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Metformin Stimulates Osteoprotegerin and Reduces RANKL Expression in Osteoblasts and Ovariectomized Rats

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ABSTRACT

Anti-diabetic drug metformin has been shown to enhance osteoblasts differentiation and inhibit osteoclast differentiation in vitro and prevent bone loss in ovariectomized (OVX) rats. But the mechanisms through which metformin regulates osteoclastogensis are not known. Osteoprotegerin (OPG) and receptor activator of nuclear factor κB ligand (RANKL) are cytokines predominantly secreted by osteoblasts and play critical roles in the differentiation and function of osteoclasts. In this study, we demonstrated that metformin dose-dependently stimulated OPG and reduced RANKL mRNA and protein expression in mouse calvarial osteoblasts and osteoblastic cell line MC3T3-E1. Inhibition of AMP-activated protein kinase (AMPK) and CaM kinase kinase (CaMKK), two targets of metformin, suppressed endogenous and metformin-induced OPG secretion in osteoblasts. Moreover, supernatant of osteoblasts treated with metformin reduced formation of tartrate resistant acid phosphatase (TRAP)-positive multi-nucleated cells in Raw264.7 cells. Most importantly, metformin significantly increased total body bone mineral density, prevented bone loss and decreased TRAP-positive cells in OVX rats proximal tibiae, accompanied with an increase of OPG and decrease of RANKL expression. These in vivo and in vitro studies suggest that metformin reduces RANKL and stimulates OPG expression in osteoblasts, further inhibits osteoclast differentiation and prevents bone loss in OVX rats. J. Cell. Biochem. 112: 2902–2909, 2011.

KEY WORDS: METFORMIN; OSTEOPROTEGERIN; OSTEOCLAST; RECEPTOR ACTIVATOR OF NUCLEAR FACTOR κB LIGAND; OSETOPOROSIS

As one of the most serious and challenging health problems in the 21st century, diabetes mellitus affects millions of individuals worldwide. Recently, increasing attention has been paid to the relationship between diabetes and osteoporotic fractures [Barrett-Connor and Holbrook, 1992]. Many clinical studies have reported that osteoporosis is one of the chronic complications associated with diabetes mellitus [Krakauer et al., 1995; Suzuki et al., 2000; Patel et al., 2008]. A higher risk of fractures was found in both type 1 and type 2 diabetes compared to the non diabetic population [Rakel et al., 2008; Khazai et al., 2009]. Moreover, both diabetes and osteoporotic fractures affect a large proportion of older adults and pose a considerable burden on healthcare resources. Therefore, it is more important and urgent to reduce the risk of fractures in diabetic subjects than in non-diabetic counterparts.

As an anti-hyperglycemic agent, metformin is commonly used in the treatment of type 2 diabetes by improving insulin resistance. Metformin could decrease the fracture rate in patients [Kanazawa, 2009] through a direct osteogenic effect on osteoblasts in culture [Cortizo et al., 2006]. Recent findings showed that metformin could prevent bone loss in ovariectomized (OVX) rats [Gao et al., 2010] and inhibit receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclast differentiation in Raw264.7 macrophage cells

Qi-Guang Mai and Zhong-Min Zhang contributed equally to this work.

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[Lee et al., 2010]. However, mechanisms underlying the differentiation and function of osteoblast and osteoclast by metformin remain unclear.

The osteoclast is a specialized macrophage polykaryon, and its differentiation is principally regulated by macrophage colony-stimulating factor (M-CSF), RANKL, and osteoprotegerin (OPG), the latter two cytokines are secreted predominantly by osteoblasts [Yasuda et al., 1998; Teitelbaum, 2000]. The RANKL, upon binding to receptor activator of nuclear factor κB (RANK) on the cell surface of osteoclasts or its precursors, functions to induce differentiation, activation and survival of osteoclasts, whereas OPG, a decoy receptor for RANKL, functions to inhibit osteoclastogenesis [Simonet et al., 1997; Tsuda et al., 1997].

Considering the important role of OPG/RANKL in osteoclastogenesis regulation and inhibitory effects of metformin in OVX-induced bone loss, we hypothesized that metformin could regulate the expression of OPG and RANKL in osteoblasts. In this study, we provided evidences for the first time that metformin reduces RANKL and stimulates OPG expression in osteoblasts in vivo and in vitro, thereby inhibits osteoclast differentiation and prevents bone loss in OVX rats.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

All cell culture media were purchased from GIBCO (Invitrogen Corp., Carlsbad, CA). Metformin, compound C, STO-609,1,25-dihydroxyvitamin D(3) (VitD3), 17-β estradiol (E2), collagenase Type-II, tartrate-resistant acid phosphatase (TRAP) staining kit were from Sigma–Aldrich Co. (St. Louis, MO). RNA extraction reagent (TRIZOL) and semi-quantitative RT-PCR kit were from TaKaRa Biotechnology Co. Ltd (Dalian, China). Mouse OPG ELISA kit was from Boster Biotech Co. Ltd (Wuhan, China). The p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, MAPK kinase 1 (MEK1) inhibitor PD98059 and the NF-κB inhibitor Bay 11-7082 were from Cell Signaling Technology (Danvers, MA). For Western blot analysis, RANKL antibody was purchased from Bioworld Technology (Louis Park, MN).

OSTEOBLASTS CULTURE

Calvarial osteoblasts were isolated from calvariae of 1-day old mice (Experimental Animal Center of Southern Medical University, Guangzhou, China) by sequential 0.25% trypsin and 0.1% collagenase Type-II digestion as described previously [Bai et al., 2005]. Cells released in the second and third digests were pooled and suspended in α-MEM medium containing 10% fetal bovine serum (FBS). The cells were timely trysinized and subcultured to prevent cell confluence.

CO-CULTURE SYSTEM AND TRAP STAINING

Calvarial osteoblasts were treated with different concentrations of metformin (50–800 μM) and VitD3 10^{-8} M for 2 days and the supernatants were collected. Raw264.7 cells were cultured with the collected supernatants for 12 days. The supernatants of osteoblasts were replaced once every 2 days. The cells were then subjected to TRAP staining following steps provided by the manufacturer, and the number of osteoclast-like TRAP-positive multi-nucleated (>3 nuclei) cells (MNCs) was counted.

ANIMALS AND TREATMENTS

A total of 45 adult Sprague–Dawley female rats (Experimental Animal Center of Southern Medical University, China) that were approximately 8–10 weeks of age and weighed an average of 180–200 g were used in this study. Within 1 week of their arrival, rats were anesthetized with intraperitoneal injection of 10% chloral hydrate (3.3 ml/kg) and underwent either a sham surgery or bilateral ovariectomization. Animals were kept in pairs under standard laboratory conditions with a 13-h light, 11-h dark cycle and a constant temperature of 25°C and humidity of 48%. Bred for 3 months with standard rat chow, they were randomly divided into three groups consisting of 15 rats each to receive a supplement diet via orogastric intubation: (1) Sham + distilled water; (2) OVX + distilled water and (3) OVX + 100 mg/kg/day metformin. After 2 months of metformin treatment, dual energy X-ray absorptiometry (Lunar PIXImus, GE Medical Systems, WI) was applied to scan the left femur of all rats to determine the levels of bone mineral density (BMD) and bone mineral content (BMC) under Nembutal (30 mg/kg) anesthesia before the rats were killed. Ten, three, and three rats were randomly taken out of the experiment (15 rats per group) for ELISA (OPG), Western blot (RANKL) analysis, and TRAP staining respectively.

HISTOLOGY OF RAT BONE

Rats proximal tibiae were fixed in 4% p-formaldehyde for 3 days, dehydrated with graded alcohols, and then all tissues were embedded for histological analysis according to standard conditions without decalcification. For Von Kossa staining, the slides were stained with 2% silver nitrate solution under UV light for 60 min, incubated in 5% sodium thiosulfate for 2 min, rinsed in distilled water and stained with Nuclear Fast Red Stain for 1 min. To quantify the number of TRAP-stained osteoclasts in vivo, TRAP staining was performed according to the manufacturer's protocol, and microscopic examinations were performed using a Nikon ECLIPSE E600 stereomicroscope (Nikon, Japan). The number of active TRAP-positive osteoclasts in bone resorption pits was normalized against the total cell number and expressed as positive cell number per total of 100 cells from each sample, so as to offset the variations of total cell number among different groups on the positive cell counting.

REAL-TIME QUANTITATIVE RT-PCR (Q-PCR) AND RT-PCR ANALYSIS

The RNA was extracted with TRIzol and DNA synthesis was performed following the protocol of the SYBR® PrimeScript TM RT-PCR kit. Primers used were as following: OPG forward 5′-AAAGCACCCTGTATAAAACA-3′, reverse 5′-CCGTTTTATCCTCTC-TAC ACT C-3′; RANKL forward 5′-CGCTCTGTTCCTGTACTT TCG-AGCG-3′, reverse 5′-TCGTGCTCCCTCCTTTCATCAGGTT-3′; GAPDH forward 5′-TGTGTCCGTCGTGGA TCTGA-3′, reverse 5′-TTGCT-GTTGAAGTCGCAG GAG-3′. Thermal cycling conditions were 30 s at 95°C, 45 cycles of 10 s at 95°C, 30 s at 58°C, and 20 s at 72°C. Relative quantification and calculation of genes expression were carried out according to the manufacture's protocol. Following primers were used for RT-PCR as analysis: TRAP forward 5′-AAA

TCA CTC TTT AAG ACC A, reverse 5′-TTA TTG AAT AGC AGT GAC AG; cathepsin K forward 5′-GGG CCA GGA TGA AAG TTG TA, reverse 5′-CCG AGC CAA GAG AGC ATA TC; GAPDH were the same as that of the q-PCR. PCR products were separated on a 2% agarose gel and stained with ethidium bromide. As an internal control for RNA quantity, the same cDNA was amplified using primers specific for GAPDH mRNA.

ELISA FOR OPG EXPRESSION ASSAY

Primary osetoblasts were serum starved for 16 h, followed by metformin (0–800 μM) stimulation for 48 h. The supernatants of osteoblasts were then collected and the levels of OPG secretion were measured by an ELISA kit according to the manufacturer's protocol.

WESTERN BLOT ANALYSIS

Osteoblasts or bone marrow cells from the rat femur were lysed in SDS sample buffer and subjected to Western blot analysis as described previously [Bai et al., 2007]. The protein level of RANKL was quantified by computer image analysis with Image J software.

STATISTICAL ANALYSIS

In vitro and in vivo data were expressed as mean ± SD and analyzed for statistical significance using the one-way ANOVA. All experiments were repeated at least three times, and representative experiments are shown. For the study, $P < 0.05$ was considered significant.

RESULTS

DOSE- AND TIME-DEPENDENT STIMULATION OF OPG MRNA EXPRESSION IN OSTEOBLASTS BY METFORMIN

To investigate the effect of metformin on OPG expression in osteoblasts, primary mouse calvarial osteoblasts and osteoblastic cell line MC3T3-E1 were treated with 0–800 μM metformin for 0–72 h, and the expression of OPG mRNA was determined by q-PCR. The results showed that treatment with 0–800 μM metformin induced a dose-dependent increase in the expression of OPG mRNA both in primary osteoblasts (Fig. 1A) and MC3T3-E1 (Fig. 1B). The stimulatory effects of metformin on OPG mRNA expression were similar with that of estradiol (E2) in both cells examined (Fig. 1A,B). Furthermore, the expression of OPG mRNA by metformin is also enhanced over time from 0 to 72 h (Fig. 1C). These results suggest that OPG mRNA expression in osteoblasts was enhanced by metformin dose- and time-dependently.

AMP-ACTIVATED PROTEIN KINASE PATHWAY IS INVOLVED IN METFORMIN-INDUCED OPG PROTEIN EXPRESSION IN OSTEOBLASTS

We further examined the ability of metformin to induce OPG protein expression in osteoblasts by ELISA. It was found that metformin increases the amount of OPG in culture medium secreted by osteoblasts in a dose-dependent manner (Fig. 2A).

To determine the specific signal transduction pathways involved in metformin-induced OPG synthesis, we observed the effects some signaling inhibitors on metformin-induced OPG (Fig. 2B). It was found that pretreatment with the AMP-activated protein kinase (AMPK) inhibitor compound C, or CaM kinase kinase (CaMKK) inhibitor STO-609, but not mitogen activated protein kinase kinase 1(MEK1) inhibitor PD98059, the p38 MAP kinase inhibitor SB203580 or the NF-κB signaling inhibitor Bay-117028, prevented the increase of OPG expression stimulated by metformin in osteoblasts (Fig. 2B,C). But compound C or STO-609 alone could also decrease endogenous level of OPG significantly (Fig. 2B,C). AMPK is a well established target of metformin while CaMKK acts as an upstream activator of AMPK. The inhibitory effect of compound C and STO-609 on endogenous and metformin-stimulated OPG secretion suggested the important role of AMPK pathway in OPG expression in osteoblasts.

METFORMIN REDUCES THE RANKL MRNA AND PROTEIN EXPRESSION IN OSTEOBLASTS

OPG is an intrinsic inhibitor of RANKL by acting as a decoy receptor for RANKL [Mundy, 2002]. Further study was carried

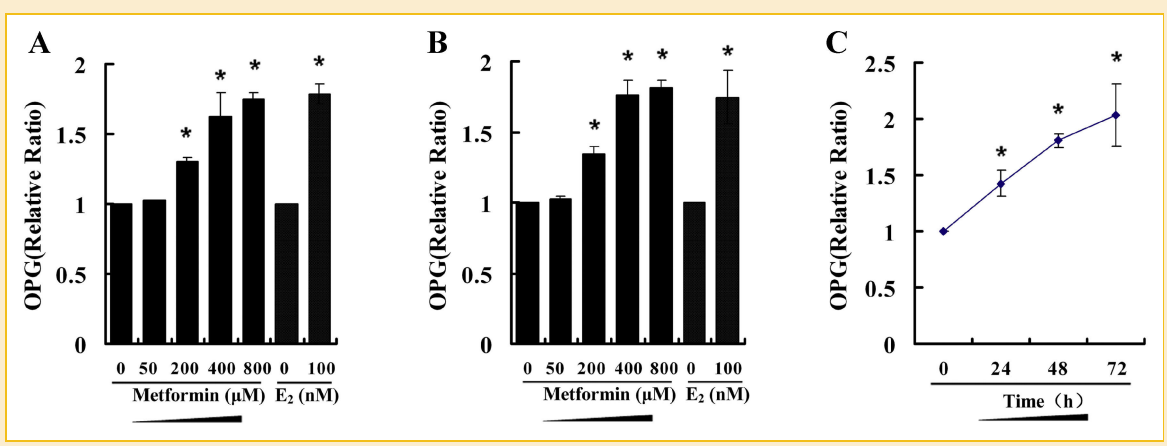

Fig. 1. Metformin stimulates OPG mRNA expression in osteoblasts and MC3T3-E1. A: Primary mouse calvarial osteoblasts and (B) MC3T3-E1 cells were serum starved for 16 h, and treated with 0–800 μM metformin or 100 nM E_2 for 48 h. C: Primary osteoblasts were incubated for the indicated times in the presence or in the absence of 800 μM metformin. OPG mRNA expression was determined by real-time quantitative PCR. Results from untreated cells were given a value of 1 and metformin or estradiol-treated cells a relative ratio. The bar graph represents the mean ± SD density (n = 3; $^*P < 0.05$ vs. control).

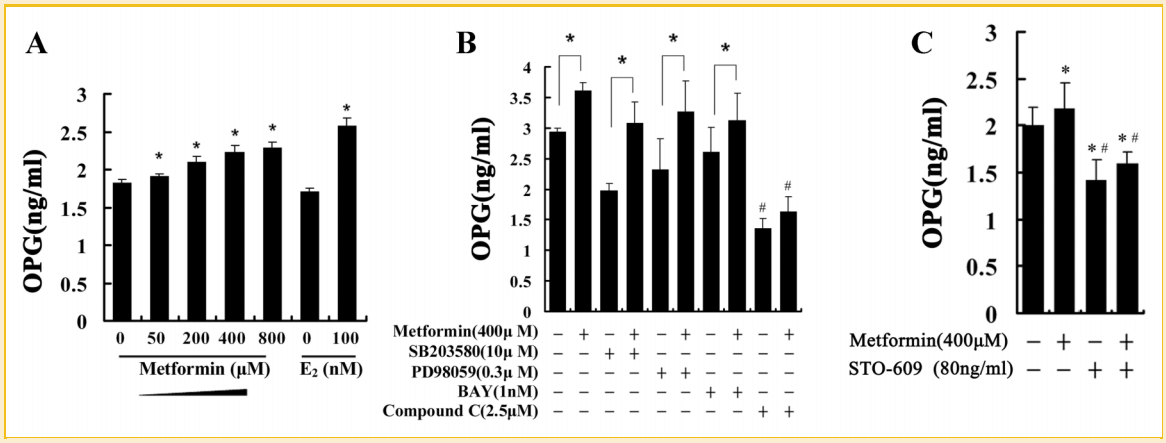

Fig. 2. AMPK signaling pathway is involved in metformin-induced OPG protein expression in osteoblasts. A: Primary osetoblasts were serum starved for 16 h, and stimulated with metformin (0–800 μM) for 48 h. Conditioned medium was harvested, and the level OPG was determined by ELISA. The bar graph represents the mean ± SD density ($^{*}P < 0.05$ vs. control). B: Osteoblasts were serum starved for 16 h, and pre-incubated with indicated inhibitors for 30 min before the addition of 400 μM of metformin for 48 h ($^{*}P < 0.05$ vs. inhibitors control, $^{\#}P < 0.05$ vs. control and metformin). C: Osteoblasts were serum starved for 16 h, and pre-incubated with 80 ng/ml STO-609 for 30 min before the addition of 400 μM of metformin for 48 h. Supernatants were then collected, and ELISA was used to measure OPG (ng/ml) levels ($^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. metformin (400 μM)).

out to determine the potential role of metformin on expression of RANKL in primary osteoblasts. It was revealed that mRNA levels of RANKL in osteoblasts are decreased by metformin treatment in a dose-dependent manner (Fig. 3A). Western blot analysis using RANKL antibody further confirmed that protein levels of RANKL in osteoblasts are reduced by metformin treatment (Fig. 3B).

These findings suggest that metformin may attenuate RANKL signaling either by direct impairment of RANKL expression or by increasing of its intrinsic inhibitor, OPG.

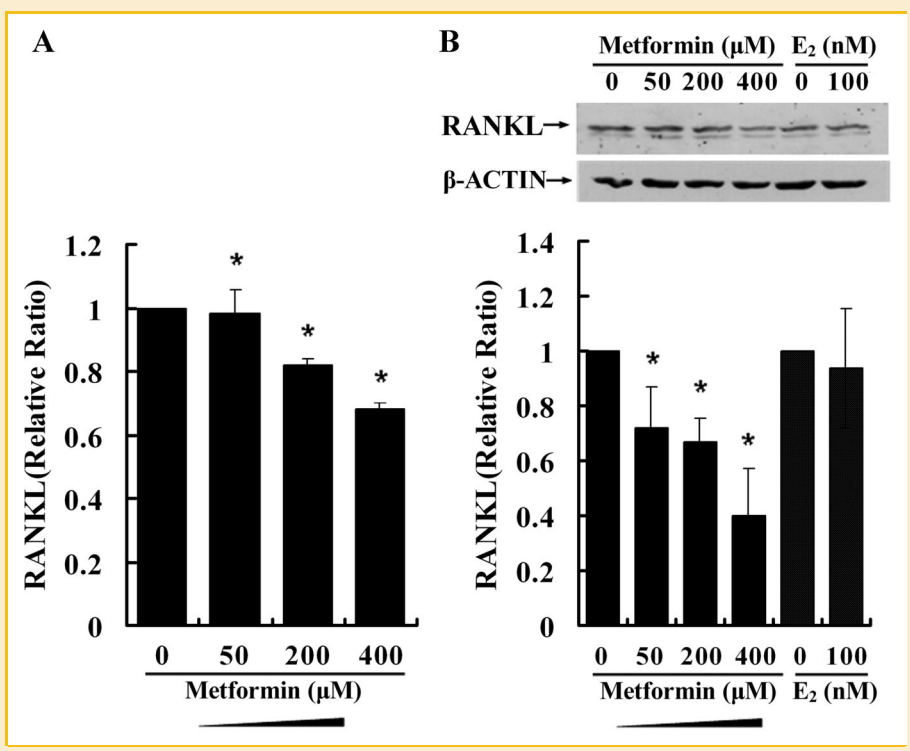

Fig. 3. Metformin reduces the RANKL mRNA and protein expression in osteoblasts. Cells were serum starved for 16 h, and stimulated with indicated doses of metformin for 48 h. RANKL mRNA (A) and protein (B) expression were analyzed by quantitative-RT-PCR or Western blot respectively. Results from untreated cells were given a value of 1 and metformin or estradiol-treated cells a relative ratio. The bar graph represents the mean ± SD density of each band normalized to actin (n = 3; $^{*}P < 0.05$ vs. control).

METFORMIN PREVENTS OSTEOCLAST DIFFERENTIATION IN VITRO

RANKL and OPG are critical factors for differentiation of osteoclasts. Hence, findings above implicated that metformin may inhibit osteoclastogenesis. To identify the possible role of metformin in osteoclast differentiation in vitro, Raw264.7 cells were cultured in the supernatants of calvarial osteoblasts treated with metformin. TRAP-positive multi-nucleated cells (MNCs) containing more than three nuclei were counted as osteoclasts under microscope. As shown in Figure 4A, we found that metformin reduced the number of TRAP-positive MNCs in a dose-dependent manner (Fig. 4B).

To confirmed the differentiation of osteoclasts in this culture system, we further examined TRAP and cathepsin K mRNA expression in Raw264.7 cells by RT-PCR. It was found that the supernatants of osteoblast increased the TRAP and cathepsin K mRNA expression in a time-dependent manner in Raw264.7 cells (Fig. 4C,D). These findings suggest that metformin may inhibit osteoclast differentiation by regulating cytokines production in osteoblasts.

METFORMIN STIMULATES OPG AND SUPPRESSES RANKL EXPRESSION IN VIVO

To confirm the effect of metformin on OPG/RANKL expression in vivo, OVX rats were treated with different doses of metformin, and then levels of OPG and RANKL were detected by ELISA and Western blot analysis respectively. The results showed that metformin could increase the serum levels of OPG and decrease RANKL expression in bone marrow cells in metformin-treated OVX rats (Fig. 5A,B). These data further confirmed that metformin stimulates OPG and inhibits RANKL expression in vivo.

METFORMIN PREVENTS BONE LOSS AND SUPPRESSES OSTEOCLASTOGENESIS IN OVX RATS

To further clarify the prevention effect of metformin on OVX-induced bone loss and impaired osteoclastogensis in vivo, bone mineral density measurement (Table I), Von Kossa and TRAP staining (Fig. 6A) were performed to examine the calcified lesion and osteoclastogensis respectively in metformin treated OVX rats. Our results demonstrated that metformin could enhance bone mineral content, bone mineral density and percent bone volume, and decrease trabecular separation in OVX rats, indicating the attenuation effect of metformin on bone loss induced by OVX (Table I and Fig. 6A). Furthermore, the normalized cell number of active TRAP-positive osteoclasts in bone resorption pits of proximal tibiae bone was 8.15 folds higher in OVX rats than in Sham rats (Fig. 6B,C). But this increase in osteoclastogenesis was markedly reduced following oral treatment of metformin (Fig. 6B,C). These

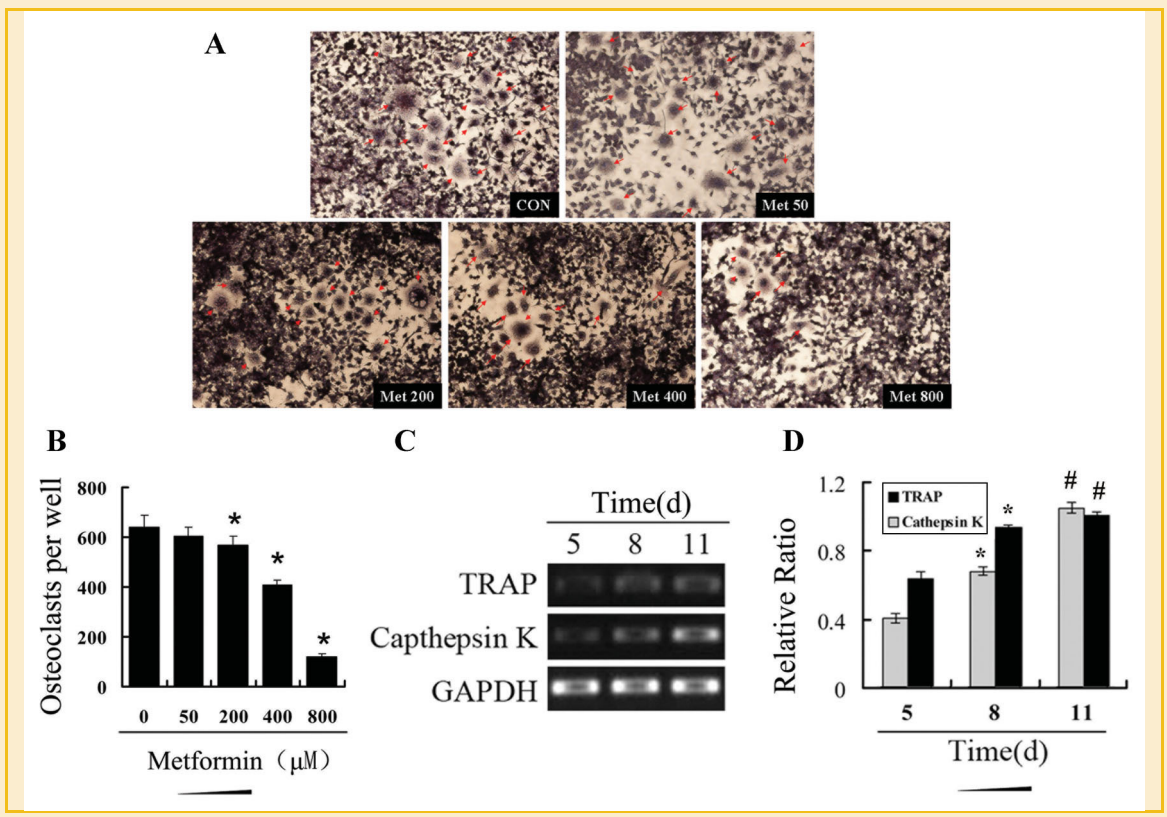

Fig. 4. Metformin suppresses osteoclast differentiation in vitro. A: Raw264.7 cells were cultured with supernatants of calvarial osteoblasts treated with indicated concentrations of metformin and VitD3 (10^{-8} M) for 12 days. The cells were fixed and stained with TRAP solution. Representative of microscopic view at a magnification of 200× were shown. B: The number of TRAP+ multi-nucleated cells was quantified ($^{*}P < 0.05$ vs. control). C: Raw264.7 cells were cultured with supernatants of calvarial osteoblasts for indicated times and the mRNA expression of TRAP and capthepsin K were analyzed by RT-PCR. GAPDH was used as control. D: Results from (C) were given a relative ratio. The bar graph represents the mean ± SD density ($n = 3$; $^{*}P < 0.05$ vs. 5 days group; $^{\#}P < 0.05$ vs. 5 days and 8 days group). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

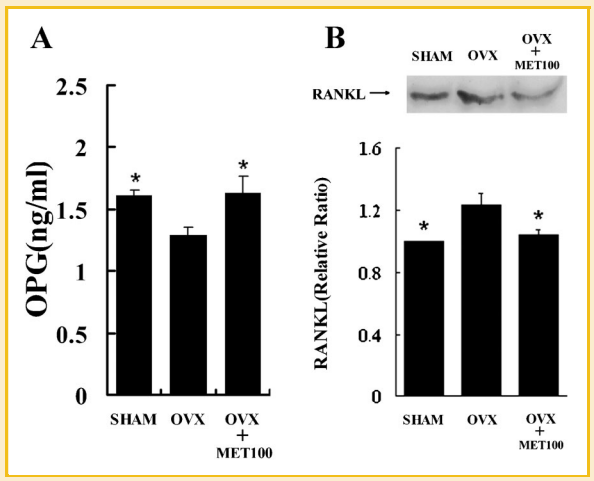

Fig. 5. Metformin stimulates OPG and inhibits RANKL expression in vivo. A: ELISA was used to measure OPG (ng/ml) levels in samples from Sprague–Dawley female rats treated with or without 100 mg/kg/day metformin for 2 months. The bar graph represents the mean ± SD density ($n = 10$; $^{*}P < 0.05$ vs. OVX). B: Western blot analysis of RANKL expression in the bone marrow from the femur of OVX rats treated with or without metformin. Results from the Sham group were given a value of 1 and OVX or metformin-treated group a relative ratio. The bar graph represents the mean ± SD density ($n = 3$; $^{*}P < 0.05$ vs. OVX). MET100, metformin (100 mg/kg/day).

results indicate that metformin is able to inhibit osteoclast differentiation and prevent cancellous bone loss in OVX rats.

DISCUSSION

Osteoclasts are the key cells in bone resorption whose differentiation is principally regulated by RANKL and OPG. Therefore, the balance between RANKL and OPG plays a significant role in homeostasis of bone metabolism. In this study, we demonstrated for the first time that anti-diabetic drug metformin enhances OPG and reduces RANKL mRNA and protein expression in mouse calvarial osteoblasts and osteoblastic cell line MC3T3-E1 dose-dependently. Moreover, metformin decreases the formation of TRAP-positive MNCs in a Raw264.7 cells and osteoblasts co-culture system, indicating inhibition of osteoclast differentiation by metformin. We further found metformin could enhance OPG and reduce RANKL expression, subsequently impair osteoclastogenesis and prevent cancellous bone loss in OVX rats.

Recently, Suzuki et al. [2005] demonstrated that serum levels of RANKL/OPG and TRAP are higher in the diabetic patients compared to healthy populations. Singh et al. [2009] also showed that serum OPG levels are significantly lower in patients with type 1 diabetes compared to normal controls. In this study, the OPG expression in osteoblasts and OVX rats was up-regulated, while RANKL expression was down-regulated by the treatment of metformin, which may reveal the possible mechanism of this drug in prevention of diabetes-related osteoporosis.

The osteogenic effect of metformin on osteoblasts has been reported by several groups [Cortizo et al., 2006; Kanazawa et al., 2008; Bak et al., 2010]. But little is known about the effect of metformin on osteoclasts. A recent report demonstrated that metformin prevented RANKL-induced osteoclasts differentiation in vitro [Gao et al., 2010]. However, study by Bak et al. [2010] reported that metformin exerted no effects on osteoclast formation induced by VitD3, lipopolysaccharides, and prostaglandin E(2) (PGE_2). In this study, we confirmed that metformin inhibits osteoclast differentiation in a Raw264.7 cells and osteoblasts co-culture system and in OVX rats. Different incubation time (12 days in our study and 6 days in Bak's report) in our study may be the cause for this discrepancy. It was recently reported that metformin could

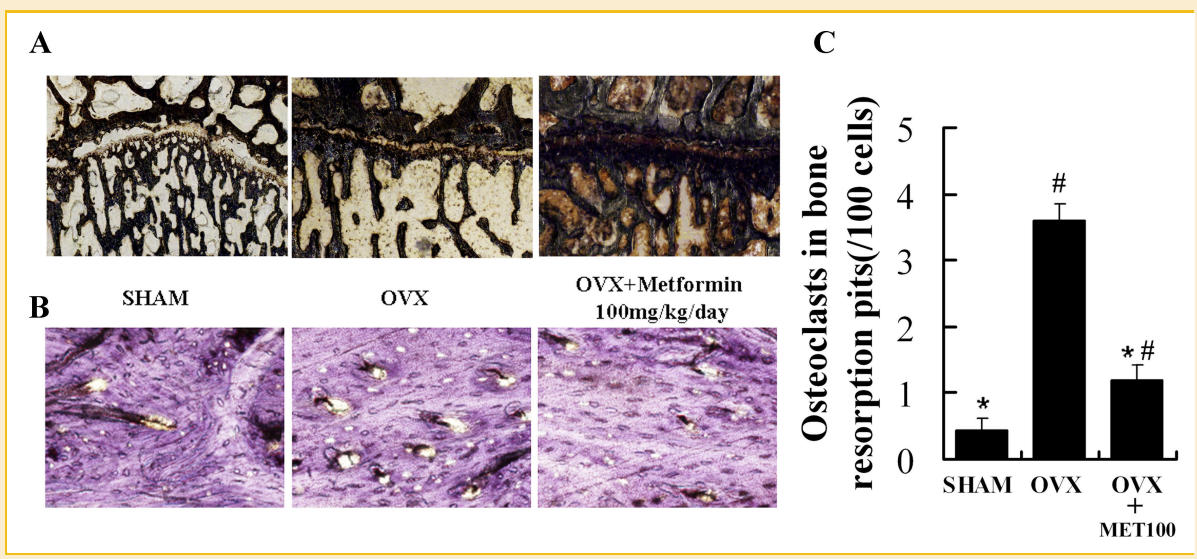

Fig. 6. Metformin suppresses osteoclastogenesis and prevents bone loss in OVX rats. A: Von Kossa staining of metaphyseal region of the proximal tibiae in OVX rats treated with or without 100 mg/kg/day of metformin (magnification, 25×). B: TRAP staining of proximal tibiae sections in OVX rats treated with or without 100 mg/kg/day of metformin (magnification, 200×). C: Quantified number of osteoclasts in resorption pits of (B). The bar graph represents the mean ± SD density ($n = 3$; $^{*}P < 0.05$ vs. OVX; $^{\#}P < 0.05$ vs. sham). MET100, metformin (100 mg/kg/day). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

TABLE I. Bone Mineral Density, Bone Mineral Content, and Bone Area of Left Femur Measured by Dual Energy X-Ray Absorptiometry (Data Are Means ± SD)

	N = 15		
Parameters	Sham	OVX	OVX + Met (100 mg/kg/day)
Bone mineral density (g/cm^2)	0.238 ± 0.021[b]	0.196 ± 0.018[a]	0.245 ± 0.048[b]
Bone mineral content (g)	0.286 ± 0.015[b]	0.238 ± 0.023[a]	0.279 ± 0.055[b]
Area (cm^2)	1.206 ± 0.064	1.204 ± 0.054	1.140 ± 0.145

[a]$P < 0.05$ versus Sham group.
[b]$P < 0.05$ versus OVX group.

prevent bone loss in OVX rats at least in part through increasing bone formation via induction of osteoblast genes core-binding factor alpha1 (Cbfa1) and the low-density lipoprotein receptor related protein 5(Lrp5) [Gao et al., 2010]. Furthermore, Lrp5 has been linked to regulate osteoblast–osteoclast coupling by affecting the RANK–RANKL and OPG pathway [Johnson, 2004]. Our study clearly demonstrated the important role of metformin in regulation of OPG/RANKL axis and inhibition of osteoclastogenesis both in vivo and in vitro. These findings support our view that metformin inhibits differentiation of osteoclasts by stimulating OPG and inhibiting RANKL production in osteoblasts.

To gain further insight into the mechanisms through which metformin regulates OPG expression, several possible signaling pathways were evaluated. AMPKs are well established to play an essential role in controlling cell proliferation and differentiation [McGee and Hargreaves, 2008]. Previous studies have shown that metformin could improve glucose metabolism via activation of AMPK [Zhou et al., 2001]. Recently, it has been shown that AMPK activation could regulate bone formation and bone mass in vitro [Shah et al., 2010]. These findings suggest that metformin could affect bone metabolism via the AMPK signaling pathway in osteoblasts [Kanazawa et al., 2008]. A variety of signaling molecules have been reported to be activated or inhibited by metformin. In this study, inhibitors of MEK1, p38 MAPK, and NF-κB signaling did not affect the action of metformin on OPG synthesis. Inhibition of AMPK or CaMKK, a upstream activator of AMPK by compound C or STO-609 respectively suppressed endogenous and metformin-stimulated OPG expression (Fig. 2B,C), suggesting the important role of AMPK pathway in OPG expression in osteoblasts. Because compound C or STO-609 alone could also reduce OPG expression, it is not clear whether AMPK mediates metformin-induced OPG expression and the possibility of indirect effect of compound C or STO-609 involved in this process could not be excluded.

In conclusion, metformin stimulates OPG synthesis and reduces RANKL expression in osteoblasts, resulting in an increase of the OPG/RANKL ratio and decrease of osteoclast differentiation in vivo and in vitro. These results suggest that metformin not only promotes osteoblast differentiation but also up-regulates OPG and down-regulates RANKL secretion in osteoblasts, subsequently prevents bone loss and osteoporosis.

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