Curcumin induces osteoblast differentiation through mild-endoplasmic reticulum stress-mediated such as BMP2 on osteoblast cells

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Abstract

Aims: Curcumin (diferuloylmethane or [1E,6E]-1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6heptadiene-3,5-dione) is a phenolic natural product derived from the rhizomes of the turmeric plant, Curcuma longa. It is reported to have various biological actions such as anti-oxidative, anti-inflammatory, and anti-cancer effects. However, the molecular mechanism of osteoblast differentiation by curcumin has not yet been reported.

Main methods: The cytotoxicity of curcumin was identified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Expression of osteogenic markers and endoplasmic reticulum (ER) stress markers in C3H1-T1/2 cells were measured using reverse-transcriptase polymerase chain reaction (RT-PCR) and Western blotting. Alkaline phosphatase (ALP) staining was performed to assess ALP activity in C3H1-T1/2 cells.

Transcriptional activity was detected using a luciferase reporter assay.

Key findings: Curcumin increased the expression of genes such as distal-less homeobox 5 (Dlx5), runt-related transcription factor 2 (Runx2), ALP, and osteocalcin (OC), which subsequently induced osteoblast differentiation in C3H1-T1/2 cells. In addition, ALP activity and mineralization was found to be increased by curcumin treatment. Curcumin also induced mild ER stress similar to bone morphogenetic protein 2 (BMP2) function in osteoblast cells. Next, we confirmed that curcumin increased mild ER stress and osteoblast differentiation similar to BMP2 in C3H1-T1/2 mesenchymal stem cells. Transient transfection studies also showed that curcumin increased ATF6-Luc activity, while decreasing the activities of CREBH-Luc and SMILE-Luc. In addition, similar to BMP2, curcumin induced the phosphorylation of Smad 1/5/9.

Significance: Overall, these results demonstrate that curcumin-induced mild ER stress increases osteoblast differentiation via ATF6 expression in C3H1-T1/2 cells.

1. Introduction

Osteoblast differentiation is highly regulated by hormones, cytokines and multiple transcription factors [1,2]. Bone morphogenetic proteins (BMPs) are cytokines that play important roles in a variety of cellular functions, among which BMP2 is the most effective inducer of osteoblast differentiation. BMP2 regulates the expression of Runx-related transcription factor 2 (Runx2) by activating the Smad 1/5/8 signaling pathway to promote osteoblast differentiation. Runx2 controls the expression of osteogenic markers, such as osteocalcin (OC), bone sialoprotein, collagen type I, and osteopontin [3]. BMP2 also activates the unfolded protein response (UPR) during osteoblast differentiation [4]. Osteogenesys is stimulated by BMP2 through pathways activated by mild endoplasmic reticulum (ER) stress involving major UPR inducers, such as protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6) [5,6,7,8,9]. In our previous study, we found that BMP2 induced osteoblast differentiation via mild ER stress-activated ATF6 and directly regulated OC expression [1].

Curcumin (diferuloylmethane or (1E,6E)-1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6heptadiene-3,5-dione) is a phenolic natural product derived from the rhizomes of the turmeric plant, Curcuma longa. It is reported to have various biological actions such as anti-oxidative, anti-inflammatory, and anti-cancer effects. However, the molecular mechanism of osteoblast differentiation by curcumin has not yet been reported.

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Significance: Overall, these results demonstrate that curcumin-induced mild ER stress increases osteoblast differentiation via ATF6 expression in C3H1-T1/2 cells.

Abbreviations: ER, endoplasmic reticulum; BMP, bone morphogenetic protein; Runx2, Runt-related transcription factor 2; OC, osteocalcin; UPR, unfolded protein response; PERK, PKR-like endoplasmic reticulum kinase; IRE-1, inositol-requiring enzyme 1; ATP, activating transcription factor; NF-κB, nuclear factor-κB; STAT, signal transducer and activator of transcription; AP-1, activated protein-1; RANKL, receptor activator of nuclear factor NF-κB ligand; DMEM, Dulbecco's Modified Eagle Medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; CREBH, CAMP-responsive element-binding protein H; ALP, alkaline phosphatase; BiP, immunoglobulin binding protein; CHOP, CEBP homologous protein; IDED, ER-degradation-enhancing α-mannosidase-like protein; OSE2, osteoblast-specific cis-acting element 2

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0024-3205/ © 2017 Published by Elsevier Inc.
methoxyphenyl)-1,6-heptadiene-3,5-dione) is a phenolic natural product isolated from the rhizomes of the turmeric plant, Curcuma longa. It has been used as a dietary spice, possess potent anti-inflammatory and anti-oxidant properties, and is used in traditional Indian medicine to treat inflammation, hepatic disorders, and sinusitis [10,11,12]. A recent study has shown that curcumin regulates ER stress by blocking cyclic adenosine monophosphate (cAMP) responsive element-binding protein H (CREBH)-mediated transactivation of target genes in hepatocytes [13]. Various transcription factors, including nuclear factor xB (NF-xB), signal transducer and activator of transcription (STAT) proteins, and activated protein-1 (AP-1), are strongly inhibited by curcumin [14,15,16]. Receptor activator of nuclear factor NF-xB ligand (RANKL) induces osteoclast differentiation through the activation of NF-xB [17,18], and treatment with curcumin suppresses both osteoclast differentiation and activation of NF-xB by RANKL [19]. However, the effect of curcumin on osteoblast differentiation has not been investigated at the cellular or molecular level.

In the present study, we investigated whether curcumin mediates osteoblast differentiation through the regulation of mild ER stress in C3H10T1/2 mesenchymal stem cells. Curcumin increased ATF6 expression and activation via Smad-mediated Runx2 expression similar to BMP2 treatment in osteoblasts. Furthermore, curcumin-activated ATF6 induced osteoblast differentiation by increasing OC expression.

2. Materials and methods

2.1. Reagents

 Dulbecco’s Modified Eagle Medium (DMEM), phosphate buffered saline (PBS), penicillin streptomycin, and 0.25% Trypsin ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from MP Biomedicals (Santa Ana, CA, USA). Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Aldrich (St. Louis, MO, USA). Various transcription factors, including nuclear factor xB (NF-xB), signal transducer and activator of transcription (STAT) proteins, and activated protein-1 (AP-1), are strongly inhibited by curcumin [14,15,16]. Receptor activator of nuclear factor NF-xB ligand (RANKL) induces osteoclast differentiation through the activation of NF-xB [17,18], and treatment with curcumin suppresses both osteoclast differentiation and activation of NF-xB by RANKL [19]. However, the effect of curcumin on osteoblast differentiation has not been investigated at the cellular or molecular level.

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2.2. Cell culture

 Mouse embryonic mesenchymal stem cell lineage C3H10T1/2 cells were maintained in DMEM with 10% FBS and 1% penicillin streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. For osteogenic differentiation, the cells were cultured in an osteogenic medium (DMEM containing 0.25 μg/mL of BMP2) and treated with curcumin (10 μM in DMEM). The C3H10T1/2 cell line was kindly provided by Prof. Koh (Chonnam National University, Republic of Korea).

2.3. Cell viability assay

 Cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) assay. Briefly, approximately 1.5 x 10⁴ cells/wells were seeded in 48-well plates and incubated for 12 h to allow the cells to adhere. Subsequently, the medium was replaced with fresh medium containing 0, 1, 3, 10, and 100 μM curcumin and incubated for a further 1, 3, 7 or 15 days. After 0.5 μg/mL of MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, the cells were incubated for another 1 h. Finally, 200 μL of DMSO was added, and the absorbance was measured at 570 nm using the Infinite 200PRO (Tecan Trading AG, Mannedorf, Switzerland).

2.4. Reverse-transcriptase PCR and real-time PCR analysis

 Total RNA was isolated from the cell cultures using the TRI-solution™ (Bio science technology, Daegu, Korea) according to the manufacturer’s instructions, and reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using 2 μg of the total RNA. Each reaction consisted of initial denaturation at 95 °C for 5 min followed by a three-step cycle: denaturation at 95 °C for 30 s, annealing at the optimal temperature for each primer pair for 30 s, and extension at 72 °C for 30 s. After 30–35 cycles, a final extension step was carried out at 72 °C for 5 min. The primers used for PCR are listed in Table 1.

2.5. Alkaline phosphatase (ALP) staining

 For ALP staining, the cultured cells were fixed with 10% formaldehyde, rinsed thrice with deionized water, and treated with the BCIP+/NBT solution (Sigma-Aldrich). After an additional washing, the stained cultures were photographed.

2.6. Transient transfection and luciferase assay

 C3H10T1/2 cells were transiently transfected with the indicated plasmids using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were harvested 24 or 48 h after transfection and assayed using the luciferase reporter assay system (Promega, Madison, WI, USA). As an internal control, the cytomegalovirus β-galactosidase plasmid was co-transfected in each transfection experiment, and the luciferase activity was normalized to the β-galactosidase activity. To confirm the effects of curcumin, the cells were transfected with ATTF6-Luc (wild and mutated types), CREBH-Luc, and Runx2-Luc vector using the Lipofectamine 2000. Firefly and renilla luciferase activities were determined using the Dual-Luciferase® Reporter Assay System (Promega, Mannheim, FRG, Germany).

2.7. Western blots

 Total cell extracts were harvested in immunoprecipitation assay buffer and then centrifuged at 12,000g for 10 min at 4 °C. Total protein was quantified using the bicinchoninic acid protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and then the proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on to a poly (vinylidene) fluoride membrane. After blocking with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20, the membrane was incubated with the specific primary antibodies for p-Smad 1/5/9 and Smad 1 (1:1000; Cell Signaling Technology, MA, USA), ATTF4, CHOP, Bip and β-actin (1:1000; Santa Cruz Biotech, Dallas, TX, USA). Signals were detected using an enhanced chemiluminescence (ECL) reagent (Advansta, Menlo Park, CA, USA) according to the manufacturer’s protocol. Densitometric analysis of the blotted membrane was performed using the Lumino-Image analyzer LAS-3000 system (Fuji Film, Tokyo, Japan).

| Table 1
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<th>Specific primers for PCR.</th>
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2.8. Statistical analysis

All experiments were repeated in triplicate, and statistical analyses were performed using Student’s t-test with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). *p values < 0.05 were considered significant. The results are presented as means ± standard error of the means (SEM) of triplicate, independent experiments.

3. Results

3.1. Curcumin increases the expression of osteogenic marker genes

Initially, to determine the concentration range of curcumin in C3H10T1/2 cells, a cytotoxicity assay was conducted. Curcumin exhibited no cytotoxic activity at concentrations up to 10 μM; however, curcumin at concentrations of 100 μM reduced cell viability (Fig. 1A). Thus, our experiments were conducted with curcumin 10 μM concentration. The effect of curcumin on the mRNA expression of osteogenic genes was determined using RT-PCR. Curcumin significantly increased the expression of Runx2 and OC mRNA (Fig. 1B). To confirm the effect of curcumin treatment on osteogenic differentiation, ALP staining was performed. ALP activities of C3H10T1/2 cells increased after curcumin treatment (Fig. 1C). In addition, curcumin increased protein expression of Runx2 on Western blot assays. However, curcumin did not regulate BMP2 protein levels (Fig. 1D). Overall, these results suggest that curcumin induced osteoblast differentiation by increasing the expression of osteogenic genes, such as Runx2, ALP, and OC.

3.2. Curcumin stimulates the expression of ATF6 along with ER stress marker genes similar to BMP2

Curcumin regulates ER stress-related gene expression in hepatocytes and cervical cancer cells [13,20]. In addition, BMP2 induces differentiation via mild ER stress in osteoblast cells [4]. Therefore, we examined whether the expression of ER stress-related genes is also regulated by curcumin in C3H10T1/2 mesenchymal cells. Initially, the expression of ER stress marker genes, such as immunoglobulin binding protein (BIP), C/EBP homologous protein (CHOP), ATF4, and ER-degradation-enhancing-a-mannidose-like protein (EDEM), was increased by curcumin (Fig. 2A). Moreover, curcumin enhanced protein expression of BIP, CHOP, and ATF4 similar to BMP2 (Fig. 2B). Interestingly, curcumin significantly increased ATF6 expression, but did not affect the expression of CREBH (Fig. 2C). To further confirm whether curcumin
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3.3. Curcumin regulates Smad 1/5/9 phosphorylation and induces Runx2 mediated increase in transcription of the ATF6 gene

The phosphorylation of Smad1/5/9 mediates BMP-induced gene transcription and osteoblast differentiation [21]. To determine whether curcumin affects Smad 1/5/9 phosphorylation in osteoblasts, C3H10T1/2 cells were treated with curcumin, and Smad 1/5/9 phosphorylation was examined using Western blot analysis. Curcumin treatment significantly induced Smad 1/5/9 phosphorylation (Fig. 3A). Several studies have found that Runx2 binds directly to the osteoblast-specific cis-acting element 2 (OSE2) ACCACA sequence and regulates the transcription of the target gene [22,23]. The promoter of the ATF6 gene also contains an OSE2 region that is located at −205 to −200 bp. A promoter study was performed using an OSE-mutated reporter construct (M-ATF6-Luc) via the activation of Runx2. Curcumin increased the luciferase activity of the wild type ATF6-Luc, but did not stimulate the M-ATF6-Luc (Fig. 3B). In addition, similar to BMP2, curcumin increased the promoter activity of BRE-Luc activity (Fig. 3C). These results suggest that curcumin induced Smad1/5/9 phosphorylation and mediated the transcription of the ATF6 gene.

In summary, these findings suggest that curcumin regulates osteoblast differentiation via Runx2 expression. In addition, similar to BMP2, curcumin increases the expression of the mild ER stress-induced ATF6 gene in C3H10T1/2 cells (Fig. 4).

4. Discussion

In this study, we demonstrated that, similar to BMP2, curcumin induced osteoblast differentiation via ATF6 expression in osteoblast cells.

BMP2 is an important regulator of osteoblast differentiation, bone development, and bone repair [24,25]. BMP2 induces ER stress via the UPR [1,4] and major transducers of UPR, including PERK, IRE1, and ATF6 [5,6,7,8,9]. BMP2 regulates osteoblast differentiation via Runx2-dependent ATF6 expression and activation of ATF6 via ER stress-induced intramembrane proteolysis. In addition, ATF6 increases OC expression by directly binding to the TGAAGT sequences on the OC promoter gene [1]. Tunicamycin, an inducer of ER stress, stimulates the expression of small heterodimer partner-interacting leucine zipper protein (SMILE) in osteoblast cells. SMILE prevents BMP2-induced osteoblast differentiation by interacting directly with Runx2, thereby inhibiting its ability to bind to the OC promoter [26]. CREBH is structurally similar to ATF6 and modulates tumor necrosis factor α (TNFα)-mediated inhibition of osteogenesis mainly by Smurf1-induced degradation of Smad1 [27,28,29].

Curcumin has potent anti-inflammatory and anti-oxidant actions in hepatic disorders, sinusitis, and a wide variety of tumor cells [10,11,12,30,31]. Curcumin regulates ER stress by blocking the CREBH-mediated transactivation of target genes in hepatocytes [13]. Additionally, it mediates ER stress-induced apoptosis and mitochondrial dysfunction by activating genes related to ER (GADD153, Calnexin, Calreticulin, PERK, IRE1, and ATF6) and mitochondrial dysfunction (TCTP, Mcl-1, Bcl-2, and Bax) which are associated with ER-stress and the UPR pathway [20,32]. Various transcription factors (NF-κB, STAT, and AP-1) are strongly inhibited by curcumin [14,15,16]. RANKL induces osteoclast differentiation through the activation of NF-κB [17,18], and treatment with curcumin suppresses both osteoclast differentiation and induction of NF-κB by RANKL [19]. Although curcumin-induced osteoblast differentiation has been studied, there are no reports of the mechanism at a molecular level [33,34].

Our results showed that curcumin regulates the expression of ER stress-related genes BIP, CHOP, ATF6, CREBH, and SMILE in osteoblasts (C3H10T1/2 cells) in a time-and dose-dependent manner. These results are consistent with those of other studies, showing that curcumin regulates ER stress [13,32]. In the present study, curcumin treatment induced ATF6 expression, which resulted in the increased expression of marker genes associated with osteoblast differentiation. The regulation of UPR by curcumin has not been reported thus far in osteoblast. In addition, curcumin increased the expression of osteogenic genes (ALP, OC, and Runx2) to promote osteoblast differentiation in C3H10T1/2 cells.

5. Conclusion

In summary, this study identified for first time the molecular mechanism underlying curcumin induced osteoblast differentiation. These findings demonstrate that curcumin is a key regulator of osteoblast differentiation by mild ER-stress in C3H10T1/2 cells.
Curcumin treatment on BRE-Luc activity. Cells were transfected with BRE-Luc and untreated control cells. All experiments were repeated at least three times independently.

Acknowledgments

The authors declare that there are no conflicts of interest.

Conflicts of interest statement

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Acknowledgments

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