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Rosiglitazone Causes Bone Loss in Mice by Suppressing Osteoblast Differentiation and Bone Formation

A. Afshan Ali, Robert S. Weinstein, Scott A. Stewart, A. Michael Parfitt, Stavros C. Manolagas, and Robert L. Jilka

Division of Endocrinology & Metabolism, Center for Osteoporosis and Metabolic Bone Diseases, Central Arkansas Veterans Healthcare System, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Because osteoblasts and marrow adipocytes are derived from a common mesenchymal progenitor, increased adipogenesis may occur at the expense of osteoblasts, leading to bone loss. Our previous *in vitro* studies indicated that activation of the proadipogenic transcription factor peroxisome proliferator-activated receptor isoform γ 2 with rosiglitazone suppressed osteoblast differentiation. Here, we show that 5-month-old Swiss-Webster mice receiving rosiglitazone for 28 d exhibited bone loss associated with an increase in marrow adipocytes, a decrease in the ratio of osteoblasts to osteoclasts, a reduction in bone formation rate, and a reduction in wall width—an index of the amount of bone formed by each team of osteoblasts. Rosiglitazone had no effect on the number of early osteoblast or osteoclast progenitors, or on osteoblast life span, but decreased the expression of the key osteoblastogenic transcription factors Runx2 and Osterix in cultures of marrow-derived mesenchymal progenitors. These effects were associated with diversion of bipotential progenitors from the osteoblast to the adipocyte lineage, and suppression of the differentiation of monopotential osteoblast progenitors. However, rosiglitazone had no effect on osteoblastic cells at later stages of differentiation. Hence, rosiglitazone attenuates osteoblast differentiation and thereby reduces bone formation rate *in vivo*, leading to bone loss. These findings provide a mechanistic explanation for the recent evidence that peroxisome proliferator-activated receptor isoform γ activation is a negative regulator of bone mass and suggest that the increased production of oxidized fatty acids with age may indeed be an important mechanism for age-related osteoporosis in humans. (*Endocrinology* 146: 1226–1235, 2005)

OSTEOBLASTS AND MARROW adipocytes are derived from a common multipotential mesenchymal stem cell (MSC) progenitor (1). It has been hypothesized that the close relationship between these lineages underlies the reciprocal relationship between increased adipocytes and decreased bone formation that occurs during aging (2–4). Indeed, age-related bone loss is associated with a deficit in the number of osteoblasts needed to replace the bone removed by osteoclasts during remodeling as evidenced by a decrease in mean wall thickness (5).

Lineage commitment of MSCs is determined by expression and/or activation of specific transcription factors: Runx2 and Osterix in the case of osteoblasts (6, 7), and CCAAT enhancer binding protein β and peroxisome proliferator-activated receptor isoform γ (PPARγ) in the case of adipocytes (8). PPARγ is broadly expressed and plays an important role not only in adipogenesis but also in glucose homeostasis, atherosclerosis, and cancer (9). It is a member of the nuclear hormone receptor family of transcription factors, and exists in two isoforms, PPARγ1 and PPARγ2, resulting from alternative promoter usage and alternative splicing. The transcriptional activity of PPARγ is induced by a variety of ligands including 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2, oxidized fatty acid metabolites, as well as synthetic thiazolidinediones like troglitazone and rosiglitazone (10–15).

Mounting evidence indicates an important role of PPARγ in skeletal metabolism. Specifically, PPARγ haploinsufficient mice exhibit increased bone mass associated with increased osteoblastogenesis and decreased adipogenesis (16). Using murine marrow-derived MSCs, as well as a model marrow-derived stromal cell line overexpressing PPARγ2, we have shown that activation of PPARγ2 with rosiglitazone stimulates adipogenesis and inhibits osteoblastogenesis, at least in part by down-regulating Runx2 (17); and that fatty acid peroxidation products such as 9,10-dihydroxyoctadecenoic acid and 9,10-epoxyoctadecenoic acid can attenuate osteoblast differentiation in a PPARγ2-dependent fashion (13). We have therefore hypothesized that increased PPARγ activation may contribute to the reduced osteoblast production and bone loss that occurs with aging.

Rosiglitazone causes bone loss in rats and mice (18, 19), but the cellular basis underlying this skeletal response has not been determined. Here, we report that adult mice receiving rosiglitazone exhibit bone loss characterized by an increase in marrow adipocytes, and a decrease in the osteoblast/osteoclast ratio, wall width, and bone formation rate. In addition, we show that rosiglitazone diverted bipotential mesenchymal progenitors from the osteoblast to the adipocyte lineage and also suppressed the differentiation of monopotential osteoblast progenitors. These inhibitory effects

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Abbreviations: aP2, Fatty acid binding protein 4; BMD, bone mineral density; CFU-OB, colony-forming unit-osteoblast; ChoB, housekeeping gene ribosomal protein S2; Ct, threshold cycle; DEXA, dual energy x-ray absorptiometry; LPL, lipoprotein lipase; MSC, mesenchymal stem cell; PPARγ, peroxisome proliferator activated receptor isoform γ; Runx2, runt-related transcription factor-2 (also known as Osf2, Cbfa1, and Aml3).

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were strongly associated with a reduction in the expression of Runx2 and Osterix.

Materials and Methods

Animals

Swiss-Webster mice were obtained from Harlan (Indianapolis, IN). The University of Arkansas for Medical Sciences Division of Laboratory Animal Medicine approved the animal use protocol. Food was blended with rosiglitazone (Tularik, Inc., South San Francisco, CA), which was dissolved at 4.25 mg/ml of a 1:3 mixture of ethanol and glyceroloctaside (Stepan Chemical, Maywood, NJ), 0.1% Tenox 20 (Eastman Chemical, Kingsport, TN) before mixing with a blender to give a concentration of 0.03 mg or 0.15 mg of rosiglitazone/g normal rodent chow (Agway RMH 3000; Arlington Heights, IL). Mice were pair fed to avoid potential artifacts secondary to the hyperphagia that has been reported to occur when rats or mice are given a thiazolidinedione (20, 21). Five-month-old mice were housed singly, and average daily consumption of vehicle chow was determined to be 5–6 g/d over a 2-wk period. Animals were fed 5 g chow/d containing vehicle (four males and two females), 5 μg/g·d rosiglitazone (two males and three females), or 25 μg/g rosiglitazone (three males and three females) for 28 d. These doses have previously been shown to give moderate to strong antihyperglycemic activity in diabetic ob/ob mice (22). By weighing the food dish before placement in the cage and after removal, it was determined that each mouse consumed more than 90% of the chow throughout the course of the experiment. Tetracycline (30 mg/kg) was administered ip at 8 and 3 d before the end of the experiment.

Bone densitometry

Dual-energy x-ray absorptiometry (DEXA) was used to determine bone mineral density (BMD) as previously described using a Hologic 2000 Plus (19, 23). Over the past 3 yr, the coefficient of variation of the measurement done on a plastic-embedded whole mouse skeleton was 1.8% (n = 202). BMD determinations were performed before initiation of the study and used to allocate the animals into groups with equivalent spinal BMD values before entry into the treatment protocol. Measurements were made again after 2 wk, and at termination of the experiment after 4 wk of treatment using the Compare program of Hologic software to ensure accurate repositioning for serial measurements. Global BMD refers to the entire skeleton excluding the skull. Spinal and hindlimb subregions were measured as previously described (23).

Bone histomorphometry

The left femur and the lumbar vertebrae (L1–L4) were fixed, embedded undecalcified in methyl methacrylate, and stained using modified Massons's trichrome (23–25). Histomorphometric measurements were performed using a computer and digitizer tablet (Osteomeasure version 3.00, Osteometrics Inc., Atlanta, GA) interfaced to a Zeiss Axioscope (Carl Zeiss Inc., Thornwood, NY) with a drawing tube attachment. The identity of the sample was unknown to the reader. Measurements were made with a Zeiss Plan-Neofluar 40 × (0.75 numerical aperture) objective on the entire secondary spongiosa, which comprised approximately 60 fields in at least three of the vertebrae from L1–L4, and approximately 30 fields in the distal femur. Variables were measured and reported as previously described (23, 25, 26) using the terminology recommended by the Histomorphometry Nomenclature Committee of American Society for Bone and Mineral Research (27). The number of fat cells within the tissue area measured, as well as individual fat cell area and diameter, were also determined. They are expressed as total fat cell number per tissue area, and the diameter of fat cells. Osteoclast and osteoblast number per tissue area are reported to facilitate direct comparison to fat cell number.

Apoptotic osteoblasts were detected by *in situ* nick end labeling using Klenow terminal deoxynucleotidyl transferase (Oncogene Research Products, Cambridge, MA) as previously described (28). Vertebrae from orchidectomized adult mice were used as a positive control. Slides were counterstained with 2% methyl green. Apoptotic osteoblasts were identified as *in situ* nick end labeling-positive cells lining the osteoid covered cancellous bone surface.

Quantification of osteoclast and osteoblast progenitors

Marrow cells were obtained from the right femur of each animal and replicate cultures established for the determination of osteoclast progenitors and colony-forming osteoblast progenitors (CFU-OB) as previously described (29, 30). Briefly, osteoclast progenitors were determined by coculturing marrow cells with UAMS-32 stromal/osteoblastic cells for 8 d in the presence 10 nM $1,25(OH)_2D_3$. Osteoclastic cells were enumerated after staining for tartrate-resistant acid phosphatase. CFU-OB number was determined by culturing cells for 25 d with irradiated guinea pig feeder cells in MEM supplemented with 15% preselected fetal bovine serum and 1 mM ascorbate-2-phosphate to promote osteoblastogenesis. Colonies containing osteoblasts were visualized by Von Kossa staining.

CFU-OB replication *in vitro* was determined as described previously (30). One aliquot of cells was used to determine CFU-OB number in the initial isolate, as described above. A second aliquot was used to establish replicate cultures of cells in type I collagen gels, which were then maintained in the absence or presence of 100 nM rosiglitazone for 6 d. The cells were dispersed using bacterial collagenase, and the number of CFU-OB within each gel was determined using the colony-forming assay described above. To calculate the fold increase in CFU-OB during culture in the collagen gels, the number of CFU-OB obtained per gel (after 6 d of culture) was divided by the number of CFU-OB initially added to the collagen gel.

The effect of rosiglitazone on adipocyte and osteoblast differentiation *in vitro* was determined by culturing bone marrow cells at 2.5×10^6 per 10 cm^2 well with irradiated guinea pig cells and maintained for 25 d in medium supplemented with 1 mM ascorbate-2-phosphate, either in the absence or presence of 100 nM rosiglitazone, added at various times after establishment of the culture. On d 25, cells were stained either with Oil Red O to visualize colonies containing adipocytes or Von Kossa stain to visualize colonies containing osteoblasts elaborating a mineralized matrix.

Quantification of gene expression in cultured bone marrow cells and osteoblastic cells

Murine bone marrow cells were cultured as described above. Osteoblastic cells were obtained from neonatal murine calvarial by collagenase digestion as previously described (31), and cultured in MEM supplemented with 15% preselected fetal bovine serum and 1 mM ascorbate-2-phosphate. Total RNA was extracted using Ultraspec reagent (Biotecx Laboratories, Inc., Houston, TX). RNA (2 μg) was reverse-transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The transcripts of interest, and that of the housekeeping gene ribosomal protein S2 (ChoB), were amplified from first-strand cDNA by real-time PCR using TaqMan Universal PCR Master Mix and Assay by Demand or Assay by Design primer and probe sets (Applied Biosystems). Amplification and detection were carried out with an ABI Prism 7300 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 quantified by subtracting the ChoB threshold cycle (Ct) value from the Ct value of the gene of interest, and expressed as $2^{-\Delta Ct}$, as described by the protocol of the manufacturer.

Statistics

Data were analyzed using SigmaStat (SPSS Science, Chicago, IL) or SAS software (SAS Institute Inc., Cary, NC). All values are reported as the mean ± SD. Differences between group means in histomorphometry studies were evaluated with Student's *t* test, or by ANOVA after *P* values were adjusted with Bonferroni's correction. Serial changes in body weight and BMD were analyzed using a model of repeated measures (32). Pearson correlation analysis of fat cell number with osteoblast number and mineralizing surface was done after adding three to total fat cell number (to avoid large negative values for mice with no adipocytes), and then taking the natural logarithm to obtain a normal distribution. Differences in group means in studies of gene expression and colony number were evaluated with Student's *t* test.

Results

Rosiglitazone causes bone loss and decreases osteoblast number and bone formation rate

Rosiglitazone was fed to adult (5 months old) male and female Swiss-Webster mice at 5 or 25 μg/g body weight·d for 28 d. Vehicle was added to the food of control mice. There was no change in body weight in mice receiving the vehicle, but both doses of rosiglitazone caused an approximately 2% increase in body weight (Fig. 1A). As reported previously (33), administration of 25 μg/g·d of rosiglitazone increased interscapular fat (Fig. 1B), indicating that drug administration was efficacious. Liver weight was unaffected (Fig. 1C).

Serial bone densitometry using DEXA revealed that after 28 d of administration, mice given rosiglitazone at 25 μg/g·d exhibited a decrease in vertebral BMD, compared with mice fed the vehicle control (Fig. 2). The same trend was noted in mice fed this dose of rosiglitazone when BMD was measured over the entire skeleton (global) and in hindlimbs, but the changes were not statistically significant. The 5 μg/g·d dose of rosiglitazone had no discernable effect on BMD. Thus, histologic and histomorphometric studies were performed only on mice fed the 25-μg dose.

Mice receiving rosiglitazone exhibited increased marrow fat in both vertebral and femoral bone compared with mice receiving the vehicle control (Fig. 3). Vertebral bone from control animals was practically devoid of adipocytes, but rosiglitazone increased their number by approximately 20-fold (Table 1). Adipocyte number in femoral metaphyseal bone of control mice was considerably higher than that of vertebral bone, and rosiglitazone caused a further 7-fold increase (Table 2). Fat cell diameter was unaffected, indicating that rosiglitazone did not enhance lipogenesis in existing adipocytes. Consistent with the densitometric findings, rosiglitazone caused loss of cancellous vertebral bone that was characterized by a decrease in trabecular width, an increase in trabecular spacing, and a decrease in trabecular number (Table 1). Similar microarchitectural changes were noted in femoral cancellous bone (Table 2).

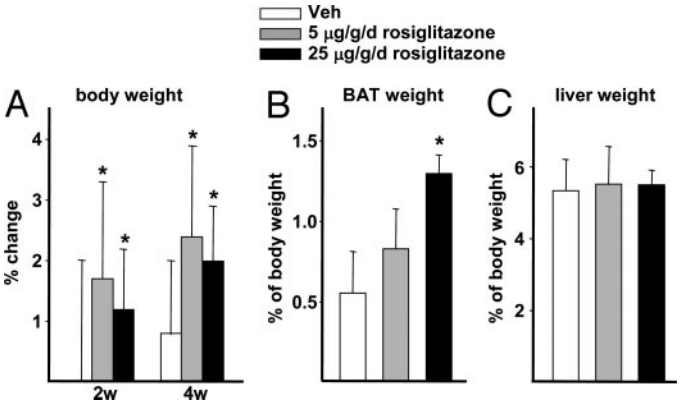

FIG. 1. Effect of rosiglitazone on body weight, interscapular brown adipose tissue (BAT) weight, and liver weight. Values represent percentage of change in body weight from baseline determined at the beginning of the experiment (A) or tissue weight at the end of the experiment (B and C). *, $P < 0.01$ *vs.* vehicle as determined by repeated measures analysis (body weight) or ANOVA (BAT and liver weight).

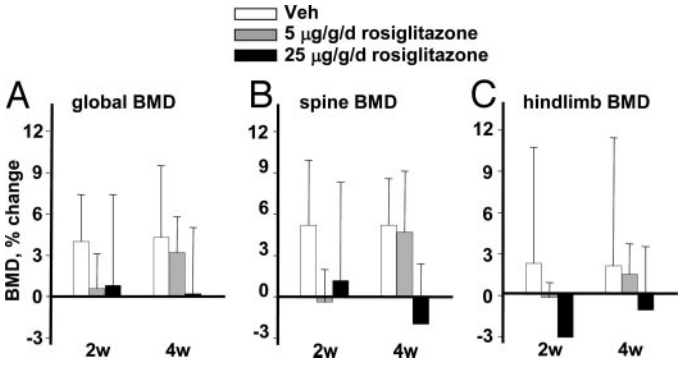

FIG. 2. Rosiglitazone causes loss of BMD. Values represent percentage of change in BMD from the baseline value determined at the beginning of the experiment. Repeated measures analysis indicated a significant group × time interaction for spinal BMD ($P = 0.007$) and indicated a stronger negative effect for the 25 μg/g dose of rosiglitazone than the 5 μg/g dose.

Rosiglitazone caused a reduction in the number of osteoblasts per square millimeter of vertebral and femoral cancellous bone (Tables 1 and 2). However, when expressed on a bone perimeter rather than bone area referent, osteoblast number did not exhibit a statistically significant decline, nor was osteoid surface affected in mice receiving rosiglitazone. The drug had no effect on the prevalence of apoptotic osteoblasts. Osteoclast number was also unaffected, whether

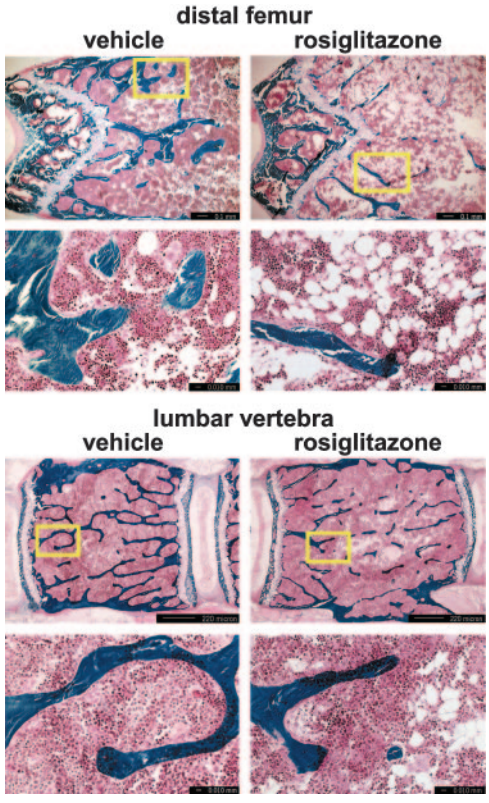

FIG. 3. Increased marrow fat and decreased cancellous bone in mice receiving rosiglitazone. Nondecalcified bone sections were stained with Masson trichrome stain. Calcified bone is stained *blue*; adipocytes were present in the *white* globular areas. The *lower panels* of each set of photomicrographs represent high-power views of the field denoted by the *yellow square* in the *upper panel*.

TABLE 1. Effect of rosiglitazone on vertebral cancellous bone

Histomorphometric measurement	Vehicle	Rosiglitazone	*P*
Bone area/tissue area (%)	14.9 ± 4.1	9.7 ± 1.3	0.01
Trabecular width (μm)	35.5 ± 3.1	31.8 ± 2.9	0.05
Trabecular spacing (μm)	211 ± 46	299 ± 22	0.005
Trabecular number (N/mm^2 tissue area)	4.2 ± 0.8	3.0 ± 0.2	0.01
Wall width (μm)	8.2 ± 1.0	6.4 ± 0.6	0.005
Osteoid width (μm)	2.2 ± 0.2	2.3 ± 0.2	NS
Osteoid perimeter/bone perimeter (%)	14.0 ± 4.3	12.6 ± 4.7	NS
Osteoblast number (N/mm^2 tissue area)	84.4 ± 30.0	45.7 ± 15.4	0.03
Osteoblast number (N/mm bone perimeter)	10.3 ± 3.9	7.5 ± 2.4	NS
Osteoblast apoptosis (%)	7.5 ± 0.8	7.7 ± 1.0	NS
Fat cell number (N/mm^2 tissue area)	6 ± 11	122 ± 50	0.001
Fat cell diameter (μm)	10.8 ± 9.9	20 ± 2.6	NS
Osteoclast number (N/mm^2 tissue area)	5.7 ± 2.7	5.1 ± 1.8	NS
Osteoclast number (N/mm bone perimeter)	1.0 ± 0.2	1.2 ± 0.3	NS
Osteoblast/osteoclast ratio (from perimeter referent)	10.2 ± 3.1	6.4 ± 3.2	0.05
Mineralizing perimeter/bone perimeter (%)	10.8 ± 4.8	4.9 ± 1.8	0.025
Mineral apposition rate (μm/d)	1.02 ± 0.1	0.9 ± 0.1	NS
Mineralization lag time (d)	3.5 ± 2.1	7.1 ± 1.8	NS
Bone formation rate/bone perimeter (μm^2/μm/d)	0.110 ± 0.050	0.046 ± 0.023	0.01

NS, Not significant.

expressed per millimeter of bone perimeter or square millimeter of bone area. However, osteoblast number in both vertebral and cancellous bone declined by 2-fold when expressed as the ratio of osteoblasts to osteoclasts, calculated from the number of each cell type per millimeter of bone perimeter. Moreover, there was an inverse correlation between adipocyte number and osteoblast number whether expressed per square millimeter of tissue area or as the ratio of osteoblasts to osteoclasts, in both vertebral and femoral cancellous bone (Table 3). The biological impact of the reduction in the ratio of osteoblasts to osteoclasts in mice receiving rosiglitazone was revealed by a decrease in vertebral bone wall width (Table 1).

These histological findings establish that rosiglitazone caused a deficit in the number of osteoblasts when compared with the number needed to refill the resorption cavities created by osteoclasts. Consistent with this, rosiglitazone caused a 2-fold decrease in bone formation rate in vertebral bone (Table 1). This was due to a reduction in mineralizing surface, rather than mineral apposition rate. Fat cell number inversely correlated with mineralizing surface (Table 3). Rosiglitazone had no effect on width of the osteoid layer between osteoblasts and mineralized bone, or on mineralization lag time, the interval between deposition and mineralization of osteoid. Therefore, the drug did not cause defective mineralization. The small amount of cancellous bone that remained in the femurs of mice fed rosiglitazone (~2–6% of tissue area) precluded ascertainment of potential changes in wall width or indices of bone formation.

Rosiglitazone inhibits the differentiation of osteoblasts from CFU-OB

Analysis of *ex vivo* cultures of mice from the experiment described above established that rosiglitazone had no effect on the number of MSCs, as measured by *ex vivo* assay of CFU-OB, or on the number of osteoclast progenitors (Fig. 4). Consistent with the lack of effect on CFU-OB number, rosiglitazone also had no effect on the replication of CFU-OB *in vitro*. Specifically, progenitors increased by 4.3 ± 0.7-fold during 6 d of culture with vehicle *vs.* 3.5 ± 0.8-fold in the presence of 100 nM rosiglitazone (n = 3 per group). Thus, the reduction in osteoblast number and bone formation rate seen in mice receiving rosiglitazone must be due to actions of the drug on progeny of MSC, as measured by CFU-OB (30).

The production of osteoblasts from MSC *in vitro* proceeds via sequential stages of replication and lineage commitment during the first 2 wk of culture, after which development of the mature matrix-synthesizing osteoblast occurs (30, 34). To determine the stage at which rosiglitazone influences osteo-

TABLE 2. Effect of rosiglitazone on distal femoral cancellous bone

Histomorphometric measurement	Vehicle	Rosiglitazone	*P*
Bone area/tissue area (%)	8.0 ± 2.3	4.2 ± 1.9	0.01
Trabecular width (μm)	39.7 ± 11.7	31.7 ± 2.1	NS
Trabecular spacing (μm)	449 ± 101	866 ± 411	0.05
Trabecular number (N/mm^2 tissue area)	2.0 ± 0.4	1.3 ± 0.7	0.05
Osteoblast number (N/mm^2 tissue area)	85.7 ± 21.0	34.6 ± 23.7	0.01
Osteoblast number (N/mm bone perimeter)	21.8 ± 7.4	14.5 ± 10.0	NS
Osteoblast apoptosis (%)	12.3 ± 1.4	13.0 ± 1.4	NS
Fat cell number (N/mm^2 tissue area)	76 ± 48	544 ± 286	0.005
Fat cell diameter (μm)	21.2 ± 6.0	18.2 ± 4.7	NS
Osteoclast number (N/mm^2 tissue area)	5.7 ± 2.7	5.1 ± 1.8	NS
Osteoclast number (N/mm bone perimeter)	1.3 ± 0.4	2.0 ± 0.7	NS
Osteoblast/osteoclast ratio (from perimeter referent)	18.2 ± 8.7	6.6 ± 3.1	0.02

NS, Not significant.

TABLE 3. Inverse correlation between marrow adipocytes and osteoblast number, as well as mineralizing surface

		Osteoblast number[a]	Osteoblast number[b]	Osteoblast/osteoclast ratio	Mineralizing perimeter
Vertebrae	Fat cell number[a]	−0.77 (0.01)	−0.58 (0.08)	−0.68 (0.03)	−0.77 (0.01)
Femur	Fat cell number[a]	−0.82 (0.004)	−0.38 (0.28)	−0.75 (0.01)	

Data from Tables 1 and 2 were analyzed by Pearson correlation.
[a], N/mm^2 tissue area; [b], N/mm bone perimeter. Correlation coefficient (r) values are shown with *P* values in *parentheses*.

blast differentiation, the drug was added on d 3, 7, 11, or 15 after establishment of *ex vivo* cultures of femoral marrow cell cultures maintained in ascorbate-2-phosphate to stimulate osteoblast differentiation. Calcium deposition and osteocalcin secretion, markers of differentiated osteoblasts, were quantified after 25 d of culture. When rosiglitazone was added on d 3 or d 7 of culture, calcium deposition and osteocalcin secretion were reduced by 90% or more, whereas only a modest inhibitory effect was observed when the drug was added on d 11 or 15 (Fig. 5A).

The influence of rosiglitazone on the differentiation of colony-forming cells was examined in more detail in a parallel set of cultures using Von Kossa and Oil Red O staining to detect the presence of osteoblasts and adipocytes, respectively. Rosiglitazone reduced the total number of colonies by approximately 50% when added on d 3 or 7 (Fig. 5B), as we had previously observed when rosiglitazone was added on the first day of culture (13); however, the drug had no effect on colony number when added after d 7. Colonies containing only adipocytes, only osteoblasts, both cell types, or neither cell type were quantified (Fig. 5, C–F). Most of the colony-forming cells gave rise to adipocytes when rosiglitazone was added on d 3, and osteoblastogenesis was practically abolished (Fig. 5, C–E). The percentage of colonies containing only adipocytes declined progressively with later addition of rosiglitazone, suggesting loss of the ability to differentiate into adipocytes with longer culture time in pro-osteogenic medium (Fig. 5E). When rosiglitazone was added on d 7, the number of colonies containing only osteoblasts was still greatly reduced, but this inhibitory effect was much less evident when the ligand was added on d 11 or 15 (Fig. 5C).

When rosiglitazone was added on d 7 or later, approximately one third of the colonies contained both osteoblasts and adipocytes (Fig. 5D). Such colonies must have arisen from a progenitor that gives rise to two kinds of daughter cells: one that can be driven into the adipocyte lineage with rosiglitazone, and one that differentiates into osteoblasts in the presence ascorbate-2-phosphate. We have designated this colony-forming cell as a bipotential progenitor but still recognize that it could differentiate into other cell types under the proper conditions. When rosiglitazone is added on d 3, few colonies containing both osteoblasts and adipocytes were observed. This indicates that rosiglitazone diverted bipotential progenitors into the adipocyte lineage. However, the ligand could also have suppressed the differentiation of osteoblasts from a monopotential osteoblastogenic daughter of the bipotential cell. Finally, about 20% of colonies contained neither osteoblasts nor adipocytes, but their number was also reduced by rosiglitazone at d 3 and 7, perhaps accounting for part of the decrease in overall colony number at these times (Fig. 5F).

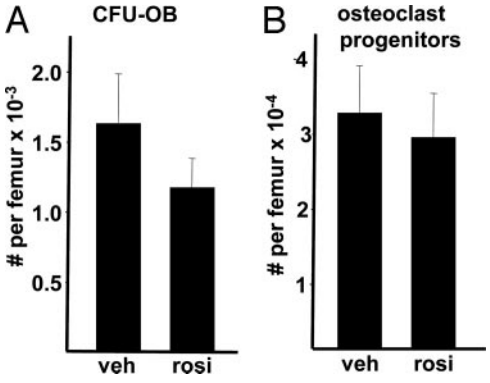

FIG. 4. Rosiglitazone has no effect on the number of osteoclast and osteoblast progenitors in the bone marrow. Femoral marrow cells were obtained from the animals receiving vehicle (veh) or rosiglitazone (rosi) and *ex vivo* femoral marrow cell cultures were established for the quantification of osteoblast progenitors (CFU-OB) (A) or osteoclast progenitors (B).

To gain additional insight on the suppressive effect of rosiglitazone on osteoblastogenesis, we examined the expression of osteoblast- and adipocyte-specific transcription factors and proteins using real-time PCR. In this experiment, the ligand was added to cultured bone marrow cells on d 7, *i.e.* before expression of the osteoblast phenotype as indicated by the lack of secretion of osteocalcin (Fig. 5A). Transcript levels were determined just before addition of the ligand, and at d 10, 15, and 25 of culture. On d 7, PPARγ2 transcripts were approximately 10-fold less than PPARγ1 transcripts, but rosiglitazone rapidly increased expression of PPARγ2 by approximately 30-fold, consistent with previous evidence that PPARγ stimulates its own expression (35). PPARγ1 transcripts were either not affected or modestly suppressed at d 15 of culture (Fig. 6). More importantly, rosiglitazone fully prevented the increase in expression of Runx2 and Osterix, which are required for osteoblast differentiation. This effect coincided with a failure to induce expression of the bone matrix proteins Col1A1, osteocalcin and bone sialoprotein, consistent with the findings of Fig. 5 showing almost complete suppression of osteoblast differentiation when rosiglitazone was added on d 7 of culture.

Rosiglitazone also down-regulated Wnt-10b expression, a response that is required for expression of the adipocyte phenotype (36) (Fig. 6). In addition, expression of the adipocyte markers fatty acid binding protein 4 (aP2), adiponectin, and lipoprotein lipase (LPL) were dramatically increased, consistent with the up-regulation of PPARγ2 and the important role of this form of the transcription factor in ligand-stimulated adipogenesis (35). These findings confirm the data of Fig. 5 showing a dramatic increase in Oil Red O-positive adipocytes when rosiglitazone was added on d 7 of culture.

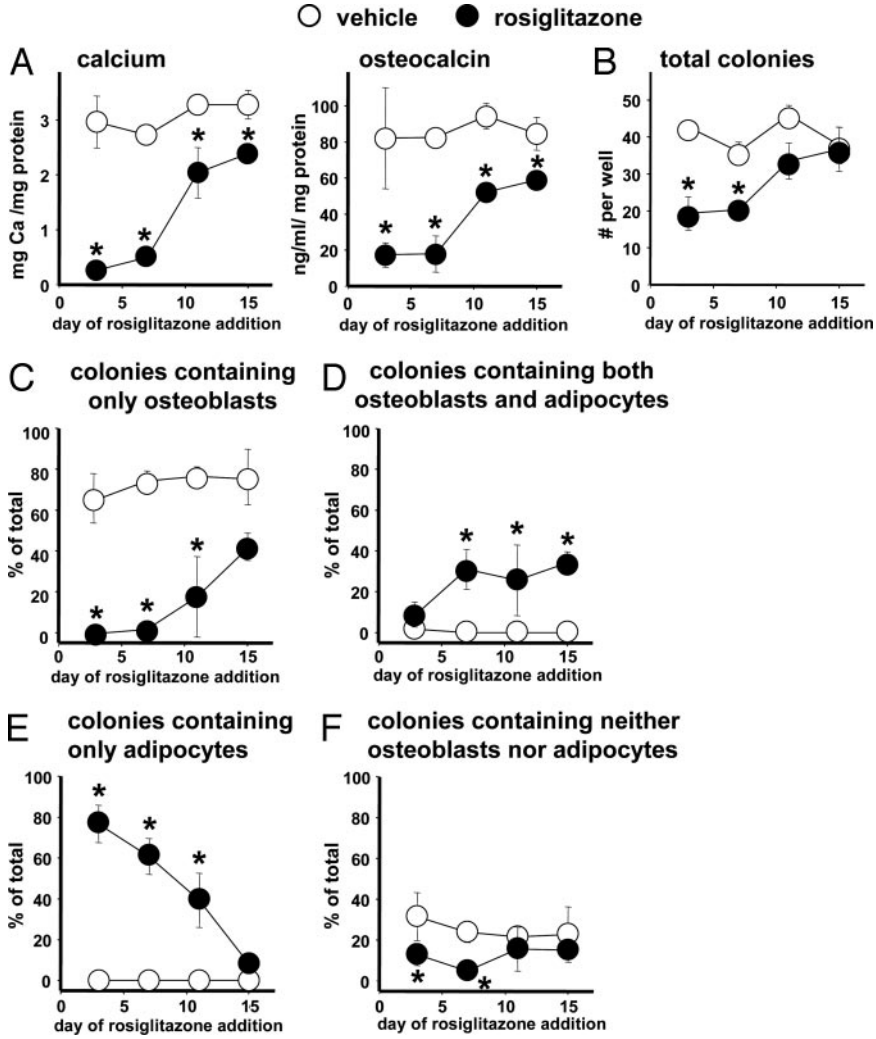

FIG. 5. Rosiglitazone inhibits osteoblastogenesis only when added in the early phase of differentiation. Marrow cell cultures from femurs of 5-month-old Swiss-Webster mice were established as described in *Materials and Methods*. Vehicle (dimethylsulfoxide) or 100 nM rosiglitazone was added to two sets of parallel cultures on d 3, 7, 11, or 15. On d 25, one set of cultures (A) was analyzed for the content of osteocalcin in the medium, and for the content of calcium in the matrix. The second set of cultures (B–F) was stained with Von Kossa to visualize colonies containing mineralizing osteoblasts and with Oil Red O to visualize adipocytes. Data shown represent the mean (±SD) of triplicate cultures. *, $P < 0.05$ *vs.* vehicle control at the same time point, by ANOVA.

In view of the evidence of Fig. 5 that rosiglitazone had no effect on osteocalcin secretion or mineralization when added to bone marrow cultures at late stages of culture, we examined the effect of rosiglitazone on gene expression in preparations of osteoblastic cells obtained from neonatal murine calvaria. In contrast to the response of bone marrow progenitors, addition of rosiglitazone did not increase PPARγ2 or decrease Wnt-10b transcripts in calvarial osteoblastic cells (Fig. 7). Moreover, transcript levels of osteoblast-specific transcription factors and matrix proteins were unaffected by the ligand. The adipocyte markers aP2, adiponectin ,and LPL were increased, but the absolute level of expression achieved by addition of rosiglitazone was approximately 20-fold lower than in bone marrow cells. This probably reflects the formation of only a few adipocytes, rather than expression of adipocyte markers in osteoblastic cells because a small number of Oil red O-positive cells were present in rosiglitazone-treated calvaria cells (not shown). These findings demonstrate that, unlike early progenitors, osteoblastic cells in the later stages of differentiation are resistant to the suppressive effects of rosiglitazone.

Discussion

We demonstrate herein that the bone loss caused by administration of rosiglitazone to mice is due at least in part to a reduction in the differentiation of osteoblasts from early progenitors. Specifically, rosiglitazone decreased the ratio of osteoblasts to osteoclasts, reduced the bone formation rate, and reduced wall width. These effects of rosiglitazone were associated with an increase in marrow adipocytes.

The cellular target of the adverse effect of rosiglitazone on the skeleton appears to be downstream of the MSC as evidenced by the lack of effect on the marrow content of CFU-OB or their replication. Moreover, rosiglitazone did not affect osteoblast life span. Instead, *in vitro* studies indicate that rosiglitazone diverts bipotential mesenchymal progenitors from the osteoblast to the adipocyte lineage, and also suppresses the differentiation of monopotential osteoblast progenitors. Consistent with our previous findings using marrow stromal cell lines (13, 17), these inhibitory effects were strongly associated with increased expression of PPARγ2 and a reduction in the expression of Runx2 and Osterix. Decreased expression of these transcription factors, which are required for osteoblastogenesis (6, 7), may explain

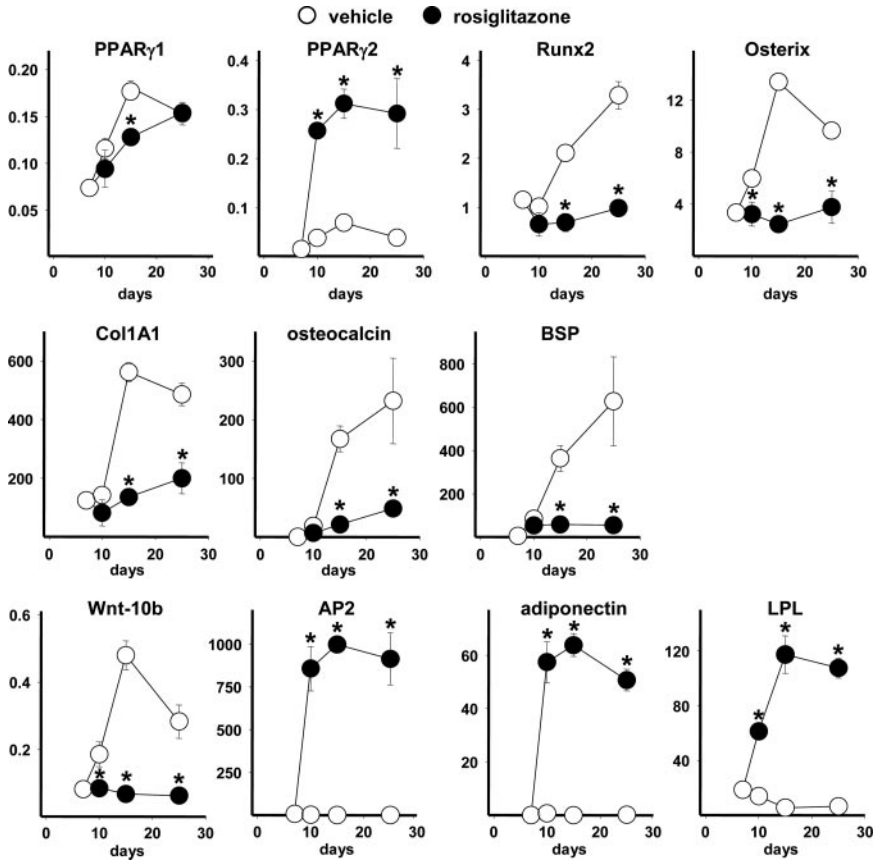

FIG. 6. Rosiglitazone reduces expression of osteoblast-specific transcription factors and matrix proteins, but up-regulates PPARγ2 and adipogenesis in cultured bone marrow cells. Marrow cell cultures were established as in Fig. 5, and vehicle (dimethylsulfoxide) or 100 nM rosiglitazone was added on d 7. RNA was isolated immediately before addition of these agents, and on d 10, 15, and 25 of culture. The indicated transcripts, and the housekeeping gene ChoB, were quantified by TaqMan PCR. Data shown represent the mean (±SD) level of expression for each transcript relative to ChoB, determined in triplicate cultures. *, $P < 0.05$ *vs.* contemporaneous vehicle control by *t* test. BSP, Bone sialoprotein.

how rosiglitazone blocked osteoblastogenesis in these cultures as evidenced by the failure to increase expression of matrix proteins like collagen1A1, osteocalcin, and bone sialoloprotein, and to deposit mineral into the extracellular matrix.

In contrast with early marrow-derived osteoblast progenitors, osteoblastic cells in the later stages of differentiation, either formed from marrow progenitors or isolated from neonatal murine calvaria, were resistant to the negative effect of rosiglitazone on the expression of the osteoblast phenotype. The reason for this difference is unclear but may be related to the inability of the ligand to increase PPARγ2 expression and/or to decrease Wnt-10b in the more differentiated osteoblastic cells.

PPARγ haploinsufficiency in mice causes an increase in bone mass associated with increased osteoblastogenesis and decreased adipogenesis from marrow-derived MSC (16), indicating that PPARγ restrains osteoblast differentiation under normal physiological circumstances. We have previously shown that certain oxidized fatty acid ligands of PPARγ suppress osteoblast differentiation *in vitro* (13). Oxidized fatty acids produced by 12/15-lipoxygenase are evidently active in the skeleton because mice lacking this enzyme exhibited increased bone mass, and administration of lipoxygenase inhibitors increased bone mass in wild-type mice (37). Thus, the adverse skeletal effects of rosiglitazone reported herein may reflect increased PPARγ-mediated restraint of bone formation beyond that already activated by endogenous oxidized fatty acids, albeit putative PPARγ-independent mechanisms of rosiglitazone action have not been excluded. The production of oxidized fatty acids increases with age (38), raising the possibility that age-related bone loss is due at least in part to an increase in the inhibitory effects of PPARγ on osteoblast differentiation. Indeed, rosiglitazone reproduced several of the histologic indices of age-related bone loss including increased marrow fat and decreased wall width (2–5). These findings highlight the need for future studies to establish whether endogenous PPARγ2 ligands are involved in age-related bone loss.

The common origin of adipocytes and osteoblasts contributes to the reciprocal changes in the number of these cells caused by rosiglitazone, but suppression of the differentiation of monopotential osteoblast progenitors may also be involved. This situation is reminiscent of the cell autonomous effects of ΔFosB overexpression that attenuates adipocyte differentiation via actions in early mesenchymal progenitors and stimulates bone formation via actions in osteoblasts (39). Moreover, reciprocal changes in marrow adipocyte and osteoblast number may not always accompany PPARγ activation. Indeed, we have shown that the ligand 9-hydroxyoctadecadienoic acid stimulates adipogenesis but does not attenuate osteoblast differentiation, whereas 9,10-epoxyoctadecenoic acid suppresses osteoblast differentiation but does not stimulate adipogenesis (13).

The reduction in the ratio of osteoblasts to osteoclasts shown here largely accounts for the reduced trabecular width, wall width, and the extent of bone surface undergoing mineralization in mice receiving rosiglitazone. However, we

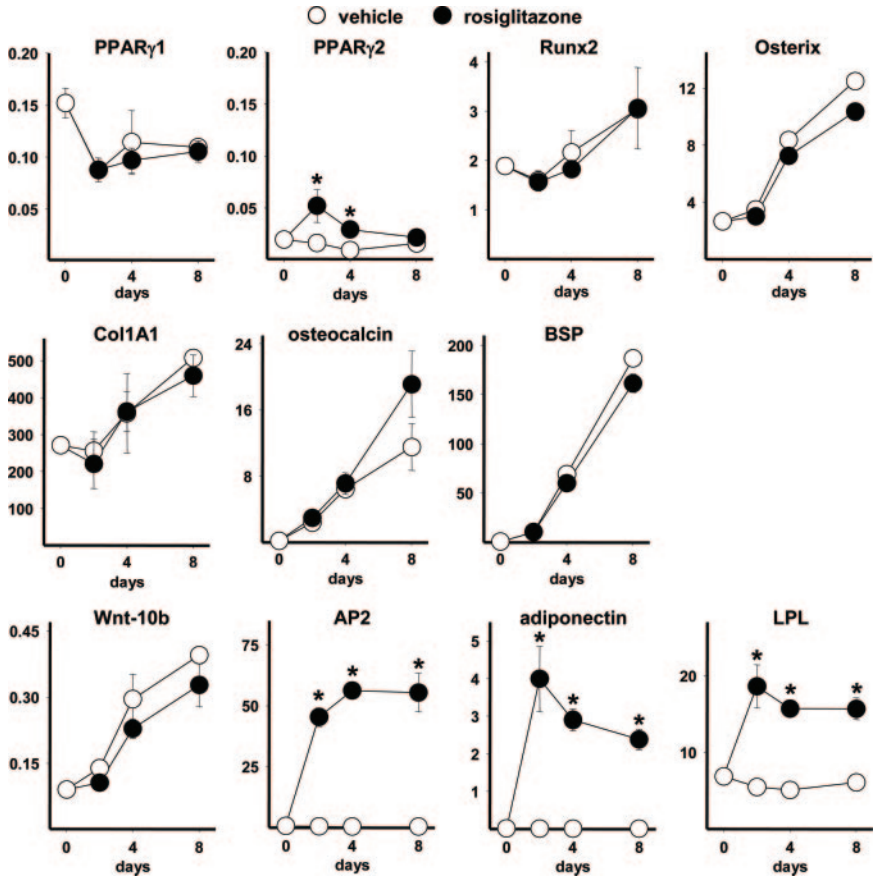

FIG. 7. Rosiglitazone has no effect on expression of osteoblast-specific transcription factors and matrix proteins in cultures of calvaria-derived osteoblastic cells. Osteoblastic cells from neonatal murine calvaria were grown to confluence, at which time vehicle (dimethylsulfoxide) or 100 nM rosiglitazone was added. RNA was isolated immediately before addition of these agents, and at 2, 4, and 8 d thereafter, and quantified by TaqMan PCR. Data shown represent the mean (±SD) level of expression for each transcript relative to ChoB, determined in triplicate cultures. *, $P < 0.05$ *vs.* contemporaneous vehicle control by *t* test. BSP, Bone sialoprotein.

also noted a decrease in trabecular number and an increase in the spacing between those remaining, indicating that some trabeculae have been removed (40). Osteoclastic perforation of a trabecular plate that was not repaired because of an osteoblast defect could be responsible, but trabeculae could have been lost due to increased bone resorption in the early phase of the experiment, leaving no trace by 28 d of how it had occurred (41). Increased resorption is inconsistent with evidence that activation of PPARγ with 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 or thiazolidinediones inhibits rather than stimulates osteoclast differentiation and bone resorption *in vitro* (42, 43), but is consistent with evidence that, in cultured human aortic smooth muscle cells, thiazolidinediones strongly suppress the synthesis of osteoprotegerin, which antagonizes the pro-osteoclastogenic effects of receptor activator of nuclear factor κB ligand (44).

In contrast with our findings, Rzonca *et al.* (19) reported that rosiglitazone reduced the mineral apposition rate in mice by 3.8-fold. Procedural differences may account for this discrepancy because we measured the entire secondary spongiosa of distal femoral bone and three lumbar vertebrae, whereas Rzonca *et al.* selected six fields in the proximal tibia—a comparatively small bone that may respond to rosiglitazone in a different fashion than other skeletal sites. Mineral apposition rate mainly reflects the vigor of individual osteoblasts, whereas mineralizing surface is a measure of the number of osteoblasts. The *in vitro* studies of the present report show that rosiglitazone inhibited the formation of osteoblasts from early progenitors, but had little if any effect on the activity of osteoblasts in the late stages of differentiation. This evidence is consistent with the reduction in mineralizing surface and lack of effect on mineral apposition rate we observed *in vivo*.

The adverse effect of thiazolidinediones on the rodent skeleton raises the possibility that this class of antidiabetic drugs could cause bone loss. Okazaki *et al.* (45) reported that, after taking troglitazone for 1 month, type II diabetics exhibited decreased bone remodeling as indicated by a reduction in the bone resorption markers deoxypyridinoline collagen type I C-terminal peptide, as well as the bone formation marker bone specific alkaline phosphatase. However, these markers returned toward baseline after 6 and 12 months of treatment (46). Most studies report normal to increased BMD and reduced fracture incidence in type II diabetics (47–49), perhaps due to low bone turnover (50). Further studies are needed to investigate whether thiazolidinediones adversely impact the seemingly favorable effects of type II diabetes on the skeleton.

In summary, we have elucidated that, as suggested by earlier *in vitro* work, rosiglitazone does indeed attenuate osteoblast differentiation and thereby reduces bone formation rate *in vivo*, leading to bone loss. Strong evidence indicates that activation of PPARγ by natural ligands, such as oxidized fatty acids, plays an important role in aging in general (38), and the development of atherosclerosis in particular (51). Furthermore, PPARγ activation is now an established negative regulator of bone mass (16, 37), and atherosclerosis and osteoporosis coexist (52). Collectively, these

lines of evidence fully justify the need for further work to establish whether increased activation of PPARγ secondary to increased level of oxidized fatty acid ligands with age represents a major mechanism for age-related osteoporosis in humans.

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Address all correspondence and requests for reprints to: Robert L. Jilka, Ph.D., Division of Endocrinology and Metabolism, Slot 587, University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, Arkansas 72205. E-mail: jilkarobertl@uams.edu.

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Note Added in Proof

While this manuscript was under review, Soroceanu *et al*. (53) reported that, similar to our findings, administration of rosiglitazone to growing mice for 90 d caused bone loss. Albeit, in contrast to our report, Soroceanu *et al*. found that the bone loss in their study was associated with a 4-fold increase in the combined prevalence of apoptotic osteoblasts and osteocytes. In our studies, we failed to detect an increase in osteoblast apoptosis (Tables 1 and 2). In addition, when we retrospectively examined vertebral sections for osteocyte apoptosis, we found no change with rosiglitazone administration (vehicle, 3.6 ± 1.3%; rosiglitazone, 4.3% ± 0.6%). The difference between the two sets of observations could be due to differences in the age of the animals or duration of treatment.

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