I exposure, GSK-3β phosphorylation returned to baseline levels. These observations extend the single time point (15 min) reported by Rommel et al. (12) and show that the GSK-3β phosphorylation effect is transient.

**IGF-I suppresses GSK-3β activity.** GSK-3β kinase activity in C2C12 myotubes was decreased by 12–33% at 1 min, 15 min, 30 min, 1 h, 12 h, and 24 h after IGF-I was added to the incubation medium (Fig. 2B).

**IGF-I increases Akt phosphorylation in C2C12 myotubes.** Two-, four-, three-, and onefold increases in Akt phosphorylation (Ser473) were detected after C2C12 myotubes were exposed to IGF-I (250 ng/ml) for 15 min, 30 min, 1 h, and 12 h, respectively (Fig. 3A). After 24 h of IGF-I exposure, Akt phosphorylation returned to baseline levels. These observations extend the single time point (15 min) reported by Rommel et al. (12) and show that the Akt phosphorylation effect is transient. In addition, PI3'-kinase inhibitors decreased Akt phosphorylation: LY-294002 (100 µM), an inhibitor of PI3'-kinase, suppressed IGF-I-induced Akt phosphorylation by 54% in myotubes (Fig. 3B), and wortmannin (150 µM), a different inhibitor of PI3'-kinase, inhibited Akt by 56% in the presence of IGF-I.

**PI3'-kinase inhibitors block GSK-3β phosphorylation.** LY-294002 (100 µM), an inhibitor of PI3'-kinase, suppressed IGF-I-induced GSK-3β phosphorylation by 67% in myotubes (Fig. 3C), and wortmannin (150 µM), a different inhibitor of PI3'-kinase, inhibited GSK-3β phosphorylation by 92% in the presence of IGF-I. These results confirm the findings of Rommel et al. (12). Constitutively active GSK-3β decreases NFAT transcripational activity. As a biological probe for the presence of nuclear NFAT, a promoter containing multiple NFAT binding sites driving a firefly luciferase reporter gene, was transfected into myoblasts. To normalize for transfection efficiencies, pRL-null Renilla luciferase was cotransfected (values ranged from 737

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**Fig. 1.** Top: increases in C2C12 myotube area after insulin-like growth factor (IGF-I; 250 ng/ml) or LiCl (10 mM) exposure. *Statistical difference from control myotubes at the P < 0.05 level. Values are means ± SE; n = 3 independent culture dish experiments performed on 3 different days. Bottom: representative examples of myotubes depicted after 5 days of exposure to either IGF-I or LiCl in differentiation medium. Arrows indicate C2C12 myotubes.
to 1,221 relative light units across all conditions). Whereas LiCl had no effect on NFAT-inducible luciferase activity, IGF-I increased luciferase activity by 145% in C2C12 myotubes (Fig. 4). However, the inclusion of a constitutively active GSK-3β (cGSK-3βA9; Ser9 mutated to an alanine) with IGF-I reversed the enhancement by IGF-I of NFAT reporter gene activity (7, 11). We therefore pursued an experiment to test whether the IGF-I-induced reduction of GSK-3β kinase activity would be associated with an increase in NFAT-responsive gene activity. The NFAT-luciferase reporter plasmid selected for these experiments contained four direct repeats of the NFAT binding site when the medium was supplemented with LiCl compared with the NFAT-only condition. A 190% increase in luciferase activity occurred when myoblasts transfected with the constitutively active pGSK-3βA9 were exposed to medium supplemented with both IGF-I and LiCl.

**DISCUSSION**

The purpose of this study was to determine whether GSK-3β plays a role in skeletal myotube hypertrophy. Of key significance was the initial finding that noncompetitive inhibition of GSK-3β by LiCl was associated with an 85% increase in myotube surface area in culture without an increase in NFAT reporter gene activity. The observation showing an increase in muscle cell size associated with GSK-3β inhibition confirms the recent results of Rommel et al. (12), who used a dominant-negative GSK-3β cDNA to produce profound C2C12 myotube hypertrophy. Our findings strongly suggest that GSK-3β inhibition, or another pathway activated and/or inhibited by LiCl, may be involved in increasing myotube size. LiCl has been widely used to assess the functional role of this enzyme in various contexts (16). Although the nonspecific effects of LiCl are limited, recent evidence does suggest that it may act as an inhibitor of casein kinase-2, p38 kinase, and mitogen-activated protein kinase kinase-2 (5). However, the LiCl-associated increase in myotube area and mitogen-activated protein kinase kinase-2 (5). However, the LiCl-associated increase in myotube area and other corroborative reports in the literature (6, 12) favor a prohypertrophic mechanism for LiCl via inhibition of GSK-3β in skeletal myotubes.

These skeletal muscle findings are similar to a recent finding for a critical role for GSK-3β as a negative regulator of cardiac hypertrophy in culture and in whole animals (1, 6, 10). For example, Haq et al. (6) observed that exposure of neonatal cardiomyocytes to LiCl reversed the inhibitory effects of cGSK-3β (GSK-3βA9 mutant) on protein synthesis and sarcomere organization. Also, in cardiac tissue, expression of cGSK-3βA9 attenuated the myocardial hypertrophy induced by the expression of a constitutively active calcineurin (1). Therefore, GSK-3β may have a role in hypertrophy of both cardiac and skeletal muscle.

An alternative approach to inhibit GSK-3β activity is to apply IGF-I to muscle cells (12). IGF-I signals the phosphorylation of Akt, which in turn inactivates GSK-3β by phosphorylation of Ser9 (10). IGF-I has been shown to produce myotube hypertrophy and increase GSK-3β phosphorylation (12). In other cell types the phosphorylation of GSK-3β by IGF-I decreases GSK-3β activity (7, 11). We therefore pursued an experiment to test whether the IGF-I-induced reduction of GSK-3β kinase activity would be associated with an increase in NFAT-responsive gene activity. The NFAT-luciferase reporter plasmid selected for these experiments contained four direct repeats of the NFAT binding sequence (−286 to −257) from the human interleukin-2 (IL-2) gene. As expected, IGF-I increased the NFAT-responsive reporter gene activity by 145% in...
C2C12 myotubes (Fig. 4). Moreover, the increase in NFAT-driven reporter gene was blocked by the simultaneous overexpression of cGSK-3βA9. Unexpectedly though, LiCl, a noncompetitive inhibitor of GSK-3β, did not enhance NFAT-inducible luciferase activity but did increase C2C12 myotube area. We interpreted the latter result to suggest that LiCl increases myotube area independently of an enhancement of NFAT-inducible transcriptional activity. Whereas cGSK-3β blocked the stimulation by IGF-I of NFAT-responsive luciferase activity, addition of LiCl to this mixture to noncompetitively inhibit GSK-3β rescued the stimulation by IGF-I of NFAT-reporter gene activity to a value seen with IGF-I alone. Because LiCl alone did not increase NFAT-responsive reporter gene activity, we infer that IGF-I in the presence of LiCl is able to enhance this NFAT-inducible marker by a GSK-3β-independent mechanism (Fig. 5).

GSK-3β has been shown to have multiple cellular targets. For example, Harwood (7) recently described GSK-3β as a “vital regulatory kinase with a plethora of significant cellular targets, some of which include cytoskeletal and transcription factor proteins.” GSK-3β is also known to effect protein synthesis by altering the phosphorylation status of the α-subunit of the eukaryotic initiation factor eIF-2. More specifically, phosphorylation of Ser540 on eIF-2α by GSK-3β results in inhibition of the GDP/GTP exchange activity of eIF-2 and an overall reduction in protein synthesis (11). Therefore, the effects of inhibition of GSK-3β kinase activity could most likely be multifactorial with regard to skeletal muscle hypertrophy.

Our current results regarding the effect of IGF-I on NFAT transcriptional activity differ from those recently published by Rommel et al. (12). They reported that the addition of 10 ng/ml IGF-I in the culture medium of C2C12 myotubes, 48 h postdifferentiation, resulted in a cytoplasmic localization of inactivated C549

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**Fig. 3.** A: increases in Akt phosphorylation in C2C12 myotubes after exposure to IGF-I (250 ng/ml) for various times. Phosphorylation status of Akt is expressed as the amount of phosphorylated Akt (Ser473) over the total amount of Akt. Representative examples of immunoblots for Akt (Ser473 and total) are depicted at bottom. Absence of the horizontal bar labeled “IGF-I” indicates that no IGF-I is present. *Statistical difference from control at the P < 0.05 level. B: inhibition of Akt phosphorylation with pharmacological inhibitors of phosphatidylinositol 3'-kinase (PI3'-kinase) activity in C2C12 myotubes after exposure to IGF-I (250 ng/ml). Akt expression was calculated in the same manner as described in A. Representative Akt (Ser473 and total) immunoblots are depicted at bottom. C, control (myotubes not receiving any inhibitors); LY, myotubes receiving LY-294002 (100 μM); W, myotubes receiving wortmannin (150 μM). *Statistical difference from control at the P < 0.05 level; †statistical difference from IGF-I at the P < 0.05 level. In A–C, values are means ± SE; n = 3 independent culture dish experiments performed on 3 different days.
NFATc1. In contrast, we observed an increase in NFAT-induced transcriptional activity following the addition of 250 ng/ml IGF-I to C2C12 myoblasts, concurrent with cell transfer into DM. Our observation thus agrees with the findings of Semsarian et al. (14).

While the reasons for this discrepancy between our report and that of Rommel et al. (12) remain unclear, plausible explanations point to the differences in IGF-I concentration and time point of growth factor exposure. Semsarian and colleagues (13, 14) observed...
C2C12 hypertrophy following the addition of 250 ng/ml IGF-I to the cell medium before or at the time of myoblast differentiation into postmitotic skeletal myotubes. Importantly, myotube hypertrophy was not noted if IGF-I was introduced at a concentration of 25 ng/ml or at additional time points beyond 24 h postdifferentiation (13). Therefore, although our methods mimicked those of Semsarian et al., who found myotube hypertrophy and NFATc1 accumulation in the nucleus at our chosen IGF-I concentration (250 ng/ml), the study of Rommel et al. (12) employed an IGF-I dosage (10 ng/ml) and time point of exposure previously shown by Semsarian et al. (13) to be ineffective in producing myotube hypertrophy. Furthermore, the NFAT-luciferase reporter assay system used in our study allows the direct measure of the transcriptional activity of various activated NFAT isoproteins. Crabtree (4) indicates that, because of the unusual NFAT-DNA binding domain, NFAT exhibits weak DNA binding and requires an unknown nuclear partner for tight association with DNA. Therefore, our utilization of an NFAT-driven reporter construct allows a direct biological measure of nuclear NFAT-inducible activity by IGF-I. In addition, Rommel et al. (12) reported the phosphorylation status and the cytoplasmic or nuclear location of only one isoform, NFATC1. There is recent evidence indicating that NFAT isoforms NFATc2 (8) and NFATc3 (9) play important roles in the control of skeletal muscle size and myogenesis, respectively, which strongly suggests that NFATc1 is not the only member of this family of transcription factors involved in the regulation of myotube hypertrophy. We therefore conclude that IGF-I increases NFAT-inducible activity.

To determine how IGF-I signals GSK-3β phosphorylation in our cells, further experiments were performed in C2C12 myotubes. Rommel et al. (12) suggested that IGF-I-induced phosphorylation of GSK-3β occurs due to activation of the PI3'-kinase/Akt pathway. Here, we confirm and extend their report of increased GSK-3β phosphorylation by our observation that the kinase activity of GSK-3β is also inhibited by IGF-I (Fig. 3B). Taken together, these observations strongly suggest that PI3'-kinase and Akt are upstream mediators of the IGF-I-mediated phosphorylation of GSK-3β in skeletal myotubes.

In summary, our results indicate that phosphorylation-mediated inhibition of GSK-3β via the PI3'-kinase/Akt cascade could play an important regulatory role in skeletal myotube hypertrophy. Noncompetitive deactivation of GSK-3β by LiCl resulted in a marked increase in skeletal myotube area without activating NFAT-inducible luciferase activity. The IGF-I-increased NFAT-inducible reporter gene activity was prevented by constitutive activation of GSK-3β. However, NFAT reporter gene activity was increased when IGF-I was placed together with LiCl and a constitutively active GSK-3β. These data show that IGF-I can produce increases in NFAT-inducible transcriptional activity independently of GSK-3β. Taken together, these data suggest that a dose of 250 ng/ml IGF-I does, in fact, induce NFAT transcriptional activity and that this increase in transcriptional activity can be attenuated by GSK-3β. Finally, our data and that of Rommel et al. (12) clearly indicate that GSK-3β can play an important regulatory role in modulating skeletal myotube size.

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REFERENCES


