Glucocorticoids and Tumor Necrosis Factor α Increase Oxidative Stress and Suppress Wnt Protein Signaling in Osteoblasts*

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Background: Glucocorticoids and tumor necrosis factor (TNF) α decrease bone mass.

Results: Oxidative stress is increased in bone-forming cells (osteoblasts) in response to glucocorticoids and TNFα.

Conclusion: Glucocorticoids and TNFα decrease osteoblast numbers via oxidative stress-dependent and -independent mechanisms.

Significance: This might help in finding treatments for osteoporosis.

Endogenous glucocorticoids (GCs) and inflammatory cytokines contribute to the age-associated loss of bone mass and strength, but the molecular mechanisms responsible for their deleterious effects on the aging skeleton are unclear. Based on evidence that oxidative stress is a causal mechanism of the insulin resistance produced by either one of these two agents, we tested the hypothesis that their adverse skeletal effects also result from increased oxidative stress. We report that administration of prednisolone to mice increased reactive oxygen species (ROS) and the phosphorylation of p66shc (an amplifier of H2O2 generation in mitochondria) in bone. Dexamethasone (Dex) and TNFα had a similar effect on osteoblastic cells in vitro. The generation of ROS by Dex and TNFα required PKCβ/p66shc signaling and was responsible for the activation of JNK and induction of apoptosis by both agents. The activity of Forkhead box O (FoxO) transcription factors was also increased in response to ROS; however, FoxO activation opposed apoptosis induced by Dex and TNFα. In addition, both agents suppressed Akt phosphorylation as well as Wnt-induced proliferation and osteoblast differentiation. However, the inhibitory actions on Wnt signaling were independent of PKCβ/p66shc. Instead, they were mediated by inhibition of Akt and stimulation of FoxOs. These results demonstrate that ROS-induced activation of a PKCβ/p66shc/JNK signaling cascade is responsible for the pro-apoptotic effects of Dex and TNFα on osteoblastic cells. Moreover, modulation of Akt and FoxOs by GCs and TNFα are cell-autonomous mechanisms of Wnt/β-catenin antagonism contributing to the adverse effects of GC excess and inflammatory cytokines on bone alike.

Glucocorticoids (GCs)2 and the inflammatory cytokine TNFα induce rapid bone loss and greatly increase the risk of fractures. Endogenous or exogenous GC excess, as well as TNFα, increase the number of osteoclasts and decrease the number of osteoblast due to reduced production of osteoblast precursors as well as premature apoptosis of mature osteoblasts (1, 2). However, the molecular mechanisms responsible for these deleterious effects remain unclear. Endogenous GC levels increase by 20–50% with age in humans and mice (3–7) because of blunting of the GC feedback inhibition of ACTH (8) as well as increased bone expression of 11β-hydroxysteroid dehydrogenase type 1, the enzyme that converts GCs from inactive to active moieties (6, 9). TNFα is a critical mediator of skeletal damage in inflammatory arthritis (10, 11). Like GCs, the levels of TNFα in the serum increase with age, and this might contribute to involutional osteoporosis (12, 13).

The progressive loss of bone with aging is characterized by an increase in osteoblast and osteocyte apoptosis as well as by a decrease in osteoblast number and bone formation rate (14). Furthermore, the age-dependent loss of bone mass is associated with a progressive increase in the levels of reactive oxygen species (ROS) and the phosphorylation of the adapter protein p66shc in bone (15). p66shc is a crucial component of a signaling pathway that is activated by increased intracellular ROS, via PKCβ phosphorylation at Ser36, and converts oxidative signals into apoptosis. Phosphorylated p66shc translocates to the mitochondria and acts as a redox enzyme to amplify ROS by generating H2O2 (16–18). Importantly, p66shc is indispensable for H2O2-induced osteoblast apoptosis (19). This along with evidence that administration of antioxidants abrogates the age-related increase in osteoblast apoptosis (20) strongly supports the notion that oxidative stress is causally related to involutional osteoporosis.

Genetic studies by our group and others have elucidated that a balance between ROS production and defense against ROS by the Forkhead box O (FoxO) family of transcription factors is...
indispensable for bone homeostasis at any age (21, 22). In mammals, the FoxO family comprises four members, of which FoxO1, FoxO3, and FoxO4 are ubiquitously expressed and relatively abundant in bone cells. FoxOs are negatively regulated by insulin and growth factors via the serine/threonine kinase Akt (23). Akt directly phosphorylates FoxOs on three conserved residues which lead to cytoplasmic retention and inhibition of FoxO-mediated transcription. In contrast to growth factors, ROS cause the retention of FoxOs in the nucleus and activation of transcription. In turn, FoxOs counteract ROS production by activating the transcription of genes involved in free radical scavenging. In several cell types including osteoblastic cells, oxidative stress also induces the association of FoxOs with β-catenin (24, 25), which is a critical component of the Wnt signaling pathway and indispensable for osteoblastogenesis (26). Activation of the low density lipoprotein receptor-related protein 5/6-frizzled receptor complex by Wnt proteins inactivates glycogen synthase kinase-3β, which prevents the proteosomal degradation of β-catenin. Upon its translocation into the nucleus, β-catenin associates with the T cell factor (TCF)/lymphoid enhancer factor family of transcription factors and regulates the expression of Wnt target genes (27). Importantly, oxidative stress induced by H2O2 promotes FoxO-mediated transcription at the expense of Wnt/TCF-mediated transcription and osteoblast differentiation (24, 28).

GCs and TNFα up-regulate cellular ROS. Indeed, oxidative stress is the main cause of the development of insulin resistance by these agents (29). Gene expression analysis in human osteoblasts exposed to dexamethasone (Dex) revealed that a significant number of transcripts related to oxidative stress are altered (30). Moreover, the generation of ROS has been implicated in the mechanisms by which TNFα increases osteoblast apoptosis (31). These agents also decrease Wnt signaling in osteoblastic cells although the underlying mechanisms are not fully elucidated (32–34). Prompted by this evidence, we tested the hypothesis that the potent suppressive effects of GC and TNFα on bone formation may be caused by increased oxidative stress and antagonism of the beneficial effects of Wnt signaling on osteoblast generation secondary to FoxO activation.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Dex, N-acetyl-l-cysteine (NAC), ebselen, etoposide, SP600125, hspidin, and H2O2 were purchased from Sigma-Aldrich. LY335351 was purchased from Axon Medchem BV (Groningen, The Netherlands). Human TNFα and mouse Wnt3a recombinant proteins were purchased from R&D Systems (Minneapolis, MN). pcDNA was purchased from Invitrogen. A reporter plasmid containing three TCF binding sites upstream of a minimal c-fos promoter driving the firefly luciferase gene (TCF-luc) (35), was provided by B. Vogelstein (John Hopkins University Medical Institutions, Baltimore, Maryland). A reporter plasmid containing six copies of daf-16 family protein-binding element (FoxO-luc) in the pGL3-basic firefly luciferase vector with a minimal TATA box (36) was provided by B. Burgering (University Medical Center, Utrecht, The Netherlands). The cDNA for Akt was provided by M. E. Greenberg (Harvard Medical School, Boston, MA).

Western Blotting and Quantitative RT-PCR Analysis—The phosphorylation status of JNK and Akt was analyzed by immunoblotting, using a mouse monoclonal antibody recognizing Thr183/Tyr185-phosphorylated JNK1 and a rabbit polyclonal antibody recognizing Ser473-phosphorylated Akt (Cell Signaling Technology). Protein levels of JNK1 and Akt were analyzed using a mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal antibody (Cell Signaling Technology), respectively. Protein levels of Shc were analyzed using a rabbit polyclonal antibody (BD Biosciences) and β-actin using a mouse monoclonal antibody (Santa Cruz Biotechnology). Quantification of the intensity of the bands was performed by chemiluminescence using a VersaDoc™ imaging system (Bio-Rad). Total RNA was extracted from cultured cells and reverse-transcribed as described previously (24). Primers and probes for the different genes were manufactured by the TaqMan® Gene Expression Assays service (Applied Biosystems). The mRNA levels were calculated by normalizing to the housekeeping gene ribosomal protein S2 using the ΔCt method (37).

**Cell Culture and Assays**—UAMS-32 cells, an osteoblastic cell line derived in our laboratory from the murine bone marrow (38), bone marrow- and calvaria-derived osteoblastic cells, and immortalized fibroblasts from wild-type (WT) and JNK1/2 (double knock-out) (dKO) mouse embryonic fibroblasts, provided by R. Davis (University of Massachusetts Medical School, Worcester, MA), were cultured in α-MEM (Invitrogen) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), penicillin (100 units/ml), streptomycin (100 µg/ml), and glucose (292 µg/ml). C2C12 cells were cultured in DMEM supplemented with 10% fetal bovine serum, antibiotics as above, and 1% sodium pyruvate. Apoptosis was measured by caspase-3 activity using a fluorogenic substrate and by the TUNEL method using the FragEL DNA fragmentation detection kit (EMD Chemicals, San Diego, CA) (39). Assays described earlier (40) were used to measure cell proliferation by BrdU incorporation. Intracellular ROS were quantified using dichlorodihydrofluorescein dye (41). Alkaline phosphatase activity in the cell lysates was determined using a buffer containing 2-amino-2-methylpropanol and p-nitrophenylphosphate (Sigma-Aldrich) as described previously (24).

**Transient Transfections and Luciferase Assay**—Cells were plated on a 48-well plate and 16 h later transfected with 0.2 µg of the respective reporter plasmid, 0.01 µg of Renilla (control reporter), and 0.2 µg of pcDNA or 0.2 µg of Akt using LipoFectamine Plus (Invitrogen). Twenty four hours later cells were treated with the different compounds for another 24 h. Luciferase activity was determined using the Dual-Luciferase Reporter® assay system (Promega), according to the manufacturer’s instructions. Light intensity was measured with a luminometer, and luciferase activity was divided by the control reporter to normalize for transfection efficiency.

**Animal Experimentation**—Animal use protocols were approved by the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences and the Central Arkansas Veterans Healthcare System. Osteoblastic cells derived from mouse calvaria were obtained from 12-week-old Mx-Cre+;FoxO1,3,4L/L (FoxO1,3,4 KO) and littermate...
Administration of GCs to Mice Increases Oxidative Stress in Bone—Based on previous findings that GCs increase ROS in a variety of cell models and that endogenous GC levels as well as oxidative stress increase in murine bone with age (6, 15), we investigated whether GCs increase ROS levels in bone. Administration of prednisolone to 5-month-old C57BL/6 male mice was associated with a 60% increase in ROS levels in the bone marrow as well as phosphorylation of p66shc in vertebral lysates (Fig. 1, A and B).

Similar to GCs, TNFα levels increase with age in several tissues (42, 43). We examined whether the same was true in bone. As shown in Fig. 1C, TNFα mRNA expression levels were increased in calvaria of 31-month-old mice compared to 8-month-old mice. Similar results were obtained in a separate experiment comparing vertebrae of 25-month-old to 6-month-old mice (Fig. 1D). The increase in TNFα with age was abrogated by administration of the antioxidants NAC or catalase for 1 month. Taken together, these results suggest that GCs increase oxidative stress in bone and that the rise in TNF levels in the bone of aged mice is due to oxidative stress.

GCs and TNFα Increase ROS in Osteoblastic Cells via p66shc Activation—To dissect the mechanism by which GCs and TNFα increase ROS in osteoblastic cells, we used the uncommitted osteoblast precursor C2C12 and the bone marrow-derived osteoblastic UAMS-32 cell lines. Dex and TNFα promoted an increase in ROS levels as early as 15 min in C2C12 cells, and this increase was sustained for up to 1 h as determined by oxidation of the redox-sensitive dye dichlorodihydrofluorescein (Fig. 2A). A similar increase in ROS was observed in UAMS-32 cells when exposed to the test agents for 30 min (Fig. 2D). We next examined whether the PKCβ/p66shc signaling pathway was altered in response to Dex or TNFα. As shown in Fig. 2B, Dex or TNFα stimulated the phosphorylation of p66shc in C2C12 cells starting at 15 min, and this effect was maintained up to 1 h. To determine whether the phosphorylation of p66shc by Dex or TNFα was dependent on ROS and PKCβ activity, we used the antioxidant NAC and the specific inhibitor of PKCβ hispidin, respectively. Both NAC and hispidin prevented the phosphorylation of p66shc induced by Dex or TNFα (Fig. 2C). Hispidin also prevented the increase in ROS caused by the two agents (Fig. 2D). A similar result was obtained with another PKCβ inhibitor, LY333531. We next tested directly whether p66shc was functionally involved in the generation of ROS by Dex or TNFα, using primary cultures of osteoblastic cells from p66shc KO mice. Whereas Dex, TNFα, and H2O2 (used as a positive control) increased ROS levels in calvaria cells derived from WT mice, deletion of p66shc prevented the increase in ROS caused by the three compounds (Fig. 2E). These results demonstrate that Dex and TNFα increase ROS and p66shc phosphorylation in osteoblastic cells and that PKCβ and p66shc are required for the pro-oxidant actions of Dex and TNFα alike.
**GCs and TNFα Activate FoxOs**—JNK is a well established target of GCs and TNFα in several cell models. JNK is also a mediator of ROS-induced FoxO activation (44–47). Based on this evidence and the findings that Dex and TNFα exert pro-oxidant actions in osteoblasts described above, we investigated whether the two agents activated FoxOs via a ROS/JNK signaling cascade. To this end, we first examined the phosphorylation status of JNK in response to Dex and TNFα. The two agents increased JNK phosphorylation, as determined by Western blotting, and this effect was attenuated by NAC (Fig. 3A). Because ROS generation by Dex or TNFα required PKC and p66<sup>shc</sup>, we examined whether the latter proteins were required for the stimulation of JNK phosphorylation. Preincubation of UAMS-32 cells with hispidin attenuated the phosphorylation of JNK by Dex and TNFα (Fig. 3D). Moreover, the phosphorylation of JNK that occurred in osteoblastic cells from WT mice was abrogated in cells from p66<sup>shc</sup> KO mice (Fig. 3B). We next determined the effect of Dex and TNFα on the transcriptional activity of FoxO using a FoxO reporter construct that contains six canonical FoxO-binding sites (FoxO-luc). As shown in Fig. 3, C and E, Dex and TNFα increased FoxO activity in C2C12 or UAMS-32 cells, and NAC prevented this effect. In line with these results hispidin or LY333531 abrogated FoxO activation by Dex and TNFα (Fig. 3D). Finally, the stimulatory effects of Dex and TNFα on FoxO activity were prevented by the JNK inhibitor SP600125 (Fig. 3E); and were abrogated in embryonic fibroblasts from mice lacking JNK1 and JNK2 (Fig. 3F). Taken together, these results indicate that Dex and TNFα activate JNK via a ROS/PKC/p66<sup>shc</sup>-dependent mechanism and that JNK is required for the activation of FoxOs by the two agents.

**ROS Mediate the Pro-apoptotic Actions of GCs and TNFα**—Prompted by the evidence that GCs and TNFα promote osteoblast apoptosis, we sought to test directly whether the pro-apoptotic actions of the two agents are mediated by ROS. To this end C2C12 cells were incubated with these agents or with the pro-apoptotic topoisomerase inhibitor etoposide, in the pres-
ence of the antioxidants NAC or ebselen. Apoptosis was quantified by caspase-3 activity. Both NAC and ebselen prevented the pro-apoptotic actions of Dex or TNFα but had no effect on etoposide-induced apoptosis (Fig. 4A). Similar findings were obtained using the osteoblastic OB-6 cell line (data not shown).

The involvement of the p66shc/JNK/FoxO signaling cascade in the pro-apoptotic actions of Dex or TNFα was tested in UAMS-32 cells in which p66shc levels were reduced by short hairpin (sh)RNA (19) and in primary calvaria cells from p66shc KO mice. Silencing of p66shc abrogated the pro-apoptotic actions of Dex and greatly attenuated the pro-apoptotic action of TNFα (Fig. 4B). Similar results were obtained with the cells from p66shc KO mice (Fig. 4C). Moreover, inhibition of JNK using SP600125 or embryonic fibroblasts from JNK1/2 dKO mice also attenuated the pro-apoptotic effect of Dex or TNFα (Fig. 4, D and E). In contrast, the pro-apoptotic actions of the two agents, as well as H2O2, were prevented in bone marrow-derived osteoblastic cells from transgenic mice overexpressing FoxO3 (Fig. 4F). These results demonstrate that the pro-apoptotic effect of Dex or TNFα in osteoblastic cells is mediated by...
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Dex and TNFα Suppress Wnt Signaling via Akt Inhibition—Akt activity inhibits FoxO and stimulates β-catenin/TCF-mediated transcription (23, 48). Moreover, both GCs and TNFα inhibit Akt phosphorylation by increasing ROS generation (29). To determine whether inhibition of Akt contributes to the negative effect of Dex and TNFα on Wnt signaling, we first examined the effects of the two agents on Akt. As shown in Fig. 6A, Dex or TNFα attenuated the phosphorylation of Akt in UAMS-32 cells, in the presence of serum. Moreover, the antioxidant NAC did not blunt the suppression of Akt phosphorylation by the two agents. We have shown previously that increasing Akt levels by transfection of a plasmid encoding this kinase in UAMS-32 cells could prevent the inhibitory actions of Dex on TCF-luc activity (49). As seen in Fig. 6B, Akt not only abrogated the negative effect of Dex under basal conditions but also in the presence of Wnt3a. Similar to the case of Dex, Akt prevented the inhibitory action of TNFα on TCF-luc activity. These results suggest that inhibition of Akt phosphorylation by Dex and TNFα is independent of ROS and contributes to the negative effects of the two agents on Wnt signaling.

**DISCUSSION**

Earlier work from our group and others has shown that an increase in endogenous GCs and inflammatory cytokines contributes to the age-associated loss of bone mass and strength (6, 11). Moreover, administration of GCs or an endogenous rise in TNFα has been causally linked to increased osteoclast numbers, decreased osteoblast numbers, and bone loss (1, 50). However, the molecular mechanisms responsible for the deleterious effects of GCs or TNFα on bone have not been fully elucidated.
The work of the present report reveals that both GCs and TNFα increase ROS in osteoblastic cells and that the effects of either one of these two agents are mediated, at least in part, by increased production of ROS. Moreover, our results support the conclusion that TNFα and GCs increase ROS by a mechanism that requires PKCβ and p66\(^{shc}\) (Fig. 6C). These findings, along with evidence that p66\(^{shc}\) amplifies ROS within the mitochondria, suggest that mitochondria might be the main source of ROS generation in response to GCs and TNFα in osteoblasts (51). Although the mitochondrial electron transport chain is the main mechanism of ROS generation, oxygen radicals can also be produced by other enzymatic sources like NADPH oxidase, xanthine oxidase, and NOS. It has been shown previously that TNFα stimulates ROS generation by the NADP oxidase 1 (Nox1) system at the cell membrane (52, 53) and that GCs increase oxidative stress by activating xanthine oxidase (54, 55). Whether p66\(^{shc}\) is the sole source of ROS generation by these agents in osteoblasts or whether ROS generated outside the mitochondria is amplified by p66\(^{shc}\) in the mitochondria requires further studies.

FIGURE 5. Inhibition of Wnt signaling by Dex and TNFα is mediated by FoxOs. A, luciferase activity in C2C12 cells transfected with a TCF-luc reporter construct and preincubated for 1 h with vehicle (veh) \(10^{-7}\) M Dex, 10 ng/ml TNFα and incubated without or with 25 ng/ml Wnt3a for 24 h. RLU, relative luminescence units. B, proliferation of C2C12 cells determined by BrdU incorporation following treatment as in A for 3 days. C, alkaline phosphatase (AP) activity in C2C12 cells following treatment as in A. D, luciferase activity in C2C12 cells transfected with a TCF-luc reporter construct and preincubated for 1 h with vehicle, 10 \(\mu\)M hispidin, or 50 nm Ly333531 followed by Dex or TNFα for another hour and incubated without or with Wnt3a for 24 h. E, luciferase activity in osteoblastic cells derived from the calvaria of control or FoxO1,3,4 KO mice transfected with a TCF-luc reporter construct and treated as in A. Bars indicate means ± S.D. (error bars) of triplicate determinations. *, \(p < 0.05\) versus vehicle; †, \(p < 0.05\) versus Wnt3a alone; #, \(p < 0.05\) versus respective vehicle.
We and others have shown that GCs or TNFα increase osteoblast and osteocyte apoptosis. Importantly, bone fragility induced by chronic GC excess is due, to a large extent, to increased osteocyte apoptosis and results from cell autonomous effects (6, 56). In view of this evidence, we have searched here for a mechanism linking ROS to the pro-apoptotic actions of GCs and TNFα. We found that the pro-apoptotic actions of GCs and TNFα are mediated by ROS. Similar to our findings, others have shown that TNFα-induced apoptosis in several cell types, including osteoblasts, is mediated by ROS (31, 57). The present work has also revealed that ROS activates a PKCβ/p66shc/JNK signaling cascade that is an essential mediator of the pro-apoptotic effects of Dex or TNFα in osteoblasts. We had shown before that GCs promote osteocyte apoptosis via a receptor-mediated mechanism that does not require gene transcription and is mediated by rapid activation of Pyk2 and JNK, followed by inside-out signaling that leads to cell detachment-induced apoptosis or anoikis (58). Based on these findings and evidence that elevated levels of ROS activate Pyk2 in several cell types, it is possible that Pyk2 activation in osteocytes is dependent on ROS/p66shc/JNK signaling. Activation of JNK plays a critical role in many ROS-dependent apoptotic processes (59–61). The evidence of the present report that the activation of JNK by Dex and TNFα is prevented by NAC or deletion of p66shc strongly suggests that ROS are indeed critical mediators of the actions of Dex and TNFα on JNK activity in osteoblasts. In support of this contention, ROS inhibits the action of phosphatases that dephosphorylate JNK leading to sustained JNK activation and consequently to apoptotic or necrotic cell death (62).

In the present work, both Dex and TNFα activated FoxOs in osteoblasts via a ROS/JNK signaling cascade. In several model organisms, JNK directly phosphorylates FoxOs in response to oxidative stress (44, 46, 47). JNK-mediated phosphorylation of FoxO4 at Thr447 and Thr451 causes the translocation of FoxO4 from the cytoplasm to the nucleus (44). Interestingly, whereas JNK promotes the apoptosis of osteoblasts, FoxOs are a defense mechanism against ROS, and FoxO3 overexpression in osteoblasts prevents apoptosis in vitro and in vivo (21). Thus, mice with targeted expression of FoxO3 in osteoblasts exhibit decreased p66shc phosphorylation in bone, decreased osteoblast and osteocyte apoptosis, and decreases osteoblast number and bone mass (21).
The anti-apoptotic effect of FoxOs on osteoblasts notwithstanding, ROS promote FoxO binding to β-catenin and thereby divert β-catenin from TCF- to FoxO-mediated transcription. This mechanism causes a decrease in osteoblastogenesis (24). However, it remains unknown whether oxidative stress-induced post-translational modifications of FoxOs promote this interaction. Hoogeboom et al. have suggested that the interaction between β-catenin and FoxOs in response to H2O2 is independent of JNK because it occurs in JNK1/JNK2-deficient cells (28). The results of the present report are in agreement with this suggestion and indicate that the attenuation of Wnt signaling by Dex and TNFα are mediated by FoxOs but are independent of JNK. Furthermore, inhibition of PKCβ activity and ROS generation, in our studies, did not influence the attenuating effect of Dex and TNFα on TCF-mediated transcription. This result adds support to the view that increased ROS production in and of itself cannot be responsible for the inhibitory effects of Dex and TNFα on β-catenin/TCF transcriptional activity.

In search for an alternative mechanism that could explain this seeming discrepancy, we found that Dex and TNFα inhibit Akt activity independent of ROS in osteoblastic cells. Furthermore, increased levels of Akt prevented the inhibitory actions of Dex and TNFα on TCF-luc activity. These results along with evidence that serum starvation, similar to Dex and TNFα, attenuates Akt and promotes the binding of β-catenin to FoxOs and concomitant inhibition of β-catenin/TCF activity suggest that Akt might override the effect of ROS on the interaction between FoxOs and β-catenin. Attenuation of Akt and activation of FoxOs in myoblasts and tenocytes by GCs cause skeletal muscle atrophy and decrease collagen I synthesis, respectively (63, 64). Interestingly, expression of a constitutively active Akt, a dominant negative glycogen synthase kinase-3β, or a stable β-catenin can block the muscle atrophy induced by Dex (65), lending support to the contention that the inhibition of β-catenin/TCF activity may be a common molecular mechanism contributing to the development not only of involutional osteoporosis, but several other pathologies (66).

Suppression of PI3K/Akt/glycogen synthase kinase-3β signaling and up-regulation of the Wnt inhibitor DKK1 have been implicated in the attenuation of Wnt signaling by GCs and TNFα in osteoblastic cells (32–34). In addition, there is evidence that TNFα suppresses RUNX2 and osterix as well as bone morphogenetic protein-induced osteoblast differentiation (2, 67–69). Hence, these alternative mechanisms may contribute to the attenuating effects of Dex and TNFα on osteoblastogenesis.

Our studies also show that TNFα levels increase in murine bone with age and that this increase is dependent on ROS. In line with these findings, we have shown earlier that H2O2 increases TNFα expression in osteoblast cell in vitro (19). Interestingly, the increase in TNFα in response to ROS is dependent on a p66shc/NFκB signaling axis and is prevented in p66shc-null osteoblasts. Based on this evidence we propose that PKCβ/p66shc signaling plays a critical role in a vicious cycle in which ROS increases TNFα which, in turn, contributes to oxidative stress by increasing ROS and the self-amplification of ROS production (Fig. 6C). In support of this contention, Neele et al. have shown that TNFα can positively autoregulate its own biosynthesis in adipose tissue, via the PKC signaling pathway and NFκB, contributing this way to the maintenance of elevated TNFα in obesity (70). GCs and TNFα evidently contribute to the increase in osteoblast and osteocyte apoptosis with age. And, by increasing ROS they perpetuate oxidative stress in the skeleton. Besides bone, GC- and TNFα-induced ROS generation contributes to many of the deleterious effects of GCs treatment or inflammation in other tissues, including insulin resistance in adipose tissue, vascular endothelial dysfunction, tendon injury, heart failure, and brain development (29, 71–73).

In closing, we propose that decreased osteoblastogenesis, due to attenuation of Akt activity and FoxO-mediated inhibition of Wnt signaling, as well as increased osteoblast apoptosis resulting from an increase in ROS and activation of a ROS/ PKCβ/p66shc/JNK cascade, represent common mechanisms underlying the adverse effects of GC excess and TNFα on bone (Fig. 6C). The combination of increased GCs and TNFα may constitute a key pathogenetic event in the dramatic decrease of bone formation in conditions such as rheumatoid arthritis, GC excess, and aging.

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REFERENCES
1. Weinstein, R. S. (2011) N. Engl. J. Med. 365, 62–70
2. Nanes, M. S. (2003) Gene 321, 1–15
3. Van Cauter, E., Leproult, R., and Kupfer, D. J. (1996) J. Clin. Endocrinol. Metab. 81, 2468–2473
4. Dennison, E., Hindmarsh, P., Fall, C., Kellingray, S., Barker, D., Phillips, D., and Cooper, C. (1999) J. Clin. Endocrinol. Metab. 84, 3058–3063
5. Reynolds, R. M., Dennison, E. M., Walker, B. R., Syddall, H. E., Wood, P. I., Andrew, R., Phillips, D. I., and Cooper, C. (2005) Calcif. Tissue Int. 77, 134–138
6. Weinstein, R. S., Wang, C., Liu, Q., Wang, Y., Almeida, M., O’Brien, C. A., Thostenson, J., Roberson, P. K., Boskey, A. L., Clemens, T. L., and Manolagas, S. C. (2010) Aging Cell 9, 147–161
7. Purnell, J. Q., Brandon, D. D., Isabelle, L. M., Loriaux, D. L., and Samuels, M. H. (2004) J. Clin. Endocrinol. Metab. 89, 281–287
8. Wilkinson, C. W., Petrie, E. C., Murray, S. R., Colasurdo, E. A., Raskind, M. A., and Peskind, E. R. (2001) J. Clin. Endocrinol. Metab. 86, 545–550
9. Cooper, M. S., Rabbitt, E. H., Goddard, P. E., Bartlett, W. A., Hewison, M., and Stewart, P. M. (2002) J. Bone Miner. Res. 17, 979–986
10. Boyce, B. F., Schwarz, E. M., and Xing, L. (2006) Curr. Opin. Rheumatol. 18, 427–432
11. Walsh, N. C., Crotti, T. N., Goldring, S. R., and Gravallese, E. M. (2005) Immunol. Rev. 208, 228–251
12. Ding, C., Parameswaran, V., Udayan, R., Burgess, J., and Jones, G. (2008) J. Clin. Endocrinol. Metab. 93, 1952–1958
13. Brunsgaard, H., Pedersen, M., and Pedersen, B. K. (2001) Curr. Opin. Endocrinol. 8, 131–136
14. Manolagas, S. C. (2010) Endocr. Rev. 31, 266–300
15. Almeida, M., Han, L., Martin-Millan, M., Plotkin, L. I., Sowter, S. A., Roberson, P. K., Kousteni, S., O’Brien, C. A., Bellido, T., Parfitt, A. M., Weinstein, R. S., Jilka, R. L., and Manolagas, S. C. (2007) J. Biol. Chem. 282, 27285–27297
16. Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L., and Pelicci, P. G. (1999) Nature 402, 309–313
17. Trinei, M., Giorgio, M., Cicalese, A., Barozzi, S., Ventura, A., Migliaccio, E., Milia, E., Padura, I. M., Raker, V. A., Maccarana, M., Petronilli, V., Minucci, S., Bernardi, P., Lanfrancone, L., and Pelicci, P. G. (2002) Oncogene 21, 3872–3878
18. Giorgio, M., Migliaccio, E., Orsini, F., Paolucci, D., Moroni, M., Contursi,
