Original Paper

Attenuation of Oxidative Stress-Induced Osteoblast Apoptosis by Curcumin is Associated with Preservation of Mitochondrial Functions and Increased Akt-GSK3 β Signaling

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Key Words

Osteoblast • Osteoporosis • Oxidative stress • Apoptosis • Mitochondrial function • Reactive oxygen species

Abstract

Background: Osteoblast apoptosis induced by oxidative stress plays a crucial role in the development and progression of osteoporosis. Curcumin, a natural antioxidant isolated from *Curcuma longa*, has highly protective effects against osteoporosis. However, the effects of curcumin on oxidative stress-induced osteoblast apoptosis remain unclear. This study aimed to explore the effect of curcumin on hydrogen peroxide (H₂O₂) induced osteoblast apoptosis and the underlying mechanisms. **Methods:** An osteoblastic cell line (Saos-2) was exposed to various concentrations of H₂O₂ with or without curcumin treatment. Cell viability was evaluated by MTT assays. The apoptosis rate was analyzed by flow cytometry and TUNEL assays. Mitochondrial ROS and membrane potential were determined using a fluorescence microscope. Mitochondrial respiratory enzyme activity was measured using a spectrophotometer. Protein levels were detected by western blotting. **Results:** Curcumin was cytoprotective because it greatly improved the viability of Saos-2 cells exposed to H₂O₂ and attenuated H₂O₂-induced apoptosis. Curcumin treatment also preserved the mitochondrial redox potential, decreased the mitochondrial oxidative status, and improved the mitochondrial membrane potential and functions. Furthermore, curcumin treatment markedly increased levels of phosphorylated protein kinase B (Akt) and phosphorylated glycogen synthase kinase-3 β (GSK3 β). **Conclusion:** Curcumin administration ameliorates oxidative stress-induced apoptosis in osteoblasts by preserving mitochondrial functions and activation of Akt-GSK3 β signaling. These data provide experimental evidence supporting the clinical use of curcumin for prevention or treatment of osteoporosis.

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Introduction

Osteoporosis is a common systemic skeletal disease characterized by decreased bone mass and architectural deterioration of bone tissues. Recent evidence indicates that oxidative stress (OS), which results from an imbalance of oxidants and antioxidants in favor of the former, is a major factor in the development and progression of osteoporosis [1, 2].

Osteoblast apoptosis plays a crucial role in bone development and maintenance, and partly contributes to diseases such as osteoporosis in sex steroid deficiency, glucocorticoid excess, and aging [3]. Many studies have demonstrated that increased OS persists in these pathological conditions, which might contribute to inhibition of osteoblast differentiation [4] and proliferation [5] or induction of cell apoptosis [6, 7]. Thus, alleviation of osteoblast apoptosis caused by OS is very important to prevent or at least delay the loss of bone mass in osteoporosis.

Mitochondria are the primary source of reactive oxygen species (ROS) and the principal sites of ROS-induced damage. Mitochondrial dysfunction essentially influences osteoblasts through the regulation of mitophagy, apoptosis, and mitochondrial DNA damage [8-10]. Improving mitochondrial functions through the application of antioxidants can prevent cytotoxicity and dysfunction in osteoblasts [9, 11]. Curcumin is a naturally occurring yellow molecule in turmeric, which demonstrates potent antioxidant and anti-inflammatory properties [12, 13]. It has been found to protect against intracellular ROS and demonstrates a free radical-scavenging activity [14]. Most importantly, curcumin has attracted considerable interest because of its preventative effects on processes related to osteoporosis. Numerous *in vitro* studies indicate that curcumin inhibits osteoclast proliferation and differentiation by suppressing ROS generation, and thus prevents bone loss in rodent models of postmenopausal osteoporosis [15-17], diabetes [18], and Alzheimer's disease [19]. Moreover, curcumin shows similar benefits in rodent models of hindlimb suspension [20] and spinal cord injury [21]. These findings suggest that curcumin affects the activity and number of osteoclasts, rather than osteoblasts, in the progression of bone loss associated with various pathological processes. Nonetheless, some studies have reported that high doses (>10 μ M) of curcumin inhibit proliferation of rat calvarial osteoblasts and human osteoblast cell death [22-24]. Gu et al. demonstrated that 10 μ M curcumin promotes osteoblastic differentiation of rat mesenchymal stem cells by upregulation of HO-1 [25]. Moreover, Xin et al. showed that 4 μ M curcumin increases osteoblastic differentiation of MC3T3-E1 cells exposed to modeled microgravity [20]. The divergence among these results was probably related to differences in the curcumin dosage, application duration, and modes of administration. Some studies indicate that curcumin treatment attenuates pathological conditions through activation of prosurvival signaling pathways [26, 27]. To a large extent, these protective effects of curcumin have been attributed to its strong activity against mitochondrial dysfunction [28, 29]. Because of its potent antioxidant properties and an anti-apoptotic activity in non-cancerous cells, we hypothesized that curcumin might effectively protect osteoblasts from oxidative damage.

Protein kinase B (Akt) is a serine/threonine protein kinase that functions as a critical regulator of cell survival and proliferation. Activated Akt inhibits glycogen synthase kinase 3 β (GSK3 β) by phosphorylation. GSK3 β mediates OS-induced apoptosis by activating Caspase-3 and promoting cytochrome c release from mitochondria [30, 31]. However, the role of Akt-GSK3 β in regulating OS-induced osteoblast apoptosis is unknown. Furthermore, studies have indicated that curcumin exerts a protective effect against apoptosis via the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway [32, 33]. However, whether curcumin treatment promotes osteoblast survival through prevention of OS-induced mitochondrial dysfunction and activation of Akt-GSK3 β signaling remains unclear.

Therefore, we examined the effects of curcumin on hydrogen peroxide (H₂O₂)-induced apoptosis of osteoblasts and its potential mechanisms. Our findings might provide new insights into the role of curcumin in OS-induced osteoblast apoptosis, thereby highlighting its potential therapeutic application for osteoporosis.

Materials and Methods

Reagents

Cell culture medium and supplements were purchased from Life Technologies (Grand Island, NY, USA). Anti-phospho-Akt (S473), anti-Akt, anti-phospho-GSK3 β (Ser9), anti-GSK3 β , anti-Bcl-2, anti-Bax, anti-Caspase-3 and anti- β -actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Chamber slides, and goat anti-rabbit and anti-mouse secondary antibodies were from Thermo Fisher Scientific (Waltham, MA, USA). MitoSOX Red (Molecular Probes, USA), tetramethylrhodamine methylester (TMRM) (Molecular Probes), and Mitotracker Green (MTGreen, Molecular Probes) were from Life Technologies. A terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) kit was from Roche (Mannheim, Germany). Curcumin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit, manganese-3-methoxy N, N'-bis (salicylidene) ethylenediamine chloride (EUK134), 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), H₂O₂, and LY294002 were from Sigma Aldrich (St. Louis, MO, USA).

Cell Culture

The human osteoblastic cell line (Saos-2) used in this study was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in McCoy's 5a modified medium supplemented with 10% fetal bovine serum and antibiotics (100 IU/ml penicillin G and 100 ng/ml streptomycin) in a humidified incubator at 37 °C with 5% CO₂. The culture medium was replenished twice per week.

Cell Treatments

Test compounds were prepared as stock solutions and diluted to the desired final concentrations immediately before use. The working concentrations of the compounds were as follows: H₂O₂ (100, 200, and 250 μ M), EUK134 (10 μ M), TDZD-8 (5 μ M), LY294002 (10 μ M), and curcumin (10, 20, 30, 40, and 50 μ M). The final concentration of the vehicle, dimethyl sulfoxide (DMSO), was less than 0.5% in all experiments. Cells were treated with or without H₂O₂ and the indicated test compounds for various times, according to the experimental protocol.

Cell Viability

Osteoblasts were seeded in 96-well plates (1 $\times$ 10⁴ cells/well) and cultured under various conditions, as indicated for each experiment. Briefly, osteoblasts were washed twice with phosphate buffered saline (PBS) and incubated in 100 μ l/well serum-free medium supplemented with 10 μ l MTT solution (5 mg/ml) at 37 °C. After 4 h, the supernatant was removed, and the formazan crystals were dissolved by incubation with 150 μ l/well DMSO for 20 min. The plates were then agitated for 15 s and absorbance at 570 nm was measured in a microplate reader.

Measurement of Apoptosis by Flow Cytometry and TUNEL assays

Apoptosis of osteoblasts was detected by Annexin-V labeled with FITC. Propidium iodide (PI; 1 μ g/ml) was used to determine cell necrosis. After exposure to various experimental conditions, cells were trypsinized and labeled with fluorochromes at 37 °C, and then cytofluorometric analysis was performed with a FACScan (Becton Dickinson, NY, USA).

Apoptosis was also evaluated by the TUNEL method. For the TUNEL assays, cells grown on a coverslip were pretreated with or without compounds in the presence of H₂O₂ (0.25 mM) for 6 hours. After treatment, the cells were washed with PBS, fixed in 4% paraformaldehyde/PBS, and permeabilized with 0.2% Triton X-100 in citrate buffer. Samples were incubated with TdT and fluorescein-labeled dUTP, counterstained with 4', 6-diamidino-2-phenylindole (DAPI), and then observed under a fluorescence microscope (Leica TCS SPE, Germany). Percentages of apoptotic cells were estimated by counting 300 cells in random fields.

Western Blot Analyses

After the indicated treatments, osteoblasts were collected and lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA). Protein concentrations were determined using a Bradford protein assay kit (Thermo Fisher Scientific). Proteins were separated by electrophoresis and transferred to a polyvinylidene difluoride membrane. Anti-phospho-Akt (1:2000), anti-Akt (1:2000), anti-phospho-GSK3 β (1:2000),

anti-GSK3 β (1:2000), anti-Bcl-2 (1:2000), anti-Bax(1:2000), anti-Caspase-3 (1:1000), and anti- β -actin antibodies were used as primary antibodies. Proteins bound by primary antibodies were visualized with an appropriate secondary antibody (1:5000), followed by addition of an enhanced chemiluminescence substrate (Thermo Fisher Scientific). The protein bands were detected using the Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA) and quantified using NIH ImageJ software (available in the public domain).

Measurements of Mitochondrial Respiratory Enzyme Activity

Mitochondrial respiration complex activity was measured in osteoblast lysates as described previously [34, 35]. Briefly, osteoblasts in 6-well plates were washed with ice-cold PBS, harvested, centrifuged, and resuspended in 50 μ l isolation buffer (225 mM D-mannitol, 75 mM sucrose, 2 mM K₂HPO₄, and 5 mM HEPES, pH 7.2). Aliquots (10–50 μ g protein) of mitochondrial membrane fractions were then assayed for respiratory complex activities. In our previous report [36], H₂O₂ significantly decreased ubiquinol cytochrome C oxidoreductase (complex III) and coenzyme Q (CoQ) activities in osteoblasts. Therefore, we only measured the activities of these respiratory chain components.

Functional Imaging Assays

Osteoblasts were seeded in chamber slides at 1×10^4 cells/well. Cells were treated with H₂O₂ and other test compounds for 1 h. Then, the cells were incubated in fresh culture medium containing 2.5 μ M MitoSOX for 30 min. To assess the mitochondrial membrane potential, cells were co-stained with MT Green (100 nM) and TMRM (100 nM) for 30 min, according to our previous study [37].

Images were captured under the fluorescence microscope. Excitation wavelengths were 543 nm for MitoSOX and TMRM and 488 nm for MT Green. Post-acquisition processing was performed with NIH ImageJ software to measure and quantify fluorescence signals. Mitochondrial fluorescence intensities were quantified by an investigator blinded to the experimental groups. More than 100 clearly identifiable mitochondria in 10–15 randomly selected cells per experiment were measured in three independent experiments.

Data Analysis

Data are presented as means $\pm$ standard error of the mean. Statistical analysis was performed using Statview software (Version 5.0.1, SAS Institute, USA). Group mean differences were assessed by the Student's t-test for two comparisons or one-way analysis of variance with a Bonferroni/Dunn post-hoc test for multiple comparisons. $P < 0.05$ was considered to be significant.

Results

H₂O₂-induced Apoptosis and Mitochondrial Dysfunction in Osteoblasts

To determine the effect of ROS on survival of Saos-2 osteoblasts, we performed MTT cell viability assays. After H₂O₂ treatment, the number of viable cells decreased in a time and dose-dependent manner (Fig. 1A). Flow cytometric analysis showed a dose-dependent increase in the incidence of apoptosis. Although early apoptosis was detected after 0.1 mM H₂O₂ treatment, higher concentrations of H₂O₂ induced late apoptosis and a slight increase in necrosis (Fig. 1B, C). These proapoptotic effects were confirmed by TUNEL staining (Fig. 1D, E).

Bcl-2 family proteins, including Bcl-2 and Bax, play important roles in initiating the mitochondrial death cascade. The role of apoptosis-related Bcl-2 gene expression in H₂O₂-induced osteoblast apoptosis was examined by immunoblot analyses (Fig. 1F, G). Compared with the control group, H₂O₂ significantly decreased the expression of Bcl-2, while no significant differences were observed in Bax expression (Fig. 1F and I). In addition, Caspase-3 cleavage is a key and irreversible event in the progression of apoptosis. Exposure of Saos-2 cells to H₂O₂ caused an increase in the expression of cleaved Caspase-3 (Fig. 1F and H). These results indicate that H₂O₂-induced cell death occurs primarily through apoptosis in Saos-2 cells.

Mitochondrial dysfunction plays a cardinal role in OS-induced dysfunction of cell proliferation, differentiation and survival in osteoblasts. Consistent with our previous study

Fig. 1. H_2O_2 -induced apoptosis in osteoblasts. (A) Cell viability was determined by MTT reduction in osteoblasts in the presence of H_2O_2 . Error bars indicate SEM (n=6). (B) and (C) Flow cytometric quantification of apoptosis. Error bars indicate SEM (n=6). (D) and (E) TUNEL staining and assay. Error bars indicate SEM (n=300). (F) Representative immunoreactive bands for Bcl-2, Bax and Caspsae-3 in osteoblasts in the presence of H_2O_2 . Quantification of immunoreactive bands for Bcl-2 (G), Bax (H) and Caspsae-3 (I) relative to β -actin. Error bars indicate SEM (n=6). H_2O_2 -induced mitochondrial dysfunction in osteoblasts. Representative images showing MitoSOX staining (J) and quantification (K) in the indicated groups. Representative images with TMRM staining (L) and quantification (M) in the indicated groups. Complex III activity (N) and CoQ activity (O) in the indicated groups. Error bars indicate SEM (n=15).

[38], the staining intensity of MitoSOX, an indicator of mitochondrial ROS, was significantly increased in osteoblasts treated with H₂O₂ (Fig. 1J, K). The mitochondrial membrane potential, as indicated by the fluorescent dye TMRM, was significantly decreased in cells treated with H₂O₂ (Fig. 1L, M).

Oxidative phosphorylation enzyme complexes are essential for mitochondrial functions. Therefore, key enzymes associated with the respiratory chain were evaluated in this study. Cells treated with H₂O₂ had significantly decreased complex III (Fig. 1N) and CoQ (Fig. 1O) activities compared with control cells.

Curcumin attenuates H₂O₂-induced Apoptosis and Mitochondrial Dysfunction in Osteoblasts

Although curcumin has shown promising anti-apoptotic effects on various types of non-cancerous cells, its effects on OS-induced osteoblast apoptosis have been unknown. To determine whether curcumin protected osteoblasts from oxidative stress, we treated Saos-2 cells with various concentrations of curcumin prior to H₂O₂ exposure. As shown in Fig. 2A, curcumin treatment (50 μ M, 9 h) was not cytotoxic in Saos-2 cells, and the reduced cell viability was significantly rescued by curcumin in a dose-dependent manner ($P < 0.001$). However, no obvious differences were observed at concentrations of $>20 \mu$ M. Thus, we chose 20 μ M as our experimental concentration. Moreover, cell apoptosis induced by H₂O₂ was significantly decreased by curcumin pretreatment ($P < 0.05$) (Fig. 2B, C). As expected, curcumin preconditioning ameliorated H₂O₂-induced suppression of Bcl-2 protein synthesis and reduced cleavage of Caspase-3 (Fig. 2D–F). These data indicate that curcumin attenuates H₂O₂-induced apoptosis in osteoblasts.

Because mitochondria are a primary source of ROS generation and ROS accumulation affects mitochondrial functions, we determined whether mitochondrial ROS generation correlated with mitochondrial dysfunction. Indeed, the MitoSOX staining intensity was significantly increased in osteoblasts treated with H₂O₂. Curcumin treatment significantly attenuated mitochondrial ROS levels, as shown by a reduced MitoSOX intensity (Fig. 2G, H).

We characterized mitochondrial functions by evaluating the membrane potential and key enzymes of the respiratory chain in osteoblasts incubated under various conditions. Compared with cells receiving only H₂O₂, osteoblasts co-treated with curcumin had significantly increased membrane potentials (Fig. 2I, J) and activities of complex III (Fig. 2K) and CoQ (Fig. 2L).

Synthetic Antioxidant Compound EUK134 prevents H₂O₂-induced Apoptosis and Mitochondrial Dysfunction in Osteoblasts

Because mitochondrial OS was implicated in causing osteoblast dysfunction, we examined whether antioxidant treatments could prevent the osteoblast dysfunction induced by H₂O₂. As shown in Fig. 3A, osteoblasts treated with EUK134 and H₂O₂ had improved cell viability compared with those treated with H₂O₂ only. EUK134 treatment significantly inhibited cell apoptosis induced by H₂O₂, as shown by TUNEL staining (Fig. 3B, C). In addition, consistent with curcumin treatment, EUK134 treatment markedly recovered Bcl-2 protein expression and decreased cleaved Caspase-3 (Fig. 3D–F) in the cells compared with the H₂O₂-treated group.

As shown in Fig. 3, EUK134 treatment significantly suppressed mitochondrial ROS levels (Fig. 3G, H) and increased the mitochondrial membrane potential (Fig. 3I, J). Consistent with these results, addition of EUK134 enhanced complex III (Fig. 3K) and CoQ (Fig. 3L) activities compared with cells receiving H₂O₂ alone. These results indicate that EUK134 prevents mitochondrial abnormalities in osteoblasts subjected to OS.

Akt-GSK3 β Signaling is involved in H₂O₂-induced Apoptosis of Osteoblasts

The Akt-GSK3 β signaling pathway is involved in OS-induced cell apoptosis [34]. To determine whether this pathway is involved in H₂O₂-induced osteoblast apoptosis, we analyzed phosphorylation of Akt and GSK3 β by immunoblotting. Compared with untreated cells, osteoblasts treated with H₂O₂ had significantly less phosphorylated Akt (0.5-fold

Fig. 2. Curcumin attenuated H₂O₂-induced apoptosis in osteoblasts. (A) Cell viability determined by MTT reduction in osteoblasts in the presence of H₂O₂ with or without curcumin. Error bars indicate SEM (n=6). (B) TUNEL staining and (C) assay after curcumin treatment. Error bars indicate SEM (n=300). (D) Representative immunoreactive bands for Bcl-2 and Caspase-3 in osteoblasts with (+) or without (-) curcumin treatment in the presence of H₂O₂ (+) or culture medium (-). Quantification of immunoreactive bands for Bcl-2 (E) and Caspase-3 (F) relative to β -actin. Error bars indicate SEM (n=6). Curcumin attenuated H₂O₂-induced mitochondrial dysfunction in osteoblasts. Representative images showing MitoSOX staining (G) and quantification (H) in the indicated groups. Representative images showing TMRM staining (I) and quantification (J) in the indicated groups. Complex III activity (K) and CoQ activity (L) in the indicated groups. Error bars indicate SEM (n=15).

decrease) (Fig. 4A, B) and GSK3 β (0.1-fold decrease) (Fig. 4C, D), while total levels of Akt and GSK3 β were unchanged. Moreover, treatment with TDZD-8, a pharmaceutical inhibitor

Fig. 3. The synthetic antioxidant EUK134 attenuated H₂O₂-induced apoptosis in osteoblasts. (A) Cell viability determined by MTT reduction in osteoblasts in the presence of H₂O₂ with or without EUK134. Error bars indicate SEM (n=6). (B) TUNEL staining and (C) assay after EUK134 treatment. Error bars indicate SEM (n=300). (D) Representative immunoreactive bands for Bcl-2 and Caspsae-3 in osteoblast with (+) or without (-) EUK134 treatment in the presence of H₂O₂ (+) or culture medium (-). Quantification of immunoreactive bands for Bcl-2 (E) and Caspsae-3 (F) relative to β -actin. EUK134 attenuated H₂O₂-induced mitochondrial dysfunction in osteoblasts. Error bars indicate SEM (n=6). Representative images showing MitoSOX staining (G) and quantification (H) in the indicated groups. Representative images showing TMRM staining (I) and quantification (J) in the indicated groups. Complex III activity (K) and CoQ activity (L) in the indicated groups. Error bars indicate SEM (n=15).

of GSK3 β (Fig. 4G), significantly inhibited the H₂O₂-induced cytotoxicity and apoptosis based on MTT assays results (Fig. 4H) and TUNEL staining (Fig. 4K, L), respectively. Furthermore,

Fig. 4. Involvement of Akt-GSK3 β signaling in H₂O₂-induced apoptosis in osteoblasts. Representative immunoblots of phospho-Akt (p-Akt) and total Akt (Akt) (A), phospho-GSK3 β (p-GSK3 β) and total GSK3 β (GSK3 β) (C) in osteoblast in the presence of H₂O₂. Quantification of immunoreactive bands for p-Akt relative to Akt (B) and p-GSK3 β relative to GSK3 β (D) in osteoblasts, after the indicated treatments, using NIH Image J software; Densitometry of immunoreactive bands for p-Akt and Akt (E), p-GSK3 β and GSK3 β (I) in osteoblasts with (+) or without (-) LY294002 treatment in the presence of H₂O₂ (+) or culture medium (-). Repre-

sentative immunoblots are shown at the bottom. (F) Cell viability determined by MTT reduction in osteoblasts with (+) or without (-) LY294002 treatment in the presence of H₂O₂ (+). Representative immunoreactive bands and relative levels of p-GSK3 β and GSK3 β (G), p-Akt and Akt (J) in osteoblasts with (+) or without (-) TDZD-8 treatment in the presence of H₂O₂ (+) or culture medium (-). Representative immunoblots are shown at the bottom. (H) Cell viability determined by MTT reduction in osteoblasts with (+) or without (-) TDZD-8 treatment in the presence of H₂O₂ (+). Error bars indicate SEM (n=6). (K) TUNEL staining and (L) assay in the indicated groups. Error bars indicate SEM (n=300). (M) Representative immunoreactive bands for Bcl-2 and Caspsae-3 in osteoblasts with (+) or without (-) TDZD-8 treatment in the presence of H₂O₂ (+) or culture medium (-). Quantification of immunoreactive bands for Bcl-2 (N) and Caspsae-3 (O) relative to β -actin. (P) Representative immunoblots of Bcl-2 and Caspsae-3 in osteoblasts with (+) or without (-) LY294002 treatment in the presence of H₂O₂ (+) or culture medium (-). (Q-R) Quantification of immunoreactive bands for Bcl-2 and Caspsae-3 relative to β -actin in osteoblast. Error bars indicate SEM (n=6).

TDZD-8 preconditioning increased Bcl-2 protein expression and decreased cleaved Caspase-3 (Fig. 4M–O). In contrast, treatment with LY294002, a PI3K inhibitor, decreased the expression of p-Akt (Fig. 4E) and significantly increased the percentage of apoptotic cells in the control group not subjected to OS (Fig. 4K, L). Consistently, western blot analysis revealed that cells incubated with LY294002 and H₂O₂ showed further increased expression of cleaved Caspase-3 (Fig. 4P and R) compared with H₂O₂ only, although the change in Bcl-2 expression had no statistical significance (Fig. 4P, Q). Moreover, it inhibited Akt phosphorylation (Fig. 4E, F) and amplified the effect of H₂O₂ on induction of apoptosis in osteoblasts (Fig. 4K, L). Conversely, LY294002 treatment significantly decreased the expression of phosphorylated GSK3 β (Fig. 4I), whereas TDZD-8 did not affect Akt expression (Fig. 4J), which further supports that Akt regulates phosphorylation of GSK-3 β . Taken together, these data demonstrate that Akt-GSK3 β signaling is involved in H₂O₂-induced apoptosis of osteoblasts.

Curcumin attenuates H₂O₂-induced Apoptosis in Osteoblasts through Akt-GSK3 β

To gain an insight into the mechanism of the preventative effects of curcumin on H₂O₂-induced osteoblast apoptosis, the Akt-GSK3 β signaling pathway was analyzed by immunoblotting. Interestingly, addition of curcumin or EUK134 resulted in significantly increased levels of p-Akt (Fig. 5A and C) and p-GSK3 β (Fig. 5B and D) during H₂O₂-induced apoptosis of osteoblasts. However, the anti-apoptotic effect of curcumin was largely abolished by administration of LY294002 (Fig. 5G–K). This result suggests that curcumin treatment is sufficient to promote Akt activation and inhibit GSK3 β during OS.

Relationship between the Akt-GSK3 β Signaling Pathway and Mitochondrial Dysfunction in OS-induced Osteoblast Apoptosis

Because Akt-GSK3 β signaling is known to function as a prosurvival signal, whether mitochondrial dysfunction is related to Akt-GSK3 β signaling pathway was determined in the OS-induced osteoblastic apoptosis model. As expected, the results showed that TDZD-8 significantly attenuated mitochondrial ROS levels (Fig. 6A, B) and increased the mitochondrial membrane potential (Fig. 6C, D) and activities of complex III (Fig. 6E) and CoQ (Fig. 6F), which were impaired by OS. However, these mitochondrial dysfunction indexes were further improved by LY294002 cotreatment with H₂O₂, although only a significant difference was observed in the mitochondrial membrane potential (Fig. 6G–L).

Discussion

Several lines of evidence demonstrate the involvement of increased OS in osteoporosis caused by aging and estrogen deficiency [39]. Moreover, osteoblast apoptosis induced by OS is critical for the development of osteoporosis [3]. In our study, we found that administration

Fig. 5. Curcumin attenuated H₂O₂-induced apoptosis in osteoblasts through Akt-GSK3 β signaling. Representative immunoblots of p-Akt and Akt (A-C), p-GSK3 β and GSK3 β (B-D) and relative levels of p-Akt and Akt, p-GSK3 β and GSK3 β in osteoblasts with (+) or without (-) curcumin/EUK134 treatment in the presence of H₂O₂ (+) or culture medium (-). (E) Cell viability determined by MTT reduction in osteoblasts treated with (+) or without (-) curcumin in the presence of H₂O₂ (+) or LY294002 (+). (F) Densitometry of immunoreactive bands for p-Akt and Akt in osteoblasts with (+) or without (-) curcumin treatment in the presence of H₂O₂ (+) or LY294002 (+). Error bars indicate SEM (n=6). (G) TUNEL staining and (H) assay in the indicated groups. Error bars indicate SEM (n=300). (I) Representative immunoreactive bands for Bcl-2 and Caspase-3 in the indicated groups; Quantification of immunoreactive bands for Bcl-2 (J) and Caspase-3 (K) relative to β -actin. Error bars indicate SEM (n=6).

Fig. 6. The relationship between Akt-GSK3 β signaling pathway and mitochondrial dysfunction in OS induced osteoblast apoptosis. Representative images showing MitoSOX staining (A) and quantification (B), TMRM staining (C) and quantification (D), Complex III activity (E) and CoQ activity (F), in osteoblasts with (+) or without (-) TDZD-8 treatment in the presence of H₂O₂ (+). Representative images showing MitoSOX staining (G) and quantification (H), TMRM staining (I) and quantification (J), Complex III activity (K) and CoQ activity (L), in osteoblasts with (+) or without (-) LY294002 treatment in the presence of H₂O₂ (+) or culture medium (-). Error bars indicate SEM (n=15).

of H₂O₂, a classical generator of OS, significantly decreased osteoblast viability and induced apoptosis. These results demonstrate that H₂O₂ exerts a profound cytotoxic effect that

reduces osteoblast survival, indicating that it causes defective bone functions. However, curcumin treatment improved cell viability by conferring resistance to apoptosis induced by OS. Furthermore, curcumin promoted osteoblast survival through prevention of OS-induced mitochondrial dysfunction and activation of Akt-GSK3 β signaling. Accordingly, our data indicate that curcumin is a beneficial agent to prevent primary or secondary osteoporosis by enhancing osteoblast survival.

Mitochondria are unique and irreplaceable organelles in eukaryotic cells, which not only act as power plants for energy production, but also play important roles in cell death, functions, and survival [40]. In osteoblasts, mitochondria are specialized for calcium transport and involved in calcification of the extracellular matrix [41]. Our previous study demonstrated that pharmaceutical and genetic blockade of dynamic-related protein 1 (Drp1), the primary regulator of mitochondrial fission, significantly protects against H₂O₂-induced osteoblast damage, as indicated by increased cell viability, improved mineralization, and restored mitochondrial functions [38]. Therefore, mitochondrial dysfunction in osteoblasts has been both directly and indirectly implicated in osteoporosis. Consistent with our previous findings [38], we observed significant reductions in the mitochondrial membrane potential and respiratory chain complex activity (complex III), while production of mitochondrial ROS was significantly increased in a cell culture model of H₂O₂-induced oxidative damage in osteoblasts. Complex III of the mitochondrial respiratory chain might play a major role in ROS production. Inhibition of electron transport causes a collapse of the proton gradient across the mitochondrial inner membrane, thereby decreasing the mitochondrial membrane potential. This effect results in opening of mitochondrial permeability transition pores (mPTPs), the release of proapoptotic proteins into the cytoplasm, and apoptotic cell death [42].

Flow cytometry and TUNEL staining combined with immunoblotting of Bcl-2 and Caspase-3 showed that H₂O₂ caused cell apoptosis in a dose and time-dependent manner in osteoblastic Saos-2 cells. These results were in agreement with a study by Yang et al. [8]. However, investigators from the Kim laboratory have shown that antimycin A induces mitochondrial dysfunction in osteoblasts through suppression of complex IV activity [43]. The discrepancies among these data might be explained by differences in cell types and oxidative stress generators. The Kim group also suggested that antimycin A causes mitochondrial depolarization and ATP depletion that might induce cell necrosis instead of apoptosis. Nonetheless, all these findings strongly support a pivotal role of mitochondrial ROS in mediating mitochondrial dysfunction leading to osteoblast apoptosis during osteoporosis. Furthermore, these observations suggest that novel therapeutic strategies to reduce mitochondrial dysfunction during osteoporosis, such as mitochondria-targeted antioxidants, might significantly prevent development of osteoblast apoptosis in osteoporotic patients.

Curcumin has several pharmacological and biological properties, and evidence has supported its potential protective function in osteoporosis [19–20]. Curcumin might decrease bone resorption by inhibition of osteoclast proliferation, differentiation, and activity as well as by promotion of osteoclast apoptosis [44–47]. Although no consistent conclusion has been reached concerning its benefits in osteogenesis, our present findings demonstrate that curcumin significantly decreases osteoblast apoptosis induced by H₂O₂ with similar effectiveness as the antioxidant compound EUK134. The cytoprotective effects of curcumin were caused not only by its antioxidant properties, but also other mechanisms. We found that pretreatment with curcumin significantly attenuated the mitochondrial dysfunction induced by H₂O₂, as evidenced by decreased mitochondrial ROS, improved complex III and CoQ activities, and an improved mitochondrial membrane potential. Recent research has revealed that curcumin might have value in prevention and therapy of numerous disorders including neurodegenerative diseases, myocardial ischemia, reperfusion injury, and diabetes. Its potential in all these disorders has been attributed to its effects against mitochondrial dysfunction induced by pathological factors [48]. Conversely, in cancer cells, curcumin induces apoptosis through mitochondrial pathways [49]. Therefore, these previous findings, in combination with our new data, support the conclusion that curcumin alleviates osteoblast

apoptosis induced by OS through prevention of mitochondrial dysfunction.

Akt is an initiator of downstream pathways that inhibit apoptosis [50]. Moreover, Akt phosphorylates GSK3 β to prevent opening of mPTPs, an important master regulator of cell death. Therefore, we examined the Akt-GSK3 β signaling pathway to elucidate mechanisms by which curcumin modulates mitochondrial functions and attenuates apoptosis in osteoblasts. We found that curcumin preconditioning significantly enhanced not only phosphorylation of Akt but also that of GSK3 β at Ser9. Previous studies have demonstrated that

GSK3 β phosphorylation at Ser9 leads to interactions with mPTP regulators and inhibits mPTP opening during reperfusion. These effects decrease activation of the cytochrome c-Caspase-3/9 apoptosis pathway, reducing cell apoptosis and protecting hepatic cells from ischemia/reperfusion injury via activation of Akt-GSK3 β signaling [51,52].

Our data indicated that blocking PI3K by LY294002, which led to downstream phosphorylation of Akt, amplified H₂O₂-induced apoptosis. The anti-apoptotic effect of curcumin was significantly abolished by LY294002, while inhibition of GSK3 β significantly suppressed apoptosis induced by H₂O₂. Taken together, these data demonstrate that curcumin alleviates osteoblast apoptosis induced by OS through activation of Akt-GSK3 β signaling. Furthermore, Akt and GSK3 β phosphorylation was associated with mitochondrial dysfunction in OS-induced osteoblast apoptosis. In our previous study [35], mitochondrial abnormalities were induced by imbalanced mitochondrial fusion and fission via a GSK3 β -Drp1-dependent mechanism. Modulation of the Drp1 pathway or inhibition of GSK3 β activity also restores hippocampal long-term potentiation impaired in diabetic mice [35]. Moreover, blockade of Drp1 rescues OS-induced osteoblast dysfunction [38]. Thus, in future studies, investigation of the GSK3 β -Drp1 pathway should provide more insights into the complex regulation of cell survival and apoptosis of osteoblasts subjected to OS.

In the present study, we used a relatively high concentration of curcumin (20 μ M) and a short treatment period. No significant adverse effects have been observed in humans who consumed 500-800 mg turmeric powder per day [53]. In long term studies, curcumin administered through diet was safe and showed protective activities. Even very high doses of curcumin (8 g/day) did not cause side effects [54]. However, there is still limited information about the potentially negative effects of curcumin on cell apoptosis and normal cell senescence [55, 56]. Therefore, further investigation will be needed to explore effective ways to increase curcumin bioavailability.

OS-induced apoptosis of osteoblasts is a complicated process with many aspects of the damage related to survival signaling and mitochondria. Therefore, our experiments addressed only some of the mechanistic pathways involved in this process. Further research will be required to investigate additional mechanisms to modulate the effects of OS on osteoblasts. In addition, we established a H₂O₂-induced oxidative cell injury model that has been frequently used in previous studies. H₂O₂ functions as both a signaling molecule [57] and cytotoxic agent under pathological conditions. The definite role of H₂O₂ in our study needs further exploration.

In conclusion, our study demonstrates that curcumin treatment attenuates OS-induced apoptosis of osteoblasts, possibly through prevention of mitochondrial dysfunction and activation of the Akt-GSK3 β signaling pathway (Fig. 7). These findings provide additional evidence for the clinical use of curcumin in prevention or treatment of osteoporosis.

Fig. 7. Working hypothesis: curcumin administration ameliorated oxidative stress-induced apoptosis in osteoblasts through preserving mitochondrial function and activating Akt-GSK3 β signaling.

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Disclosure Statement

The authors declare no conflict of interest.

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