Glucocorticoids Inhibit the Transcriptional Activity of LEF/TCF in Differentiating Osteoblasts in a Glycogen Synthase Kinase-3β-dependent and -independent Manner*

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Glucocorticoids, widely used as immune suppressors, cause osteoporosis by inhibiting bone formation. In MC3T3-E1 osteoblast-like cultures, dexamethasone (DEX) activates glycogen synthase kinase-3β (GSK3β) and inhibits a differentiation-related cell cycle that occurs at a commitment stage immediately after confluence. Here we show that DEX inhibition of the differentiation-related cell cycle is associated with a decrease in β-catenin levels and inhibition of LEF/TCF-mediated transcription. These inhibitory activities are no longer observed in the presence of lithium, a GSK3β inhibitor. DEX decreased the serum-responsive phosphorylation of protein kinase B/Akt-Ser473 within minutes, and this inhibition was also observed after 12 h. When the phosphatidylinositol 3-kinase (PI3K)/Akt pathway was inhibited by wortmannin, DEX no longer inhibited β-catenin levels. Furthermore, DEX-mediated inhibition of LEF/TCF transcriptional activity was attenuated in the presence of dominant negative forms of either PI3K or protein kinase B/Akt. These results suggest cross-talk between the PI3K/Akt and Wnt signaling pathways. Consistent with a role for Wnt signaling in the osteoblast differentiation-related cell cycle, wortmannin partially negated the DEX inhibition of this cell cycle. DEX also induced histone deacetylase (HDAC) 1, which is known to inhibit LEF/TCF transcriptional activity. Overexpression of HDAC1 negated the inhibitory effect of DEX on LEF/TCF transcriptional activity. In the presence of trichostatin A, a deacetylase inhibitor, DEX-mediated inhibition of the differentiation-related cell cycle was partially negated. When administered together, wortmannin and trichostatin A completely negated the inhibitory effect of DEX on the differentiation-related cell cycle. These results suggest that inhibition of a PI3K/Akt/GSK3β/β-catenin/LEF axis and stimulation of HDAC1 cooperate to mediate the inhibitory effect of DEX on Wnt signaling and the osteoblast differentiation-related cell cycle.

Glucocorticoids (GCs)† are used extensively for the treatment of autoimmune and inflammatory diseases. In addition, GCs are used in combination with other drugs to reduce inflammation associated with hematologic and other cancers and to prevent implant rejection. A major side effect of GC treatment is rapid bone loss and increased risk of fracture (1). Although bone resorption has been suggested to contribute to GC-induced osteoporosis (2), it is now clear that the main mechanism underlying long-term GC-induced bone loss is the impairment of osteoblast function and bone formation (3–5).

One of the most effective culture systems for the investigation of inhibitory effects of GCs on osteoblasts is the MC3T3-E1 model. In fact, this is the only reported culture system in which GCs strongly inhibit mineralization (6, 7). We have refined a commitment stage during osteoblast differentiation in MC3T3-E1 cultures, during which the cells are susceptible to GC inhibition (8). At the commitment stage, unique cell cycle machinery is activated. Unlike cell cycle progression in pre-confluent cultures, the differentiation-related cell cycle is inhibited by GCs, concomitant with the abandonment of the differentiation program (7).

In our pursuit of mechanisms driving the differentiation-related post-confluent cell cycle, we concentrated on the Wnt signaling pathway. The second messenger engaged by the Wnt signaling pathway to activate cell cycle-regulatory genes such as c-myc (9) is β-catenin, which also plays a role in cell-cell and cell-matrix interactions (10, 11). Wnt signaling may therefore link cell-cell and cell-matrix interactions occurring in post-confluent cultures and the cell cycle. Importantly, Wnt proteins have been shown to promote post-confluent proliferation in cultured cells (12). We hypothesized that the Wnt signaling pathway plays a critical role in the osteoblast differentiation-related cell cycle and in the aforementioned inhibitory effects of GCs (13). A key negative regulator in the Wnt signaling pathway is the serine/threonine kinase GSK3β, which phosphorylates and promotes the degradation of β-catenin in quiescent cells. The tumor suppressors APC and axin form complexes with GSK3β and β-catenin, thus promoting the activity of GSK3β on β-catenin (14–17).

In addition to the phosphorylation of β-catenin, GSK3β may also impede cell cycle progression by phosphorylating and marking for degradation substrates outside the Wnt signaling pathway, including the proto-oncogenes c-Myc and cyclin D1 (18, 19). These substrates must first undergo priming by another kinase in order to correctly associate with GSK3β (20). Stimulation of the cell cycle with growth factors that activate the phosphatidylinositol 3-kinase (PI3K/Akt pathway results in phosphorylation of GSK3β on Ser9 (21–23). [pSer9]GSK3β is mouse calvarial osteoblast; NFκB, nuclear factor κB; HDAC, histone deacetylase; CBP, cAMP-response element-binding protein (CREB)-binding protein.
unable to correctly associate with substrates that had been phosphorylated by the priming kinase and is thus inactive with respect to these substrates (24). Recently, it has been shown that, similar to substrates outside the Wnt signaling pathway, β-catenin also undergoes priming before it is phosphorylated by GSK3β (25).

The inhibition of the differentiation-related cell cycle in GC-treated MC3T3-E1 osteoblasts is associated with activation of GSK3β, inhibition of its Ser17 phosphorylation, and accelerated degradation of c-Myc (13). Furthermore, administration of lithium, a specific inhibitor of GSK3β, to GC-treated MC3T3-E1 cultures rescued c-Myc and the differentiation-related cell cycle (13). Here we show that the GC-mediated activation of GSK3β also results in inhibition of the Wnt signaling pathway and LEF/TCF transcriptional activity. We demonstrate that GCs inhibit phosphorylation of protein kinase B (PKB/Akt) on Ser473 and thus disrupt input from the PI3K/Akt pathway into the Wnt signaling pathway at the level of GSK3β.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Three culture models were employed in this study. A highly osteoblastic MC3T3-E1 subclone was isolated previously (7) and used between passage 7 and 11. The results did not depend on the passage number within this range. Newborn mouse calvarial osteoblast (NeMCO) cultures were prepared from 6-day-old mice by successive collagenase digestion (26). Human osteoblast cultures were obtained previously from vertebral bodies of subjects undergoing neurosurgical procedures (27), and frozen cell stocks were thawed for the present study. All cells were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum (Omega, Tarzana, CA). At confluence, 50 μm ascorbic acid and 10 μm β-glycerophosphate were added to promote differentiation (7). Dexamethasone (DEX, 1 μM), wortmannin (230 nM), and trichostatin A (TSA; 50 nM) were administered with the last medium change.

**Transfection and Luciferase Assays**—Cells were plated in 6-well plates (80,000 cells/well) and transiently transfected with a total of 5 μg of plasmid DNA using the calcium phosphate-co-precipitation method of Chen and Okayama (28). The cells were harvested 20–48 h after feeding and lysed in "reporter lysis buffer" (Promega). Luciferase activity was determined using a microtiter plate luminometer (MLX Dynex Technologies), and effects of DEX were tested for statistical significance using Student's t test.

**Protein Extracts**—Proteins were extracted using the following procedures. High salt extraction was performed according to the method of Ito et al. (29). Briefly, cells were suspended in buffer containing 10 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.25 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, 10 μg/ml leupeptin, 1 mM Na3VO4, and 0.1 mM phenylmethylsulfonyl fluoride. The extraction was incubated on ice for 5 min. The nuclear suspension was homogenized and centrifuged at 20,000 × g for 1 h to remove cell debris, and the supernatant was used for Western analysis. Triton extraction was performed according to the method of Su et al. (30). Cells were suspended in buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM EDTA, and protease and phosphatase inhibitors as described above. Suspensions were homogenized and centrifuged at 20,000 × g for 30 min to remove cell debris. Nuclear extracts were prepared essentially according to Verona et al. (31). Cell pellets were suspended in two packed cell volumes of hypotonic buffer containing 10 mM HEPES (pH 7.5), 10 mM KCl, 3 mM MgCl2, 1 mM EDTA (pH 8.0), and protease and phosphatase inhibitors as described above. Cells were left to swell on ice for 10 min and then vortexed for 10 s and spun at 500 × g for 5 min. The nuclear pellet was washed twice with hypotonic buffer and lysed in two nuclear pellet volumes of lysis buffer containing 100 mM HEPES (pH 7.4), 0.5 M KCl, 5 mM MgCl2, 28% glycerol, and protease and phosphatase inhibitors as described above. The nuclear suspension was homogenized and centrifuged at 20,000 × g for 1 h to remove cell debris. Protein concentrations were determined using the micro BCA protein assay kit (Pierce).

**Western Analysis**—Protein (60–100 μg) was subjected to SDS-PAGE and transferred to a 0.2-μm nitrocellulose membrane using a Mini Trans-Blot Transfer Cell (Bio-Rad). Immunodetection was performed using ECL (Amersham Biosciences) according to the manufacturer's recommendations, followed by exposure of the membrane to x-ray film. Results were quantitated by photodensitometry using an Alpha Innotech 2000 system (Alpha Innotech Corp.). For each experiment, the control value was set at 1 to calculate the relative signal in DEX-treated cells. Effects of DEX were tested for statistical significance using the Mann-Whitney test.

**Flow Cytometry**—Cell cycle analysis was performed according to the method of Darzykiewicz et al. (32). Briefly, cells were lightly trypsinized, resuspended in Hanks' buffer, fixed in cold 70% ethanol, washed with Hanks' buffer, and suspended in 1 ml of Hanks' buffer containing 20 μM propidium iodide and 5 Kunitz units of DNase-free RNase A. Cell cycle profiles were determined using an EPICS Profile Analyzer, and effects of DEX were tested for statistical significance using Student's t test.

**Reagents**—Tissue culture reagents were purchased from Invitrogen. DEX, wortmannin, and trichostatin A were from Sigma. Anti-β-catenin antibodies (C19220) were from Transduction Laboratories. Anti-Akt antibodies (catalog no. 9272) and anti-βSer473Akt antibodies (catalog no. 4051) were from Cell Signaling Technology. Anti-HDAC1 antibodies (sc-8410) were from Santa Cruz Biotechnology. The following constructs were used in transient transfection assays: LEF/TCF reporter constructs (9), NFκB reporter construct and expression vectors for p65 NFκB (33), HDAC1 (34), and dominant negative PI3K and PKB/Akt (from David K. Ann; University of Southern California).

**RESULTS**

**GCs Inhibit LEF/TCF-mediated Transcription via GSK3β**—We have shown previously that GC-mediated inhibition of the osteoblast differentiation-related cell cycle occurs via activation of GSK3β and proteasomal degradation of c-myc (13). Because GSK3β is also a negative regulator of the Wnt signaling pathway, we decided to test whether GCs inhibit LEF/TCF-mediated activity, the transcriptional outcome of the Wnt signaling pathway. MC3T3-E1 osteoblasts were transfected with TBE_g-luc, which contains four LEF/TCF binding sites that control transcription of a luciferase reporter gene (9). Post-confluent proliferating cells undergoing commitment were treated with DEX and subjected to luciferase assay. As shown in Fig. 1A, DEX inhibited LEF/TCF-mediated transcription by 2-fold, whereas TBE_m-luc, which contains four mutated LEF/TCF sites, was not inhibited (Fig. 1C). Unlike cultures undergoing commitment, administration of DEX to pre-confluent proliferating MC3T3-E1 cells did not inhibit LEF/TCF-mediated transcription (Fig. 1B).

It has been shown that GSK3β facilitates the transcriptional activity of p65-NFκB (35). If DEX inhibition of LEF/TCF-mediated activity were a result of GSK3β activation, one would expect the presence of lithium, a specific inhibitor of GSK3β. Indeed, in seven independent experiments, one of which is shown in Fig. 1G, the inhibition of LEF/TCF transcription by DEX was reduced by an average of 5-fold in the presence of lithium as compared with potassium, which was used as control. These results suggest that GSK3β mediates the inhibition of LEF/TCF transcriptional activity in GC-treated differentiating osteoblasts.

**GCs Inhibit PKB/Akt Phosphorylation in Differentiating Osteoblasts**—We have shown previously that GC activation of GSK3β correlates with a decrease in the inhibitory phosphorylation of GSK3β-Ser9 (13). The primary enzyme that phosphorylates GSK3β on Ser9 is PKB/Akt, which is stimulated in response to serum and PI3K activation (23, 36–38). Phosphorylation of PKB/Akt on serine 473 is a hallmark of enzyme activation (39). We therefore measured Akt-Ser473 phosphorylation in DEX-inhibited osteoblast cultures. In MC3T3-E1 cells, the only reported osteoblast culture system in which GCs inhibit terminal differentiation, DEX decreased the phosphoryl-
the PI3K/Akt pathway mediates in part the inhibitory effect of DEX on LEF/TCF transcriptional activity.

The PI3K/Akt Pathway Mediates DEX-induced Decrease in β-Catenin—The observation that DEX inhibition of LEF/TCF transcriptional activity is counteracted by lithium (Fig. 1G) suggests that DEX may have abrogated Wnt signaling through activation of GSK3β. To confirm this interpretation, we measured the effect of DEX on β-catenin levels in post-confluent MC3T3-E1 proliferating cultures. As shown in the left panels of Fig. 3, A and B, β-catenin levels were indeed reduced in response to DEX. Furthermore, β-catenin levels were not decreased by DEX in the presence of lithium (Fig. 3A, right panel), indicating that GCs indeed reduce β-catenin via GSK3β activation.

The destructive activity of GSK3β on β-catenin is thought to be primarily regulated by Wnt ligands and disheveled, which destabilize the β-catenin destruction complex (14–17). Here, we propose that the action of PKB/Akt on GSK3β also contributes to the regulation of β-catenin levels and would therefore mediate the inhibitory effect of GCs on the Wnt signaling pathway. To examine the interaction between the PI3K/Akt and Wnt signaling pathways, we tested whether DEX could still inhibit β-catenin levels in the presence of wortmannin, an inhibitor of the PI3K/Akt pathway. DEX no longer reduced β-catenin levels in the presence of wortmannin, whereas inhibition was preserved in the presence of Me2SO used as vehicle (Fig. 3B, DMSO). These results demonstrate cross-talk between the PI3K/Akt and the Wnt signaling pathways, placing GSK3β at an intersection through which extra-cellular signals upstream of PI3K/Akt would cooperate with Wnt signaling. In differentiating osteoblasts, this intersection is blocked by GCs (see Fig. 7).

DEX Targets the Wnt Signaling Pathway in Primary Osteoblast Cultures—Although the MC3T3-E1 cell culture model is ideal for the investigation of adverse effects of GCs in osteoblasts, we also tested whether DEX reduces β-catenin levels in NeMCO cultures under conditions established by Noh and Frenkel.2 DEX decreased β-catenin levels in NeMCO cultures (Fig. 3C) and in differentiating bone marrow-derived human mesenchymal stem cell cultures (data not shown) in a manner comparable with that seen in the MC3T3-E1 model (Fig. 3, A and B). Furthermore, GSK3β mediated DEX attenuation of cell cycle progression in NeMCO cultures: after 9 h of treatment, the percentage of S-phase cells was insignificantly reduced by only 10%, followed by a 27% inhibition at the 15 h time point and a 7-fold inhibition after 5 days of treatment (Fig. 3D). When the NeMCO cultures were co-treated with lithium, a GSK3β inhibitor, DEX no longer attenuated cell cycle progression (Fig. 3D). Thus, the Wnt signaling pathway is intercepted by DEX in both MC3T3-E1 and NeMCO cultures.

Histone Deacetylase I Contributes to GC Inhibition of LEF/TCF Transcriptional Activity—Although our data suggest that GCs inhibit LEF/TCF transcriptional activity through a PI3K/Akt pathway, histone deacetylase I is also required for the inhibitory activity of DEX on LEF/TCF transcriptional activity.

Initially, we tested the effect of the histone deacetylase inhibitors CBP on DEX-mediated repression of LEF/TCF transcriptional activity. As shown in Fig. 4A, co-transfection of CBP did not influence the DEX-mediated repression of TBE-Luc. We also tested
whether the DEX-mediated repression would be compromised when endogenous CBP is unable to co-activate LEF/TCF. To this end, cells were co-transfected with TBE4-luc along with p53, which has been shown to sequester CBP from interaction with LEF/TCF transcription factors (46). However, in the presence of p53, DEX still repressed LEF/TCF transcriptional activity to the same extent seen with the empty vector control (Fig. 4B), although the basal activity was reduced 2.2-fold by p53 (data not shown). These results suggest that CBP does not play a significant role in GC inhibition of LEF/TCF transcriptional activity in differentiating osteoblasts.

We next asked whether HDAC1, a LEF/TCF co-repressor (42–45), could be involved in the DEX-mediated inhibition of LEF/TCF transcriptional activity. Co-transfection of HDAC1 suppressed TBE4-luc activity and attenuated the inhibitory effect of DEX (Fig. 5A). This suggested to us that GCs could have induced HDAC1 in differentiating osteoblasts. Indeed, as demonstrated by Western analysis, DEX-treated cells had 1.9-fold more HDAC1 than did untreated cells (Fig. 5A). Thus, induction of HDAC1 likely contributes to DEX inhibition of LEF/TCF transcriptional activity.

Abrogation of the PI3K/Akt Pathway and Stimulation of HDAC Cooperate to Inhibit the Differentiation-related Cell Cycle in GC-treated Osteoblasts—We have shown previously that in the MC3T3-E1 osteoblast model, DEX inhibits cell cycle progression specifically in post-confluent cultures undergoing commitment to mineralization (7). We hypothesized that this GC-sensitive differentiation-related cell cycle was primarily
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Mechanisms underlying GC-induced osteoporosis are poorly understood. The present study focuses on a commitment stage during development of the osteoblast phenotype in the non-transformed MC3T3-E1 model, in which cell cycle progression is sustained in post-confluent cultures (7). We have shown previously that specifically at this stage, GCs inhibit both the osteoblast differentiation-related cell cycle by GCs (Fig. 7). Driven by the Wnt signaling pathway (13). In the preceding experiments, we showed that the inhibition of the PI3K/Akt pathway and induction of HDAC each contributed to GC-mediated inhibition of LEF/TCF transcriptional activity. If these activities of GCs are causally related to the inhibition of the cell cycle, then manipulation of the PI3K/Akt pathway and HDAC activity could be expected to negate the effect of GCs on the differentiation-related cell cycle at least partially. We therefore tested the effect of DEX on the cell cycle profile in the absence and presence of wortmannin, a PI3K inhibitor, and TSA, a HDAC inhibitor. As shown in Fig. 6A, wortmannin and DEX each reduced the percentage of post-confluent MC3T3-E1 cells in S phase from 15% to 2.9%. Importantly, whereas DEX reduced the percentage of post-confluent S-phase cells by 5.2-fold when the PI3K/Akt pathway was intact, DEX inhibition was attenuated to only 2.4-fold in the presence of wortmannin (Fig. 6C). These results suggest that the PI3K/Akt pathway is critically important for DEX repression of the osteoblast differentiation-related cell. Interestingly, wortmannin did not inhibit the percentage of cells in S-phase in pre-confluent MC3T3-E1 cultures (Fig. 6B). The residual DEX inhibition of the cell cycle in wortmannin-treated post-confluent cells (Fig. 6C) could have resulted from induction of HDAC1 (Fig. 5B). To address this possibility, we measured the effect of DEX on the cell cycle profile in the presence of both wortmannin and TSA. DEX no longer decreased the percentage of S-phase cells in the presence of both inhibitors (Fig. 6C), suggesting that HDAC stimulation contributes to DEX-mediated inhibition of the cell cycle. We conclude that inhibition of the PI3K/Akt pathway and stimulation of HDAC cooperatively mediate the inhibition of the osteoblast differentiation-related cell cycle by GCs (Fig. 7).

DISCUSSION

Mechanisms underlying GC-induced osteoporosis are poorly understood. The present study focuses on a commitment stage during development of the osteoblast phenotype in the non-transformed MC3T3-E1 model, in which cell cycle progression is sustained in post-confluent cultures (7). We have shown previously that specifically at this stage, GCs inhibit both the osteoblast differentiation-related cell cycle by GCs (Fig. 7).

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GSK3β may attenuate cell cycle progression by phosphorylating substrates in and outside of the Wnt signaling pathway (Fig. 7). Within the Wnt signaling pathway, GSK3β phosphorylates and promotes the degradation of β-catenin (14–17). Outside the Wnt signaling pathway, GSK3β phosphorylates and promotes the degradation of substrates such as the proto-oncogene c-Myc (18). Progression of the osteoblast differentiation-related cell cycle can be promoted by interference with the activity of GSK3β on these substrates. This can be achieved in two ways (Fig. 7). First, Wnt signaling results in disassembly of the β-catenin destruction complex and rescue of β-catenin from proteasomal degradation. Second, growth factors that activate the PI3K/Akt pathway inhibit GSK3β phospho-

The results of the present study indeed suggest that the PI3K/Akt pathway plays a role in the GC inhibition of the Wnt signaling pathway. GCs decreased β-catenin levels, and this inhibition was negated not only by inhibition of GSK3β (lithium), but also by inhibition of the PI3K/Akt pathway (wortmannin) (Fig. 3). Furthermore, we demonstrated that DEX inhibits Ser473-Akt phosphorylation in MC3T3-E1 as well as NeMCO and human osteoblast cultures. When examined at the level of LEF/TCF transcriptional activity, again we observed that the DEX-mediated inhibition of the Wnt reporter TBE4-luc was abrogated not only by lithium, but also by dominant-negative forms of both PI3K and PKB/Akt (Figs. 1G and 2C). The notion that the PI3K/Akt pathway influences the activity of GSK3β not only on substrates such as c-Myc, but also on β-catenin, is consistent with the recent discovery that, like c-Myc, β-catenin undergoes priming phosphorylation before it is phosphorylated by GSK3β (25). It remains to be seen what determines the ability of the PI3K/Akt pathway to influence the Wnt signaling pathway. In MC3T3-E1 cells, the PI3K/Akt/GSK3β/β-catenin/LEF axis (Fig. 7) appears to be specific for the commitment stage. It may also be operative in other cell systems, where tight association between activation of the PI3K/Akt pathway and β-catenin-mediated activation of LEF/TCF has been demonstrated (47–50). It is noteworthy that the response of β-catenin to GCs was abrogated not only by lithium, but also by dominant-negative GSK3β (Fig. 2C).

The role of the Wnt signaling pathway in the regulation of bone mass in vivo has been well demonstrated by the altered bone mass phenotypes in mice and humans carrying mutations in the Wnt co-receptor LRPs (26, 52–55). The consequences of LRPs inactivation could be very similar to the effect of GCs emerging from our studies: inhibition of a cell cycle that becomes critically dependent on Wnt signaling during a distinct stage of osteoblast differentiation. Understanding how this cell cycle is controlled may provide unique opportunities in drug design for the treatment of medical conditions in which Wnt-dependent regulation of the cell cycle goes awry. These conditions may include GC-induced and other osteoporoses as well as cancer.

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