Mechanical Regulation of Glycogen Synthase Kinase 3β (GSK3β) in Mesenchymal Stem Cells Is Dependent on Akt Protein Serine 473 Phosphorylation via mTORC2 Protein*

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Mechanical signals can inactivate glycogen synthase kinase 3β (GSK3β), resulting in stabilization of β-catenin. This signaling cascade is necessary for the inhibition of adipogenesis in mesenchymal stem cells (MSC) that is produced by a daily strain regimen. We investigated whether Akt is the mechanically activated kinase responsible for phosphorylation and inactivation of GSK3β in MSC. Mechanical strain (2% magnitude, 0.17 Hz) induced phosphorylation of Akt at Ser-473 and Thr-308 in parallel with phosphorylation of GSK3β at Ser-9. Inhibiting Akt (Akt1/2 kinase inhibitor treatment or Akt knockdown) prevented strain-induced phosphorylation of GSK3β at Ser-9. Inhibition of PI3K prevented Thr-308 phosphorylation, but strain-induced Ser-473 phosphorylation was measurable and induced phosphorylation of GSK3β, suggesting that Ser-473 phosphorylation is sufficient for the downstream mecanorresponse. As Rictor/mTORC2 (mammalian target of rapamycin complex 2) is known to transduce phosphorylation of Akt at Ser-473 by insulin, we investigated whether it contributes to strain-induced Ser-473 phosphorylation. Phosphorylation of Ser-473 by both mechanical and insulin treatment in MSC was prevented by the mTOR inhibitor KU0063794. When mTORC2 was blocked, mechanical GSK3β inactivation was prevented, whereas insulin inhibition of GSK3β was still measured in the absence of Ser-473 phosphorylation, presumably through phosphorylation of Akt at Thr-308. In sum, mechanical input initiates a signaling cascade that is uniquely dependent on mTORC2 activation and phosphorylation of Akt at Ser-473, an effect sufficient to cause inactivation of GSK3β. Thus, mechanical regulation of GSK3β downstream of Akt is dependent on phosphorylation of Akt at Ser-473 in a manner distinct from that of growth factors. As such, Akt reveals itself to be a pleiotropic signaling molecule whose downstream targets are differentially regulated depending upon the nature of the activating input.

The mesenchymal stem cell (MSC)3 pool resident in bone marrow serves as a critical repository for lineages that support bone remodeling throughout life. A multitude of environmental factors, including hormones, growth factors, and biophysical stimuli, influence MSC lineage allocation, leading to an inversely proportional commitment of MSC between adipogenic and osteoblastic lineages (1). In rodents, exercise regimens have been shown to reduce marrow adiposity and increase the number of committed osteoprogenitors (2, 3). In vitro, mechanical stimulation directly restrains adipogenic differentiation of mesenchymal progenitors (4, 5). Understanding the mechanical signaling pathways critical to this unique anti-adipogenic response should help define new strategies for preserving MSC potential.

Inhibition of MSC adipogenesis is mediated by activation of β-catenin. In contradistinction to Wnt signaling, where β-catenin is protected from degradation through LRP5/6 (LDL receptor-related protein 5/6)-mediated sequestration of glycogen synthase kinase 3β (GSK3β) (6), mechanical preservation of β-catenin occurs downstream of GSK3β inactivation through phosphorylation at Ser-9, which prevents GSK3β targeting of β-catenin for degradation (7, 8). The mechanical effect on GSK3β is independent of signaling through LRP5/6 (9, 10). Along with GSK3β phosphorylation, mechanical input also activates Akt/protein kinase B. Although Akt is known to transduce insulin inactivation of GSK3β (11), an upstream role for Akt in mechanical regulation of GSK3β has not been proven.

Akt signaling influences a broad range of cellular functions (12). As such, Akt can be activated by a diverse set of stimuli, including growth factors, cytokines, and mechanical signals. The Akt protein contains three domains, an N-terminal pleckstrin homology domain, a central kinase domain, and a C-terminal regulatory domain that contains a hydrophobic motif (13). Full activation of Akt is a multistep process that requires recruitment of Akt to the plasma membrane via its pleckstrin homology domain coupled with phosphorylation of two key sites, Thr-308 in the kinase domain and Ser-473 in the hydrophobic motif (14). Membrane recruitment of Akt is dependent on the PI3K second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3). PKCθ (3-phosphoinositide-dependent kinase-1) localized at the membrane in response to PIP3 synthesis then phosphorylates Akt at Thr-308. The kinase responsible for Ser-473 phosphorylation appears to be cell- and process-specific. Integrin-linked kinase (ILK) can modulate Akt activation (15, 16), and its association with integrins, which are necessary to perception of the physical environment (17), suggests that it could be regulated by mechanical stimuli. PKCθ, which can be activated by mechanical signals (18, 19), has been

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1 The abbreviations used are: MSC, mesenchymal stem cell(s); GSK3β, glycogen synthase kinase 3β; PIP3, phosphatidylinositol 3,4,5-trisphosphate; ILK, integrin-linked kinase; mdMSC, marrow-derived MSC; Akti-1/2, Akt1/2 kinase inhibitor.
implicated in the regulation of Akt activity by growth factors (20). Recently, mTORC2 (mammalian target of rapamycin complex 2) was shown to have a unique role in Akt Ser-473 phosphorylation in response to insulin stimulation (21), an effect that appears to be consequent to PI3K activation, causing Akt relocation to the plasma membrane (22).

In this work, we used undifferentiated marrow-derived MSC (mdMSC) to demonstrate that mechanical inhibition of GSK3β is dependent on activation of Akt through Ser-473 phosphorylation. Mechanical strain-dependent phosphorylation of Akt at Ser-473 required both PKC and mTORC2 but was independent of signaling through either PI3K or ILK. Furthermore, the dependence of GSK3β inhibition on Akt Ser-473 phosphorylation downstream of mTORC2 was found to be specific to the mechanical effect, as insulin could inactivate GSK3β despite disruption of Akt Ser-473 phosphorylation. These differences indicate that mechanical inhibition of GSK3β downstream of Akt activation is distinct from inhibition by insulin and suggest how unique cellular responses can be regulated through a common kinase.

EXPERIMENTAL PROCEDURES

Reagents—FBS was obtained from HyClone (Logan, UT). Culture medium, glutamine, and antibiotics were purchased from Invitrogen. LY294002, Akt1/2 kinase inhibitor (Akti-1/2), and rapamycin were obtained from Sigma-Aldrich. KU0063794 was obtained from Selleck Chemicals LLC (Houston, TX). Calphostin C was purchased from Tocris Bioscience (Ellisville, MO), and Gö6976 was from Calbiochem. The siRNA transfection reagent PepMute Plus was purchased from SignaGen Laboratories (Ijamsville, MD).

Cell Culture—mdMSC were generated from C57BL/6 wild-type mice using the procedure of Peister et al. (23). mdMSC were then plated at 3000 cells/cm² in Iscove’s modified Dulbecco’s medium containing 10% FBS and 100 μg/ml penicillin/streptomycin for expansion from passages 5 to 15. For experiments, mdMSC were seeded at 5000–10,000 cells/cm² in growth medium (α-minimal essential medium, 10% FBS, and antibiotics). The following pharmacologic agents were used: the Akt inhibitor Akti-1/2 (40 μM), the PI3K inhibitor LY294002 (50 μM), the PKC inhibitors calphostin C (1 μM) and Gö6976 (0.1–2.5 μM), and the mTOR inhibitors KU0063794 (2 μM) and rapamycin (30 nm). Akti-1/2, also known as Akt inhibitor VIII, is a pleckstrin homology domain-dependent inhibitor that is selective for Akt isoforms 1 and 2. Each agent or its appropriate vehicle was added to cultures 1 h prior to strain initiation or insulin addition and remained in the culture medium throughout the experiment. For experiments using calphostin C, cells were exposed to 1 h of light following addition of this agent. For experiments using LY294002, KU006379, or rapamycin, growth medium was replaced with serum-free medium for 4 h prior to addition of the agent.

Transient Transfection with siRNA—siRNAs targeting murine ILK and Akt were purchased from Invitrogen. mdMSC were transfected with specific siRNA or a control siRNA (scrambled siRNA) at a concentration of 20 nM using the PepMute Plus reagent in growth medium for 6–18 h, followed by replacement with fresh growth medium. Experiments were initiated 72 h after transfection.

Mechanical Strain—mdMSC were plated on 6-well Bioflex collagen I-coated plates (Flexcell International Corp., Hillsborough, NC). Uniform biaxial strain was applied (2% magnitude, 0.17 Hz) using the Flexcell FX-4000 system.

Western Blotting—Whole cell lysates were prepared as described previously (4, 7), and protein (5–20 μg) was separated on a polyacrylamide gel and then transferred to PVDF membrane. The following antibodies were used: GSK3β (Chemicon, Billerica, MA) and phospho-GSK3β Ser-9, phospho-Akt Ser-473, phospho-Akt Thr-308, Akt, and ILK1 (Cell Signaling, Danvers, MA). Horseradish peroxidase-conjugated secondary antibody was detected by chemiluminescence. Images were acquired with a Hewlett-Packard Scanjet, and densitometry was determined using NIH ImageJ 1.37v.

Statistical Analysis—Results are expressed as the mean ± S.E. Significance was determined by Student’s t test or two-way analysis of variance where appropriate (GraphPad Prism). All experiments were replicated at least once. Densitometry data were compiled from three separate experiments.

RESULTS

Mechanical Strain Induces Rapid Activation of Akt in mdMSC—Mechanical regulation of Akt and GSK3β was evaluated in undifferentiated mdMSC. Phosphorylation of Akt at two key sites, Thr-308 and Ser-473, consistent with enhanced activation, was measured 30 min after beginning strain (Fig. 1A). Akt phosphorylation returned to basal levels by 2 h despite continued mechanical input. Phosphorylation of GSK3β at Ser-9, which causes its inactivation and was shown to be a necessary step for mechanical activation of β-catenin (10), increased during strain application in parallel with changes in phosphorylated Akt. To confirm the strain-induced changes in Akt and GSK3β phosphorylation, densitometry was performed in a series of experiments after 45 min of strain application. Strain significantly increased phosphorylation of Akt at Ser-473 to 218 ± 17% above the unstrained control level and that of GSK3β at Ser-9 to 161 ± 12% above the control level (Fig. 1B).

We next queried whether insulin stimulation would promote activation of Akt and consequent GSK3β inhibition in mdMSC similar to accepted insulin-responsive tissues (11, 24). Insulin increased phosphorylation of Akt at both Thr-308 and Ser-473, as well as phosphorylation of GSK3β at Ser-9, at 30 min (Fig. 1C). Contrasting with the mechanical response, Akt and GSK3β phosphorylation was more intensely activated by insulin and remained elevated at 2 h of insulin treatment.

Mechanical Inhibition of GSK3β Is Dependent on Akt Activation—To demonstrate a role for Akt in mechanical inactivation of GSK3β, mdMSC were treated with the allosteric inhibitor Akti-1/2. Akti-1/2 (40 μM) decreased basal phosphorylation of Akt (Fig. 2A). Mechanical phosphorylation of Akt was disrupted in the presence of Akti-1/2, and, importantly, phosphorylation of GSK3β at Ser-9 in response to strain also did not occur. Densitometry showed that strain significantly increased phospho-GSK3β/total GSK3β to 153 ± 15% of the unstrained control level in vehicle-treated mdMSC (Fig. 2B) but did not
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**FIGURE 1.** Mechanical strain induces rapid phosphorylation of Akt. A, mdMSC were subjected to strain for 0.5–2 h, and cellular proteins were immunoblotted for phosphorylated Akt and GSK3β. B, densitometric analysis of phosphorylated Akt (Ser-473) normalized to total Akt and phosphorylated GSK3β (Ser-9) normalized to total GSK3β (n = six experiments) for mdMSC subjected to strain for 45 min. *, significant difference from the unstrained control (p < 0.001). C, immunoblots of cultures treated with insulin (100 nM) for 0.5–2 h. Densitometry showed that strain applied for 60 min increased phospho-Akt Ser-473/total Akt to 197 ± 23 and 206 ± 44% of the unstrained control level in mdMSC treated with scrambled siRNA (siSCR) or siRNA targeting Akt (siAkt) and then cultured for 3 days. 

Mechanical inhibition of GSK3β does not require PI3K

**FIGURE 2.** Mechanical inhibition of GSK3β is dependent on Akt activation. A, immunoblots of mdMSC subjected to strain for 45 min following treatment with the Akt inhibitor Akti-1/2 (40 μM). B, densitometric analysis of phosphorylated GSK3β (Ser-9) normalized to total GSK3β (n = four experiments). *, significant difference from the unstrained control (p < 0.05). C, immunoblots of total cellular proteins from mdMSC transfected with nonsense siRNA (siSCR) or siRNA targeting Akt (siAkt) and then cultured for 3 days before application of strain for 45 min. D, densitometric analysis of phosphorylated GSK3β (Ser-9) normalized to total GSK3β (n = three experiments). *, significant difference from the unstrained control (p < 0.01).

Significantly change the level in Akti-1/2-treated cells (69 ± 20% of the control level).

To confirm the critical function of Akt, the protein was knocked down using siRNA (Fig. 2C). In cells where total Akt protein was reduced by 70%, mechanical strain failed to phosphorylate GSK3β. This was verified by densitometry (Fig. 2D).

**Strain-dependent Phosphorylation of GSK3β in mdMSC.** Does Not Require PI3K—PI3K mediates insulin-induced phosphorylation of Akt by inducing its membrane recruitment via the second messenger PIP3, in contrast, inhibition of PI3K does not prevent mechanical inactivation of GSK3β in osteoblasts (7). The PI3K inhibitor LY294002 (50 μM) reduced basal levels of phosphorylated Akt in mdMSC, leaving total Akt unchanged (Fig. 3A). LY294002 prevented strain-induced phosphorylation of Akt at Thr-308 but not at Ser-473. Importantly, mechanical inactivation of GSK3β was unaffected by PI3K inhibition. This indicates that phosphorylation of Akt at Ser-473 is sufficient for downstream mechanical effects. In contrast, phosphorylation of Akt at both Ser-473 and Thr-308 in response to insulin stimulation was blocked in the presence of LY294002 (Fig. 3B), reflecting the known dependence of Ser-473 phosphorylation on PI3K (22) and distinguishing growth factor-induced from mechanically induced signaling paradigms.

**ILK Does Not Contribute to Akt Activation by Strain**—As the phosphorylation of Akt at Ser-473 was found to be of primary importance for mechanical inhibition of GSK3β, ILK was considered as a candidate kinase (16). Knockdown of ILK by siRNA (>60% at 72 h) did not prevent mechanical activation of Akt or downstream GSK3β phosphorylation in mdMSC (Fig. 4A). Densitometry showed that strain applied for 60 min increased phospho-Akt Ser-473/total Akt to 197 ± 23 and 206 ± 44% of the unstrained control level in mdMSC treated with scrambled and ILK siRNAs, respectively, and increased phospho-GSK3β/total GSK3β to 221 ± 54 and 254 ± 11% of the control level in scrambled and ILK siRNA-transfected cells, respectively (Fig. 4B). Similarly, ILK was not necessary for an insulin response. Addition of insulin increased phosphorylation of Akt and GSK3β in mdMSC transfected with siRNA to ILK (data not shown).

**PKC Mediates Akt Phosphorylation at Ser-473**—PKC has been shown to influence Akt activation by growth factors (20), and mechanical stimulation activates specific PKC isoforms (18). The global PKC inhibitor calphostin C was used to evaluate a role for PKC in strain activation of Akt in mdMSC. Treatment with light-activated calphostin C (1 μM) reduced basal Ser-473 phosphorylation, whereas total Akt was unchanged (Fig. 5A). Calphostin C disrupted strain-induced phosphorylation of Akt at Ser-473 but not at Thr-308. Importantly, mechanical inhibition of GSK3β was blocked by calphostin C treatment, as verified by densitometry (Fig. 5B), confirming that Ser-473 of Akt is essential for mechanical inhibition of GSK3β.
Strain significantly increased phospho-GSK3β/total GSK3β to 164 ± 4% of the unstrained control level in vehicle-treated mdMSC but did not significantly change the level in calphostin C-treated cells (79 ± 10% of the control level).

The inhibitor Gö6976 was used to specifically inactivate conventional PKC isoforms. Phosphorylation of both Akt and GSK3β was increased by mechanical strain in the presence of Gö6976 (Fig. 5C) at all concentrations (0.1–2.5 μM) tested, suggesting that a novel or atypical PKC isoform, rather than a conventional isoform, is involved in mechanically induced phosphorylation of Akt at Ser-473.

mTORC2 Is Required for Mechanical Activation of Akt—Rictor/mTORC2 is known to specifically transduce insulin-induced Akt phosphorylation at Ser-473 (21). Here, the mTOR inhibitor KU0063794 (2 μM) reduced basal levels of phospho-Akt in mdMSC, leaving total Akt unchanged (Fig. 6A). KU0063794 prevented strain-induced phosphorylation of Akt at both Ser-473 and Thr-308. Importantly, mechanical inactivation of GSK3β was disrupted with mTOR inhibition, as confirmed by densitometry (Fig. 6B). Strain significantly increased phospho-GSK3β/total GSK3β to 147 ± 12% of the unstrained control level in vehicle-treated mdMSC but did not significantly change the level in KU0063794-treated cells (94 ± 6% of the control level). To distinguish between possible contributions of Raptor/mTORC1 and Rictor/mTORC2 to strain-induced phosphorylation of Akt, mdMSC were treated acutely with rapamycin (30 nM) to specifically inhibit Raptor/mTORC1. Both mechanical activation of Akt and downstream GSK3β phosphorylation occurred in the presence of rapamycin (Fig. 6C), underlining the importance of the Ser-473 activation site for the mechanical effect.

mTOR inhibition with KU0063794 disrupted insulin-induced phosphorylation of Akt at Ser-473 (Fig. 6D), but an increase in Akt phosphorylation at Thr-308 did occur, although it was less than the insulin-induced increase when the mTOR inhibitor was absent. In contrast to disruption of strain-induced phosphorylation of GSK3β by KU0063794, insulin inactivation of GSK3β was unaffected by mTOR inhibition, presumably due to a predominant effect of Thr-308 phosphorylation. Insulin-induced changes in Akt and GSK3β phosphorylation also pro-
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DISCUSSION

Akt is known to be a critical signaling molecule, in part because of its involvement in multiple diseases, as well as its role in transducing diverse incoming information, including growth factors, cytokines, and biophysical stimuli (12). Mechanical activation of Akt has been associated with increased proliferation in multiple cell models (25, 26). Here, we have shown that Akt is subject to mechanical control in undifferentiated mdMSC and acts as the definitive upstream regulator of mechanical inhibition of GSK3β. Mechanical regulation of Akt in mdMSC was uniquely dependent on transient phosphorylation of Ser-473 downstream of mTORC2, which was sufficient to cause GSK3β inactivation. As GSK3β is a control node for adipogenic differentiation of MSC (10), an understanding of this mechanical signaling pathway may suggest new strategies for controlling adipogenesis and preserving MSC potential for higher order tissues.

Although application of mechanical strain led to a transient phosphorylation of Akt at both Thr-308 and Ser-473, it was determined that mechanically induced phosphorylation of these two sites was separable and could be independently regulated. Thus, phosphorylation of Ser-473 did not require concurrent phosphorylation of Thr-308 by PI3K, as reported previously for Akt activation by growth factors (27, 28). Although maximal Akt activation requires phosphorylation of both sites (24), recent studies demonstrate that phosphorylation of Thr-308 enables sufficient activation of Akt to allow it to modulate a subset of its downstream targets, including GSK3β (27, 29). In mdMSC, mechanical inactivation of GSK3β was dependent primarily on strain-induced phosphorylation of Ser-473. Conversely, insulin-induced phosphorylation of Akt at Thr-308 was sufficient to phosphorylate GSK3β when phosphorylation at Ser-473 was prevented by treatment with the mTOR inhibitor KU0063794. Interestingly, the basal GSK3β Ser-9 level was insensitive to conditions that caused a marked reduction in the basal phospho-Akt Ser-473 level, such as treatment with Akti-1/2 or calphostin C. This suggests that other kinases are involved in regulating basal phosphorylation of GSK3β in mdMSC; indeed, protein kinase A and p70 ribosomal S6 kinase have been suggested to phosphorylate Ser-9 of GSK3β (30, 31).

In contrast to the well described phosphorylation of Akt at Thr-308 by PDK1, multiple kinases have been implicated in regulation of Ser-473 (13). ILK, considered a candidate due to its association with integrins (32) and its ability to enhance Akt activation via Ser-473 (15, 16), did not play a role in the mecha-
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noresponse studied here. Rather, we found that mTORC2 was responsible for strain-induced Ser-473 phosphorylation. This novel finding is one of the first showing independent activation of mTORC2 (22) and adds to the repertoire of mechanical targets. That KU0063794 blocked mechanical phosphorylation at Thr-308 contrasts with effects in an mTORC2 knock-out mouse model, where Thr-308 phosphorylation was unaffected (27). However, our result is consistent with a prior report demonstrating that pharmacologic inhibition of mTORC2 function did disrupt Akt Thr-308 phosphorylation (33), confirming a cooperativity between phosphorylation at sites 308 and 473 (34). Because the site at which mTORC2 phosphorylates Akt was shown to be sufficient for downstream GSK3β inactivation, we have established this kinase as a novel and critical regulator of the mechanical response in mdMSC.

The PKC family has been linked to regulation of Akt activity (20), with multiple PKC isoforms involved. In mdMSC, we found that PKC inhibition led to a loss of Akt Ser-473 phosphorylation by strain, suggesting that PKC could be a necessary cofactor for activation of mTORC2 in mdMSC. Alternatively, PKC modulation of Ser-473 phosphorylation could occur downstream of mTORC2 activation, as suggested by a recent report on PKCβII effects in 3T3-L1 adipocytes (35).

mdMSC exhibited a robust response to insulin stimulation with respect to Akt and GSK3β phosphorylation. The signaling pathway upstream of these phosphorylation events was consistent with insulin signaling in other cell types, as Akt phosphorylation was dependent on both PI3K activity and mTORC2. Although phosphorylation of Akt at Ser-473 was similarly dependent on mTORC2 in response to strain, the mechanical effect did not require activation of PI3K. A recent finding demonstrated that full-length Akt binds to phosphatidylserine, enriched in the inner leaflet of the plasma membrane, even in the absence of PIP3 (36). The proposed mechanism for this binding involves electrostatic interaction, which has been shown to mediate cellular localization of proteins to phosphatidylserine (37). Thus, interaction of Akt with membrane-associated phosphatidylserine may be sufficient to mediate strain-induced phosphorylation of Akt at Ser-473 by mTORC2 in the absence of PI3K activation.

The differential dependence on PI3K of insulin and strain could further suggest that these two stimuli activate distinct pools of Akt in mdMSC. In contrast to membrane translocation of Akt facilitated by PIP3 binding that precedes its activation by insulin, mechanical strain may activate Akt localized at the membrane. The involvement of Akt in focal adhesions, proposed sites for transduction of mechanical stimuli (38), supports this possibility.

In sum, mechanical input initiates a signaling cascade that requires mTORC2 activation to phosphorylate Akt at Ser-473, which in turn induces phosphorylation of GSK3β at Ser-9 even in the absence of Akt Thr-308 phosphorylation. Our data indicate that the mechanical regulation of GSK3β downstream of Akt is uniquely dependent on Akt phosphorylation at Ser-473 in a manner distinct from that of insulin signaling, suggesting that mechanical Akt/GSK3β coupling involves molecules already present at the membrane. These differences provide clues as to the unique processes by which mechanical input, conscripting common signaling pathways, regulates MSC function.

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