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Communication

Metformin regulates osteoblast and adipocyte differentiation of rat mesenchymal stem cells

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Abstract

Metformin is widely used for the treatment of type 2 diabetes mellitus. In this study, we evaluated the effects of metformin on the osteoblast and adipocyte differentiation of rat marrow mesenchymal stem cells (MSCs) in culture. Treatment of MSCs in osteoblastic or adipogenic medium with 100 μM metformin for 21 days led to an increased mRNA expression of the osteoblast markers but a decreased mRNA expression of the adipocyte markers in the MSCs. Metformin markedly stimulated deposition of mineralized nodules and blocked the formation of cytoplasmic lipid droplets. In addition, alkaline phosphate activity and Western blot analysis for core binding factor a1 (Cbfa1) and peroxisome proliferator-activated receptor gamma 2 (PPARγ2) proteins also confirmed that metformin inhibited adipocyte differentiation and promoted osteoblast differentiation. The reciprocal relationship between osteoblastic and adipogenic differentiation suggests that metformin may regulate osteoblastic and adipogenic differentiation through inhibition of PPARγ.

Introduction

Recent studies have reported bone loss in patients with diabetes (KraKauer et al 1995; Chau et al 2003; Carnevale et al 2004). Diabetes may affect bone metabolism through multiple pathways, including obesity, insulin levels, hyperglycaemia and advanced glycation end products in extracellular matrix proteins (Katayama et al 1996; McCarthy et al 1997). Treatment with thiazolidinediones may increase fracture risk, at least in older women (Schwartz & Sellmeyer 2008). Metformin is widely used for the treatment of type 2 diabetes mellitus; however, little is known about its potential effects on bones. Because of the inhibitory influence of this medication on glycation reactions in-vivo, a recent hypothesis proposed that metformin may decrease the risk for osteoporotic bone fractures in diabetic patients (Yamagishi et al 2005). To our knowledge, only one study has investigated the effect of metformin on the growth and development of two osteoblast-like cell lines: UMR106 and MC3T3E1 (Cortizo et al 2006). Metformin was found to have a direct osteogenic effect on osteoblast-like cells. It increased type-I collagen production in both cell lines and stimulated alkaline phosphatase (ALP) activity in MC3T3E1 osteoblasts.

Bone marrow contains a population of mesenchymal stem cells (MSCs) that undergo osteoblastic and adipogenic differentiation following stimulation from certain biochemical and biomechanical signals, both in-vivo and in-vitro (Pittenger et al 1999). The bone metabolic conditions are mainly determined by the biological balance between bone formation and marrow adipogenesis (Nuttall & Gimble 2004). In fact, marrow adipocytes share common MSCs with bone-forming osteoblasts, and these two cell types may affect each other's actual number and activity. Previous studies have shown that osteoblast special genes play an important role in the osteoblastic differentiation of MSCs; activation of these genes such as core binding factor a1 (Cbfa1) and low-density lipoprotein (LDL) receptor-related protein 5 (Lrp5) has been identified as well-established transduction mechanisms for various interventions acting on bone formation (Ducy et al 1997; Tang et al 2006). While adipocyte genes – peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding protein alpha (C/EBPα) and adipocyte lipid-binding protein (aP2) – play a hierarchically dominant role in the determination of adipogenic differentiation of MSCs through suppressing the expression of osteoblastic transcription factors and thus controlling the adipocyte/osteoblast relationship within the bone cavity (Nuttall & Gimble 2004; Backesjo et al 2006).

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Because of the reciprocal relationship between osteoblast and adipocyte differentiation, our study was designed to examine how metformin may affect the osteoblastic and adipogenic differentiation from rat marrow MSCs in culture. In addition, we have investigated the possible mechanisms underlying the action of metformin, such as the down-regulation of PPARγ and up-regulation of Cbfa1.

Materials and Methods

Materials

Alpha-minimum essential medium (α-MEM), trypsin-EDTA and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Metformin was from Calbiochem (San Diego, CA, USA), rabbit anti-PPARγ2 monoclonal antibody was from Genetex (San Antonio, TX, USA), and rabbit anti-Cbfa1 polyclonal antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The osteoblastic factors were 50 mg L^{-1} L-ascorbic acid, 10^{-8} mol L^{-1} dexamethasone, 10 mmol L^{-1} β-glycerophosphate and 10 mmol L^{-1} Vitamin D3. The adipogenic factors were 1 μmol L^{-1} dexamethasone, 100 μmol L^{-1} indometacin, 0.5 mmol L^{-1} 3-isobutyl-1-methylzanthine and 10 mg mL^{-1} insulin (all obtained from Sigma (St Louis, MO, USA)). Other common chemicals such as penicillin/streptomycin, 4-*p*-nitrophenylphosphate and Alizarin red S (ARS) and oil red O dyes were obtained from Sigma.

Isolation and culture of rat MSCs

The rat MSCs used in the study were harvested from the femurs and tibias of Sprague–Dawley rats, weighing between 220 and 250 g. Primary cultures of the cells were established by a modification of the method previously described by Sugiura et al (2004). Briefly, the released marrow cells were cultured in a basal medium containing α-MEM with 10% FBS and 100 μL mL^{-1} penicillin/streptomycin, at 37°C in a 5% CO_2 air atmosphere. At 80% cell confluence, the cells were passaged and subcultured in osteoblastic or adipogenic medium with or without 100 μM metformin, which was the dose that effectively stimulated ALP activity, type-I collagen production and calcium deposition in osteoblast-like cell lines (Cortizo et al 2006). The medium was changed every 3 days throughout the study. Cells were photographed with a phase-contrast microscope (Olympus IX70, Olympus Optical Co., Tokyo, Japan).

All experimental procedures using rats in the study were approved by the Animal Care Committee of Sichuan University.

Cell proliferation assay

The MTT (3-{4,5-dimethylthiazol-2yl}-2,5-diphenyl-2H-tetrazolium-bromide) assay was used to evaluate the proliferation of MSCs in osteoblastic and adipogenic medium with or without 100 μM metformin. Briefly, the medium was discarded, and MTT solution was added. After 4 h' incubation at 37°C in a 5% CO_2 air atmosphere, MTT was removed and the precipitated formazan was dissolved in DMSO. The optical density (OD) of the solution was measured at 570 nm. The MTT assay was performed once a day for 10 days.

ALP activity assay and ARS staining for osteoblastic differentiation cells

ALP activity was measured after 5, 10 and 15 days using a method reported by Leskela et al (2006). Briefly, the cells were incubated in osteoblastic medium with or without metformin, then rinsed with phosphate-buffered saline (PBS) and digested by trypsinization. After three freeze–thaw cycles, ALP activity was measured at 405 nm, using *p*-nitrophenyl phosphate as a substrate. The protein content was quantified by the Bradford assay (Bio-Rad, Hercules, CA, USA), and the ALP activity was expressed as units mg^{-1} protein.

Mineralization nodules in the extracellular matrix of MSCs were evaluated using ARS staining. After 21 days, cells were washed with PBS, fixed in 70% ethanol at −20°C for 1 h, then rinsed in distilled water and stained with ARS for 10 min. Finally, the stained cells were rinsed with distilled water and PBS. After removing the distilled water, the red dye was eluted in 10% cetylpyridinium chloride monohydrate for 30 min. The OD of the solution was measured at 540 nm (Choi et al 2005).

Oil red O staining for adipogenic differentiation cells

Adipogenic differentiation was assessed with oil red O staining of the cells cultured in adipogenic medium with or without metformin. After 21 days, the cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. The cells were then washed with 3% isopropanol, followed by staining with a newly filtered oil red O staining solution for 10 min. After washes with distilled water, the cells were destained in 100% isopropanol for 15 min. The OD of the destained solution was measured at 500 nm (Choi et al 2005).

Total RNA extraction and real-time RT-PCR

Total RNA was isolated from the cells cultured in osteoblastic and adipogenic medium with or without metformin for 21 days; cDNA was subsequently prepared from RNA using RevertAid First Strand cDNA Synthesis kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions.

Real-time RT-PCR using SYBR Green I (Invitrogen, Carlsbad, CA, USA) was performed to quantify the mRNA expression of osteoblastic marker genes in osteoblastic differentiation cells and adipocyte-specific genes in adipogenic differentiation cells, including Cbfa1/Runx2, COL-I, Lrp5, PPARγ, C/EBPα and aP2/ALBP. The primers are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous housekeeping gene. The PCR reaction system (30 μL) included 1 μL cDNA sample, 1μL of each primer (10 μM), 0.36 μL dNTP (25 mM), 1 μL 1 × SYBR Green (Invitrogen), and PCR-grade water. Amplification reactions were conducted under the following conditions: an initial step at 94°C for 2 min, then 45 cycles at 94°C for 20 s, 53°C for 30 s and 60°C for 40 s. Relative expression of the real time RT-PCR product was evaluated using the comparative Ct method. Expression levels for each target gene were normalized to GAPDH mRNA.

Table 1 Sequences of primers used in this study

Gene	Forward primer sequence	Reverse primer sequence	GeneBank accession	Size (bp)
Cbfa1	5′-GGACGAGGCAAGAGTTTCA-3′	5′-CTGTCTGTGCCTTCTTGGTT-3′	**XM346016**	123
Lrp5	5′-CCGCCAGCAGCAGATGAT-3′	5′-GGATGGGCTGAGAACTCCT-3′	**XM215187**	135
COL-I	5′-ATCAAGGTCTACTGCAACAT-3′	5′-CAGGATCGGAACCTTCGCTT-3′	**XM213440**	178
PPARγ	5′-GTCTCACAATGCCATCAGGTT-3′	5′-TTCAGCTGGTCGATATCACT-3′	**NM013124**	90
C/EBPα	5′-GAGACGCAGCAGAAGGTGTT-3′	5′-TCACGCGCAGTTGCCCAT-3′	**NM012524**	153
aP2	5′-GACAGGAAAGTGAAGAGCATC-3′	5′-ATGACACATTCCACCACCAG-3′	**NM053365**	128

Western blot analysis of Cbfa1 and PPARγ2

Western blot analysis was performed to assess protein expression of Cbfa1 (in osteoblast-differentiating cells) and PPARγ2 (in adipogenic differentiation cells) after 21 days. Proteins (50 μg per lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad). After washing and blocking, the membranes were incubated with primary antibody for 2 h and with a horseradish-peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz) for 1 h at 37°C. Western blot was performed using an enhanced chemiluminescence detection system (Western Lighting, Perkin-Elmer Life Sciences, Boston, MA, USA). The images of the blots captured on Kodak X-ray films (Kodak, Rochester, NY, USA) were quantified using densitometry software (Kodak 1D Image Analysis Software) after scanning of the films. β-actin was used as the internal control, and the results were expressed as the ratio of target protein to β-actin.

Statistical analysis

Results are presented as mean ± s.d. The Mann–Whitney U test was performed to compare the difference between the metformin group and control group using SPSS 12.0 (Chicago, IL, USA); $P < 0.05$ was considered statistically significant.

Results

Effect of metformin on osteoblast differentiation

To investigate the effect of metformin on osteoblastic differentiation of MSCs, the cells were cultured in osteoblastic medium with or without metformin treatment for 21 days. The MTT assay indicated a positive effect of metformin on the number of MSCs in the osteoblastic medium. During the 10 days of culture, the cell number was always higher in the metformin group than in the control group ($P < 0.05$; Figure 1). ALP activities reached significantly higher levels in the metformin-treated cells after 10 and 15 days in culture ($P < 0.05$; Figure 2). Mineralization nodules were observed after 21 days' culture. By contrast, MSCs with metformin treatment demonstrated more nodules in the culture and more calcium deposition in mineralized nodules, as examined by ARS staining. The ARS elusion solutions were read at 540 nm with an ELISA reader, and the cells with metformin treatment showed a higher OD than the cells without metformin ($P < 0.05$; Figure 3).

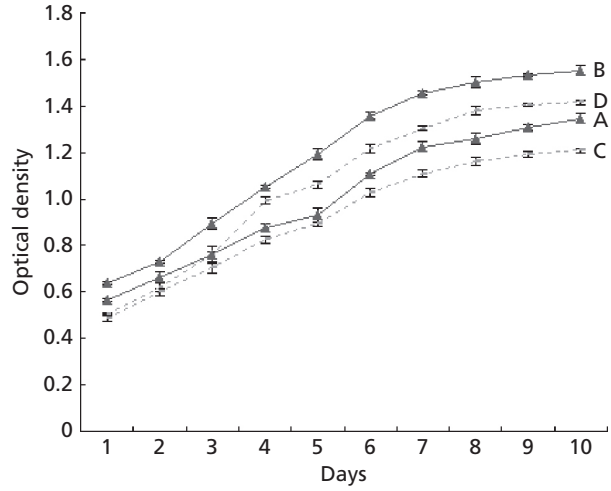

Figure 1 MTT assay of rat mesenchymal stem cell proliferation in osteoblastic medium without (A) or with (B) metformin, and in adipogenic medium without (C) or with (D) metformin.

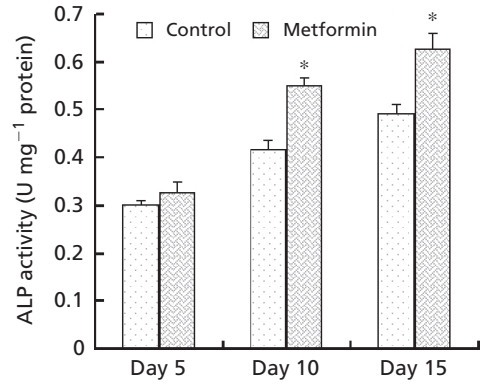

Figure 2 Alkaline phosphatase activity of rat mesenchymal stem cells cultured in osteoblastic medium with or without metformin. *$P < 0.05$ vs control group.

The effect of metformin on osteoblast differentiation was further examined by testing the mRNA levels of osteoblastic marker genes using real-time RT-PCR. The metformin-containing medium yielded higher levels of COL-I, Cbfa1/Runx2 and Lrp5 than the metformin-free medium ($P < 0.05$; Figure 4). In addition, Western blot analysis using anti-Cbfa1 antibody showed similar results to real-time RT-PCR, with a significant increase in Cbfa1 protein after metformin treatment (Figure 5).

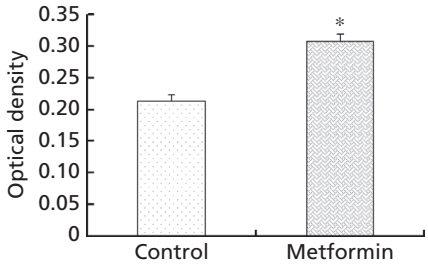

Figure 3 Effect of metformin on the optical density of the Alizarin red S destaining solution measured at 540 nm. *$P < 0.05$ vs control group.

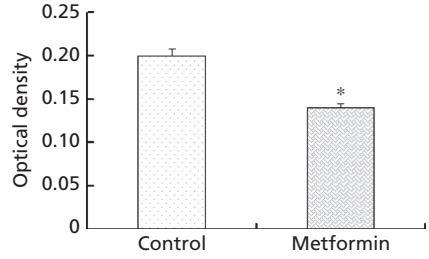

Figure 6 Effect of metformin on the optical density of the oil red O destaining solution measured at 500 nm. *$P < 0.05$ vs control group.

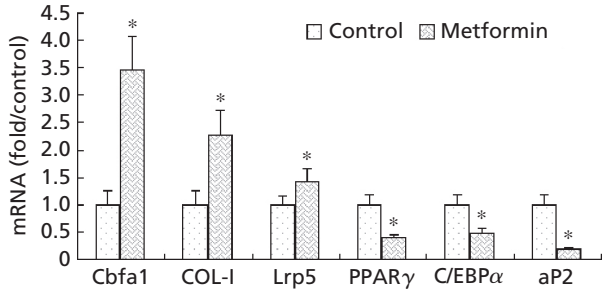

Figure 4 Real-time RT-PCR analysis of the mRNA expression of osteoblastic specific genes (Cbfa1, COL-I, Lrp5) in osteoblastic differentiation cells and adipose-specific genes (PPARγ, C/EBPα and aP2) in adipogenic differentiation cells after 21 days, with or without metformin treatment. *$P < 0.05$ vs control group.

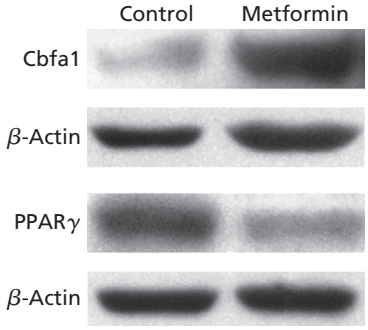

Figure 5 Western blot analysis for Cbfa1 expression (a marker of osteoblast differentiation) and PPARγ expression (a marker of adipogenic differentiation) after 21 days.

Effect of metformin on adipogenic differentiation

The effect of metformin on adipogenic differentiation was examined in the cells cultured in adipogenic medium with or without metformin for 21 days. The number of MSCs increased with time over 10 days' culture in both groups. There was no significant difference between the groups during the first 2 days but from the third day on, metformin induced more cells ($P < 0.05$; Figure 1). Quantification of adipogenic markers by real-time RT-PCR showed lower mRNA levels of PPARγ, C/EBPα and aP2 in the cells treated with metformin (Figure 4). The decrease of aP2 (late marker) was most remarkable; its mRNA was approximately one-fifth of that in cells grown in the absence of metformin. The inhibitory effects of metformin on adipocyte differentiation was further tested by Western blot analysis of PPARγ2 protein expression, the main transcription factor during the differentiation of MSCs into adipocytes. Metformin (100 μM) induced a significant decrease in PPARγ2 protein after 21 days' culture (Figure 5). Cytoplasmic lipid droplets of mature adipocytes were less extensive in cells cultured in metformin-containing medium, with a lower OD of the oil red O destaining solution compared with those cultured in metformin-free medium ($P < 0.05$; Figure 6).

Discussion

Previous investigations have described decreased bone formation and remodelling in diabetic patients (KraKauer et al 1995; Chau et al 2003; Carnevale et al 2004). This low bone mass in diabetic patients may be specifically caused by diabetes and anti-diabetic medications, or may just exist as a concurrent disease, unrelated to diabetes or anti-diabetic medications. In the next few years, the number of diabetic patients is expected to increase to approximately 200 million globally because of the rising incidence of obesity and an ageing population (Zhang et al 2008). Thus, it is necessary to investigate the effects of commonly used anti-diabetic drugs on bone metabolism.

Metformin is widely used as monotherapy or combination therapy for type 2 diabetes. This drug improves hyperglycaemia and insulin resistance and also reduce LDL cholesterol. Several studies have investigated the effects of metformin on cardiomyocytes (Kewalramani et al 2006), intestine (Wilcock & Bailey 1991) and adipose tissue (Rouru et al 1993), but little is known about its potential effect(s) on bone. In a case–control study, metformin was associated with decreased fractures in diabetic patients (Vestergaard et al 2005). The influence of metformin on osteogenic actions of osteoblasts in culture was further demonstrated in an in-vitro study (Cortizo et al 2006), which showed that treatment of two osteoblast-like cells (UMR106 and MC3T3E1) with metformin (25–500 μM) for 24 h led to a dose-dependent increase in cell proliferation. Metformin also promoted osteoblastic differentiation, increasing type-I collagen production in both lines and ALP activity in MC3T3E1 osteoblasts.

In our study, metformin enhanced osteoblast differentiation of rat MSCs, as demonstrated by the marked increase in extracellular matrix mineralization and increased expression of osteoblastic marker genes. Compared with MSCs grown in osteoblastic medium without metformin, 100 μM metformin in osteoblastic medium augmented ALP activity, increased mineralization nodule depositions, increased Cbfa1 expression at both mRNA and protein levels, and increased expressions of COL-I and Lrp5 mRNA.

Cbfa1/Runx2 has been identified as a key transcription factor associated with osteoblast differentiation. It regulates the expression of several genes responsible for the osteoblast phenotype, including ALP, osteocalcin and osteopontin (Ducy et al 1997). Cbfa1/Runx2-transduced bone marrow stromal cells could facilitate bone formation in-vitro and in-vivo (Zheng et al 2004). Some preclinical studies also found that Runx2$^{-/-}$ calvarial cells failed to differentiate into osteoblasts, even in the presence of bone morphogenetic protein BMP2. In this study, some well-established osteoblastic markers were assessed to explore the possible mechanisms underlying the action of metformin on MSCs. The results suggested that metformin could exert positive influence on osteoblast differentiation through increasing the expression of Cbfa1/Runx2. Nevertheless, the multiple signalling pathways converging on Cbfa1 were not evaluated directly. Cortizo et al (2000, 2006) proposed that such actions may be partly mediated by activation/redistribution of extracellular signal-regulated kinases ERK1/2 and induction of endothelial/inducible nitric oxide synthase. Further studies are necessary to investigate whether metformin treatment is capable of modifying bone growth factors secreted by MSCs or osteoblasts in culture, and how metformin exerts its osteoblastic influence through regulating special signal pathways, such as mitogen-activated protein kinase and protein kinase A pathways.

Age-related low bone mass is always accompanied by an increase in marrow adipose tissue. An animal study suggested that with aging there is an increase in adipocyte differentiation from MSCs (Kajkenova et al 1997). In fact, an increase in marrow adipocytes can be observed in all conditions that lead to bone loss, such as ovariectomy, immobilization or treatment with glucocorticoids (Nuttall & Gimble 2004). Bone metabolism is a dynamic balance between osteogenesis and adipogenesis, depending on the differentiation directions of bone marrow MSCs. Thus, our study also focused on the effect of metformin on the differentiation of rat MSCs into adipogenic lineages, in order to illuminate the effect of this drug on bone metabolism. Two main transcription factors, PPARγ and C/EBPα, have been considered as the key elements for the differentiation of MSCs into adipocytes. The expression of one of them is required for adipogenic differentiation to proceed (Duque et al 2004; Backesjo et al 2006). A recent report by Akune et al (2004) has shown that PPARγ deficiency promotes osteoblastogenesis. The author observed that embryonic stem cells from PPARγ-deficient mice spontaneously underwent osteogenesis but failed to undergo adipogenesis. Moreover, in-vitro studies have shown that bone marrow stromal cells isolated from PPAR$\gamma^{-/+}$ mice exhibited a two-fold reduction in adipogenesis and a three-fold increase in osteogenesis. PPARγ can convert stromal cells from a plastic osteoblastic phenotype that reversibly expresses adipocyte characteristics to terminally differentiated adipocyte (Lecka-Czernik et al 1999, 2002). These results suggest that PPARγ may play a hierarchically dominant role in determining the fate of mesenchymal progenitors (Nuttall & Gimble 2004) due to its ability to inhibit the expression of other lineage-specific transcription factors.

In order to address this issue in particular, we assessed the expression of PPARγ to explore the mechanism of action of metformin on the adipogenic differentiation of MSCs in culture. Similar investigations have been conducted on thiazolidinediones, which are used as insulin-sensitizing drugs to treat diabetic patients with insulin resistance. As synthetic ligands of PPARγ, thiazolidinediones directly bind and activate PPARγ (Lehmann et al 1995), stimulating adipocyte differentiation and blocking osteoblast differentiation (Lecka-Czernik et al 1999, 2002). A possible risk for development of low bone mass could be envisaged on long-term treatment with thiazolidinediones (Backesjo et al 2006). In contrast to the action of the thiazolidinediones, our present study demonstrated that metformin inhibited the process of adipogenic differentiation of MSCs in culture, as evidenced by a decrease in expression of adipocyte-specific genes. To our knowledge, it is the first time that the effect of metformin on adipogenic differentiation of MSCs has been studied. In addition to the enhanced expression of Cbfa1, metformin markedly reduced the expression of PPARγ. Thus, in contrast to thiazolidinediones, metformin may be beneficial for bone metabolism.

Conclusions

The results have demonstrated for the first time that metformin can inhibit adipocyte differentiation and promote osteoblast differentiation of rat MSCs. The decreased adipogenic differentiation may be mediated through inhibition of PPARγ, and the increased osteoblast differentiation could be caused by inhibition of PPARγ, either alone or in conjunction with other mechanisms that regulate osteoblastic differentiation. The potential benefits of metformin on bone metabolism require further research in terms of effects on bone mineral density and subsequent fracture risk in patients with type 2 diabetes.

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