Glucocorticoids Act Directly on Osteoblasts and Osteocytes to Induce Their Apoptosis and Reduce Bone Formation and Strength

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Whether the negative impact of excess glucocorticoids on the skeleton is due to direct effects on bone cells, indirect effects on extraskeletal tissues, or both is unknown. To determine the contribution of direct effects of glucocorticoids on osteoblastic/osteocytic cells *in vivo*, we blocked glucocorticoid action on these cells via transgenic expression of 11β-hydroxysteroid dehydrogenase type 2, an enzyme that inactivates glucocorticoids. Osteoblast/osteocyte-specific expression was achieved by insertion of the 11β-hydroxysteroid dehydrogenase type 2 cDNA downstream from the osteoblast-specific osteocalcin promoter. The transgene did not affect normal bone development or turnover as demonstrated by identical bone density, strength, and histomorphometry in adult transgenic and wild-type animals. Administration of excess glucocorticoids induced equivalent bone loss in wild-type and transgenic mice. As expected, cancellous osteoclasts were unaffected by the transgene. However, the increase in osteoblast apoptosis that occurred in wild-type mice was prevented in transgenic mice. Consistent with this, osteoblasts, osteoid area, and bone formation rate were significantly higher in glucocorticoid-treated transgenic mice compared with glucocorticoid-treated wild-type mice. Glucocorticoid-induced osteocyte apoptosis was also prevented in transgenic mice. Strikingly, the loss of vertebral compression strength observed in glucocorticoid-treated wild-type mice was prevented in the transgenic mice, despite equivalent bone loss. These results demonstrate for the first time that excess glucocorticoids directly affect bone forming cells *in vivo*. Furthermore, our results suggest that glucocorticoid-induced loss of bone strength results in part from increased death of osteocytes, independent of bone loss. (*Endocrinology* 145: 1835–1841, 2004)

Т HE ADVERSE EFFECTS of glucocorticoid excess on the skeleton may be mediated by direct actions on bone cells, actions on extraskeletal tissues, or both (1). Glucocorticoid-induced bone loss occurs in two phases in humans and mice: a rapid, early phase in which bone mass is lost due to excessive bone resorption and a slower, later phase in which bone is lost due to inadequate bone formation (2–5). *In vitro* studies indicate that glucocorticoids act directly on differentiated osteoclasts to extend their life span and on osteoblasts to stimulate their apoptosis (4, 6). However, it is unknown whether glucocorticoids act directly on these cells *in vivo* or whether action on non-bone cells may contribute to changes in osteoblast and osteoclast numbers and/or activity via altered production of growth factors or cytokines.

In mineralocorticoid-sensitive tissues, such as the kidney, colon, and salivary glands, mineralocorticoid receptors (MR) have equal affinity for aldosterone and glucocorticoids. Therefore, to obtain the required aldosterone selectivity in the presence of the 1000-fold greater concentration of glucocorticoids, rapid pre-receptor oxidative inactivation of glucocorticoids occurs via 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), an enzyme expressed in mineralocorticoid-sensitive tissues. 11β-HSD2 is a 42-kDa, high-affinity, nicotinamide adenine dinucleotide-dependent enzyme, which converts biologically active glucocorticoids to their inert 11-keto metabolites (7, 8). We reasoned that transgenic expression of 11β-HSD2 specifically in mature osteoblasts and osteocytes would render them resistant to glucocorticoid action, and thus dissect the component of the adverse effects of glucocorticoid excess that results from direct action on these cells as opposed to actions on osteoclasts or tissues other than bone.

In the studies described herein, we overexpressed 11β-HSD2 in transgenic mice using the murine osteocalcin gene 2 (OG2) promoter, which is active only in mature osteoblasts and osteocytes (9, 10). As expected from our previous studies (4), demonstrating that the initial rapid phase of bone loss is due to glucocorticoid action on osteoclasts, the OG2–11β-HSD2 transgene did not affect glucocorticoid-induced bone loss. However, mice harboring the transgene were protected from glucocorticoid-induced apoptosis of osteoblasts and osteocytes. Prevention of osteoblast/osteocyte apoptosis in turn resulted in the preservation of cancellous osteoblasts and osteoid production thereby preventing the decrease in bone formation. More strikingly, bone strength was preserved in the transgenic mice despite loss of bone mass, suggesting that osteocyte viability independently contributes to bone strength.

Materials and Methods

Conditional expression and in vitro apoptosis assay

Conditional expression of 11β-HSD2 in the MLO-Y4 osteocytic cell line (11) was accomplished by infecting the cells with a retrovirus expressing the tetracycline-regulated transcription factor, tTA (TET-OFF), as previously described (12). A retroviral construct for conditional expression of 11β-HSD2 was prepared by inserting a 2.0-kb *Bam*HI-*Apa*I fragment containing the human 11β-HSD2 cDNA into the *Hpa*I site of pST (12). Retrovirus generated from this construct was used to infect the MLO-Y4 cells expressing the tTA protein to create a pool of cells in which the absence of doxycycline activated expression of the 11β-HSD2 protein. All retroviral supernatants were generated by transient transfection of Phoenix ampho packaging cells (13) using LipofectAMINE (Life Technologies, Gaithersburg, MD). Infections and immunoblots of the transduced cells, using antibodies directed against 11β-HSD2 and β-actin, were performed as previously described (7, 12). Apoptotic cells were quantified by trypan blue dye exclusion (6). We have previously demonstrated that use of trypan blue exclusion to quantitate the apoptosis of MLO-Y4 cells induced by these agents yielded levels of apoptosis similar to those detected by other methods such as nuclear morphology and caspase 3 activation (6).

Generation of transgenic mice

Constitutive, osteoblastic lineage-specific expression of 11β-HSD2 was achieved by placing the human 11β-HSD2 cDNA downstream of the osteoblast-specific 1.3-kb murine osteocalcin promoter. To supply heterologous introns and a polyadenylation site, and thus increase the likelihood of transgene expression, a human GH (hGH) minigene (14) was inserted downstream of the 11β-HSD2 cDNA. The start-codon in the hGH minigene has been inactivated so that it does not produce a functional GH protein. Purified DNA was microinjected into fertilized C57BL/6 eggs at the National Institute of Child Health and Human Development transgenic mouse development facility at the University of Alabama at Birmingham (Birmingham, AL). Transgenic founders were identified by PCR using the following primer sequences: HSD-forward (5′-CACTTCATGCCCACTGCCGATGCC-3′) and hGH-reverse (5′-GGTGAGCTGTCCACAGGACCCTG-3′). Transgenic mice were maintained in the C57BL/6 genetic background. All protocols involving these mice and their wild-type littermates 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.

Assessment of transgene expression

Total RNA from tibia, calvaria, vertebra L6, liver, and spleen of wild-type and OG2–11β-HSD2 transgenic mice was extracted using Ultraspec reagent (Biotecx Laboratories, Inc., Houston, TX) following instructions of the manufacturer. Equal amounts of RNA (2 μg) from each sample were reverse-transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Human 11β-HSD2, osteocalcin, or a housekeeping gene, ribosomal protein S2 (ChoB), were subsequently amplified from the first strand cDNA by real-time PCR using TaqMan Universal PCR Master Mix (Applied Biosystems). The following primer and probe sets were used: human 11β-HSD2, forward 5′-GCTTCAAGACAGAGTCAGTGAGAAA-3′, reverse 5′-AGGTTG-GCCAGCAGCAATT-3′, probe 5′-CGTGGGTCAGTGGGA-3′; osteocalcin, forward 5′-GCTGCCTCTGTCTCTCTGA-3′, reverse 5′-TGCTTG-GACATGAAGGCTTTG-3′, probe 5′-AAGCCCAGCGGCC-3′; and ChoB, forward 5′-CCCAGGATGGCGACGAT-3′, reverse 5′-CCGAAT-GCTGTAATGGCGTAT-3′, probe 5′-TCCAGAGCAGGATCC-3′. PCR amplification and detection were carried out on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) as follows: 5-min denaturation at 95 C for 10 min, 40 cycles of amplification including denaturation at 94 C for 15 sec and annealing/extension at 60 C for 1 min. Gene expression was quantitated using the comparative threshold cycle (Ct) method by subtracting the ChoB Ct value from the 11β-HSD2 or osteocalcin Ct value, because amplification efficiencies of 11β-HSD2, osteocalcin, and ChoB were equal (data not shown).

Immunohistochemistry

Lumbar vertebra (L1–L4) from three transgenic and three wild-type animals were fixed overnight in modified phosphate-buffered formalin (Surgipath, Richmond, IL) and decalcified in 5% EDTA for 1 wk. Vertebra were then embedded in paraffin, and 5 μM longitudinal sections were taken. Sections of human kidney were used as positive controls. After paraffin removal and rehydration, antigens were recovered by microwaving the sections in 0.1 M citrate buffer (pH 6.0) for 4 min at 48 C. The sections were then treated with 0.05% pepsin in 0.1 N HCl, at 37 C for 30 min followed by quenching in 3% H_2O_2 in methanol for 5 min. Sections were washed twice in PBS and blocked for 20 min in 2.5% normal horse serum. Antihuman 11β-HSD2 antibody (Alpha Diagnostic International, San Antonio, TX) was diluted to 7.5 μg/ml in PBS and incubated with the sections for 1 h at 37 C followed by rinsing and additional incubation for 20 min with biotinylated antirabbit IgG. Ready-to-use Elite ABC reagent (VectaStain, Vector Laboratories, Burlingame, CA) was applied for 20 min, washed thoroughly, and developed with diaminobenzidine (Lab Vision, Freemont, CA). Diaminobenzidine staining was enhanced by a 2-min incubation in 0.15% copper sulfate. Sections were counterstained with 2% methyl green for 2 min, dehydrated, and mounted.

Bone mineral density (BMD) determinations and glucocorticoid administration

Spinal bone mineral density was measured using dual-energy x-ray absorptiometry as previously described (Hologic, Inc., Bedford, MA) (3, 4, 15, 16). Determinations were done at 2-wk intervals to identify the peak adult bone mass of the mice, which occurred between 3 and 4 months of age. Measurements were then repeated to allocate the animals into groups with equivalent spinal BMD values. Slow-release pellets releasing 2.1 mg/kg·d prednisolone or placebo were implanted sc as previously described (3).

Bone histomorphometry

Lumber vertebra 1–4 from animals receiving prednisolone or placebo were fixed and embedded undecalcified in methyl methacrylate, and the histomorphometric examination was performed using the OsteoMeasure Analysis System (OsteoMetrics, Inc., Decatur, GA) as previously described (3, 4, 15). Apoptosis of osteoblasts and osteocytes was detected by *in situ* nick-end labeling using the Klenow enzyme (Oncogene Research Products, Cambridge, MA) in sections counterstained with 2% methyl green as previously described (2). To calculate the extent of osteoblast and osteocyte apoptosis, an average of 386 ± 222 (SD) osteoblasts and 1826 ± 416 (SD) osteocytes were counted per vertebral bone section.

Biomechanical testing

The load-bearing properties of the fifth lumbar vertebrae were measured on the day the mice were killed as previously described (15). After preseating with less than 0.5 Newtons of applied load, vertebrae were compressed between screw-driven loading platens using a lower-platen, miniature spherical seat that minimized shear by adjusting to irregularities in the end plates of the specimens (17). The length, width, and depth of the bones were recorded with a digital caliper at a resolution of 0.01 mm (Mitutoyo no. 500-196, Ace Tools, Ft. Smith, AR). The mechanical properties were normalized for vertebral size and ultimate strength or stress (Newtons/square millimeters; in megapascals) was calculated.

Statistics

To evaluate treatment effects for the various measurements, one-way ANOVA was used. Levene's test was used to test for homogeneity of variance of a given parameter. The Shapiro-Wilk test was performed to investigate the assumption of normality. When either the homogeneity of variance or normality assumptions required under ANOVA were not satisfied, transformations were done or the Kruskal-Wallis test was used. Bonferroni's or Dunnett's method was used to control the experiment-wise error rate, depending on whether or not all comparisons

were against a control. All comparisons were defined *a priori*. $P < 0.05$ was considered significant.

Results

Overexpression of 11β-HSD2 blocks glucocorticoid action

To determine whether ectopic expression of 11β-HSD2 could block the action of glucocorticoids *in vitro*, we used MLO-Y4 osteocytic cells (11). MLO-Y4 cells were selected for this purpose based on previous studies showing that they readily undergo programmed cell death after a 6-h exposure to dexamethasone (6). Specifically, in this *in vitro* experiment, we controlled the expression of the 11β-HSD2 gene by means of a tetracycline-regulated expression system in which removal of the tetracycline derivative doxycycline from the culture medium dramatically stimulates the expression of the gene of interest. Indeed, as shown in Fig. 1A, removal of doxycycline led to a marked up-regulation of the 11β-HSD2 protein, as assessed by immunoblot analysis. The specificity of the conditional expression of the transgene in the prevention of glucocorticoid action was established by the finding that overexpression of 11β-HSD2 completely blocked dexamethasone-induced apoptosis but had no effect on apoptosis induced by etoposide or TNFα (Fig. 1B).

Generation of transgenic mice expressing 11β-HSD2 in osteoblastic cells

Based on the ability of the ectopic expression of 11β-HSD2 to block glucocorticoid-induced apoptosis *in vitro*, we generated transgenic mice with a construct consisting of the human 11β-HSD2 inserted downstream from the murine OG2 promoter, which is expressed almost exclusively in cells of the osteoblastic lineage, including osteocytes (Fig. 2A) (9, 10). A single founder was identified and maintained in the C57BL/6 strain. Offspring hemizygous for the OG2–11β-HSD2 transgene were obtained at the expected Mendelian frequency. Growth and development were the same as wild-type littermates. Real-time PCR analysis of mRNA from tibia, calvaria, vertebra, liver, and spleen of transgenic and wild-type mice indicated that expression of the transgene faith-

fully coincided with that of the endogenous osteocalcin gene (Fig. 2B). Consistent with the expression of the 11β-HSD2 mRNA only in bone, human 11β-HSD2 protein was detected specifically in osteoblasts and osteocytes present in vertebral cancellous bone of the transgenic, but not wild-type, mice (Fig. 2, C and D). The specificity of the anti-11β-HSD2 antibody was further confirmed by demonstrating that it stained human kidney tubules and collecting ducts, which express high levels of this protein, but not in glomeruli, which do not (Fig. 2E).

Overexpression of 11β-HSD2 prevents osteocyte apoptosis and preserves bone strength

Spinal BMD determinations, performed at weekly intervals over a 28-d period in mature (4 months old) transgenic and wild-type control mice, revealed no difference (Fig. 3A). In addition, at 5 months of age, vertebral compression strength (Fig. 3B) and bone histomorphometric measurements, including the percentage of cancellous bone covered by osteoblasts, were identical in transgenic animals and wild-type controls (Table 1). These results indicate that blockade of glucocorticoid action in osteocalcin-expressing cells (mature osteoblasts and osteocytes) does not alter skeletal development, peak adult bone mass, or bone cell number.

In the absence of a skeletal impact of the transgene during normal development and growth, we next challenged the animals with a 28-d course of prednisolone treatment. Wild-type and OG2–11β-HSD2 mice exhibited a similar decrease in spinal BMD soon after administration of prednisolone (Fig. 4A). Consistent with this finding, the percentage of cancellous bone covered by osteoclasts did not differ between transgenic mice and wild-type controls (Fig. 4C), indicating that, as expected, the transgene did not affect osteoclasts.

Strikingly, however, although prednisolone increased osteoblast apoptosis in wild-type mice, this phenomenon was completely prevented in transgenic mice (Fig. 4B). Consistent with this finding, cancellous osteoid area, osteoblast perim-
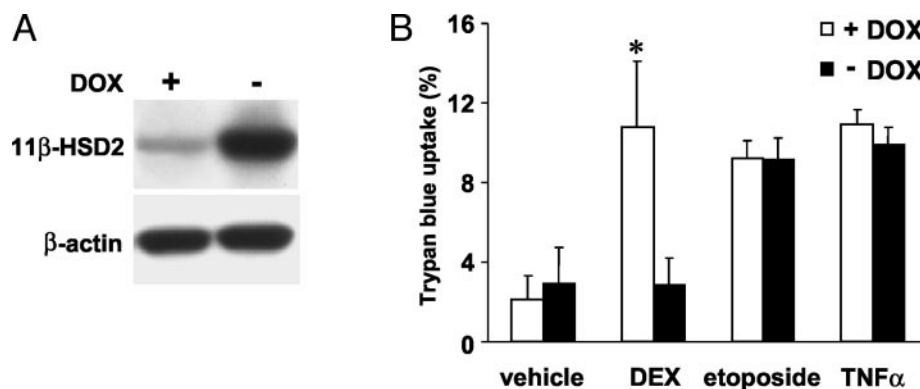
FIG. 1. Conditional expression of 11β-HSD2 in the MLO-Y4 osteocytic cell line blocks glucocorticoid-induced apoptosis. A, MLO-Y4 cells conditionally expressing 11β-HSD2 were cultured in the presence or absence of doxycycline (DOX) for 48 h after which protein extracts were prepared and analyzed by immunoblot with antibodies to 11β-HSD2 and β-actin. B, MLO-Y4 cells conditionally expressing 11β-HSD2 were cultured as in panel A and subsequently treated with dexamethasone, etoposide, or TNFα for 6 h. Values indicate the mean percentage of trypan *blue* staining cells determined from three separate wells ± SD. *White bars*, 100 ng/ml DOX; *black bars*, no DOX. *, $P < 0.05$ by one-way ANOVA *vs.* dexamethasone in the absence of DOX.
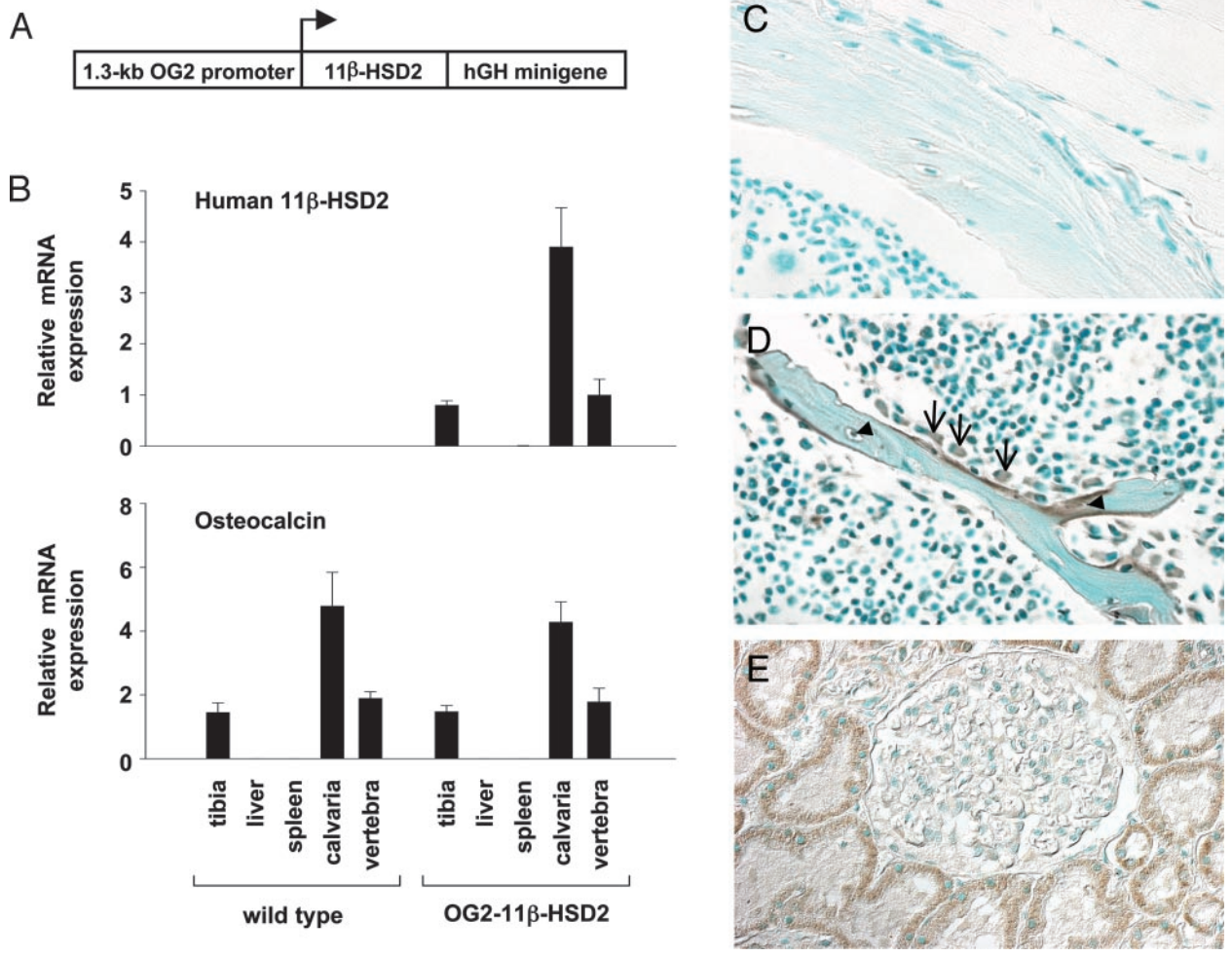
FIG. 2. Generation of OG2-HSD2 transgenic mice. A, The construct used to generate transgenic mice consisted of the 1.3-kb murine osteocalcin promoter (OG2) upstream from the human 11β-HSD2 cDNA. The construct also included a hGH minigene downstream from the 11β-HSD2 cDNA. B, Total RNA (2 μg), prepared from tibia, liver, spleen, calvaria, and vertebra of wild-type or OG2–11β-HSD2 transgenic mice, was reverse-transcribed and subsequently amplified by real-time PCR for 11β-HSD2, osteocalcin, and ribosomal protein S2 (ChoB). 11β-HSD2 and osteocalcin values, relative to ChoB, are given as the mean ± SD of analyses performed on RNA samples from five wild-type and five transgenic mice. C–E, Antihuman 11β-HSD2 antibody was incubated with sections of wild-type vertebra (C), transgenic vertebra (D), or human kidney (E). The *dark brown* staining indicates the presence of the human 11β-HSD2 protein in murine osteoblasts (*arrows*) and osteocytes (*arrowheads*) from transgenic animals (D) and in human tubules and collecting ducts but not glomeruli (E) (original magnification, ×400).

FIG. 3. Osteoblast-specific expression of 11β-HSD2 *in vivo* does not affect normal bone development or growth. A, Serial measurements of spinal BMD in wild-type (*gray line*) or OG2–11β-HSD2 (*orange line*) mice were performed beginning at 120 d of age. Values indicate the mean of five to seven animals per group ± SD. B, Vertebral compression strength (strength) was determined in the animals shown in panel A at 150 d of age. MPa, Megapascals.
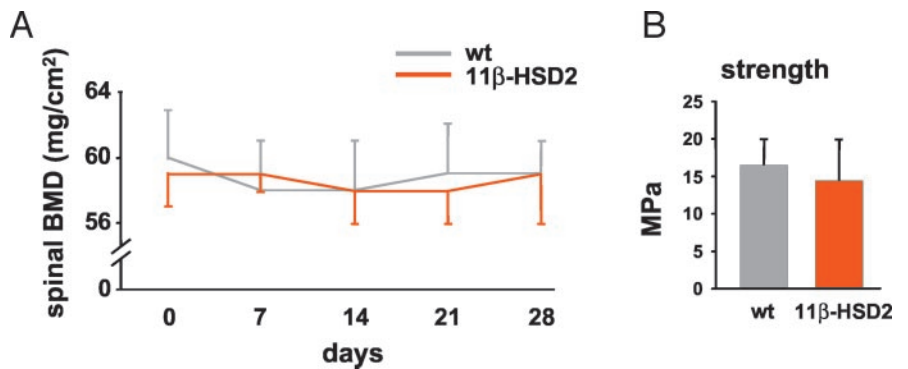
eter, bone formation rate, and activation frequency were unaffected in prednisolone-treated transgenic mice (Fig. 4C). OG2–11β-HSD2 transgenic mice were also protected from prednisolone-induced apoptosis of cancellous osteocytes

(Fig. 4D). Furthermore, despite the similar loss of BMD in both wild-type and transgenic animals, vertebral compression strength was preserved in the transgenic mice when compared with wild-type (Fig. 4E).

TABLE 1. The OG2-11β-HSD2 transgene does not alter vertebral cancellous bone histology under basal conditions

Histomorphometric determination	Wild type	OG2-11β-HSD2
Bone area/tissue area (%)	13.8 ± 1.9	15.0 ± 2.5
Trabecular width (μm)	38.7 ± 4.5	40.7 ± 7.3
Trabecular spacing (μm)	247 ± 59	233 ± 38
Trabecular number (per mm)	3.6 ± 0.6	3.7 ± 0.6
Osteoid area/bone area (%)	1.6 ± 1.1	2.4 ± 1.9
Osteoid width (μm)	2.6 ± 0.6	3.0 ± 0.9
Osteoblast perimeter/bone perimeter (%)	10.4 ± 5.6	15.0 ± 11.4
Osteoclast perimeter/bone perimeter (%)	3.8 ± 2.6	4.2 ± 3.1
Mineral appositional rate (μm/d)	1.30 ± 0.44	1.23 ± 0.36
Bone formation rate/bone perimeter (μm^2/μm/d)	0.14 ± 0.16	0.17 ± 0.16

Data shown are the mean ± SD. There are six animals per group.

Discussion

To determine the contribution of direct glucocorticoid action on osteoblasts and osteocytes to the overall adverse effects of a pharmacologic excess of these agents on the skeleton, we expressed the enzyme 11β-HSD2 specifically in osteocalcin-expressing cells in transgenic mice. Whereas glucocorticoid excess induced a similar loss of BMD in wild-type and transgenic mice, osteoblasts and osteocytes were protected from steroid-induced apoptosis in the transgenic mice. Likewise, the transgenic mice, in difference to the wild-type controls, were protected from reductions in osteoid area, osteoblasts, bone formation rate, activation frequency, and compression strength. These results provide the first *in vivo* evidence that the proapoptotic effects of glucocorticoids on osteoblasts and osteocytes are indeed the result of direct actions of glucocorticoids on these two cell types. Furthermore, these results confirm that the initial loss of bone that occurs with this pharmacologic insult does not result from direct glucocorticoid action on osteoblasts and osteocytes. More importantly, our results suggest for the first time that osteocyte survival may contribute to bone strength independently of BMD.

Glucocorticoids promote differentiation of rat and human osteoblastic cells *in vitro* under some conditions (18). On the other hand, they inhibit proliferation and differentiation of murine osteoblasts and are not required for mineralized nodule formation in murine bone marrow cultures (19, 20). However, it is unknown whether glucocorticoids are needed *in vivo* for the differentiation of mesenchymal stem cell progenitors to osteoblasts or for the function of the differentiated osteoblast. Our finding that BMD and histomorphometric parameters were normal in adult OG2–11β-HSD2 mice suggests that endogenous glucocorticoid action in osteocalcin-expressing cells is not required for normal skeletal development. Nonetheless, this finding does not preclude an action of glucocorticoids on osteoblast progenitors before the stage when they express osteocalcin. Indeed, in a recent report by Sher *et al.* (21), female mice harboring a 2.3-kb Col1a1 promoter-11β-HSD2 transgene exhibited reduced vertebral bone volume and trabecular number. These effects however, were not observed in males or in the long bones of either sex, suggesting that endogenous glucocorticoids may posi-

tively affect murine bone in a site- and sex-specific manner. Because the Col1a1 promoter is active at an earlier stage of osteoblast differentiation than the osteocalcin promoter (22), our finding that blockade of endogenous glucocorticoid action in osteocalcin-expressing cells did not alter bone mass, strength, or histology suggests that in our transgenic animal model glucocorticoid inactivation occurred in a stage of osteoblast differentiation that no longer requires endogenous glucocorticoids. From our studies, we cannot exclude the possibility that the transgene might have affected the skeleton during growth. Nonetheless, because the transgene had no detectable effect on the BMD, strength, size, weight, or bone histology of 5-month-old mice, any effects that might have occurred during growth must have been very small or reversible.

The observation that glucocorticoids act directly on osteoblasts and osteocytes *in vivo* to stimulate their apoptosis is consistent with previous *in vitro* studies showing that pharmacologic levels of glucocorticoids induce apoptosis of primary osteoblasts as well as osteoblastic and osteocytic cell lines (6). Moreover, this observation is consistent with evidence that the glucocorticoid-induced increase in osteoblast apoptosis contributes to the decrease in bone formation rate, as does a decrease in osteoblastogenesis (3). Nonetheless, whether decreased osteoblastogenesis is a result of direct actions on mesenchymal osteoblast precursors, as opposed to an indirect effect on other cell types in the bone marrow, remains unknown, because osteocalcin, and thus the transgene used in our experiments, is strongly expressed only in fully differentiated osteoblasts and osteocytes (9).

The results of the present study indicate that glucocorticoid action on osteoblasts and osteocytes is not required for the early bone loss caused by this class of steroid hormones. We have shown previously that glucocorticoids transiently increase osteoclast number after 7–10 d of administration, when osteoclastogenesis is decreased, due to an antiapoptotic effect on mature osteoclasts, and this increase is associated with the early loss of bone (3, 4). In addition to direct actions, glucocorticoids may increase osteoclast survival in part via suppression of osteoprotegerin (OPG) production by osteoblastic cells (23), thereby increasing the available amount of receptor activator of nuclear factor-κB ligand, and inhibiting osteoclast apoptosis (24). Assuming that glucocorticoid suppression of OPG is indeed important for the glucocorticoid-induced increase in osteoclasts, our results argue that the target of the suppressive effect of glucocorticoids on OPG must be a cell that is different from the mature, osteocalcin-expressing cell, because loss of bone mass did occur in the prednisolone-treated OG2–11β-HSD2 mice despite the fact that glucocorticoid action was blocked in osteoblasts and osteocytes.

Because cancellous osteoblasts and bone formation rate were not compromised in the prednisolone-treated transgenic mice, one might have expected that these mice would not lose as much BMD as the wild-type controls, but they did. Why is this? The early rapid loss of bone is due primarily to osteoclastic bone resorption (4), which was of course not affected by the transgene. Moreover, there was not sufficient time for the differences in osteoblast perimeter and bone formation rate to result in an increase in BMD in the trans-
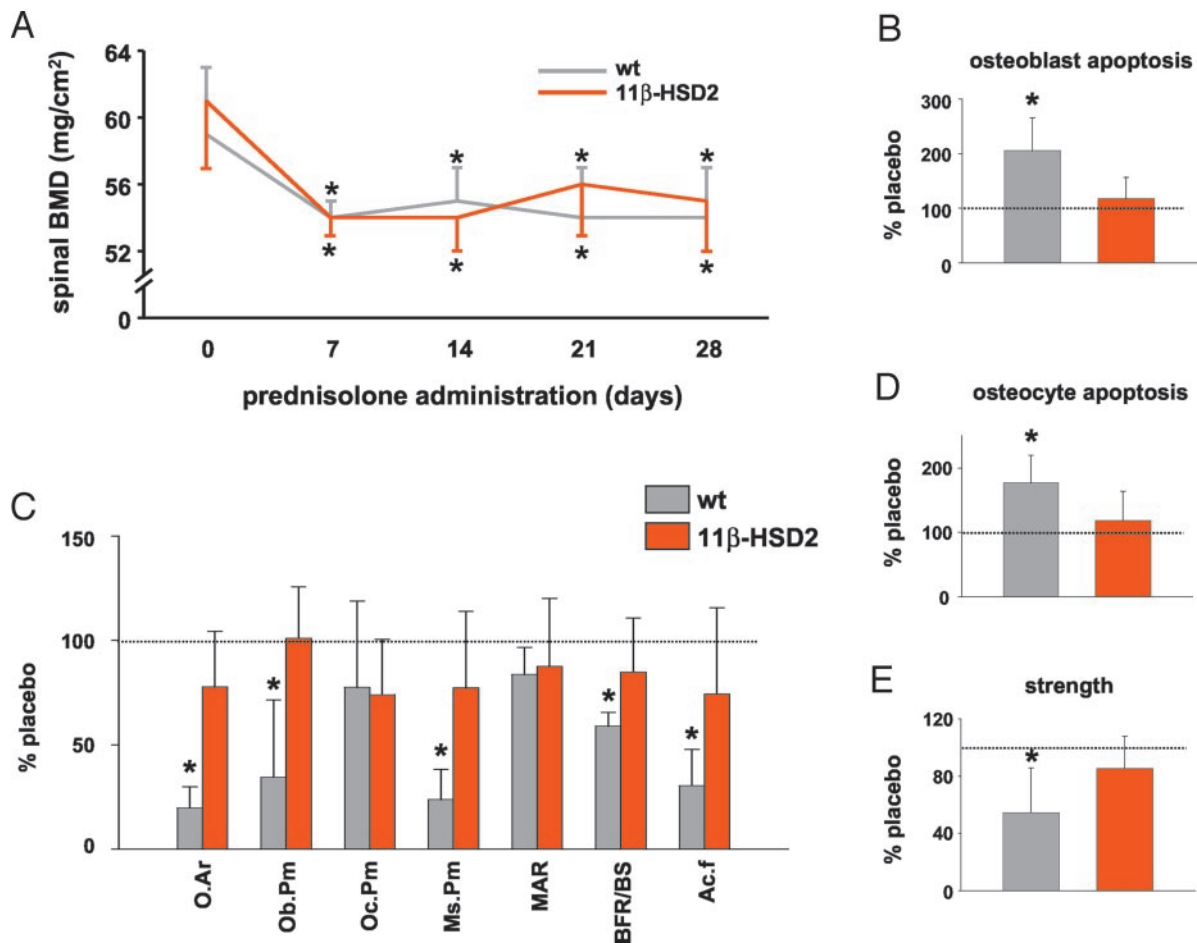
FIG. 4. Osteoblast-specific expression of 11β-HSD2 prevents glucocorticoid-induced osteoblast and osteocyte apoptosis as well as loss of bone strength. A, Serial measurement of spinal BMD of 120-d-old wild-type or OG2–11β-HSD2 mice was performed weekly after implantation of prednisolone pellets. The spinal BMD of placebo-treated mice did not change significantly during the 28-d experiment (shown in Fig. 3A). *, $P < 0.05$ *vs.* placebo. B, Osteoblast apoptosis was determined in the same animals indicated in panels 3A and 4A, at the end of the experiment (150 d). *, $P < 0.05$ *vs.* placebo. C, In the same animals, osteoid area (O.Ar), osteoblast perimeter (Ob.Pm), osteoclast perimeter (Oc.Pm), mineralizing surface (Ms.Pm), mineral appositional rate (MAR), bone formation rate (BFR/BS), and activation frequency (Ac.f) were determined. The 1/(square root) transformation was used to test BFR/BS and Ac.f. The Wilcoxon rank sum test was used to test O.Ar, Ob.Pm, Oc.Pm, and Ms.Pm. *, $P < 0.05$ *vs.* placebo. D, Cancellous osteocyte apoptosis was determined in the same animals indicated in panels 3A and 4A, at the end of the experiment (150 d). *, $P < 0.05$ *vs.* placebo. E, Vertebral compression strength (strength) was determined in the animals indicated above. *, $P < 0.05$ *vs.* placebo. In all panels, *gray lines* and *bars* indicate wild-type and *orange lines* and *bars* indicate OG2–11β-HSD2 mice. *Dotted lines* indicate 100% of placebo.

genic mice, relative to wild-type controls, because osteoblasts require a 3- to 4-fold longer time period to form bone than osteoclast need to resorb it (3, 4).

Most unexpectedly, prevention of osteocyte apoptosis in our studies was associated with a significant preservation of bone strength in the prednisolone-treated OG2–11β-HSD2 mice. This striking observation leads us to speculate that osteocytes contribute to bone strength independently of bone mass. In support of this idea, in humans treated with glucocorticoids, the rate of fracture increases within 3 months of the initiation of therapy, even before significant loss of BMD (25, 26). Specifically, BMD declines by 0.6–6% per year in patients receiving excess glucocorticoids, but the relative risk of fracture increases more rapidly, escalating by as much as 75% within the first 3 months after initiation of steroid therapy (25). Consistent with this, in some (27–29) but not all (30–32) studies, glucocorticoid-induced fractures occur at

higher BMD values than those found in patients with other kinds of osteoporosis (27, 28), indicating that the adverse effects of glucocorticoid excess on bone may be due, in part, to abnormalities separate from the decline in bone mass. Furthermore, even when a difference in the relationship between BMD and fracture threshold was not confirmed, fractures in glucocorticoid-treated patients were more likely to be multiple than in other forms of osteoporosis (30). Finally, abundant apoptotic osteocytes are typical of glucocorticoid-induced osteonecrosis of the hip (33). In this disorder, collapse of the femoral head occurs despite radiographic evidence of increased bone density (34), thus supplying additional evidence of a glucocorticoid-induced decrease in bone strength independent of changes in bone mass.

The mechanism by which osteocytes contribute to bone strength is unknown but may be related to bone quality (26). Loss of osteocytes may disrupt the osteocyte-canalicular net-

work resulting in a failure to detect signals that normally stimulate replacement of damaged bone. Accumulation of damaged bone would in turn lead to a decrease in bone strength without a decrease in bone mass. Disruption of the osteocyte-canalicular network, via osteocyte apoptosis, would also be expected to disrupt fluid flow within the network that could adversely affect the material properties of the surrounding bone, independently of changes in bone remodeling or microarchitecture.

In conclusion, the evidence presented herein demonstrates that osteoblasts and osteocytes are direct targets of glucocorticoid action *in vivo* and that excess levels of this steroid hormone directly induce apoptosis of these cell types. Moreover, our results raise for the first time the possibility that osteocyte viability might be an independent determinant of bone strength.

Acknowledgments

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