ORIGINAL ARTICLE

JBMR

Selective Glucocorticoid Receptor Modulation Maintains Bone Mineral Density in Mice

Sylvia Thiele,[1] Nicole Ziegler,[1] Elena Tsourdi,[1] Karolien De Bosscher,[2,3] Jan P Tuckermann,[4] Lorenz C Hofbauer,[1,5]* and Martina Rauner[1]*

[1]Division of Endocrinology and Metabolic Bone Diseases, Department of Medicine III, Technical University, Dresden, Germany
[2]Department of Medical Protein Research, VIB, Ghent, Belgium
[3]Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium
[4]Leibniz Institute of Aging Research, Fritz Lipmann Institute, Jena, Germany
[5]DFG Research Center and Cluster of Excellence for Regenerative Therapies, Technical University, Dresden, Germany

ABSTRACT

Glucocorticoids (GCs) are potent anti-inflammatory drugs, but their use is limited by their adverse effects on the skeleton. Compound A (CpdA) is a novel GC receptor modulator with the potential for an improved risk/benefit profile. We tested the effects of CpdA on bone in a mouse model of GC-induced bone loss. Bone loss was induced in FVB/N mice by implanting slow-release pellets containing either vehicle, prednisolone (PRED) (3.5 mg), or CpdA (3.5 mg). After 4 weeks, mice were killed to examine the effects on the skeleton using quantitative computed tomography, bone histomorphometry, serum markers of bone turnover, and gene expression analysis. To assess the underlying mechanisms, in vitro studies were performed with human bone marrow stromal cells (BMSCs) and murine osteocyte-like cells (MLO-Y4 cells). PRED reduced the total and trabecular bone density in the femur by 9% and 24% and in the spine by 11% and 20%, respectively, whereas CpdA did not influence these parameters. Histomorphometry confirmed these results and further showed that the mineral apposition rate was decreased by PRED whereas the number of osteoclasts was increased. Decreased bone formation was paralleled by a decline in serum procollagen type 1 N-terminal peptide (P1NP), reduced skeletal expression of osteoblast markers, and increased serum levels of the osteoblast inhibitor dickkopf-1 (DKK-1). In addition, serum CTX-1 and the skeletal receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) ratio were increased by PRED. None of these effects were observed with CpdA. Consistent with the in vivo data, CpdA did not increase the RANKL/OPG ratio in MLO-Y4 cells or the expression of DKK-1 in bone tissue, BMSCs, and osteocytes. Finally, CpdA also failed to transactivate DKK-1 expression in bone tissue, BMSCs, and osteocytes. This study underlines the bone-sparing potential of CpdA and suggests that by preventing increases in the RANKL/OPG ratio or DKK-1 in osteoblast lineage cells, GC-induced bone loss may be ameliorated. © 2012 American Society for Bone and Mineral Research.

KEY WORDS: GLUCOCORTICOID-INDUCED OSTEOPOROSIS; BONE REMODELING; COMPOUND A; DKK-1; RANKL

Introduction

Osteoporosis is a frequent disease that leads to an increased risk of fractures caused by a systemic impairment of bone mass, strength, and microarchitecture.[1] The most common form of secondary osteoporosis is glucocorticoid-induced osteoporosis (GIO), which affects up to 50% of patients receiving glucocorticoids (GCs). GC therapy is widely used for the treatment of various diseases, including autoimmune and inflammatory disorders and malignancies, and is associated with detrimental effects on bone caused by suppression of bone formation and stimulation of bone resorption.[2,3] One of the underlying mechanisms of increased bone resorption is the increase in the receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) ratio in osteoblastic cells.[4,5] We previously showed that pharmacological inhibition of RANKL by denosumab, an anti-RANKL antibody, prevented bone loss in a mouse model of GIO,[6] indicating that this pathway is crucial for the induction of GC-induced bone loss.

Besides stimulating bone resorption, GCs also inhibit bone formation.[3,7] Several pathways have been identified to be affected by GCs, including the Wnt pathway.[2,8] Wnts are secreted glycoproteins involved in morphogenesis, embryogenesis, and cellular differentiation, and are important regulators of

Received in original form February 27, 2012; revised form June 4, 2012; accepted June 12, 2012. Published online June 19, 2012.
Address correspondence to: Martina Rauner, PhD, Division of Endocrinology, Diabetes and Bone Diseases, Department of Medicine III, Fetscherstraße 74, D-01307 Dresden, Germany. E-mail: martina.rauner@uniklinikum-dresden.de
*LCH and MR are joint senior authors.
For a Commentary on this article, please see Cooper et al. (J Bone Miner Res. 2012;27:2238–2241. DOI: 10.1002/jbmr.1753).

Journal of Bone and Mineral Research, Vol. 27, No. 11, November 2012, pp 2242–2250
DOI: 10.1002/jbmr.1688

bone biology. The Wnt signal is tightly regulated by endogenous inhibitors including members of the dickkopf (DKK) and secreted frizzled-related protein (sFRP) family, Wnt inhibitory factor-1, and sclerostin. Ohnaka and colleagues[9] recently showed that dexamethasone (DEX) increased DKK-1 expression in primary cultured human osteoblasts. Moreover, inhibition of DKK-1 with RNAi prevented GC-induced bone loss in mice, indicating an important role of DKK-1 in the suppression of osteoblast functions by GCs.[10,11] Besides DKK-1, GCs also increase sFRP-1 and thereby suppress osteogenesis.[9,10,12] While effective antiresorptive therapies are available, GIO still remains a clinical challenge because bone formation is suppressed and bone-anabolic drugs are limited. Therefore, extensive efforts are aimed at developing selective GC receptor (GR) agonists (SEGRAs) as novel therapies with an improved risk/benefit ratio.

The concept of SEGRAs is based on the fact that they largely mediate their effects via transrepression by GR monomers and not through transactivation by GR dimers, which are considered to induce several side effects. Among others, Compound A (CpdA) is one well-investigated agent that mediates its effects by binding the GR.[13] Several studies have already demonstrated the potent anti-inflammatory effects of CpdA in various inflammatory mouse models including arthritis and multiple sclerosis.[14–16] However, none of these studies have addressed the in vivo effects of CpdA on the skeleton. In initial studies on bone cells, we showed that, in contrast to conventional GC, CpdA does not increase the RANKL/OPG ratio in bone marrow stromal cells or the numbers of osteoclasts in vitro.[16] Moreover, although DEX was shown to inhibit osteoblast differentiation through suppressing interleukin-11 (IL-11), CpdA did not affect the production of IL-11 or osteoblast differentiation.[17]

Here, we assessed the effects of CpdA on bone metabolism in an experimental model of GIO and show that CpdA displays a bone-sparing profile as determined by bone density measurements, bone histomorphometry, serum markers of bone remodeling, and bone cell analysis. Its inability to simulate the RANKL/OPG ratio in osteocytes or DKK-1 in osteoblasts may be the mechanistic basis for its bone-sparing effects.

Subjects and Methods

Induction of glucocorticoid-induced bone loss in mice

We purchased female FVB/N mice from Janvier and housed them under institutional guidelines. All mice ($n = 24$) were fed a standard diet with water *ad libitum*. The local animal care committee approved all animal procedures. GC-induced bone loss was induced in 6-month-old female FVB/N mice by implanting 60-day slow-release pellets (Innovative Research of America, Sarasota, FL, USA) containing either vehicle, prednisolone (PRED) (3.5 mg), or CpdA (3.5 mg; synthesized according to Louw and colleagues[18]). At 7 days and 3 days before sacrifice, mice received two calcein (15 mg/kg; Sigma-Aldrich, Hamburg, Germany) injections intraperitoneally to identify newly formed bone. After a total of 4 weeks, mice were killed to examine the effects on the skeleton.

Structural and histological bone analyses

Bone mineral density, bone mass, and bone microarchitecture were assessed in the distal femur and fourth lumbar vertebra by peripheral quantitative computed tomography (pQCT; Stratec, Pforzheim, Germany) and bone histmorphometry. The measurements were made with a voxel size of 70 μm at the distal femur (two slices at the trabecular region located 1 mm from the growth plate, one at the mid-diaphysis located 6 mm from the growth plate) and the 4th vertebral body (two slices in the mid-vertebral body). The position of the growth plate was determined with the help of a scout view image. For the measurement of trabecular bone mineral density, regions of interest were set and contour mode 1 and peel mode 20 were used. A threshold of 710 mg/cm^3 was used to calculate cortical bone mineral density. Quality control measurements were performed using the standard and cone phantom, which was supplied by the manufacturer.

For the histomorphometric analysis, the 3rd vertebral body was fixed in formalin and dehydrated in an ascending ethanol series. Then bones were embedded in methylmethacrylate (Technovit 9100 NEW, Heraeus, Wehrheim, Germany) and cut into 4 μm sections for subsequent staining and 7 μm sections for assessing the fluorescence labels. The sections were stained with von Kossa to analyze bone volume over total volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th). Tartrate-resistant acid phosphatase (TRAP) staining was used to assess the number of osteoclasts per bone surface (Oc.N/BS), and unstained sections were analyzed using fluorescence microscopy to determine the mineral apposition rate (MAR) and the bone formation rate (BFR/BS) using the two fluorescent labels. Histomorphometric analysis was performed with the Osteomeasure software (OsteoMetrics, Decatur, GA, USA). The measurements, terminology, and units used for histomorphometric analysis were those recommended by the Nomenclature Committee of the American Society of Bone and Mineral Research.[19]

Analysis of serum markers

Procollagen type 1 N-terminal peptide (P1NP), a serum marker of bone formation, and the bone resorption marker C-terminal collagen crosslinks (CTX-1) were measured in the serum of mice using commercially available ELISAs (IDS, Frankfurt, Germany).

Culture of human bone marrow-derived osteoblasts and osteocytes

Primary human bone marrow stromal cells (BMSCs) were collected from healthy donors (aged 22–49 years, mixed gender) following Institutional Review Board approval and after obtaining written informed consent, and cultured according to modifications of previously reported methods.[16] Cells were maintained in DMEM (Invitrogen, Darmstadt, Germany) with 10% fetal calf serum (FCS) (Lonza, Cologne, Germany) and 1% penicillin/streptomycin (PAA, Cölbe, Germany) and were used in passages 3 to 5. To generate osteogenic cells, 70% confluent cells were switched to basal medium supplemented with 100 μM ascorbate phosphate, 5 mM β-glycerol phosphate, and 10 nM

DEX (all from Sigma-Aldrich, Hamburg, Germany) for 21 days. Before treatment, cells were switched to starving media overnight and then treated with DEX (Sigma-Aldrich) or CpdA (Calbiochem, Darmstadt, Germany) at concentrations as indicated in the appropriate figure (Fig. 5) for 48 hours and 72 hours, respectively. In some experiments, cells were pretreated for 1 hour with 1 μM RU-486 (Sigma-Aldrich), an established GR antagonist.[16]

The murine osteocyte-like cell line MLO-Y4 (kindly provided by Lynda F. Bonewald, Kansas City, MO, USA) were cultured on collagen-coated cell culture flasks (Greiner BioOne, Frickenhausen, Germany) in α-modified essential medium (α-MEM) with 10% FCS and 1% antibiotics/antimycotics. Before treatment, cells were switched to starving media overnight and then treated with DEX or CpdA (both 0.1 μM) for 48 hours. For the time-kinetic experiment, cells were treated either for 3 hours, 6 hours, 12 hours, 24 hours, or 48 hours using 0.1 μM DEX. In some experiments, cells were pretreated for 1 hour with 1 μM RU-486.[16]

RNA isolation, RT, and real-time PCR

RNA was extracted from the femur by crushing the flushed bone in liquid nitrogen and collecting the bone powder in Trifast (Peqlab, Erlangen, Germany). For the isolation of RNA from the spleen, the spleen was minced and subjected to Biocoll density centrifugation (1.077 g/mL; Biochrom, Berlin, Germany). The mononuclear cell fraction was lysed in Trifast (Peqlab). RNA from human BMSCs was isolated using Trifast (Peqlab) after washing twice with PBS. RNA isolation was performed according to the manufacturer's protocol. RNA from murine osteocytes was isolated using the HighPure RNA extraction kit from Roche (Mannheim, Germany) according to the manufacturer's instructions. Five-hundred nanograms (500 ng) of RNA were reverse-transcribed using Superscript II (Invitrogen, Darmstadt, Germany) and subsequently used for SYBR green-based real-time PCR reactions using a standard protocol (Applied Biosystems, Carlsbad, CA, USA). Primer sequences are summarized in Table 1. PCR conditions were 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles with 95°C for 15 seconds and 60°C for 1 minute. The melting curve was assessed in the following program: 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 30 seconds. The results were calculated applying the relative quantification method ΔΔCT and are presented in *x*-fold increase relative to β-actin.

Table 1. Primers Used for Semiquantitative Analyses of Gene Expression

Targeted gene	Primer sequences (5′–3′)
hu *ACTB*	CCAACCGCGAGAAGATGA
	CCAGAGGCGTACAGGGATAG
mu *ACTB*	GATCTGGCACCACACCTTCT
	GGGGTGTTGAAGGTCTCAAA
mu *ALP*	ATCCAACTGACACCAAGCAG
	TGAGCGGTTCCAAACATACC
hu *DKK-1*	AGCACCTTGGATGGGTATTC
	CACACTTGACCTTCTTTCAGGAC
mu *DKK-1*	GAGGGGAAATTGAGGAAAGC
	AGCCTTCTTGTCCTTTGGTG
mu *IL-6*	ACTTCCATCCAGTTGCCTTC
	ATTTCCACGATTTCCCAGAG
mu *OCN*	GCGCTCTGTCTCTCTGACCT
	ACCTTATTGCCCTCCTGCTT
mu *OPG*	CCTTGCCCTGACCACTCTTA
	ACACTGGGCTGCAATACACA
mu *OSCAR*	CACACACACCTGGCACCTAC
	GAGACCATCAAAGGCAGAGC
mu *RANKL*	GCAGAAGGAACTGCAACACA
	GATGGTGAGGTGTGCAAATG
mu *RUNX2*	CCCAGCCACCTTTACCTACA
	TATGGAGTGCTGCTGGTCTG
mu *TNF-α*	CCTCTTCTCATTCCTGCTTGTG
	CACTTGGTGGTTTGCTACGAC
mu *TRAP*	ACTTGCGACCATTGTTAGCC
	AGAGGGATCCATGAAGTTGC

hu = human; mu = murine; ACTB = beta-actin; ALP = alkaline phosphatase, DKK-1 = Dickkopf-1; IL-6 = interleukin 6; OCN = osteocalcin; OPG = osteoprotegerin; OSCAR = osteoclast-associated receptor; RANKL = receptor activator of nuclear factor κB ligand; RUNX2 = runt-related transcription factor 2; TNF-α = tumor necrosis factor-α; TRAP = tartrate resistant acid phosphatase; f = forward primer; r = reverse primer.

Immunofluorescence

MLO-Y4 cells were grown on glass slides in α-MEM for 3 days. After a 48-hour treatment with 0.1 μM DEX, CpdA, or 1 μM RU-486 in serum-reduced medium (1% FCS), cells were washed with PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 30 minutes. The glass slides were exposed to an anti-murine phycoerythrin (PE)-labeled RANKL antibody (12-5952-81; ebioscience, Frankfurt, Germany) for 48 hours (1:50). After three washing steps, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) (AppliChem, Darmstadt, Germany) for 5 minutes and washed again three times. Thereafter, glass slides were embedded in a small droplet of mounting medium (Dako, Hamburg, Germany). Slides were examined using a Zeiss Axio Imager M.1 fluorescence microscope (Carl Zeiss, Jena, Germany), and photographs were taken and processed with the AxioVision 3.1 program.

Flow cytometry

MLO-Y4 cells were grown on six-well-plates in α-MEM for 3 days. After a 48-hour treatment with 0.1 μM DEX, CpdA, or 1 μM RU-486 in serum-reduced medium (1% FCS), cells were washed with PBS and blocked with 3% BSA in PBS containing 5% FCS for 30 minutes. Then the cells were exposed to an anti-murine PE-labeled RANKL antibody (12-5952-81; ebioscience) for 30 minutes (1:50). After three washing steps with PBS/FCS (5%), RANKL-positive cells were measured using FACSCalibur (BD Biosciences, Heidelberg, Germany). CellQuest Pro (BD Biosciences) was used for analyzing the obtained data.

Statistical analysis

Results are presented as means ± SD. All experiments were repeated at least three times. Statistical evaluations were

performed using a Student's *t* test or one-way ANOVA. Values of $p < 0.05$ were considered statistically significant.

Results

CpdA maintains bone mass and density in mice

In order to assess the influence of the selective GR agonist, CpdA, on bone, we implanted slow-release pellets in FVB/N mice and analyzed bone parameters. After a pilot study (data not shown) with mice of different strains (FVB/N and C57BL/6) and different ages (3 and 6 months old), as well as with different pellet doses (range, 1.25–30 mg), we identified 6-month-old female FVB/N mice and 60-day slow-release pellets containing 3.5 mg of the respective substance as a suitable GIO model. Peripheral QCT measurement showed that, in contrast to PRED, which significantly reduced the total density in the femur by 9% ($p < 0.05$; Fig. 1*A*) and in the vertebral body by 11% ($p < 0.01$; Fig. 1*C*), CpdA did not influence these parameters. The trabecular bone density of these two compartments was also not reduced by CpdA (Fig. 1*B*, *D*), whereas PRED reduced bone mineral density in the femur by 24% ($p < 0.05$) and in the vertebral body by 20% ($p < 0.01$), respectively. These results were confirmed by histomorphometry. The vertebral bone volume/tissue volume (BV/TV) was decreased by 21% ($p < 0.05$) in mice receiving the PRED pellets, whereas the CpdA-treated mice showed no reduction (Fig. 1*E*). In addition, the structural parameters trabecular number, thickness, and separation were not affected by CpdA (Fig. 1*F–H*), but were diminished by PRED. As an established surrogate of bone strength, we determined the cross-sectional moment of inertia (CSMI) of the femur and found that it was decreased by 8.3% by PRED treatment, but was not changed by CpdA ($p = 0.055$; Fig. 2). Thus, PRED reduced bone mineral density and lowered CSMI-based bone strength in mice, whereas the values of the CpdA-treated mice were almost equal to that of placebo-treated mice, indicating that PRED may impair bone strength.

CpdA has no negative effects on bone formation or resorption parameters

We next set out to investigate whether the neutral effect of CpdA on bone is mediated via support of osteoblast or osteoclast functions, respectively. Therefore, we performed structural and histological bone analysis. Using fluorescence microscopy we determined the mineral apposition rate (MAR), which was decreased by 50% ($p < 0.05$) in mice receiving the PRED pellets but not by CpdA (Fig. 3*A*). Representative calcein double labels are shown for each treatment group (Fig. 3*B*). Bone sections of vehicle and CpdA-treated mice exhibited a larger space between the calcein labels and a stronger signal compared to the sections of the PRED treated mice. In line with the histological data, the negative effects of PRED on bone formation were also shown by a 34% ($p < 0.01$) decrease of the serum bone formation marker P1NP (Fig. 3*C*). For the assessment of the osteoclast number per bone surface (Oc.N/BS), TRAP staining was performed. PRED induced a threefold increase ($p < 0.01$) in the number of TRAP-positive osteoclasts, whereas CpdA showed no such effect (Fig. 3*D*, *E*). Additionally, these results were confirmed by the serum marker CTX-1. PRED was able to increase this bone resorption marker by 26% ($p < 0.05$), whereas CpdA did not induce CTX-1 (Fig. 3*F*). Hence, these results indicate that, in contrast to PRED, CpdA neither impairs bone formation nor stimulates bone resorption.

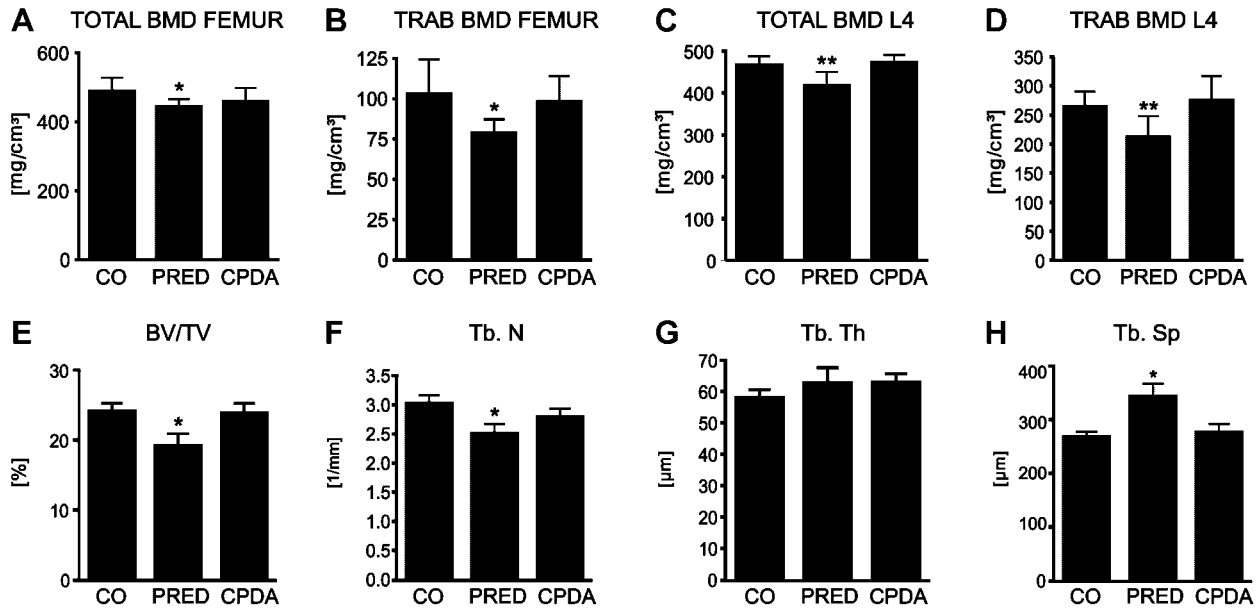

Fig. 1. Compound A (CpdA) maintains bone density and structural parameters in mice. Peripheral quantitative computed tomography (pQCT) was performed to analyze the total bone mineral density in the femur (*A*) and 4th lumbar vertebrae (*C*), as well as the trabecular density in the femur (*B*) and in the vertebral bodies of treated mice (*D*) (vehicle [CO], prednisolone [PRED] or CpdA); $n = 6–7$. Using von Kossa staining, we determined the bone volume over tissue volume (BV/TV; *E*), the trabecular number (Tb.N; *F*), thickness (Tb.Th; *G*), and separation (Tb.Sp; *H*); $n = 6–7$; $^{*}p < 0.05$; $^{**}p < 0.01$ versus CO.

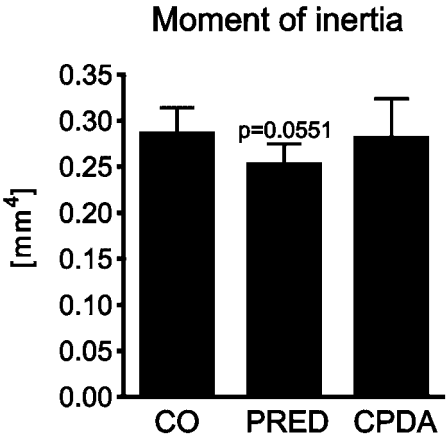

Fig. 2. Compound A (CpdA) maintains the cross-sectional moment of inertia, an indicator of bone strength, in mice. Peripheral quantitative computed tomography (pQCT) was performed to analyze the cross-sectional moment of inertia in the femur of treated mice (vehicle [CO], prednisolone [PRED], or CpdA); $n = 6–7$; $p = 0.0551$ versus CO.

CpdA does not influence osteoblast and osteoclast markers in the bone tissue

To confirm that CpdA does not affect bone remodeling parameters, we next investigated the expression of genes involved in bone formation and resorption processes in the bone tissue. While CpdA did not influence the bone formation markers runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and osteocalcin (OCN), PRED decreased them by 64%, 59%, and 45% ($p < 0.05$; Fig. 4*A–C*), respectively. In addition, PRED, but not CpdA, increased the RANKL/OPG ratio fivefold ($p < 0.05$; Fig. 4*D*). This effect was largely due to a significant downregulation of OPG (vehicle [CO]: 1.642 ± 0.859; PRED: 0.723 ± 0.370; CPDA: 1.618 ± 0.745) and concurrent upregulation of RANKL (CO: 2.150 ± 1.734; PRED: 3.498 ± 1.378; CPDA: 2.212 ± 0.648). However, the expression of the osteoclast marker genes osteoclast-associated receptor (OSCAR) and TRAP was not changed by either substance (Fig. 4*E*, *F*). Thus, these results indicate that CpdA has no influence on osteoblast and osteoclast markers in the bone tissue. Because CpdA did not suppress bone remodeling parameters, we next determined the expression of proinflammatory cytokines to verify its efficacy. Indeed, CpdA suppressed the gene expression levels of tumor necrosis factor (TNF)-α (–35%) and IL-6 (–56%; $p < 0.01$) in mononuclear cell extracts from the spleen (Table 2). Similarly, CpdA was effective in reducing lipopolysaccharide (LPS)-induced expression of TNF-α and IL-6 in bone marrow stromal cells (Table 2).

CpdA does not increase the RANKL/OPG ratio in osteocyte-like cells

After having established that CpdA does not influence the RANKL/OPG ratio in murine bone tissue and the recent discovery that osteocytes are the main source of RANKL in bone,[26,27] we asked whether DEX and CpdA modify this ratio in osteocytes. DEX treatment of MLO-Y4 cells increased the mRNA levels of RANKL ninefold ($p < 0.01$) whereas CpdA did not (Fig. 5*A*). Additionally, DEX decreased OPG mRNA levels by 59% ($p < 0.05$; Fig. 5*B*). As a consequence, the RANKL/OPG ratio was increased 22-fold ($p < 0.001$) by DEX but remained unchanged after CpdA treatment. The induction of the ratio was reversed by pretreatment with the GR antagonist RU-486 (Fig. 5*C*). To determine whether the RANKL-inducing effect of DEX is direct or indirect, we performed time kinetics suggesting that the effects are indeed direct, as the RANKL/OPG ratio is already increased fourfold ($p < 0.01$) after 3 hours and peaked at 24 hours of stimulation (Fig. 5*D*). The RANKL-inducing effect of DEX was also shown by immunofluorescence staining with an anti-RANKL antibody (Fig. 5*E*) as well as by flow cytometry (Fig. 5*F*). Here,

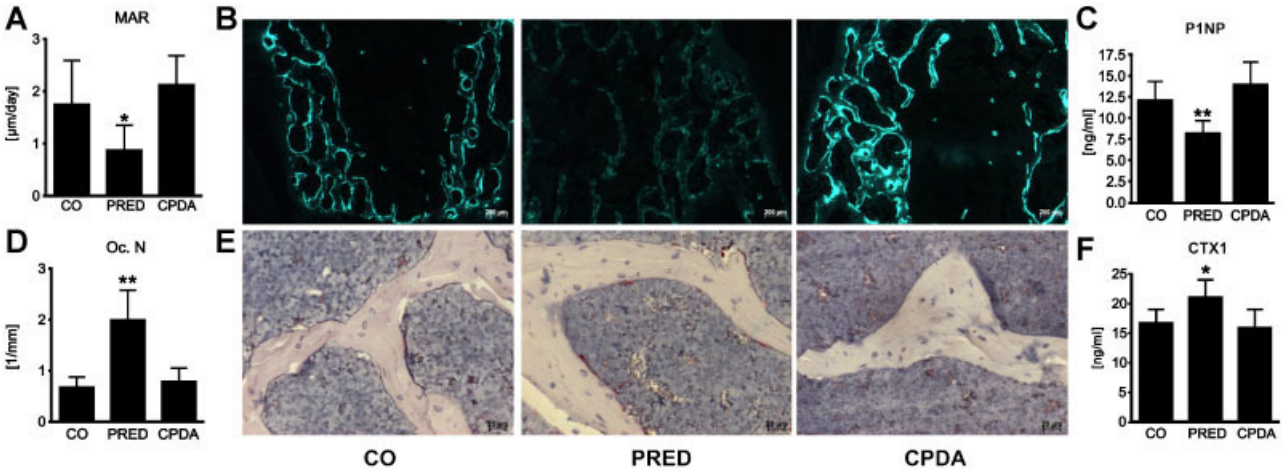

Fig. 3. Histomorphometric and serum osteoblast and osteoclast parameters were not changed by compound A (CpdA). Calcein double labeling was performed to determine the influence of prednisolone (PRED) and compound A (CpdA) on the mineral apposition rate (MAR) (*A*); $n = 7–8$. Representative double labels for each treatment group are shown (*B*). Serum procollagen type 1 amino-terminal propeptide (P1NP) was assessed using a commercially available ELISA (*C*); $n = 6–8$. Using tartrate-resistant acid phosphatase (TRAP) staining we determined the number of osteoclasts (Oc.N) in the treated animals (*E*); $n = 3–4$. Representative images are shown (*D*). Serum carboxy-terminal collagen crosslinks (CTX-1) was measured using ELISA (*F*); $n = 6–8$; $^{*}p < 0.05$; $^{**}p < 0.01$ versus CO.

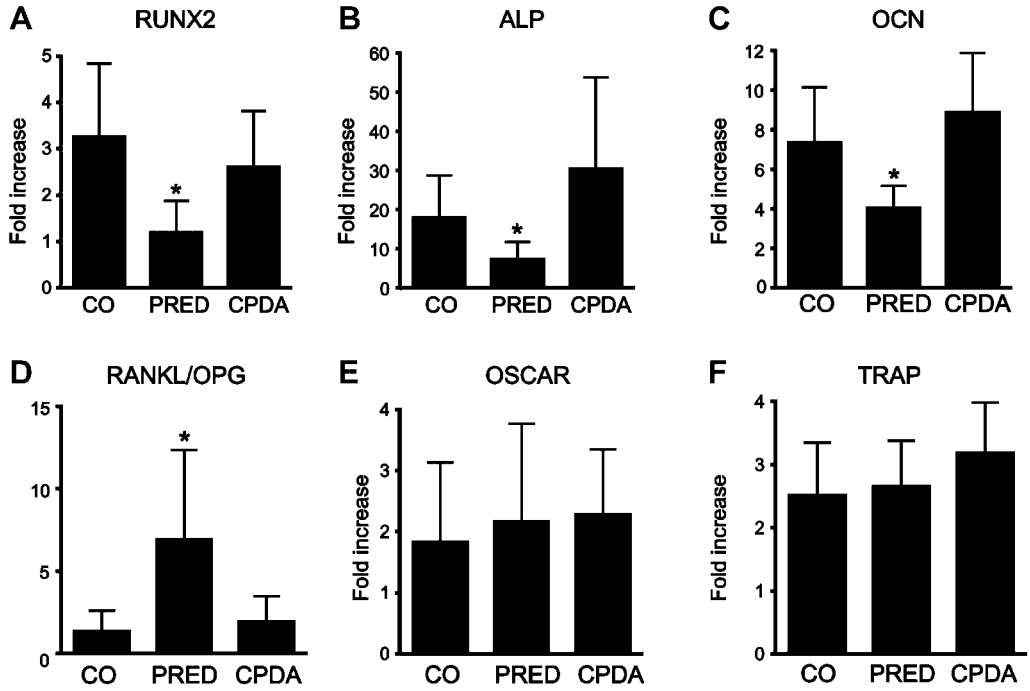

Fig. 4. Compound A (CpdA) does not influence osteoblast and osteoclast markers in the bone tissue. Real-time PCR analysis was performed for runt-related transcription factor 2 (RUNX2; *A*), alkaline phosphatase (ALP; *B*), and osteocalcin (OCN; *C*) as well as osteoclast associated receptor (OSCAR; *E*) and tartrate-resistant acid phosphatase (TRAP; *F*) in murine femoral bone tissue (vehicle [CO], prednisolone [PRED], or CpdA). The receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) ratio (*D*) was determined by dividing RANKL expression by OPG expression as determined by real-time PCR. Gene expression levels were normalized to β-actin. $n = 6–7$; $^{*}p < 0.05$ versus CO.

RANKL was increased threefold ($p < 0.001$) in DEX-treated cells. Those data show again that the induction could be reversed by pretreatment with RU-486 and that CpdA had no influence on RANKL production. Thus, these data indicate that the bone-sparing effects of CpdA may in part be mediated by maintaining a physiological RANKL/OPG balance.

Table 2. Expression Levels of Proinflammatory Genes in Murine Bone Marrow Stromal Cells and Murine Spleen Tissue

Tissue	IL-6	TNF-α
Murine bone marrow stromal cells		
CO	1.17 ± 1.53	1.83 ± 0.66
DEX	0.48 ± 0.24	1.13 ± 0.44
CPDA	0.62 ± 0.35	$0.61 \pm 0.32^{*}$
LPS	121 ± 41.3	23.3 ± 4.75
LPS + DEX	$3.87 \pm 0.83^{\#\#}$	$5.70 \pm 0.50^{\#\#\#}$
LPS + CPDA	$3.00 \pm 1.89^{\#\#}$	$4.21 \pm 1.64^{\#\#\#}$
Murine spleen		
CO	0.55 ± 0.250	1.27 ± 0.25
PRED	$0.28 \pm 0.11^{*}$	$0.90 \pm 0.36^{*}$
CPDA	$0.24 \pm 0.14^{**}$	$0.82 \pm 0.24^{**}$

IL-6 = interleukin 6; TNF-α = tumor necrosis factor-α; CO = control; DEX = dexamethasone; CPDA = compound A; LPS = lipopolysaccharide; PRED = prednisolone.

$^{*}p < 0.05$ versus CO.

$^{**}p < 0.01$ versus CO.

$^{\#\#}p < 0.01$ versus LPS.

$^{\#\#\#}p < 0.001$ versus LPS.

CpdA does not stimulate DKK-1

To gain further insight into the underlying mechanisms of GC-induced suppression of bone formation, we studied one important inhibitor of the Wnt signaling pathway, DKK-1. Therefore, we determined the expression of DKK-1 mRNA levels in bone tissue of treated mice (vehicle, PRED, or CpdA), as well as in human BMSCs and murine osteocytes after treatment with DEX or CpdA. The mRNA expression of DKK-1 in bone tissue was increased twofold ($p < 0.05$) in mice treated with PRED, whereas CpdA did not modulate DKK-1 mRNA levels (Fig. 6*A*). Similarly, DEX upregulated DKK-1 threefold ($p < 0.05$) in human BMSCs and twofold ($p < 0.01$) in MLO-Y4 cells, whereas CpdA exerted no effect on DKK-1 (Fig. 6*B*, *C*). Human BMSCs further showed a dose- and time-dependent increase of DKK-1 expression after DEX treatment (Fig. 6*D*, *E*). This effect was blocked by pretreating the cells with RU-486 (Fig. 6*F*). Hence, these data suggest that, in contrast to PRED, CpdA does not enhance DKK-1 expression in bone cells.

Discussion

One of the most common and serious side effects of GC treatment is osteoporosis, which predisposes patients to increased osteoporotic fractures occurring in up to 50% of GC treated patients.[3,20] Here, we hypothesized whether the use of CpdA, a dissociated GR modulator, has fewer adverse effects on bone metabolism. Indeed, CpdA showed bone-sparing potential in an experimental model of GC-induced bone loss, which may

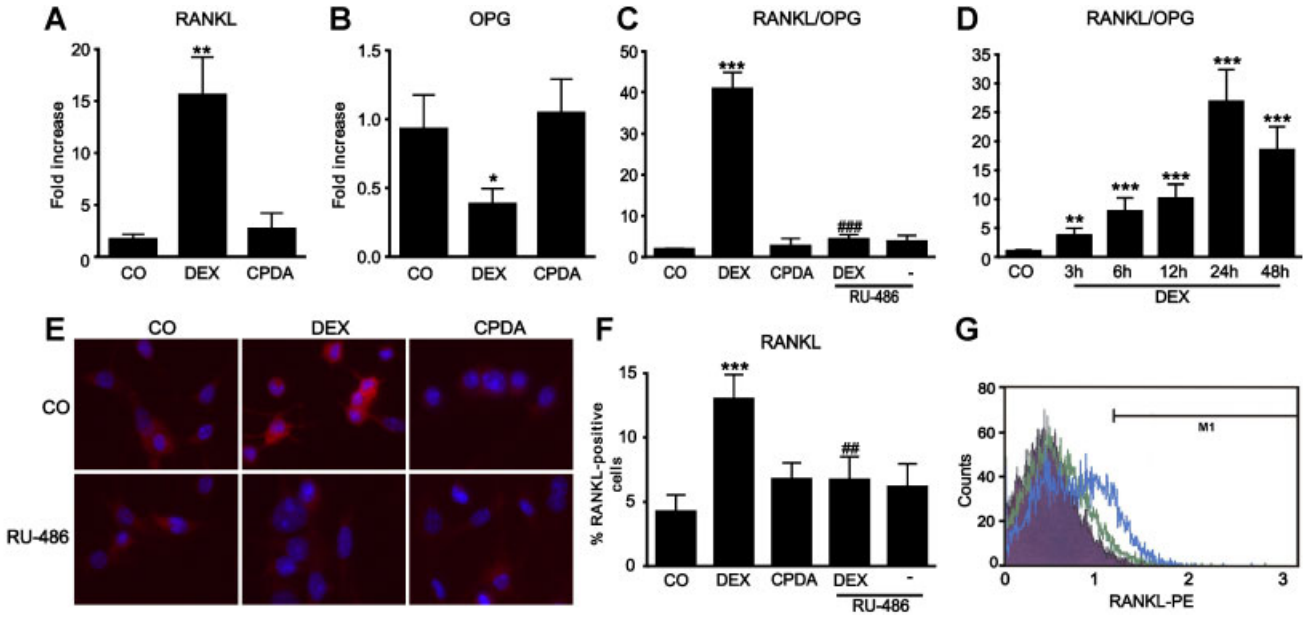

Fig. 5. Compound A (CpdA) does not increase the receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) ratio in murine osteocytes. Real-time PCR analysis was performed to determine the mRNA levels of RANKL (*A*) and OPG (*B*) in murine osteocytes (MLO-Y4 cells) after treatment with dexamethasone (DEX; 0.1 μM) or CpdA (0.1 μM). (*C*) The RANKL/OPG ratio was calculated by dividing RANKL expression by OPG expression as determined by real-time PCR. To inhibit glucocorticoid actions, cells were pretreated with RU-486 (1 μM); $n = 3$. (*D*) The RANKL/OPG ratio was also calculated after treatment with DEX (0.1 μM) for different durations (3–48 hours); $n = 4$. (*E*) For immunofluorescence staining of RANKL MLO-Y4 cells were cultured for 3 days and after serum starving for 24 hours, cells were stimulated for 48 hours with 0.1 μM DEX or CpdA and/or 1 μM RU-486; immunofluorescence images show RANKL stained in red; nuclei were stained with DAPI (blue); magnification: ×40. (*F*) Using flow cytometry we determined the percentage of RANKL-positive cells. (*G*) Representative histogram of unstained cells (purple) and RANKL-PE-stained control (green)- and DEX-treated (blue) cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus CO; $^{\#\#\#}p < 0.001$ versus DEX.

result from preserving osteoblast functions and maintaining a physiological RANKL/OPG balance.

Although previous studies demonstrated the potent anti-inflammatory effects of CpdA in experimental models of autoimmune neuritis,[21] multiple sclerosis,[22] and collagen-induced arthritis,[14] none of these studies have addressed its effects on bone. To examine the skeletal influence of CpdA, we compared its effects on bone metabolism in an experimental model of GC-induced osteoporosis and showed that CpdA does not reduce bone mineral density and trabecular parameters, whereas PRED decreased total and trabecular bone mineral density in the femur and 4th lumbar vertebrae. Furthermore, while PRED lowered the cross-sectional moment of inertia, an indicator of bone strength, CpdA did not affect this parameter. These bone-sparing effects are in accordance with a previous study by Owen and colleagues[23] showing that the selective GR modulator AL-438 has growth plate sparing effects in chondrocytes and ex vivo cultures of fetal metatarsal organ cultures. The current findings that CpdA does not affect bone formation or resorption parameters are in full agreement with our previous in vitro findings, in which CpdA induced proliferation and reduced apoptosis of mature osteoblasts, and maintained osteoblast differentiation, even when treated at higher doses.[16,17]

The balance of RANKL and OPG is critical for maintaining bone mass. Although the induction of the RANKL/OPG ratio by GCs has been reported controversially,[4,5,16,24] inhibiting excessive RANKL by neutralizing antibodies prevents GC-induced bone loss, suggesting that this pathway is critical for the induction of GIO.[4,6] Here, we showed that PRED treatment increased the RANKL/OPG ratio and was accompanied by an increased number and activity of osteoclasts. Of note, we found a twofold increase in the number of osteoclasts after PRED treatment whereas previous studies only showed a smaller or no increase in osteoclast numbers.[6,7] The reason for this discrepancy is currently unknown but it is interesting to note that the FVB/N mouse strain used here, which showed a twofold induction of osteoclasts, also lost the highest amount of bone density (11%), while C57BL/6 and Swiss Webster mice, which only lose about 6% to 7% bone density, show no significant alterations of osteoclast numbers.[6,7] Thus, although other factors might also contribute to the regulation of osteoclasts in GIO, the varying regulation of osteoclast numbers by GCs may depend on the mouse strains used.

In our previous studies we already showed that, in contrast to conventional GCs, CpdA does not increase the RANKL/OPG ratio in MSC-derived osteoblasts, fibroblast-like synoviocytes or SaOS-2 cells.[16,17] Similar positive effects on maintaining the RANKL/OPG ratio were reported for other SEGRAs, including AL-438 and ZK 216348.[25] Due to the recent discovery that osteocytes produce more RANKL than osteoblasts and thereby contribute to bone remodeling in adults,[26,27] we turned our attention to osteocytes.[28] Here, we show that CpdA also maintains the RANKL/OPG ratio in the osteocyte-like MLO-Y4 cells, which is in contrast to the about 25-fold induction by DEX. The induction of RANKL by DEX was further shown to be

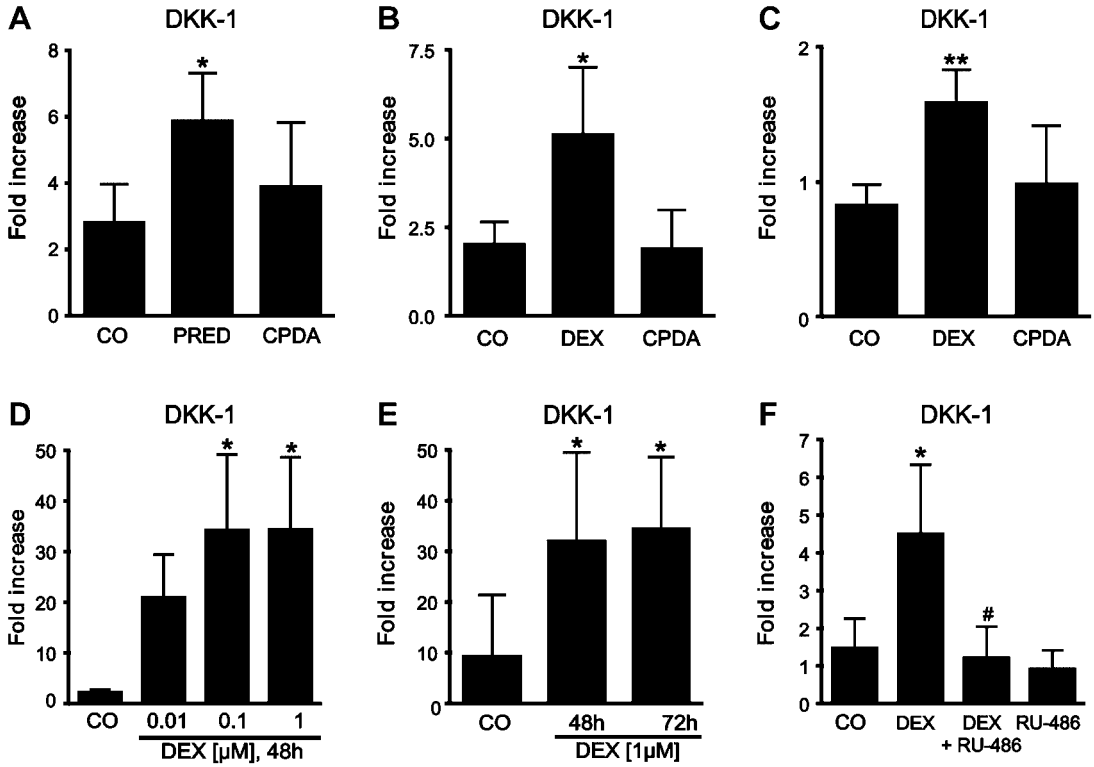

Fig. 6. Compound A (CpdA) does not stimulate dickkopf-1 (DKK-1) levels. Gene expression analysis of DKK-1 mRNA expression in murine femoral bone tissue ($n = 6$–8; *A*) as well as in human bone marrow stromal cells (BMSCs; *B*) and in murine osteocyte-like cells (MLO-Y4; *C*), which were treated with either dexamethasone (DEX) or CpdA (both 0.1 μM). DKK-1 expression levels were also determined in BMSCs after treatment with different doses of DEX (0.01–1 μM; *D*) and for different time points (*E*). To inhibit glucocorticoid actions, cells were pretreated with RU-486 (1 μM; *F*). $n = 3$–4; $^{*}p < 0.05$; $^{**}p < 0.01$ versus CO; $\#p < 0.05$ versus DEX.

mediated directly, as the RANKL/OPG ratio was significantly increased as early as 3 hours after stimulation. Thus, these results indicate that osteocytes may play an important role in GC-induced bone loss by controlling the RANKL/OPG ratio.

In our earlier studies, we have shown that the suppression of osteoblast differentiation by DEX is mediated via IL-11.[17] In contrast, CpdA did not affect IL-11 expression; hence, providing a possible explanation for the osteoblast-friendly actions of CpdA. In this study, we addressed the influence of GC on the Wnt inhibitor DKK-1, because Wnt signaling also plays an important role in osteoblast differentiation and bone formation.[11] We showed that DEX treatment of human BMSCs as well as of murine osteocytes enhanced DKK-1 expression significantly, which is consistent with previous studies.[9,10,12] Moreover, inhibition of DKK-1 with RNAi prevented GC-induced bone loss in mice, indicating an important role of DKK-1 in the suppression of osteoblast functions by GCs.[9–11] However, CpdA did not increase DKK-1 expression in vivo or in vitro. Thus, the inability to enhance DKK-1 may reflect an important mechanism to prevent GC-induced bone loss.

In conclusion, our results indicate that, in contrast to conventional GC, CpdA has bone-sparing properties at several levels, including bone mass, histomorphometry, remodeling, and cellular analyses. Whether the superiority of SEGRAs such as CpdA in experimental models of GC-induced bone loss can also be translated into clinical applications remains to be determined.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

We thank Patrick Boehme and Ina Gloe for excellent technical assistance. This work was supported by the European Calcified Tissue Society/AMGEN 2011 fellowship, the MedDrive start-up grant from the TU Dresden to MR, the Elsbeth Bonhoff Foundation to MR and LCH, Deutsche Forschungsgemeinschaft grants RA1923/1-1, HO1875/8-1 (SPP 1468 Immunobone) to LCH and MR, TU220/5-1 and TU220/6-1 to JPT, and Boehringer Stiftung to JPT, and the DFG Research Center for Regenerative Therapies Dresden to LCH. KDB holds a postdoctoral fellowship at FWO-Vlaanderen.

Authors' roles: ST, LCH, and MR contributed to the study conception and design. ST, NZ, ET, and MR collected data and performed data analysis. Data were interpreted by ST, NZ, ET, KDB, JPT, LCH, and MR. ST and MR drafted the manuscript. All authors critically revised the content of the manuscript

and approved its final version. MR takes responsibility for the integrity of the data analysis.

References

1. Rachner TD, Khosla S, Hofbauer LC. Osteoporosis: now and the future. Lancet. 2011;377:1276–87.
2. Hofbauer LC, Rauner M. Minireview: live and let die: molecular effects of glucocorticoids on bone cells. Mol Endocrinol. 2009;23:1525–31.
3. Canalis E, Mazziotti G, Giustina A, Bilezikian JP. Glucocorticoid-induced osteoporosis: pathophysiology and therapy. Osteoporos Int. 2007;18:1319–28.
4. Hofbauer LC, Gori F, Riggs BL, Lacey DL, Dunstan CR, Spelsberg TC, Khosla S. Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. Endocrinology. 1999;140:4382–9.
5. Kondo T, Kitazawa R, Yamaguchi A, Kitazawa S. Dexamethasone promotes osteoclastogenesis by inhibiting osteoprotegerin through multiple levels. J Cell Biochem. 2008;103:335–45.
6. Hofbauer LC, Zeitz U, Schoppet M, Skalicky M, Schüler C, Stolina M, Kostenuik PJ, Erben RG. Prevention of glucocorticoid-induced bone loss in mice by inhibition of RANKL. Arthritis Rheum. 2009;60: 1427–37.
7. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest. 1998;102:274–82.
8. Rauch A, Seitz S, Baschant U, Schilling AF, Illing A, Stride B, Kirilov M, Mandic V, Takacz A, Schmidt-Ullrich R, Ostermay S, Schinke T, Spanbroek R, Zaiss MM, Angel PE, Lerner UH, David JP, Reichardt HM, Amling M, Schütz G, Tuckermann JP. Glucocorticoids suppress bone formation by attenuating osteoblast differentiation via the monomeric glucocorticoid receptor. Cell Metab. 2010;11:517–31.
9. Ohnaka K, Taniguchi H, Kawate H, Nawata H, Takayanagi R. Glucocorticoid enhances the expression of dickkopf-1 in human osteoblasts: novel mechanism of glucocorticoid-induced osteoporosis. Biochem Biophys Res Commun. 2004;318:259–64.
10. Wang FS, Ko JY, Yeh DW, Ke HC, Wu HL. Modulation of Dickkopf-1 attenuates glucocorticoid induction of osteoblast apoptosis, adipocytic differentiation, and bone mass loss. Endocrinology. 2008;149: 1793–801.
11. Krishnan V, Bryant HU, Macdougald OA. Regulation of bone mass by Wnt signaling. J Clin Invest. 2006;116:1202–9.
12. Mak W, Shao X, Dunstan CR, Seibel MJ, Zhou H. Biphasic glucocorticoid-dependent regulation of Wnt expression and its inhibitors in mature osteoblastic cells. Calcif Tissue Int. 2009;85:538–45.
13. De Bosscher K, Vanden Berghe W, Beck IM, Van Molle W, Hennuyer N, Hapgood J, Libert C, Staels B, Louw A, Haegeman G. A fully dissociated compound of plant origin for inflammatory gene repression. Proc Natl Acad Sci U S A. 2005;102:15827–32.
14. Dewint P, Gossye V, De Bosscher K, Vanden Berghe W, Van Beneden K, Deforce D, Van Calenbergh S, Müller-Ladner U, Vander Cruyssen B, Verbruggen G, Haegeman G, Elewaut D. A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis. J Immunol. 2008;180:2608–15.
15. van Loo G, Sze M, Bougarne N, Praet J, Mc Guire C, Ullrich A, Haegeman G, Prinz M, Beyaert R, De Bosscher K. Antiinflammatory properties of a plant-derived nonsteroidal, dissociated glucocorticoid receptor modulator in experimental autoimmune encephalomyelitis. Mol Endocrinol. 2010;24:310–22.
16. Rauner M, Goettsch C, Stein N, Thiele S, Bornhaeuser M, De Bosscher K, Haegeman G, Tuckermann J, Hofbauer LC. Dissociation of osteogenic and immunological effects by the selective glucocorticoid receptor agonist, compound A, in human bone marrow stromal cells. Endocrinology. 2011;152:103–12.
17. Rauch A, Gossye V, Bracke D, Gevaert E, Jacques P, Van Beneden K, Vandooren B, Rauner M, Hofbauer LC, Haegeman G, Elewaut D, Tuckermann JP, De Bosscher K. An anti-inflammatory selective glucocorticoid receptor modulator preserves osteoblast differentiation. FASEB J. 2011;25:1323–32.
18. Louw A, Swart P, de Kock SS, van der Merwe KJ. Mechanism for the stabilization in vivo of the aziridine precursor --(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride by serum proteins. Biochem Pharmacol. 1997;53:189–97.
19. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res. 1987; 2:595–610.
20. Compston J. Glucocorticoid-induced osteoporosis. Horm Res. 2003; 60(Suppl 3):77–9.
21. Zhang Z, Zhang ZY, Schluesener HJ. Compound A, a plant origin ligand of glucocorticoid receptors, increases regulatory T cells and M2 macrophages to attenuate experimental autoimmune neuritis with reduced side effects. J Immunol. 2009;183:3081–91.
22. Wüst S, Tischner D, John M, Tuckermann JP, Menzfeld C, Hanisch UK, van den Brandt J, Lühder F, Reichardt HM. Therapeutic and adverse effects of a non-steroidal glucocorticoid receptor ligand in a mouse model of multiple sclerosis. PLoS One. 2009;4:e8202.
23. Owen HC, Miner JN, Ahmed SF, Farquharson C. The growth plate sparing effects of the selective glucocorticoid receptor modulator, AL-438. Mol Cell Endocrinol. 2007;264:164–70.
24. Jia D, O'Brien CA, Stewart SA, Manolagas SC, Weinstein RS. Glucocorticoids act directly on osteoclasts to increase their life span and reduce bone density. Endocrinology. 2006;147:5592–9.
25. Humphrey EL, Williams JH, Davie MW, Marshall MJ. Effects of dissociated glucocorticoids on OPG and RANKL in osteoblastic cells. Bone. 2006;38:652–61.
26. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, Bonewald LF, Kodama T, Wutz A, Wagner EF, Penninger JM, Takayanagi H. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med. 2011;17:1231–4.
27. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. Nat Med. 2011; 17:1235–41.
28. Bonewald LF. The amazing osteocyte. J Bone Miner Res. 2011;26: 229–38.