

Inhibition of PPAR γ by bisphenol A diglycidyl ether ameliorates dexamethasone-induced osteoporosis in a mouse model

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Abstract

Objectives: Bisphenol A diglycidyl ether (BADGE) is an antagonist for PPAR γ that reduces bone marrow adiposity and increases bone formation in some animal models of osteoporosis and osteonecrosis. However, the effect of BADGE treatment on glucocorticoid-induced osteoporosis is unknown. This study investigated the preventive effects of BADGE on steroid-induced osteoporosis in mice.

Methods: Thirty-six female C57BL/6J mice were randomly divided into normal (phosphate-buffered saline), model (50 mg/kg dexamethasone sodium phosphate [Dex]), and BADGE (30 mg/kg of BADGE, combined with Dex) groups. All groups received intraperitoneal injections of their treatments, daily for 4 weeks. Protein and mRNA expression levels of gene markers were measured. Micro-computed tomography was used to measure physical parameters of femurs. Bone histomorphology was analyzed by hematoxylin and eosin staining. ELISA was used to measure serum osteocalcin and C-terminal telopeptide of type I collagen (CTX-I).

Results: Glucocorticoid treatment enlarged the marrow fat, concomitant with bone deterioration; BADGE treatment reversed steroid-induced marrow adiposity. Compared with the model group, BADGE treatment improved bone quality and increased bone volume, while increasing osteogenic markers and reducing adipogenic markers at both mRNA and protein levels; moreover, it reduced serum CTX-I and increased serum osteocalcin.

Conclusion: BADGE treatment ameliorates glucocorticoid-induced osteoporosis by inhibiting PPAR γ .

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Keywords

BADGE, bone loss, marrow fat, PPAR γ , Runx2, glucocorticoid, osteoporosis, mice

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Introduction

Steroid-related osteoporosis is a common side effect of steroid therapy.¹ Approximately 30% to 50% of patients experience considerable bone loss under long-term glucocorticoid (GC) treatment.^{2,3} Both the endogenous overproduction of cortisol and chronic glucocorticoid therapy are associated with marked bone marrow adiposity and low bone mineral density, which increase the risk of fracture.⁴ Fractures in osteoporotic individuals are associated with increased mortality compared with that of healthy individuals; thus, steroid-related osteoporosis are the focus of pharmaceutical therapeutics and regenerative medicine.⁵

Marrow fat is reportedly increased in patients with glucocorticoid-related osteoporosis.^{6–8} High concentrations and long durations of steroid treatment can induce bone marrow mesenchymal stem cells (BMSCs) to differentiate into a small number of osteoblasts and a large number of adipocytes.^{9,10} When BMSCs differentiate into adipocytes, they can no longer be induced to differentiate into osteoblasts. The BMSCs of glucocorticoid-related osteoporotic mice tend to differentiate into adipocytes, but not osteoblasts.^{11,12} Thus, inhibition of bone marrow adipogenesis presumably could prevent or limit bone loss, based on the close relationship between adipocyte and osteoblast differentiation.

The process of adipocyte differentiation is regulated by many factors. PPAR γ , a master regulator of adipocyte differentiation, is closely involved in the adipocyte differentiation of BMSCs. Overexpression of

PPAR γ increases adipocyte differentiation and decreases osteoblast differentiation, which leads to increased lipid generation and reduced bone formation.^{13,14} Akune et al.^{15,16} showed that PPAR γ deletion reduced marrow fat and increased bone mass. Bisphenol A diglycidyl ether (BADGE), an antagonist of PPAR γ , has been reported to inhibit adipogenesis both in vitro and in vivo.^{17,18} In mice with age-related osteoporosis, BADGE treatment resulted in reduced marrow adipogenesis, concomitant with increasing levels of osteoblastogenesis and bone formation.¹⁹ Moreover, BADGE treatment improved bone formation, concomitant with reduced marrow adiposity in rabbits with steroid-related osteonecrosis.²⁰ However, the effect of BADGE treatment on glucocorticoid-related osteoporosis is unknown. This study was performed to investigate whether BADGE treatment could prevent dexamethasone-induced bone deterioration, and to explore the potential underlying mechanisms of this effect.

Materials and methods

Steroid-induced bone loss in mice

This study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (no. SY02518072). Thirty-six female C57BL/6J mice (8 weeks old; weight, 21 ± 2.4 g) were housed in a standard animal research environment with consistent temperature and humidity; they had free access to drinking water and food. One week after acclimation to the

environment, the mice were randomly divided into three groups—the model group (n=12) received intraperitoneal injections of 50 mg/kg of dexamethasone sodium phosphate (Dex), daily for 4 weeks;²¹ the normal group (n=12) received intraperitoneal injections of phosphate-buffered saline; and the BADGE group (n=12) received intraperitoneal injections of 30 mg/kg of BADGE, combined with Dex, daily for 4 weeks.²² At the endpoint, animals were anesthetized with pentobarbital sodium and blood samples were obtained; animals were then euthanized by an overdose of pentobarbital sodium. Femurs were dissected from the euthanized mice for analysis in this study.

Micro-computed tomography (μ CT) analysis

Left femurs were fixed in 4% paraformaldehyde overnight and scanned with a SCANCO MEDICAL μ CT 50 (SCANCO, Brüttsellen, Zurich, Switzerland) to quantify structural parameters. The following parameters were used: X-ray intensity, 100 kVp, 98 μ A; slice thickness, 10 μ m; rotation between frames, 0.9°; scanning resolution, 1024 \times 1024; exposure time, 300 ms; and scanning regions, 7 to 10 mm of the distal femurs. Two-dimensional images of the femurs were used for three-dimensional reconstruction. Measurements were made of the trabecular thickness (Tb.Th), bone mineral density (BMD), trabecular number (Tb.N), and bone volume/total volume (BV/TV).

Histopathology

After left femurs were fixed in 4% paraformaldehyde for 24 hours, they were decalcified in 10% ethylenediaminetetraacetic acid and then embedded in paraffin. The blocks of paraffin were then cut into 4- μ m sections and stained with hematoxylin and eosin.

The adipocyte number (Ad.N/MV), adipocyte volume (Ad.V/MV), and adipocyte size (Ad.Size/MV) were calculated as in the study by Ko et al.²³

PCR analysis

Total RNA from right femurs was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biomedical Technology, Beijing, China). Subsequently, 2 μ L cDNA was amplified by using the StepOne™ Real-Time PCR kit (Life Technologies, Beijing, China), in the TC-XP thermocycler (BIOER TECHNOLOGY, Hangzhou, China); primers were specific for the following genes: *PPAR γ* : forward, 5'-TGTCTCACAATGC CATCAGGT-3' and reverse, 5'-TGTGAT CTCTTGACACGGCTT-3'; *aP2 (FABP4)*: forward, 5'-TTGGTCACCATCCGGTCA GA-3' and reverse, 5'-CATTCCACCACCA GCTTGTCA-3'; *Runx2*: forward, 5'-GCGG TGCAAACCTTTCTCCAG-3' and reverse, 5'-ACCCAGTCCCTGTTTTAGT TGT-3'; and *osteocalcin (OCN)*: forward, 5'-CCTT GGGTTCTGACTGGGTG-3' and reverse, 5'-GGCCACTTACCCAAG GTAGC-3'. *β -actin* served as the internal standard. Relative gene expression levels were quantified using the $\Delta\Delta$ Ct method.

Western blot

Total protein from right femurs was crushed under liquid nitrogen conditions and prepared in ice-cold RIPA lysis buffer. Protein extracts were resolved by using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 10% non-fat milk, then incubated with primary antibodies overnight at 4°C; this was followed by incubation at

room temperature with horseradish peroxidase-labelled goat anti-rabbit IgG (1:10,000 dilution in 5% skim milk; Cat. No. AS1058; Aspen, Wuhan, China). Protein blots were analyzed using an image analysis system (AlphaEaseFC; Alpha Innotech Corporation, San Leandro, CA, USA). An anti- β -actin antibody was used as a loading control (ab37168, 1:10,000 dilution in 5% skim milk; Abcam, Cambridge, MA, USA). The primary antibodies used were anti-PPAR γ (ab59256, 1:500 dilution in 5% skim milk), anti-OCN (ab23981, 1:1000 dilution in 5% skim milk), and anti-RUNX2 (ab93876, 1:500 dilution in 5% skim milk) (Abcam).

ELISA

To evaluate the levels of bone resorption and formation markers in all mice, serum levels of mouse OCN and C-terminal telopeptides of type I collagen (CTX-1) were measured with ELISA kits (Bio-Swamp, Wuhan, China), in accordance with the manufacturer's instructions.

Statistical analysis

For comparison, two-tailed t-test or one-way analysis of variance were conducted using Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). The Student–Newman–Keuls method was used for multiple comparisons among groups. Data are presented as the mean \pm standard deviation. Differences with $P < 0.05$ were considered statistically significant.

Results

BADGE treatment ameliorated glucocorticoid-induced bone mass loss

μ CT analyses showed that Dex treatment led to significantly reduced trabecular

BV/TV and BMD, as well as reduced Tb.N and Tb.th (Figure 1a,b). Thus, BADGE treatment improved BMD and trabecular BV/TV, while ameliorating the cortical bone reduction and bone mass loss, compared with those parameters in the model group ($P < 0.05$ for all comparisons, Figure 1a,b).

BADGE treatment reduced glucocorticoid-induced bone marrow fat

As shown in Figure 2a and b, more fat cells accumulated in the bone marrow in the steroid-treated group than in the normal group. Thus, BADGE treatment ameliorated glucocorticoid-induced marrow adiposity.

BADGE treatment increased expression levels of osteogenic markers, concomitant with reduced expression levels of adipogenic markers

As shown in Figure 3, compared with the normal group, Dex treatment increased the expression levels of adipogenic markers PPAR γ and *aP2*, but reduced the expression levels of osteogenic markers Runx2 and osteocalcin. The BADGE group exhibited increased expression levels of Runx2 and OCN, as well as reduced expression levels of PPAR γ at both the protein and mRNA levels, compared with the model group ($P < 0.05$ for all comparisons). The mRNA expression level of *aP2* was also significantly reduced in the BADGE group, compared with the model group ($P < 0.05$).

BADGE treatment reduced the level of bone resorption marker concomitant with increased level of bone formation marker

We analyzed the effects of BADGE on the serum levels of a total bone resorption marker, CTX-1, and a bone formation marker, OCN. As shown in Figure 4, compared with the normal group, Dex

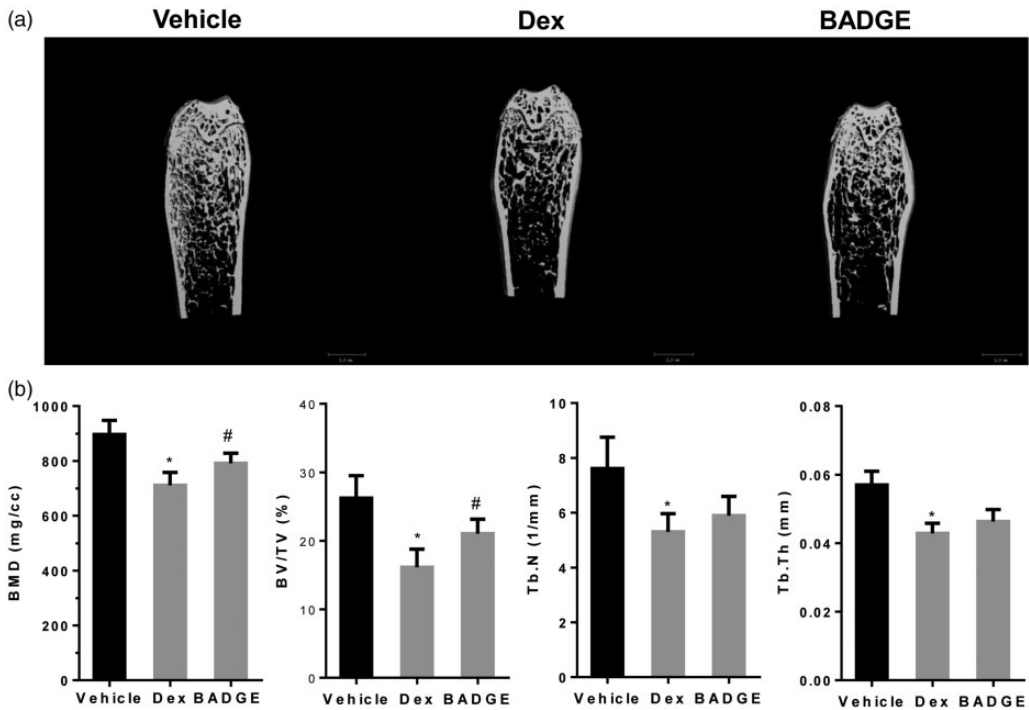


Figure 1. BADGE ameliorates glucocorticoid induced bone mass loss. (a) Representative two-dimensional reconstruction of the femur. (b) Micro-computed tomography revealed that, compared with the normal group, dexamethasone treatment reduced Tb.Th, BV/TV, BMD, and Tb.N. Compared with the model group, BADGE treatment significantly increased trabecular BV/TV and BMD ($n = 10$). # $P < 0.05$ vs. model group and * $P < 0.05$ vs. normal group. BADGE, bisphenol A diglycidyl ether; Tb.Th, trabecular thickness; BV/TV, bone volume/total volume; BMD, bone mineral density; Tb.N, trabecular number.

treatment increased the serum level of CTX-1 and reduced the serum level of OCN. BADGE treatment significantly reduced the serum level of CTX-1 and increased the serum level of OCN, compared with the model group ($P < 0.05$). The increased bone mass with BADGE treatment was demonstrated to partly result from increased bone formation, concomitant with inhibition of bone resorption.

Discussion

In the current study, BADGE, a PPAR γ inhibitor, was found to ameliorate steroid-induced osteoporosis in a mouse model. BADGE treatment increased bone mass,

concomitant with reduced infiltration of marrow fat. Expression levels of osteogenic markers were increased, whereas expression levels of adipogenic markers were reduced. Moreover, BADGE treatment reduced the serum level of CTX-1 and increased the serum level of OCN.

Zhang et al.²⁴ demonstrated that BMSCs derived from osteoporotic mice had significantly lower osteogenesis potential and higher adipogenesis potential, compared with those parameters in normal BMSCs; moreover, human mesenchymal stem cell (hMSC)-derived osteoblasts co-cultured with hMSC-derived adipocytes expressed typical amounts of adipogenic genes, but low amounts of osteogenic markers.

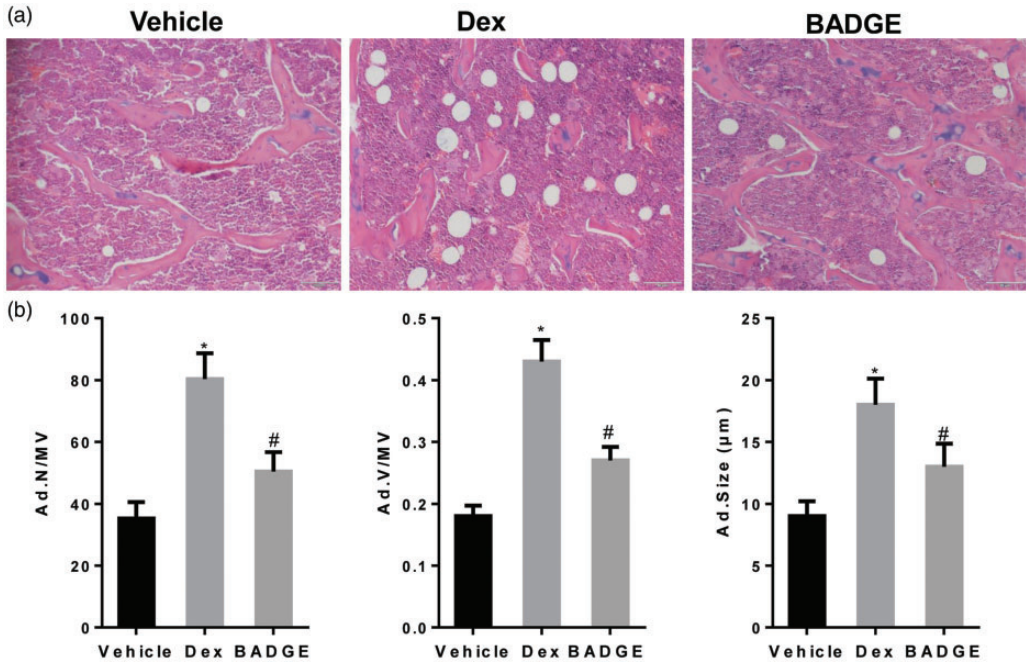


Figure 2. BADGE treatment reduces glucocorticoid-induced bone marrow fat. (a) Images captured at original magnifications of $\times 20$. In the model group, many fat cells accumulated in the bone marrow, whereas BADGE treatment significantly reduced the volume of marrow fat. (b) BADGE treatment reduced Ad.Size/MV, Ad.V/MV, and Ad.N/MV, which were promoted by dexamethasone in bone tissue. # $P < 0.05$ vs. model group and * $P < 0.05$ vs. normal group. BADGE, bisphenol A diglycidyl ether; Ad.Size/MV, adipocyte size; Ad.V/MV, adipocyte volume; Ad.N/MV, adipocyte number.

Martin et al.²⁵ showed that human MSC-derived adipocytes secrete extracellular vesicles that can be transferred to osteoblasts; these vesicles contain anti-osteogenic miRNAs and adipocyte-specific mRNAs.

PPAR γ stimulates adipogenesis at the expense of osteoblastogenesis.²⁶ PPAR γ deletion induces high bone mass and low marrow fat,¹⁵ while activation of PPAR γ can induce bone loss and stimulate bone marrow adiposity.^{27,28} Steroid treatment reportedly increases the gene expression of PPAR γ , both in vivo and in vitro.¹⁰ BADGE treatment inhibits adipogenesis and reduces fatty marrow;^{17,18} notably, BADGE treatment has been shown to increase osteoblastogenesis in mouse bone marrow stromal cells in vitro. Gustavo et al.¹⁷ indicated that BADGE treatment

increased bone mass and osteoblastogenesis in C57BL/6 mice. However, Li et al.²⁹ reported that early BADGE treatment could not increase bone formation in ovariectomized rats, although it could reduce marrow adiposity. Recently, Yuan et al.²⁰ reported that BADGE treatment improved bone formation, concomitant with reduced marrow adiposity, in a rabbit model of steroid-related osteonecrosis. In the current study of mice with glucocorticoid-induced osteoporosis, we found that BADGE treatment increased bone mass and reduced marrow fat infiltration. Importantly, BADGE treatment increased the expression levels of Runx2 and OCN, while reducing the expression levels of PPAR γ and aP2.

Glucocorticoid treatment increased the serum level of CTX-1 and reduced the

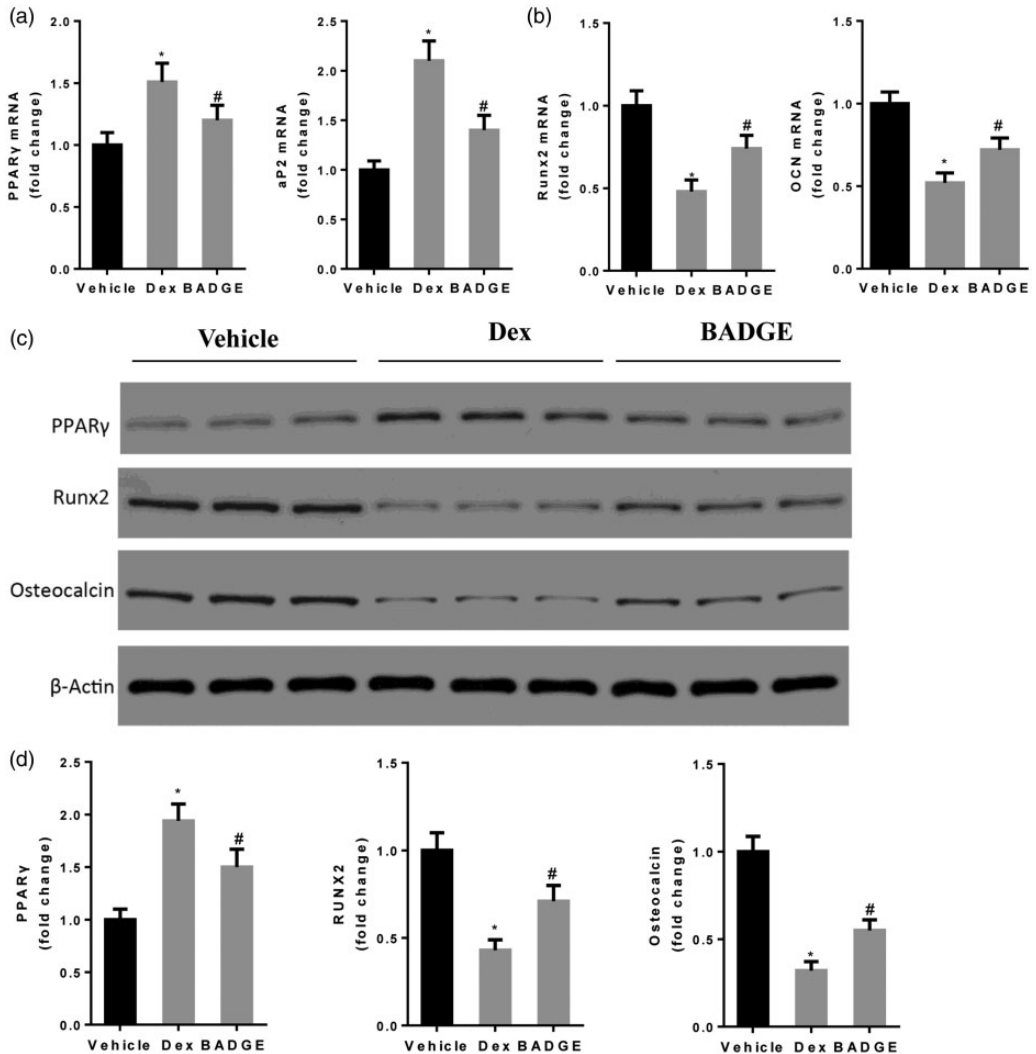


Figure 3. BADGE treatment increases the expression levels of osteogenic markers and reduces the expression levels of adipogenic markers. (a) BADGE treatment significantly reduced mRNA expression levels of PPAR γ and aP2 ($n = 12$). (b) BADGE treatment increased mRNA expression levels of OCN and Runx2 ($n = 12$). (c, d) Compared with the model group, BADGE treatment reduced the protein expression level of PPAR γ and increased the protein expression levels of OCN and Runx2. * $P < 0.05$ vs. normal group and # $P < 0.05$ vs. model group. BADGE, bisphenol A diglycidyl ether; OCN, osteocalcin.

level of OCN, a bone formation marker.⁴ Zhu et al.³⁰ reported that adipocytes differentiated from BMSCs significantly promoted osteoclast formation. Wan and colleagues reported that PPAR γ regulates osteoclastogenesis in mice, and that

PPAR γ deletion reduced osteoclast differentiation. Treatment with antagonists of PPAR γ , such as GW9662 and T0070907, reduced osteoclast gene expression. In contrast, osteoclast differentiation was increased by treatment with rosiglitazone,

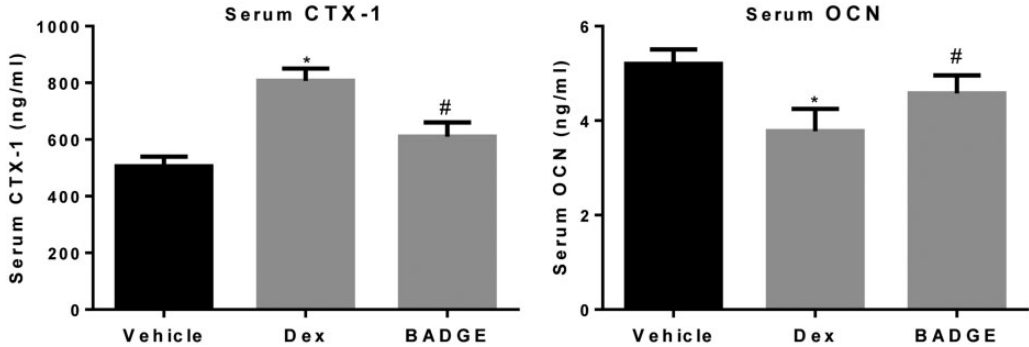


Figure 4. BADGE treatment reduces the serum level of CTX-1 while increasing the serum level of OCN. Compared with the normal group, the serum level of CTX-1 was higher and the serum level of OCN was lower in the model group ($P < 0.05$). BADGE treatment reduced the serum level of CTX-1 and increased the serum level of OCN, compared with the model group ($n = 12$ for all analyses). # $P < 0.05$ vs. model group and * $P < 0.05$ vs. normal group. BADGE, bisphenol A diglycidyl ether; OCN, osteocalcin; CTX-1, C-terminal telopeptide of type I collagen

an agonist of PPAR- γ .³¹ However, Zhao et al.³² reported that both rosiglitazone and pioglitazone treatment inhibited osteoclastogenesis in a dose-dependent manner. In the current study, we also analyzed the effects of BADGE treatment on serum levels of CTX-1 and OCN. We found that dexamethasone treatment reduced the serum level of OCN and increased the serum level of CTX-1. Compared with the model group, BADGE treatment reduced the serum level of CTX-1 and increased the serum level of OCN. However, we could not distinguish whether BADGE reduced bone resorption markers through effective suppression of fatty marrow or by directly affecting osteoclast differentiation in bone. Further studies are needed to identify the direct effects of BADGE treatment on osteoclast differentiation.

A limitation of our study was that it assessed the preventive effect of BADGE treatment on glucocorticoid-induced osteoporosis, but did not assess its treatment effect. Further studies are thus needed to determine the treatment effect of BADGE on glucocorticoid-induced osteoporosis. BADGE may be effective for clinical

prevention or treatment of glucocorticoid-induced osteoporosis in the near future.

Taken together, our data showed that the inhibition of PPAR γ by BADGE ameliorated glucocorticoid-induced osteoporosis. BADGE treatment increased bone mass, concomitant with reduced infiltration of marrow fat. Treatment with BADGE increased the transcription of osteogenic genes and reduced the transcription of adipogenic genes. Moreover, BADGE administration reduced the serum level of the bone resorption marker, CTX-1, and increased the serum level of the bone formation marker, OCN, which may have contributed to the increase in bone mass.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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