Original Article

Bezafibrate enhances proliferation and differentiation of osteoblastic MC3T3-E1 cells via AMPK and eNOS activation

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Aim: To investigate the effects of bezafibrate on the proliferation and differentiation of osteoblastic MC3T3-E1 cells, and to determine the signaling pathway underlying the effects.

Methods: MC3T3-E1 cells, a mouse osteoblastic cell line, were used. Cell viability and proliferation were examined using MTT assay and colorimetric BrdU incorporation assay, respectively. NO production was evaluated using the Griess reagent. The mRNA expression of ALP, collagen I, osteocalcin, BMP-2, and Runx-2 was measured using real-time PCR. Western blot analysis was used to detect the expression of AMPK and eNOS proteins.

Results: Bezafibrate increased the viability and proliferation of MC3T3-E1 cells in a dose- and time-dependent manner. Bezafibrate (100 $\mu\text{mol/L}$) significantly enhanced osteoblastic mineralization and expression of the differentiation markers ALP, collagen I and osteocalcin. Bezafibrate (100 $\mu\text{mol/L}$) increased phosphorylation of AMPK and eNOS, which led to an increase of NO production by 4.08-fold, and upregulating BMP-2 and Runx-2 mRNA expression. These effects could be blocked by AMPK inhibitor compound C (5 $\mu\text{mol/L}$), or the PPAR β inhibitor GSK0660 (0.5 $\mu\text{mol/L}$), but not by the PPAR α inhibitor MK886 (10 $\mu\text{mol/L}$). Furthermore, GSK0660, compound C, or N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME, 1 mmol/L) could reverse the stimulatory effects of bezafibrate (100 $\mu\text{mol/L}$) on osteoblast proliferation and differentiation, whereas MK886 only inhibited bezafibrate-induced osteoblast proliferation.

Conclusion: Bezafibrate stimulates proliferation and differentiation of MC3T3-E1 cells, mainly via a PPAR β -dependent mechanism. The drug might be beneficial for osteoporosis by promoting bone formation.

Keywords: bezafibrate; osteoblast; MC3T3-E1 cell; AMPK; eNOS; PPAR α ; PPAR β

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Introduction

Osteoporosis is a major public health problem, characterized by low bone mass and micro-architectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture due to imbalance between bone resorption by osteoclasts and bone formation by osteoblasts. Bone formation is likely to be affected by reductions of osteoblast proliferation, differentiation and lifespan^[1]. Hypercholesterolemia was reported to be associated with lower bone mineral density (BMD)^[2, 3]. An animal model study has also demonstrated some detrimental effects of dyslipidemia on bone metabolism^[4]. Fibrate use, including bezafibrate, was associated with a reduced odds of vertebral fractures in men

and women^[5]. Bezafibrate was shown to increase bone mass in intact male rats principally through increasing periosteal bone formation^[6]. A growing body of evidence suggests that bezafibrate plays an important role in bone metabolism. However, it is not clear whether these changes result from the direct effects of bezafibrate on bone or from changes in lipid levels.

Bezafibrate, a dual ligand for peroxisome proliferator-activated receptors α (PPAR α) and PPAR β ^[7], is a lipid-lowering drug widely used to treat hypertriglyceridemia. In addition, fibrates, including bezafibrate, were reported in both clinical and basic research to reduce the progression of atherosclerotic lesions^[8] and improve endothelial function^[9]. Fenofibrate, a PPAR α agonist, stimulates eNOS phosphorylation and nitric oxide (NO) production through AMP-activated protein kinase (AMPK) activation^[10, 11]. GW501516, a PPAR β agonist, also activates AMPK and stimulates glucose uptake in skeletal muscle cells^[12].

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AMPK is a heterotrimeric enzyme complex consisting of one catalytic α subunit, two regulatory β subunits and a γ subunit. AMPK is recognized as a regulator of energy homeostasis and is known to be expressed ubiquitously, including in bone. Pharmacological AMPK activators, 5-aminoimidazole-4-carboxamide- β -D-ribose nucleoside (AICAR) or metformin, could promote the differentiation and mineralization of osteoblastic MC3T3-E1 cells^[13, 14]. It has recently been shown that AMPK activity regulates bone formation *in vitro* and the maintenance of bone mass *in vivo*^[15]. These previous findings together suggested that AMPK could affect bone metabolism. AMPK has been shown to increase eNOS activity and contribute to NO production in endothelial cells^[16]. NO is also a signaling molecule constitutively produced in bone cells. The source of NO production in bone cells is largely due to eNOS, which was constitutively expressed in bone. The eNOS isoform seems to play a key role in regulating osteoblast activity and bone formation since eNOS knockout mice have osteoporosis due to defective bone formation^[17, 18].

Because these data have been obtained from non-osteoblastic cells, it is still unclear whether bezafibrate could activate AMPK and eNOS in osteoblasts. A model commonly used to study osteogenic development is the MC3T3-E1 osteoblast-like cell line because in culture, these cells are characterized by distinct proliferative and differentiated stages, thereby reproducing a temporal program consistent with osteoblast differentiation as it occurs during *in vivo* bone formation^[19].

Thus, the objective of the present study was to investigate whether bezafibrate promotes the proliferation and differentiation of osteoblastic MC3T3-E1 cells. In addition, we also investigated possible mechanisms of action of bezafibrate, such as the AMPK-eNOS signaling pathway, and the effect of PPAR α or PPAR β .

Materials and methods

Reagents

Chemicals including bezafibrate, GSK0660, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), glycerol 2-phosphate disodium salt hydrate (β -GP), ascorbic acid, *p*-nitrophenol (*p*-NP), and *p*-nitrophenylphosphate (*p*-NPP) were purchased from Sigma (St Louis, MO, USA). Compound C and MK886 were purchased from Calbiochem (Darmstadt, Germany). Fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, and α -minimal essential medium (α -MEM) were obtained from Gibco-BRL (Grand island, NY, USA). For Western blot analysis, total and phospho (p)-AMPK α (Thr-172), total and p-eNOS (Ser-1177), and total and p-acetyl-CoA carboxylase (ACC) (Ser-79) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were of the highest grade available commercially.

Cell culture

MC3T3-E1 cells, a mouse osteoblastic cell line, were obtained from American Type Culture Collection. MC3T3-E1 cells were cultured in α -MEM supplemented with 10% FBS and 1%

penicillin-streptomycin in 5% CO₂ at 37 °C. The medium was changed three times a week. When cells reached 80% confluence two or three days after plating, they were subsequently cultured for 7–28 d in differentiation medium consisting of 90% α -MEM, 10% FBS, 50 mg/L ascorbic acid, and 10 mmol/L β -GP. Cultured cells were analyzed on d 0, 7, 14, 21, 28 after reaching 80% confluence.

Measurement of cell viability and proliferation

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) dye. MC3T3-E1 cells were incubated with bezafibrate (1–1000 μ mol/L) for 24, 48, or 72 h, and were pretreated with the AMPK inhibitor compound C (5 μ mol/L), PPAR β inhibitor GSK0660 (0.5 μ mol/L), PPAR α inhibitor MK886 (10 μ mol/L), or NOS inhibitor L-NAME (1000 μ mol/L) followed by bezafibrate (100 μ mol/L) incubation for 48 h. After the incubations, 10 μ L of MTT (Sigma-Aldrich) was added to each well of a 96-well microplate, and the microplates were placed in an incubator at 37 °C for 4 h. One hundred fifty microliters of dimethyl sulfoxide (DMSO) was added to all wells and mixed thoroughly to lyse the cells and dissolve the dark blue crystals. After 10 min, the absorbance was measured at 570 nm using a microplate reader (Bio-Rad iMARK, Japan).

Cell proliferation was determined by the colorimetric bromodeoxyuridine (BrdU) incorporation assay (Millipore, USA). A quantity of 3×10^3 cells were seeded in triplicate into a 96 well plate. BrdU solution (20 μ L) was added to the cells and incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO₂ in air. After removing the culture medium, the cells were fixed, and the DNA was denatured in one step by using the Fixing solution. Incorporated BrdU was detected by an anti-BrdU monoclonal antibody. The immune complex was detected by a subsequent TMB Peroxidase Substrate reaction and the absorbance was measured at 450/540 nm using a microplate reader.

RNA isolation and real-time PCR

To investigate the expression of alkaline phosphatase (ALP), collagen I (Col-I), osteocalcin, bone morphogenetic protein-2 (BMP-2), and runt related transcription factor 2 (Runx-2) mRNA in MC3T3-E1 cells incubated with bezafibrate (100 μ mol/L) for 0, 7, 14, 21, or 28 d in differentiation medium, SYBR green chemistry was used to perform quantitative determinations of the mRNAs. Total RNA was extracted from cultured MC3T3-E1 cells using the TRIzol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's recommended protocol. The concentration and purity of total RNA were calculated with the absorbance at 260 and 280 nm. Total RNA (1 μ g) was employed for the synthesis of first strand cDNA (cDNA synthesis kit; Toyobo, Japan). PCR primers (Table 1) were designed using the Primer 5.0 software. Real-time PCR was performed using 1 μ L of cDNA in a 25 μ L reaction volume with the ABI PRISM 7500 (Applied Biosystems, USA). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the SYBR

Table 1. Primers used for real-time quantitative PCR.

Name	Primer sequence	Size (bp)	Accession No
β-actin	5'-TCTTGGGTATGGAATCCTGTG-3' 5'-AGGTCTTTACGGATGTCACG-3'	82	NM_007393.2
Osteocalcin	5'-TGCTTGTGACGAGCTATCAG-3' 5'-GAGGACAGGGAGGATCAAGT-3'	149	NM_001032298.2
BMP-2	5'-GGTCACAGATAAGGCCATTGC-3' 5'-GCTTCCGCTGTTGTGTTTG-3'	102	NM_007553.2
Runx-2	5'-AAGTGGGTGCAAACTTCTT-3' 5'-TCTCGGTGGCTGGTAGTGA-3'	90	NM_009820.2
ALP	5'-AACCCAGACACAAGCATTCC-3' 5'-GAGAGCGAAGGGTCAGTCAG-3'	151	NM_007431.1
Col-I	5'-AGAGCATGACCGATGGATTG-3' 5'-CCTTCTTGAGGTTGCCAGTC-3'	177	NM_007742.3

PCR kit (Toyobo, Japan) to allow for quantitative detection of the PCR product. The PCR reactions were carried out under the following conditions: 95 °C for 60 s, 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s. All reactions were run in triplicate and analyzed by the $2^{-\Delta\Delta CT}$ method^[20]. β-Actin was used as the internal control gene.

Assay of mineralization

To examine the mineralization of the MC3T3-E1 cells, the cells were seeded at 5×10^4 per well in 24-well plates with differentiation medium containing vehicle (DMSO) or bezafibrate (100 μmol/L). After 21 d, the formation of mineralizing plaques was visualized by Alizarin red staining. Cells were washed with PBS 3 times and fixed in 10% formaldehyde for 1 h at 4 °C. After 3 washes with distilled water, the cells were stained in 1% Alizarin red solution (pH 4.3) for 30 min. The remaining dye was washed out by several washes with distilled water. To quantify matrix mineralization, Alizarin red stained cultures were incubated in 100 mmol/L cetylpyridinium chloride for 1 h to solubilize and release calcium-bound Alizarin red into solution. The absorbance of the released Alizarin red was measured at 550 nm.

Assay of alkaline phosphatase activity

The MC3T3-E1 cells were pretreated with compound C (5 μmol/L), GSK0660 (0.5 μmol/L), MK886 (10 μmol/L), or L-NAME (1000 μmol/L) for 1 h, followed by incubation with bezafibrate (100 μmol/L) for 7 d in differentiation medium. ALP enzyme activity was determined by measuring the hydrolysis of p-NPP according to the manufacturer's instructions (Sigma, St Louis, MO, USA). In brief, cells in 24-well plates were rinsed three times with PBS and then sonicated in 0.1% Triton X-100. The protein assays were performed with the bicinchoninic acid (BCA) protein assay reagent. Cell lysates containing 2 μg of total protein were added to 200 μL of the p-NPP solution. The plate was incubated in the dark for approximately 30 min at room temperature. The reaction was stopped by 3 mol/L NaOH, and the absorbance was measured

at 405 nm using the Bio-Rad microplate reader. A standard curve was prepared with p-NP.

Western blot analyses

For Western blotting, the MC3T3-E1 cells were plated in 6 cm dishes. Cells were treated with bezafibrate (100 μmol/L) for 0, 15, 30, 60, 120, or 240 min and were pretreated with compound C (5 μmol/L), GSK0660 (0.5 μmol/L), or MK886 (10 μmol/L), followed by incubation with bezafibrate (100 μmol/L) for 120 min. MC3T3-E1 cells were washed twice with ice-cold PBS and then resuspended in lysis buffer (RIPA, Beyone, China) containing 1% NP-40, 0.1% SDS, 5 mmol/L EDTA, 0.5% sodium deoxycholate, 1 mmol/L sodium orthovanadate, and 1 mmol/L phenylmethylsulfonyl fluoride. The cell lysates were then sonicated for 30 s. For each sample, 30 μg of protein, assessed by a BCA protein assay (Bio-Rad, Mississauga, Ontario, Canada), was run on a 7% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). After being blocked with 5% non-fat milk/TBST, the membrane was incubated overnight at 4 °C with p-AMPKα, total-AMPKα, p-ACC, total-ACC, p-Akt, total-Akt, p-eNOS, and total-eNOS antibodies at a 1:1000 dilution. Specific antibody binding was detected by a 1:2000 dilution of corresponding horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 1 h at room temperature. The blots were then washed, and the signal was visualized by an HRP chemiluminescent substrate reagent kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. After stripping of p-AMPKα, p-eNOS or p-ACC, total-AMPKα, total-eNOS or total-ACC, immunoreactivity was determined in the same membrane. The band intensity was quantified by densitometric analysis using IMAGE J software (National Institutes of Health, USA).

Assessment of NO production

Cells were grown in 24-well plates to measure NO production, which was assessed by the measurement of the stable end-product of NO oxidation, nitrite. MC3T3-E1 cells were incubated with bezafibrate (100 μmol/L) for 0, 30, 60, 120, or 240 min and were pretreated with compound C (5 μmol/L) followed by bezafibrate for 240 min. Nitrite was measured using a total nitric oxide assay kit (Beyotime, Haimen, China). Briefly, a standard curve was prepared with standard nitrite solutions in α-MEM medium, covering a concentration range of 2–50 μmol/L. The standard solutions or cell supernatants were reacted with nitrate reductase for 40 min in a 96-well plate, and Griess reagent I and Griess reagent II were then added. After a 10 min incubation at room temperature, the absorbance was measured at 540 nm, and nitrite concentrations were determined from the standard curve made using a solution of KNO₂ dissolved in medium.

Statistical analysis

Data were expressed as mean ± SEM and analyzed by SPSS 16.0 software. Statistical evaluations for the differences between groups were performed by two-tailed independent Student's

t-tests or a one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD). $P < 0.05$ was considered statistically significant.

Results

Effect of bezafibrate on the viability and proliferation of MC3T3-E1 cells

To investigate the effect of bezafibrate on osteoblastic MC3T3-E1 cell growth, cell viability was assessed using the MTT assay and cell proliferation was assessed using the BrdU assay after treating cells with bezafibrate (1–1000 $\mu\text{mol/L}$) for 24–72 h. We found that treating the cells with bezafibrate led to a dose- and time-dependent increase in cell viability (Figure 1A) and cell proliferation (Figure 1B). The maximal effect on cell viability was reached at the concentrations of 500 (134% of basal) and 100 (159% of basal) $\mu\text{mol/L}$ at 24 and 72 h, respectively. The maximal effect on cell proliferation was reached at a concentration of 100 $\mu\text{mol/L}$ at 48 (121% of basal) and 72 (132% of basal) h.

Effect of bezafibrate on the differentiation and mineralization of MC3T3-E1 cells

To investigate the effect of bezafibrate on osteoblast differentiation, the mRNA expression levels of ALP, Col-I and osteocalcin on d 0, 7, 14, 21, and 28 were evaluated by real-time PCR (Figure 2A, 2B, and 2C). Bezafibrate (100 $\mu\text{mol/L}$) increased the mRNA expression levels of ALP on d 7 and 14 by 1.89- and 1.46-fold, respectively ($P < 0.05$). Bezafibrate also increased the mRNA expression levels of Col-I on d 7, 14, and 21 by 1.88-, 1.40-, and 1.5-fold, respectively ($P < 0.05$), as well as the mRNA expression levels of osteocalcin on d 14 and 21 by 2.04- and 1.65-fold, respectively ($P < 0.05$).

In contrast, after 21 d of exposure to the osteogenic induction medium, bezafibrate (100 $\mu\text{mol/L}$) showed marked and diffuse mineralization nodules (Figure 2D). A 1.25-fold

Figure 1. Effects of bezafibrate on viability and proliferation differentiation of MC3T3-E1 cells. MC3T3-E1 cells were treated with bezafibrate (1–1000 $\mu\text{mol/L}$) at 24, 48, or 72 h. (A) Cell viability was determined using the MTT assays. Results represent as the percentage of cell viability with the control ($n=4$). (B) Cell proliferation was determined using the colorimetric BrdU incorporation assays. Results represent as arbitrary units by optical absorption ($n=3$). $^aP < 0.05$, $^bP < 0.01$ compared to the control group

increase in the extent of mineralization was also observed by spectrophotometric readings (Figure 2E).

Figure 2. Effects of bezafibrate on differentiation and mineralization of MC3T3-E1 cells. (A, B, C) Cells were treated with vehicle (con) or bezafibrate (beza) (100 $\mu\text{mol/L}$) for the time periods indicated after the cells reached 80% confluence. Total RNA was collected on d 0, 7, 14, 21, and 28. ALP, Col-1, and osteocalcin (OCN) mRNA expression were evaluated by real-time PCR. Results were expressed as fold increase over the control values. (D) Photomicrograph of mineralized nodules stained by Alizarin red staining at the differentiation d 21 (Original magnification: $\times 100$). (E) Quantification of Alizarin red staining with cetylpyridinium chloride. The absorbance of the released Alizarin red was measured at 550 nm. $^bP < 0.05$ compared to the control group ($n=3$).

Effect of bezafibrate on eNOS phosphorylation and NO production in MC3T3-E1 cells

To investigate whether bezafibrate can induce eNOS phosphorylation and NO production in osteoblastic MC3T3-E1 cells, bezafibrate was added after the cells reached 80% confluence with different time points and dosages. Western blot analysis was performed to determine the expression of p-eNOS and eNOS. Treatment of MC3T3-E1 cells with bezafibrate resulted in a time- and dose-dependent activation of eNOS phosphorylation, reaching approximately 1.58-fold that of the control at 100 $\mu\text{mol/L}$ (Figure 3A) and 2.05-fold that of the baseline level at 60 min (Figure 3B). The total amount of eNOS was unchanged throughout this experiment. Furthermore, bezafibrate-induced NO production increased markedly in osteoblasts in a time-dependent manner (Figure 3C).

Effects of bezafibrate on the AMPK and Akt signaling pathway in MC3T3-E1 cells

To investigate whether bezafibrate can activate phosphorylation of AMPK and Akt, the upstream kinases for eNOS, bezafibrate (100 $\mu\text{mol/L}$), was added to osteoblastic MC3T3-E1 cells at various time points (0, 15, 30, 60, 120, and 240 min). Western blot analysis revealed that the phosphorylation levels of AMPK and ACC, which is a well-established downstream target of AMPK^[21], rose gradually in a time-dependent manner. The maximum effect of bezafibrate on AMPK and ACC appeared after a 2-h incubation, which was 1.34- and 1.75-fold, respectively, that of the baseline level (Figure 4A and 4B), whereas the phosphorylation level of Akt remained unchanged throughout the time course (Figure 4A and 4C). These results indicate that treatment with bezafibrate can activate the AMPK signaling pathway.

To further address whether the AMPK signaling pathway is involved in bezafibrate-induced upregulation of eNOS phosphorylation and NO production, we pretreated MC3T3-E1 cells with the AMPK inhibitor compound C (5 $\mu\text{mol/L}$). Compared to the control group, bezafibrate (100 $\mu\text{mol/L}$) enhanced the phosphorylation level of eNOS by 1.46-fold, which was suppressed by 37.68% when pretreated with compound C, compared to the group treated with bezafibrate alone ($P < 0.01$, Figure 5A). Compared to the control group, bezafibrate (100 $\mu\text{mol/L}$) enhanced NO production by 4.08-fold, which was suppressed by 60.8% when pretreated with compound C, compared to the group treated with bezafibrate alone ($P < 0.01$, Figure 5B). These results indicate that AMPK activation is essential for bezafibrate-induced upregulation of eNOS phosphorylation and NO production.

Effects of bezafibrate on PPAR α and PPAR β signal transduction pathways

To investigate whether PPAR α and PPAR β signaling pathways are involved in bezafibrate-induced upregulation of AMPK and eNOS phosphorylation, the MC3T3-E1 cells were pretreated with the PPAR α inhibitor MK886 (10 $\mu\text{mol/L}$) and PPAR β inhibitor GSK0660 (0.5 $\mu\text{mol/L}$), and the level of AMPK and eNOS phosphorylation was then evaluated

Figure 3. Effect of bezafibrate on eNOS phosphorylation levels and NO production in MC3T3-E1 cells. (A) MC3T3-E1 cells were treated with indicated concentration of bezafibrate for 2 h and immunoblotted with anti-phosphorylated eNOS (Ser-1177) and anti-eNOS antibodies. (B) MC3T3-E1 cells were treated with bezafibrate (100 $\mu\text{mol/L}$) for indicated duration and immunoblotted with anti-phosphorylated eNOS (Ser-1177) and anti-eNOS antibodies. Bar graph showing semi-quantitative results by ratio of phosphorylated eNOS to total eNOS scanning density values and were normalized by setting the control as 1. (C) MC3T3-E1 cells were treated with bezafibrate (100 $\mu\text{mol/L}$) for indicated duration and NO production was evaluated using the Griess reagent. ^c $P < 0.01$ compared to the control group ($n = 3$).

by Western blotting (Figure 6). Pretreatment with GSK0660 reduced the levels of AMPK and eNOS phosphorylation by 34.86% ($P < 0.05$) and 48.18% ($P < 0.01$) respectively, compared to the group treated with bezafibrate alone. Pretreatment with MK886 slightly reduced the level of AMPK and eNOS phos-

Figure 4. Effects of bezafibrate on AMPK, ACC, and Akt phosphorylation levels in MC3T3-E1 cells. (A) MC3T3-E1 cells were treated with bezafibrate (100 $\mu\text{mol/L}$) for indicated duration and immunoblotted with anti-phosphorylated AMPK, ACC, or Akt antibodies. (B and C) Bar graph showing semi-quantitative results by ratio of phosphorylated antibody to total antibody scanning density values and were normalized by setting the control group as 1. ^b $P<0.05$, ^c $P<0.01$ compared to the control group ($n=3$).

phorylation by 12.58% ($P>0.05$) and 15.25% ($P>0.05$) respectively. These results indicate that bezafibrate-induced up-regulation of AMPK and eNOS phosphorylation is partially dependent on PPAR β .

Effects of bezafibrate on BMP-2 and Runx-2 expression in MC3T3-E1 cells

Next, we investigated the effect of bezafibrate on BMP-2 and Runx-2 mRNA expression by real-time PCR. After addition of bezafibrate, BMP-2 mRNA expression was increased to 1.80- and 1.74-fold ($P<0.05$) of the control group by bezafibrate at d 7 and 14, respectively (Figure 7A), whereas Runx-2 mRNA expression was increased to 1.51-, 1.55-, and 1.64-fold ($P<0.05$)

Figure 5. Effects of AMPK inhibitor compound C on bezafibrate-induced upregulation of eNOS phosphorylation and NO production. (A) MC3T3-E1 cells were pretreated with 5 $\mu\text{mol/L}$ compound C (CC) 1 h, followed by incubation with or without 100 $\mu\text{mol/L}$ bezafibrate for 2 h, and then immunoblotted with anti-phosphorylated eNOS antibody. Data were normalized by setting the untreated control group as 1. (B) MC3T3-E1 cells were treated with or without 100 $\mu\text{mol/L}$ bezafibrate in the presence or absence of 5 $\mu\text{mol/L}$ compound C for 4 h and NO production was evaluated using the Griess reagent. ^c $P<0.01$ compared to the control group; ^f $P<0.01$ compared to the group treated with bezafibrate alone ($n=3$).

at d 7, 14, and 21, respectively (Figure 7B). Compared to the group treated with bezafibrate alone, pretreatment with the PPAR β inhibitor GSK0660 reduced BMP-2 and Runx-2 mRNA expression by 40.76% ($P<0.01$) and 35.22% ($P<0.01$), respectively. Pretreatment with the AMPK inhibitor compound C reduced BMP-2 and Runx-2 mRNA expression by 37.82% ($P<0.01$) and 40.15% ($P<0.01$), respectively (Figure 7C and 7D). However, pretreatment with the PPAR α inhibitor MK886 only slightly reduced BMP-2 and Runx-2 mRNA expression by 6.40% ($P>0.05$) and 8.85% ($P>0.05$), respectively (Figure 7C and 7D). These results indicate that the expression of BMP-2 and Runx-2 is affected largely by PPAR β and AMPK activity.

Effects of blocking different signaling molecules on bezafibrate-induced MC3T3-E1 cell proliferation and differentiation

To further investigate whether the PPAR α , PPAR β , AMPK, or eNOS signaling pathways participate in the bezafibrate-induced enhancement of osteoblast proliferation and differentiation, the MC3T3-E1 cells were pretreated with the PPAR α inhibitor MK886, PPAR β inhibitor GSK0660, AMPK inhibitor

Figure 6. Effects of bezafibrate on PPAR α and PPAR β signal transduction pathways. MC3T3-E1 cells were pretreated with 10 μ mol/L MK886 (MK) or 0.5 μ mol/L GSK0660 (GSK) 1 h, followed by incubation with or without 100 μ mol/L bezafibrate (beza) for 2 h, and then immunoblotted with anti-phosphorylated AMPK or eNOS antibody. Bar graph showing semi-quantitative results by ratio of phosphorylated antibody to total antibody scanning density values and were normalized by setting the control group as 1. ^c $P < 0.01$ compared to the control group; ^e $P < 0.05$, ^f $P < 0.01$ compared to the group treated with bezafibrate alone ($n = 3$).

Compound C, or NOS inhibitor *L*-NAME. Pretreatment with compound C, MK886, GSK0660, or *L*-NAME inhibited bezafibrate-induced cell viability by 28.3%, 43.8%, 40.5%, or 41.8% (Figure 8A), respectively, and cell proliferation by 15.5%,

38.2%, 28.1%, or 36.4% (Figure 8B), respectively ($P < 0.01$), compared to the group treated with bezafibrate alone for 48 h. ALP activity and ALP mRNA expression in response to MK886, compound C, GSK0660, or *L*-NAME were also examined (Figure 8C and 8D). Pretreatment with compound C (2 and 5 μ mol/L), *L*-NAME (100 and 1000 μ mol/L), or GSK0660 (0.1 and 0.5 μ mol/L) reversed the bezafibrate-induced enhancement of ALP activity on d 7 ($P < 0.05$), whereas MK886 (5 or 10 μ mol/L) slightly reversed it ($P > 0.05$). In addition, pretreatment with compound C (5 μ mol/L), GSK0660 (0.5 μ mol/L), or *L*-NAME (1000 μ mol/L) partly reversed the bezafibrate-induced enhancement of ALP mRNA expression on d 7 ($P < 0.05$), whereas MK886 (10 μ mol/L) had no significant effect ($P > 0.05$).

Discussion

Results from this study suggest that bezafibrate can induce the proliferation and differentiation of osteoblastic MC3T3-E1 cells and augment the expression of BMP-2 and Runx-2 in the cells. Moreover, bezafibrate could enhance NO production as well as the phosphorylation of AMPK and eNOS, which can be reversed by the PPAR β inhibitor GSK0660, but not the PPAR α inhibitor MK886. Thus, bezafibrate seems to promote the proliferation and differentiation of MC3T3-E1 cells partly through the PPAR β -AMPK-eNOS signaling pathway.

Fibrates, including fenofibrate and bezafibrate, are a group of drugs widely used for the treatment of hypertriglyceridemia and hypercholesterolemia. Recently published data have shown that bezafibrate was able to increase the number of osteoblastic colonies formed from rat bone marrow stromal cells (BMSCs)^[6]. It has been reported that the inhibition of NO synthesis by NMMA markedly decreases the proliferation of the osteoblast-like cell line MG63^[22] and primary fetal calvarial osteoblasts^[23]. Furthermore, the addition of NO through donor compounds stimulates osteoblastic MC3T3-E1 cell proliferation^[24]. Our results are consistent with their reports and

Figure 7. Effects of bezafibrate on BMP-2 and Runx-2 mRNA expression in MC3T3-E1 cells. (A and B) MC3T3-E1 cells were treated with vehicle (con) or bezafibrate (beza) (100 μ mol/L) for indicated time period. Total RNA was collected on d 0, 7, 14, 21, and 28. BMP-2 and Runx-2 mRNA expression was evaluated by real-time PCR. (C and D) MC3T3-E1 cells were pretreated with 10 μ mol/L MK886 (MK), 0.5 μ mol/L GSK0660 (GSK), or 5 μ mol/L compound C (CC) 1 h, followed by incubation with or without 100 μ mol/L bezafibrate (beza) for 7 d, and were then collected to determine the mRNA levels of BMP-2 and Runx-2. Results were expressed as fold increase over the control group values. ^b $P < 0.05$, ^c $P < 0.01$ compared to the control group; ^f $P < 0.01$ compared to the group treated with bezafibrate alone ($n = 3$).

Figure 8. Effects of inhibitors of different signaling molecules on bezafibrate-induced proliferation and differentiation in MC3T3-E1 cells. (A and B) MC3T3-E1 cells were pretreated with 10 μmol/L MK886 (MK), 0.5 μmol/L GSK0660 (GSK), 5 μmol/L compound C (CC) or 1000 μmol/L L-NAME for 1 h, followed by treatment with or without 100 μmol/L bezafibrate (beza) for 48 h. Cell viability was determined using the MTT assays. Results represent as the percentage of cell viability compared with the untreated control group ($n=4$). Cell proliferation was determined using the colorimetric BrdU incorporation assays. Results represent as arbitrary units by optical absorption ($n=3$). (C and D) MC3T3-E1 cells were pretreated with MK, GSK, or CC with 1 h, followed by treated with 100 μmol/L bezafibrate for 7 d. ALP activity of MC3T3-E1 cells were measured using the p-NPP assay. ALP mRNA expression was evaluated by real-time PCR. Results were expressed as fold increase over the control group values ($n=3$). ^c $P<0.01$ compared to the control group; ^e $P<0.05$, ^f $P<0.01$ compared to the group treated with bezafibrate alone.

indicate that bezafibrate significantly stimulated osteoblastic MC3T3-E1 cell proliferation. Both PPAR α and PPAR β could be involved in bezafibrate-induced osteoblastic proliferation, and this action could be associated with eNOS activity and NO production. However, the mechanisms by which PPAR α may be involved in cell proliferation need to be investigated in future experiments.

In this study, we demonstrated that bezafibrate can promote mineralization and the expression of osteoblastic differentiation markers in MC3T3-E1 cells, including ALP, Col-I and osteocalcin, which was consistent with previous finding that fenofibrate stimulated osteoblast differentiation^[25]. These results further confirmed the role of fibrates in stimulating bone formation. It has been demonstrated that both PPAR α and PPAR β can be activated by bezafibrate^[26]. In our study, we found that the PPAR β inhibitor GSK0660, not the PPAR α inhibitor MK886, could partly but significantly reverse the bezafibrate-induced osteoblast differentiation. Thus, our study suggests that the effects of bezafibrate are in part dependent on PPAR β and are not dependent on PPAR α , in MC3T3-E1 cells.

We next examined the mechanisms involved in bezafibrate-induced osteoblast proliferation and differentiation. Previous studies have shown that bezafibrate can enhance phosphorylation of eNOS (Ser1177), which increases eNOS activity and then contributes to increased NO production in endothelial cells^[27]. NO has pleiotropic effects on bone cells *in vitro*. In osteoblast and osteoblast-like cells, NO promotes differentiation^[28] and increases proliferation^[29]. Long-term therapy with NO will not only increase BMD but may also decrease fracture rates^[17]. Estrogen is known to enhance proliferation and differentiation of osteoblastic cells by stimulating eNOS

activity^[17]. These findings indicate that eNOS and NO play an important role in osteoblast differentiation and function. In the present study, bezafibrate was found to significantly enhance eNOS phosphorylation and NO production in osteoblastic MC3T3-E1 cells.

The phosphorylation of eNOS is known to be regulated by various kinases. In endothelial cells, AMPK has been shown to phosphorylate and activate eNOS^[16, 30]. Another study showed that the PI3K/Akt signal pathway was involved in bezafibrate-mediated upregulation of eNOS phosphorylation^[31]. To investigate whether the AMPK or Akt pathway is involved in bezafibrate-induced eNOS phosphorylation in osteoblasts, we examined the effects of bezafibrate on AMPK and Akt phosphorylation. In this study, we found that treatment of MC3T3-E1 cells with bezafibrate led to an increase in the phosphorylation of AMPK and ACC, a well-characterized substrate of AMPK^[21], whereas no changes were observed in the level of Akt phosphorylation. Additionally, the AMPK inhibitor compound C was found to suppress bezafibrate-induced eNOS phosphorylation and NO production. These data suggest a possible role of AMPK as an upstream kinase of bezafibrate-induced eNOS phosphorylation and NO production in osteoblasts.

However, the mechanisms involved in bezafibrate-activated AMPK are still unknown. Bezafibrate was reported to reduce circulating cholesterol and affect hepatic cholesterol metabolism through a PPAR α -independent mechanism in mice^[32]. In addition, PPAR α is also not required for bezafibrate-mediated lipid metabolism since the PPAR β isoform is predominant in the small intestine^[33]. However, Wang *et al*^[31] showed that bezafibrate-induced up-regulation of eNOS phosphorylation was fully inhibited by the PPAR α inhibitor MK886 in

bovine endothelial cells. In the present study, we indicated that bezafibrate activated AMPK and eNOS partly via PPAR β , not PPAR α . It is presumable that these inconsistent results might be due to the differences in the expression of PPAR α and PPAR β in various cells. The mRNA expression level of PPAR β is much higher than that of PPAR α mRNA in MC3T3-E1 cells^[34]. Additionally, the potency of bezafibrate at the murine PPAR α and PPAR β is remarkably similar because the EC₅₀ for bezafibrate is 90 and 110 μ mol/L, respectively^[26]. Therefore, the bezafibrate-induced activation of PPAR β may contribute to its beneficial effects in osteoblastic MC3T3-E1 cells.

BMP-2, a member of the transforming growth factor- β superfamily, regulates the differentiation of various cells involved in cartilage and bone formation during fracture repair^[35]. Runx-2 has been widely recognized as the master osteogenic transcription factor because Runx-2-knockout mice display complete absence of bone due to arrested osteoblast maturation; it also plays a critical role in osteoblast marker gene expression, including Col-1, BSP, and osteocalcin^[36]. The activation of AMPK promotes the differentiation of osteoblastic cells via increasing eNOS and BMP-2 expression^[37]. The activation of eNOS and NO activity might lead to BMP-2 protein increases^[38]. The present study found that bezafibrate enhanced BMP-2 and Runx-2 expression in the early stages of osteoblast differentiation in osteoblastic MC3T3-E1 cells, which was reversed by the PPAR β inhibitor GSK0660 or the AMPK inhibitor compound C, but not the PPAR α inhibitor MK886. Thus, it is possible that bezafibrate enhances BMP-2 and Runx-2 expression at least partly via activation of PPAR β and AMPK.

Osteoporosis is associated with atherosclerosis and vascular calcification^[39]. Dyslipidemia appears to participate in the pathogenesis of bone and vascular diseases^[40]. A number of studies have suggested a positive relationship between BMD and triglyceride levels^[3, 41]. Hormone replacement therapy has beneficial effects in the prevention of both atherosclerosis and osteoporosis^[42]. Bisphosphonates, which inhibit bone resorption, are used for the treatment of osteoporosis, whereas the statins, which inhibit cholesterol biosynthesis, are used for the treatment of atherosclerosis. Some clinical data suggest that statins can increase bone density^[38], whereas bisphosphonates may have a beneficial effect on plasma lipid levels and on the atherosclerotic process^[43]. Previous studies suggested that agents modulating AMPK can not only cure dyslipidemia and atherosclerosis^[30] but also promote bone formation^[37]. In this study, we also found that bezafibrate might be a candidate drug promoting bone formation.

In summary, these results show that bezafibrate can enhance the proliferation and differentiation of MC3T3-E1 cells, partly via the activation of PPAR β , but not PPAR α , which could be mediated by the activation of AMPK and eNOS, leading to increased BMP-2 and Runx-2 expression. This study provides evidence that bezafibrate might be beneficial for osteoporosis by promoting bone formation.

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Author contribution

Xing ZHONG and Guo-hong WEI performed the research and wrote the paper; Xing ZHONG, Ling-ling XIU, Guo-hong WEI, Yuan-yuan LIU, Yan-bing LI, and Lei SU analyzed the data; Ling-ling XIU, Xiao-pei CAO, and Hai-peng XIAO designed the study and wrote the paper.

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