Simvastatin protects human osteosarcoma cells from oxidative stress-induced apoptosis through mitochondrial-mediated signaling

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Abstract. Apoptosis of osteoblasts has been proposed as the common basis of osteoporosis, with oxidative stress as the major cause. This study was performed to investigate the protective effect of simvastatin (0.001-0.1 μM) on 100 μM hydrogen peroxide (H_2O_2)-mediated oxidative stress-induced apoptosis in human osteosarcoma (MG63) cells and the molecular mechanisms involved. Cell apoptosis was evaluated by observation of morphological changes and Annexin V-propidium iodide double staining followed by flow cytometric analysis. MTS assays were used to evaluate cell viability. To investigate the underlying molecular mechanisms, the expression of caspase-3, caspase-9 and Bcl-2 were analyzed by Western blotting. Following stimulation with H_2O_2 for 24 h, a high proportion of MG63 cells underwent apoptosis, while a dose-dependent inhibition of apoptosis was observed in the presence of simvastatin. A significant, dose-dependent reduction in the expression of caspase-3 and caspase-9 protein induced by H_2O_2 in MG63 cells was observed in response to simvastatin and the Bcl-2 levels were increased. In conclusion, simvastatin protects MG63 cells from oxidative stress-induced apoptosis through downregulation of caspase-3 and caspase-9 activation and upregulation of Bcl-2 expression, suggesting a protective effect in osteoporosis.

Introduction

Osteoporosis has been defined as a systemic skeletal disease characterized by gradual loss and microarchitectural deterioration of bone tissue, resulting in increased bone fragility and susceptibility to fracture (1). Osteoblasts are responsible for bone formation while osteoclasts are involved in bone resorption. Conditions such as postmenopausal osteoporosis are associated with significant changes in bone turnover; bone formation decreases and bone resorption increases or remains the same, resulting in net bone loss (2,3).

Oxidative stress, resulting from excessive levels of reactive oxygen species (ROS), represents a major cause of cellular damage and death in a plethora of pathological conditions, including osteoporosis. This is associated with distinct increases in blood levels of oxidative stress markers (4-6). The main oxygen species responsible for oxidative stress are hydrogen peroxide (H_2O_2), the free radical superoxide anion (O_2^-) and the hydroxyl radical (OH^-). Osteoblasts produce antioxidants such as glutathione peroxidase that protect against ROS (7) and osteoclast-generated superoxide contributes to bone degradation (8). In ovariectomized rats, a model of postmenopausal osteoporosis, increased levels of lipid peroxidation and H_2O_2 and decreased levels of enzymatic antioxidants were detected in tissue homogenates from the femora (9). Furthermore, antioxidant enzyme GPX1 gene polymorphisms are associated with low bone mineral density (BMD) and increased bone turnover markers (10).

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-COA) reductase inhibitor, simvastatin, is a widely used cholesterol-lowering drug that inhibits hepatic cholesterol biosynthesis. Recent studies have shown the beneficial effects of statins on BMD (11,12). It has been suggested that a number of statin drugs, including simvastatin, increase the mRNA expression of bone morphogenetic protein 2 (BMP-2) in osteoblasts and are involved in promoting osteoblast differentiation with a subsequent increase in bone formation, when injected subcutaneously over the murine calvaria (13). Numerous animal and human studies have been performed to elucidate the clinical importance of statins. The majority of experimental and epidemiological studies have shown statins to have beneficial effects on bone metabolism, as evaluated by BMD (14-16) and fracture risk (17-19). Statins have therefore been proposed as potential agents in the treatment of osteoporosis.

The MG63 human osteogenic sarcoma cell line is widely studied as a typical example of human osteoblast-like cells (20,21). This study aimed to evaluate the effects of simv-

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astatin on MG63 cell death in response to oxidative stress by investigation of Bcl-2 and caspase-9 expression and caspase-3 activity.

Materials and methods

Reagents and antibodies. H_2O_2 was purchased from Sigma (St. Louis, MO, USA) and prepared immediately prior to use in phosphate-buffered saline (PBS) at 100 μM. A stock solution of simvastatin (Zhejiang Xinchang Pharmaceutical Co., Ltd.) dissolved in dimethyl sulfoxide (DMSO) at 10 U/ml was prepared and stored at -30°C. Working solutions of simvastatin (0.1, 0.01 and 0.001 μM) were prepared from the stock. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxym ethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was purchased from Promega (San Luis Obispo, CA, USA). Annexin V/FITC kits were purchased from Bender MedSystems GmbH (Vienna, Austria). Antibodies used for Western blot analysis were rabbit anti-active caspase-3 and caspase-9 polyclonal antibodies (Chemicon, Temecula, CA, USA) recognizing only the cleaved large subunit (17 kDa, caspase-3; 37 kDa, caspase-9), and rabbit anti-Bcl-2 polyclonal antibodies were purchased from Cell Signaling Technologies (Beverly, MA, USA). Anti-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caspase-3 and caspase-9 colorimetric activity assay kits and recombinant active caspase-3, and caspase-9 standards were purchased from Chemicon.

Cell culture and treatment. The osteosarcoma cell line, MG63 cell, was obtained from the State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Zhejiang University, China. MG63 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified 5% CO_2 atmosphere at 37°C. The cells were passaged every 3 days. MG63 cells were seeded at a density of 2×10^6 cells/dish in 100-mm dishes in DMEM with 2% calf serum. Confluent cell cultures were incubated in the presence or absence of simvastatin (0, 0.001, 0.01 and 0.1 μM) for 2 h followed by stimulation with 100 μM H_2O_2 and further incubation for 24 h. MG63 cells were observed for morphological changes.

Flow cytometric analysis. Recovery of cells was monitored by examination of the levels of apoptosis 24 h following H_2O_2 treatment. Annexin V binding and propidium iodine (PI) staining were determined by flow cytometry. Cells were treated with 100 μM H_2O_2 for 24 h, washed with ice-cold PBS and double-stained with FITC-conjugated Annexin V protein and PI for 20 min. Flow cytometry was performed with a 488-nm laser coupled to a cell sorter (FacsCalibur; BD Biosciences, San Jose, CA, USA). Cells stained with PI and Annexin V were considered necrotic, while cells stained only with Annexin V were considered apoptotic.

MTS assay. MG63 cells (5,000 cells/well) were seeded in 96-well microtiter plates. Following incubation with 0.001, 0.01 and 0.1 μM simvastatin for 2 h, cells were treated with 100 μM H_2O_2 for a further 24 h. Subsequently, 20 μl MTS
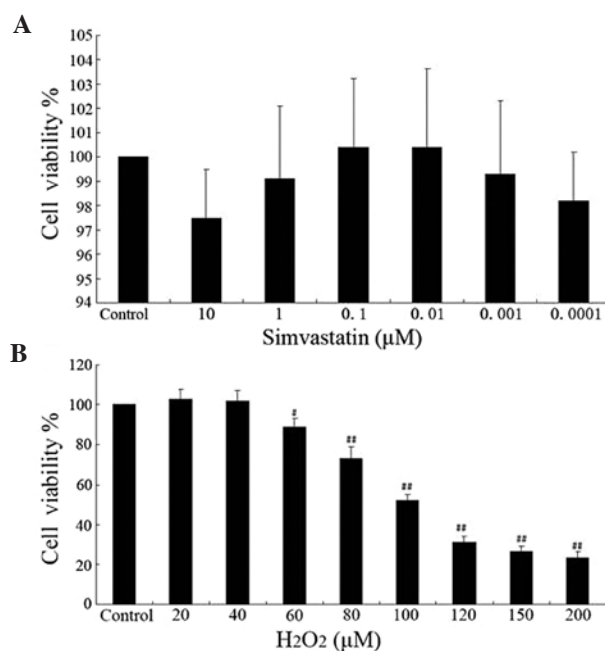

Figure 1. Determination of experimental conditions. MG63 cells were cultured with various concentrations of (A) simvastatin or (B) H_2O_2 for 24 h and cell viability was assessed by MTS assay. #$p<0.05$ compared with control group, ##$p<0.01$ compared with control group.

solution was added to each well, and the plates were incubated for 3 h at 37°C. The absorbance was measured at 490 nm and used to calculate the relative ratio of cell viability.

Western blot analysis. Protein expression of activated caspase-3, caspase-9 and Bcl-2 was analyzed by Western blotting. Cytoplasmic extracts were prepared in lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.9), 0.5% Triton X-100, 0.6% NP-40, and protease inhibitors, 1 mg/ml leupeptin, 1 mg/ml pepstatin A and 2 mg/ml aprotinin. Protein content was determined using a detergent compatible (DC) protein assay kit (Bio-Rad, Richmond, CA, USA). Protein (40 μg mixed with 2X SDS sample buffer) was separated by 10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) prior to incubation for 2 h in blocking buffer (PBS with 7.5% non-fat dry milk, 2% BSA, 0.1% Tween). Active caspase-3, caspase-9 and Bcl-2 proteins were detected by incubation with appropriate primary antibodies (1:400 in blocking buffer) overnight at 4°C. Subsequently, membranes were washed (PBS with 0.1% Tween-20) and incubated with peroxidase-conjugated goat anti-rabbit IgG (Pierce, 1:10000 dilution in blocking buffer) for 1 h at room temperature. Membranes were then washed in PBS and developed using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Uppsala, Sweden).

Statistical analysis. All experiments were performed on three or more occasions and results are expressed as the mean ± SD. Statistical significance was analyzed by one-way analysis of variance (ANOVA) and $p<0.05$ was considered to be statistically significant.
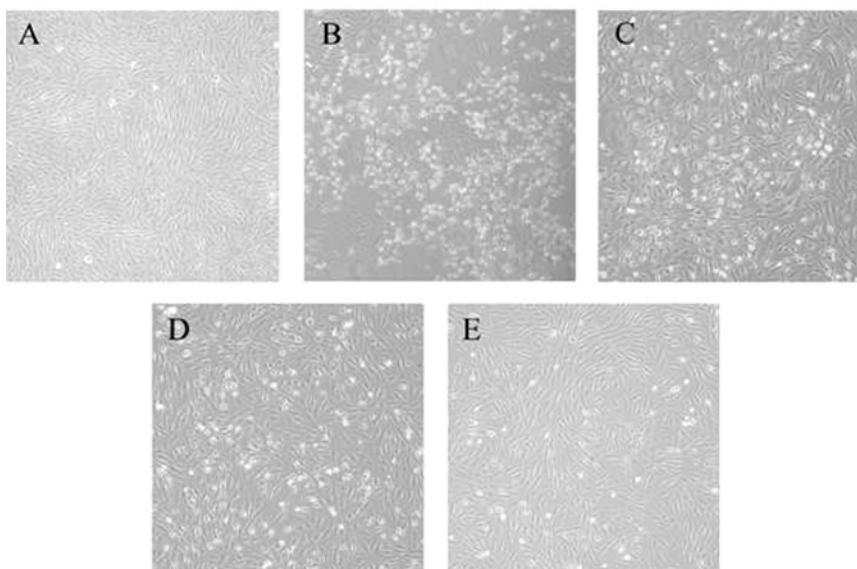
Figure 2. Simvastatin inhibited morphological changes of MG63 cells induced by H_2O_2. When treated with H_2O_2 (100 μM) for 24 h, a large proportion of cells exhibited signs of apoptosis such as detachment and cytoplasmic condensation leading to rounding. (A) MG63 cells without H_2O_2 or simvastatin treatment (control). (B) MG63 cells treated with 100 μM H_2O_2. (C-E) MG63 cells treated with 100 μM H_2O_2 and 0.001, 0.01 or 0.1 μM simvastatin, respectively. (magnification, x10).

Results

Selection of conditions for simvastatin and H_2O_2 treatment. MG63 cells were cultured in DMEM supplemented with simvastatin (0.0001, 0.001, 0.01, 0.1, 1 or 10 μM) for 24 h. No detrimental effects on cells were observed (Fig. 1A). Three concentrations of simvastatin were selected (0.001, 0.01 and 0.1 μM) for further investigation based on these observations. The working concentration of H_2O_2 was determined in a series of dose-response MTS assays of viability. Treatment with increasing concentrations of H_2O_2 for 24 h caused a dose-dependent loss of cell viability (Fig. 1B). Treatment with 100 μM H_2O_2 reduced cell viability (survival rate, 52.2±3.0%). This concentration was used in subsequent experiments for the investigation of the effects of simvastatin on apoptosis.

Morphological changes of MG63 cells. Morphological changes in MG63 cells treated with H_2O_2 (100 μM) were observed in the presence or absence of simvastatin (0.001, 0.01 and 0.1 μmol/l). Following H_2O_2 treatment, a high proportion of cells exhibited apoptosis-like morphological changes, such as detachment, and cytoplasmic condensation leading to rounding. However, the proportion of cells with abnormal morphology suggestive of apoptosis decreased with increasing simvastatin concentration (Fig. 2).

Simvastatin blocked MG63 cell apoptosis induced by H_2O_2. The effects of simvastatin on H_2O_2-induced cell apoptosis were quantified by flow cytometric analysis, and the percentage of apoptotic cells detected by Annexin V-FITC and PI double staining. Cell viability was determined by MTS assay. Significantly increased apoptosis was observed in MG63 cells treated with 100 μM H_2O_2 compared with the control (27.77±2.31 vs. 1.9±0.98%, p<0.01). The percentages of apoptotic cells treated with H_2O_2 and simvastatin at 0.001,
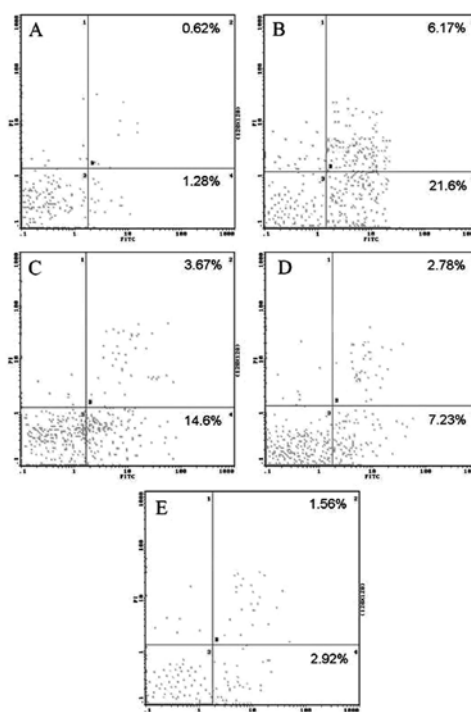

Figure 3. Simvastatin blocked H_2O_2-induced apoptosis in MG63 cells. Apoptotic cells were detected by Annexin V and propidium iodine double staining. (A) Untreated MG63 cells (control). (B) MG63 cells treated with 100 μM H_2O_2. (C-E) MG63 cells treated with 100 μM H_2O_2 and 0.001, 0.01 or 0.1 μM simvastatin, respectively.

0.01 or 0.1 μM were 18.27±1.72, 10.01±2.11 and 4.48±1.55%, respectively. Simvastatin significantly reduced the percentage of apoptotic cells in a dose-dependent manner compared with the H_2O_2 treated cells (Fig. 3). Significantly decreased viability was observed by MTS assay in MG63 cells treated
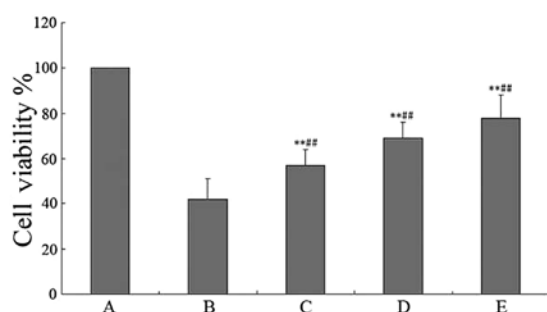
Figure 4. Simvastatin blocked apoptosis of MG63 cells induced by H_2O_2. Cell viability was evaluated by MTS assay. Simvastatin significantly increased cell viability compared with H_2O_2-treated cells in a dose-dependent manner (p<0.01 at all three concentrations). (A) Untreated MG63 cells (control). (B) MG63 cells treated with 100 μM H_2O_2. (C-E) MG63 cells treated with 100 μM H_2O_2 and 0.001, 0.01 or 0.1 μM simvastatin, respectively; **p<0.01 compared with H_2O_2 alone; ##p<0.01 compared with the control.

with 100 μM H_2O_2 compared with the control (42.3±9.1 vs. 99.5±2.3%, p<0.01). The percentages of viable cells following treatment with H_2O_2 and simvastatin at 0.001, 0.01 or 0.1 μM were 57.2±6.9, 69.4±7.0 and 78.7±10.4%, respectively. Simvastatin significantly increased the percentage of cell viability in a dose-dependent manner compared with the H_2O_2-treated cells (p<0.01 at all three concentrations) (Fig. 4).

Simvastatin prevented H_2O_2-induced downregulation of Bcl-2 and upregulation of caspase-3 and caspase-9 activation. The mechanism underlying the observed protective effects of simvastatin on MG63 cells against H_2O_2-induced apoptosis was investigated by analysis of the cleaved form of caspase-3 (17 kDa), caspase-9 (37 kDa) and Bcl-2 (28 kDa) by Western blot analysis. Simvastatin at concentrations >0.001 μM significantly upregulated the protein expression of caspase-3 and caspase-9 in MG63 cells following 24 h of H_2O_2 treatment and prevented H_2O_2-induced downregulation of Bcl-2. A direct correlation was observed between the magnitude of this effect and the concentration of simvastatin (Fig. 5).

Discussion

ROS are free radical-containing molecules derived from oxygen and its products. Recent studies have reported increased ROS as a significant factor in the occurrence of senile, post-menopausal and other types of primary osteoporosis (6,22). A negative correlation has been reported between the level of oxidative stress and bone density in the elderly (6). Related data showed that ROS promoted activation of osteoclast and bone resorption (23), and inhibited proliferation and differentiation of osteoblasts (24). In this study a dose-dependent reduction in viability was detected by MTS assay in cells treated with increasing concentrations of H_2O_2 for 24 h. Based on these observations cells were treated with 100 μM H_2O_2 in subsequent experiments.

Statins are 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-COA) reductase inhibitors, widely used in the clinic for regulation of cholesterol levels in the blood. In recent years, statins have been shown to stimulate osteoblast proliferation *in vitro*, and to promote new bone formation in the skull and os
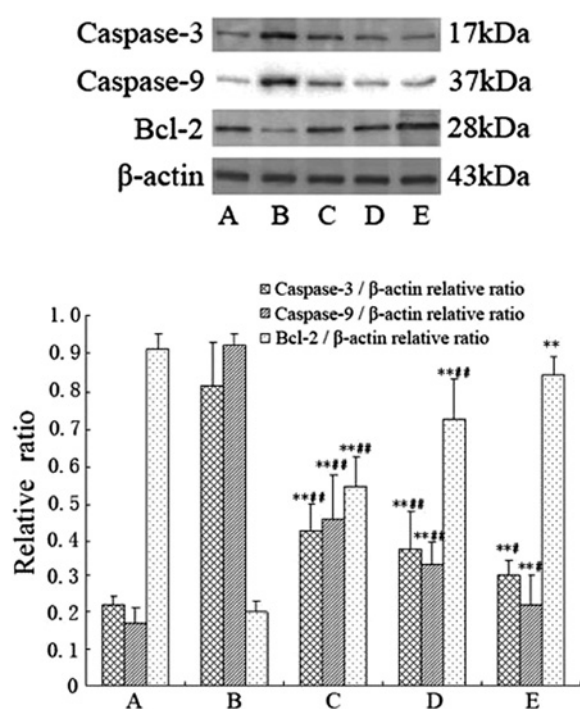

Figure 5. Effect of simvastatin on the expression of proteins in H_2O_2-treated MG63 cells. The expression of caspase-3, active caspase-9 and Bcl-2 in treated MG63 cells was analyzed by Western blot analysis. Top panel: Ratio of the band of interest and the β-actin or α-tubulin band. Bottom panel: (A) Untreated MG63 cells (control). (B) MG63 cells treated with 100 μM H_2O_2. (C-E) MG63 cells treated with 100 μM H_2O_2 and 0.001, 0.01 or 0.1 μM simvastatin, respectively; **p<0.01 compared with H_2O_2 treatment alone, ##p<0.01 compared with control.

integumentale of newborn rats (13). Statins stimulate increased expression of the BMP-2 gene in osteoblasts, and simvastatin has been suggested to activate the protein kinase pathway and resist the inhibitory action of TNF-α on BMP-2-induced osteoblast differentiation by regulating Smad signaling and Ras/Rho mitosis (25,26). Statins are associated with a variety of other activities including induction of osteoblast differentiation (27), resistance of the inhibitory effects on osteoblast growth and enhancement of osteoclast activity induced by IL-1, IL-6, TNF-α and other inflammatory factors (28), inhibition of matrix metalloproteinase activity (29), upregulation of vascular endothelial growth factor (VEGF) (30), enhanced activity of endothelial nitric oxide synthase (eNOS) (31) and inhibition of differentiation of adipose cells from multipotential stem cells (32).

In this study, no significant differences in the growth and cytotoxicity of MG63 cells in the simvastatin-treated groups (0.001, 0.01, 0.1, 1, and 10 μM) were observed compared to those of the control group. Simvastatin doses of 0.001, 0.01, and 0.1 μM were chosen as the experimental model conditions, which was in accordance with the conventional C_{max} dose (ng/ml) used in the clinic. Observation of cell morphology and viability revealed that the proportion of apoptotic cells induced by H_2O_2 decreased with increasing simvastatin concentration. Similarly, flow cytometric data confirmed simvastatin-associated inhibition of MG63 cells apoptosis induced by H_2O_2. It may be speculated that these protective effects contribute to the antioxidant activity of this drug.

Apoptosis progresses via a series of complex procedures regulated by caspases, which are a group of proteases known as apoptosis effectors (33). Caspases are activated by three main activation pathways: the mitochondrial (34), death receptor (35) and endoplasmic reticulum pathways (36). Activation of caspase-9, the most upstream protease in the mitochondrial pathway is particularly significant in activation of the whole endogenous apoptosis pathway. Following release from mitochondria, cytochrome c combines and activates apoptotic protease activating factor 1 (Apaf-1) in a deoxyadenosine triphosphate (dATP)-dependent process. Activated Apaf-1 combines with and activates caspase-9 proenzyme, which cleaves the caspase-3 proenzyme to produce the activated caspase-3 tetramer (37), which is the most critical effect protease (38).

Bcl-2 functions as a key regulator of apoptosis by modulation of mitochondrial function (39). ROS increase mitochondrial permeability, resulting in cytochrome c release. Bcl-2 family member proteins regulate the release of mitochondrial cytochrome c during oxidative stress in MG63. Cell survival is enhanced when Bcl-2 expression is relatively high. Caspases are downstream of the Bcl-2 family in the apoptotic cascade. Pro-caspase-3 is cleaved by active caspase-9 to active caspase-3, which along with caspases-6 and -7 are 'executioner' caspases, which activate a DNase responsible for the fragmentation of oligonucleosomal DNA. In this study simvastatin was shown to upregulate H_2O_2-induced expression of Bcl-2 with concomitant downregulation of active caspase-9 expression and delayed reduction in caspase-3 activation. These results indicated that the anti-apoptotic effect of simvastatin was due to mitigated H_2O_2 stress-induced mitochondrial dysfunction.

In conclusion, this study demonstrated that simvastatin rescues MG63 cells from H_2O_2-induced cell death, and that this protective effect is related to its antioxidant effects. Notably, the mechanism by which simvastatin prevents apoptosis appears to involve downregulation of H_2O_2-induced caspase-3 and caspase-9 activation and upregulation of Bcl-2 expression. These *in vitro* data suggest that simvastatin protects MG63 cells against H_2O_2-induced apoptosis by preventing activation of the mitochondrial pathway, thus suggesting that simvastatin may have a significant role in preventing oxidant-induced osteoporosis. However, further investigation is required to determine the upstream molecular mechanisms as well as the *in vivo* relevance of these observations.

Acknowledgements

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