Psoralen stimulates osteoblast proliferation through the activation of nuclear factor-κB-mitogen-activated protein kinase signaling

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Abstract. Osteoporosis is a systemic skeletal disease that leads to increased bone fragility and susceptibility to fracture. Approximately 50% of postmenopausal women develop osteoporosis as a result of postmenopausal estrogen deficiency. To reduce fractures related to osteoporosis in women, previous studies have focused on therapeutic strategies that aim to increase bone formation or decrease bone resorption. However, pharmacological agents that aim to improve bone fracture susceptibility exhibit side effects. Current studies are investigating natural alternatives that possess the benefits of selective estrogen receptor modulators (SERMs) without the adverse effects. Recent studies have indicated that phytoestrogen may be an ideal natural SERM for the treatment of osteoporosis. In Chinese herbal medicine, psoralen, as the predominant substance of Psoralea corylifolia, is considered to be a phytoestrogen and is used as a remedy for osteoporosis. A number of studies have demonstrated the efficacy of psoralen in bone formation. However, the pathways and underlying molecular mechanisms that participate in psoralen-induced osteoblast proliferation are not well understood. In the present study, hFOB1.19 cells were treated with psoralen at different concentrations (0, 5, 10, 15 and 20 µM) for 0,24, 36, 48 and 72 h, respectively. Reverse transcription-quantitative polymerase chain reaction and western blot assays were performed to detect glucose transporter 3 (GLUT3) expression. A cell counting kit-8 assay was used to analyze cell proliferation. In addition the effects of mitogen activated protein kinase inhibitors on extracellular signal-regulated kinase (ERK), phosphorylated (p)-ERK, p38, c-Jun N-terminal kinase (JNK) and p-JNK expressions and cell proliferation were measured, as was the effect of nuclear factor (NF)-κB inhibitor on P65 and GLUT3 expressions and cell proliferation. The results indicated that psoralen stimulates hFOB1.19 cell proliferation in a dose-dependent manner (P<0.05). Phospho-ERK, p38 and JNK were markedly increased by psoralen compared with the control group (P<0.05), and the specific inhibitors of ERK (SCH772984), p38 (SB203580) and JNK (SP600125) reversed the stimulatory effects of psoralen on signal marker phosphorylation (P<0.05). The rate of psoralen-induced cell proliferation was significantly suppressed by inhibitors of ERK, JNK and p38 compared with psoralen treatment alone (P<0.05). In addition, psoralen stimulated osteoblast proliferation via the NF-κB signaling pathway. Therefore, the present findings suggest that psoralen may be a potential natural alternative to SERMs in the treatment of osteoporosis and fractures.

Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mass and poor quality bone tissue, which leads to increased bone fragility and susceptibility to fracture (1). Osteoporosis is a leading chronic disease, and the fracture risk associated with osteoporosis increases exponentially with age. (2) In the current aging society, osteoporosis has become a substantial burden for healthcare services and individuals. In 2005, 17 billion dollars was required for the treatment of incident fractures in the United States, in which women accounted for 71% of fractures and 75% of costs (3). Additionally, ~50% of postmenopausal women may have osteoporosis and sustain an osteoporotic fracture as a result of postmenopausal estrogen deficiency (4).

Osteoporosis treatment for women has developed over recent years. To reduce the rate of osteoporosis-related fractures in women, current therapeutics principally focus on increasing bone formation or decreasing bone resorption (5). A number of medicines also aim to restore bone loss by inducing osteoblasts to form new bone tissue (6). Bisphosphonates and related therapeutics have been demonstrated to reduce the risk...
of fractures and are currently the most widely-used agents for the treatment of osteoporosis (7). Postmenopausal women with osteoporosis may also be treated with hormone replacement therapy (HRT). Drugs including tamoxifen, raloxifene and levormeloxifene are types of HRT that target estrogen receptors (ERs) or exert direct effects on the bone to reduce fracture and breast cancer risk (8). The positive effects of raloxifene on the quality and mass of bone have been indicated in a number of preclinical studies (9-11). In a long-term study, Delmas et al (12) demonstrated that long-term treatment with raloxifene induced a significant reduction in vertebral fractures in women with osteoporosis. However, HRTs, as selective estrogen receptor modulators (SERMs), exhibit side effects, and have been associated with an increased risk of coronary heart disease, venous thromboembolic events and exacerbation of menopausal symptoms (13). It has been documented that a consistent number of women taking SERMs exhibit gynecologic symptoms, such as endometrial hyperplasia (14). Therefore, natural alternatives that exert the therapeutic effects of SERMs without the associated side effects are of current interest.

Phytoestrogen are a class of non-steroidal compounds that are of plant origin or obtained from the metabolism of precursors present in plants (15). Phytoestrogens are considered to be an ideal natural SERM and have been demonstrated to stimulate osteoblast formation in vitro (16,17). In epidemiological findings, Cassidy (18) observed that natural isoflavones, as a subclass of phytoestrogens, had protective effects against the development of osteoporosis. In Chinese herbal medicine, psoralen, as the primary substance of Psoralea corylifolia, has been identified as a phytoestrogen and is used as a remedy for osteoporosis (19). Several studies have demonstrated the stimulatory effects of psoralen in bone formation. For instance, Miura et al (20) observed that crude fractions of P. corylifolia seeds stimulated rat bone calcification and increased bone quality in vivo. In vitro, the crude ethanol extract of P. corylifolia stimulated osteoblast-like differentiation of the UMR-106 osteosarcoma cells (21). Furthermore, Wong and Rabie (22) documented that psoralen was able to promote local novel bone formation in vivo. However, the pathways and underlying molecular mechanisms that participate in osteoblast formation are not well understood. In the present study, human fetal osteoblastic 1.19 (hFOB1.19) cells were treated with psoralen in vitro to identify the function of psoralen on osteoblasts and the signaling pathways involved.

Materials and methods

hFOB1.19 cell culture and treatment. hFOB1.19 cells purchased from American Type Culture Collection (Manassas, VA, USA) were cultured as described previously (23). Briefly, 1x10^5 cells were plated into 96-well plate and incubated in medium composed of 1:1 Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich; P8399) and Ham's F12 (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 0.3 mg/ml geneticin (EMD Millipore, Billerica, MA, USA) and 10% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO, USA) in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h. Different concentrations of psoralen (0, 5, 10, 15 and 20 μM; Sigma-Aldrich; P8399) were used for a cell proliferation assay. For time course assays, 1x10^4 cells were seeded into 96-well plate in 1:1 DMEM/Ham's F12 with 0.3 mg/ml geneticin and 10% FBS at 37°C. Cells were treated with PBS (control group) or 15 μM psoralen for 0, 24, 36, 48 and 72 h at 37°C in a humidified atmosphere containing 5% CO_2. In mitogen-activated protein kinase (MAPK) signaling analysis, cells (1x10^4) were seeded into 6-well plates in 1:1 DMEM/Ham's F12 with 0.3 mg/ml geneticin and 10% FBS at 37°C. Cells were treated with the working concentration 10 μM p38 inhibitor (SB203580) for 30 min, 10 μM ERK inhibitor (SCH772984) for 16 h, 5 μM JNK inhibitor (SP600125) for 12 or 5 μM nuclear factor (NF)-κB inhibitor (PDTC; all from Sigma-Aldrich; Merck KGaA) for 12 h in DMEM: Ham's F12 medium.

Cell counting kit-8 (CCK-8) assay. A CCK-8 assay was performed to determine the effects of psoralen on hFOB1.19 cell proliferation, according to the manufacturer's instructions. Briefly, hFOB1.19 cells (2x10^3 cells/well) were seeded in 96-well plates in the medium composed of 1:1 DMEM and Ham's F12 supplemented with 0.3 mg/ml geneticin and 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were treated with different concentrations of psoralen (0, 5, 10, 15 and 20 μM) for 36 h. The same amount of medium served as the control group. Cells were washed with cold phosphate buffered saline (PBS), and 10 μl cell counting kit-8 (CCK-8: WST-8 Dojindo Molecular Technologies, Inc., Rockville, MD, USA) was added to each well and incubated for 2 h at 37°C. Absorbance was measured at 450 nm using a microplate reader.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from hFOB1.19 cells from the proliferation assay, time course assay and MAPK signaling assay groups. cDNA was acquired using a Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc.). The thermocycling conditions were 25°C for 5 min, 42°C for 60 min and 70°C for 10 mins. qPCR was performed using an ABI PRISM 7500 Real-Time PCR system with SYBR Premix Ex TaqTM (Takara Biotechnology Co., Ltd., Dalian, China). Thermocycling conditions were as follows: 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. The glucose transporter 3 (GLUT3) gene, which is closely related to cell proliferation, was amplified with the following specific primers: Forward, 5'-CGG CTT CCT CAT TAC CTT C-3' and reverse, 5' -GGC ACG ACT TAG CCT CAT TAC CTT C-3'. The relative levels of mRNA expression were calculated using the 2^(-ΔΔCT) method normalized to GAPDH (26). All experiments were repeated three times.

Western blotting. Cells from the control group (treatment with equivalent PBS) and treatment group were lysed with radiolabelling precipitation assay buffer (Sigma-Aldrich; Merck KGaA), according to the manufacturer's instructions. A bicinchoninic
Psoralen markedly decreased the proliferation of cells when compared with the 15 µM treatment group (P<0.01 vs. 0 µM; Fig. 1C). As a result, 15 µM psoralen was chosen as the optimal dose for subsequent experiments.

Psoralen stimulates hFOB1.19 cell proliferation in a time-dependent manner. hFOB1.19 cells treated with 15 µM psoralen exhibited increased levels of GLUT3 mRNA expression in a time-dependent manner between 24-48 h; however, treatment for 72 h decreased the levels of GLUT3 mRNA (P<0.01 vs. 0 h; Fig. 1D). Similar results were observed in a western blot analysis of GLUT3 protein expression (Fig. 1E). In addition, a CCK-8 assay demonstrated that treatment with 15 µM psoralen increased cell proliferation in a time-dependent manner between 24-48 h, while treatment for 72 h lead to a decrease in cell proliferation when compared to cells treated for 48 h (P<0.01 vs. 0 h; Fig. 1F). These results indicated that 15 µM psoralen for 48 h was the optimal treatment.

To verify the effect of psoralen on hFOB1.19 cell proliferation, 15 µM psoralen was administered to hFOB1.19 cells for 48 h. Following treatment with 15 µM psoralen for 48 h, levels of GLUT3 mRNA were significantly increased in the psoralen treatment group compared to untreated controls (P=0.002641; Fig. 2A). Similar alterations in the levels of GLUT3 protein expression were observed by western blot analysis (Fig. 2B). Furthermore, treatment with 15 µM psoralen significantly promoted the proliferation of hFOB1.19 cells between 24-72 h when compared with the control group (P<0.05; Fig. 2C).

Psoralen stimulates hFOB1.19 cell proliferation depends on NK-κB-MAPK signaling. MAPK signaling pathways have been demonstrated to participate in osteoblast proliferation (28). Previous studies have demonstrated the effect of psoralen on osteoblast proliferation in vitro (29,30). To elucidate the mechanism of action of psoralen on osteoblast proliferation, the effects of psoralen on three primary signaling pathways, namely extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and MAPK, were determined by evaluating signal pathway activity and cell proliferation (Fig. 3). As depicted in Fig. 3A, after treatment with 15 µM psoralen for 48 h, levels of activated phospho-ERK and p65 were markedly increased compared with the control group. Analysis of p38 and JNK exhibited similar results (Fig. 3B and D, respectively). To verify whether MAPKs were involved in cell proliferation, hFOB 1.19 cells were treated with psoralen and inhibitors of p38, ERK, JNK or NF-xB and the effects on signaling activity and cell proliferation were evaluated. As depicted in Fig. 3A, B and D, treatment with specific inhibitors of ERK (SCH772984), p38 (SB203580) and JNK (SP600125) reversed the stimulatory effects of psoralen on signal marker phosphorylation. In addition, the rate of psoralen-induced cell proliferation was significantly suppressed by inhibitors of ERK (P=0.004676), JNK (P=0.001535) and p38 (P=0.001828) compared with psoralen treatment alone. Results also indicated that 15 µM psoralen stimulated osteoblast proliferation through the NF-xB signaling pathway. Notably, western blotting indicated that the expression of p65, as an NF-xB signal marker (31), similar to the activities of ERK and JNK following treatment with psoralen and signaling inhibitors (Fig. 3A and D). Thus, it was suspected that the NF-xB pathway may be involved.
Figure 1. Time and dose-dependent effects of psoralen on hFOB1.19 cell proliferation. (A) RT-qPCR and (B) western blot analysis of GLUT3 expression at the mRNA and protein levels, respectively, in hFOB1.19 cells at psoralen concentrations of 0, 5, 10, 15 and 20 µM. GAPDH was used as a control. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 µM. (C) A CCK-8 assay of hFOB1.19 cell proliferation following treatment with 0, 5, 10, 15 and 20 µM psoralen. **P<0.01 and ***P<0.001 vs. 0 µM. (D) RT-qPCR and (E) western blot analysis of GLUT3 expression in hFOB1.19 cells treated with 15 µM psoralen at 0, 24, 36, 48 and 72 h. GAPDH was used as a control. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 h. (F) A CCK-8 assay of hFOB1.19 cell proliferation at 0, 24, 36, 48 and 72 h after treatment with 15 µM psoralen. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 h. hFOB1.19, human fetal osteoblastic 1.19; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GLUT3, glucose transporter 3; CCK-8, cell counting kit-8.

Figure 2. Effect of psoralen on hFOB1.19 cell proliferation. (A) RT-qPCR and (B) western blot analysis of GLUT3 expression at the mRNA and protein levels in hFOB1.19 cells treated with or without 15 µM psoralen for 48 h. GAPDH was used as a control. Western blotting indicated that 15 µM psoralen stimulated the expression of GLUT3 protein in osteoblast cells. (C) A CCK-8 assay indicated that psoralen significantly increased cell proliferation compared with the control group at 24, 36, 48, and 72 h. *P<0.05 vs. control. hFOB1.19, human fetal osteoblastic 1.19; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GLUT3, glucose transporter 3; CCK-8, cell counting kit-8.
Figure 3. (A-D) Effect of MAPK inhibitors on psoralen-induced proliferation in osteoblast cells. Western blotting was used to measure the protein expression of (A) ERK, P-ERK, (B) p38, P-p38, (D) JNK and P-JNK following treatment with psoralen and MAPK inhibitors. Levels of p65, GLUT3 and GAPDH expression were also determined. GAPDH was used as a control. (C) A CCK-8 assay indicated that MAPK inhibitors significantly suppressed psoralen-induced proliferation. MAPK, mitogen-activated protein kinase; SCH772984, ERK inhibitor; SB203580, P38 inhibitor; SP600125, JNK inhibitor; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; GLUT3, glucose transporter 3; P-, phosphorylated; CCK-8, cell counting kit-8.

Figure 4. Effect of NF-κB inhibitor on psoralen-induced osteoblast proliferation. hFOB1.19 cells were incubated with or without PDTC in the presence or absence of 15 μM psoralen. (A) Level of P65 and GLUT3 in the cell extracts were measured by western blotting. When PDTC was added, levels of P65 and GLUT3 were markedly decreased compared with the psoralen-induced group. GAPDH was used as a control. (B) A CCK-8 assay indicated that PDTC significantly suppressed psoralen-induced proliferation. NF-κB, nuclear factor-κB; hFOB1.19, human fetal osteoblastic 1.19; PDTC, NF-κB inhibitor; GLUT3, glucose transporter 3; CCK-8, cell counting kit-8.
NF-κB knockout mice exhibit bone disorder (32). In the majority of cells, NF-κB is present as a latent-form in the cytoplasm and stimulates the transcription of target genes in the nucleus (33). NF-κB is involved in cellular responses to various stimuli (34). To determine whether psoralen induced NF-κB signaling, a NF-κB signal pathway inhibitor (PDTC) was administered to hFOB 1.19 cells following psoralen treatment. As depicted in Fig. 4A, cells treated with psoralen exhibited increased levels of GLUT3 and p65 compared to controls. In turn, when cells were treated with psoralen and PDTC inhibitor, the expressions of GLUT3 and p65 were reduced when compared to cells treated with psoralen alone. Furthermore, the proliferation of cells was significantly decreased when cells were treated with psoralen and PDTC inhibitor (P=0.010386 vs. psoralen group; Fig. 4B). As similar results were obtained for NF-κB and MAPK pathway analysis, this suggested that psoralen stimulated osteoblast proliferation through NF-κB-MAPK signaling.

Discussion

Osteoblasts are mesenchymal cells and are responsible for bone matrix synthesis, secretion and mineralization during the bone remodeling process (35). Osteoblasts serve a key role in maintaining bone mass and reducing bone loss. During the proliferation stage, osteoblasts secrete type I collagen to aid mineralization and reduce bone loss (36). Osteoblasts also secrete osteoprotegerin, which blocks receptor activator NF-κB ligand interactions and prevents bone resorption (37).

Osteoblast proliferation is typically evaluated through measurements of total cellular protein, alkaline phosphatase activity and type I collagen secretion or GLUT3 expression (38). GLUT3 is a transporter with a high affinity for glucose and is essential for cell proliferation (39). Bell et al (40) observed that GLUT3 was expressed in osteoblasts and that glucose uptake in osteoblasts was mediated by GLUT1 and 3.

In the present study, the mRNA and protein expression levels of GLUT3 were upregulated with increasing doses of psoralen, though cell toxicity was induced at the highest dose of 20 μM psoralen. In addition, the mRNA and protein expression levels of GLUT3 were upregulated with time. Collectively, results indicated that osteoblast proliferation was increased in a dose and time-dependent manner.

Components within the MAPK, ERK and JNK signaling pathways, including insulin, glucocorticoid, hyperbaric oxygen and fibroblast growth factor-6, have been identified as major promoters of osteoblast proliferation in a number of studies (41-43). Song et al (44) demonstrated that icarin, a flavonoid glucoside isolated from P. corylifolia, promoted MC3T3-E1 osteoblast proliferation by inducing the activation of ERK and JNK, but exerted no effect on the activity of p38 kinase. By contrast, Luo et al (45) observed that adiponectin was able to promote osteoblast differentiation through the p38 pathway, and that adiponectin stimulated osteoblast proliferation by inducing JNK activation, but not ERK1/2, in osteoblasts. MAPK pathways serve key roles in cell proliferation, and more specifically, these pathways are considered to serve vital roles in osteoblast proliferation and differentiation (44).

A previous in vivo study indicated that bone morphogenetic proteins (BMPs) controlled osteoblast proliferation and induced bone formation (46). BMP-2 has been demonstrated to induce the activation of alkaline phosphatase genes, which serve an essential role in osteoblast differentiation (47). Kong et al (48) suggested that interactions with BMP and the MAPK signaling pathway may be involved in osteoblast proliferation.

Psoralen, as a type of furanocoumarin, is the predominant component of the leguminous plant P. corylifolia (49). In a recent study, psoralen has been indicated to possess bacteriostatic, anti-tumor, hemostatic and cardiovascular effects, and promote bone formation while inhibiting bone resorption (50). In addition, Tang et al (51) observed that psoralen upregulated the expression of BMP-2 to stimulate mouse calvarial osteoblast differentiation. In the present study, psoralen stimulated osteoblast proliferation through the ERK/MAPK, JNK/MAPK and p38/MAPK pathways. Furthermore, psoralen significantly increased the expression of NF-κB. This result may indicate that NF-κB was the pathway by which psoralen induced cell proliferation. Two explanations may account for these observations. First, the MAPK pathway, as an important pathway involved in the process of cell proliferation, may have stimulated osteoblasts directly. Second, psoralen-induced MAPK activation may have stimulated the BMP pathway to promote osteoblast proliferation.

In conclusion, the present findings indicated that psoralen stimulated osteoblast proliferation through the activation of NF-κB and MAPK signaling. Thus, psoralen may be a viable therapeutic agent in the treatment of osteoporosis.

References


