Pterostilbene promotes the osteogenic differentiation of MC3T3-E1 cells inhibited by H$_2$O$_2$
by activating the AMPK signaling pathways

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Abstract

Osteoporosis is one of the common degenerative skeletal diseases whose essential mechanism is the imbalance between bone resorption and formation. Currently, the close coordination of signaling pathways at genetic level during bone development is a hot topic of osteoporosis research. The present study is focused on whether pterostilbene protects against hydrogen peroxide (H$_2$O$_2$)-induced osteoblastic cell apoptosis, oxidative stress, and reveals the related underlying mechanisms. MC3T3-E1 osteoblastic cells were cultured and treated with different concentrations of H$_2$O$_2$ (0–1 mM) along with or without pterostilbene, and MTT assay or Annexin V-FITC/propidium iodide staining was applied for measuring cell viability and apoptosis. The in vitro cellular antioxidant analysis was performed using 2,7-dichlorodihydrofluorescein diacetate assay and enzyme-linked-immunosorbent serologic assay against glutathione peroxidase and malondialdehyde. In addition, cellular alkaline phosphatase activity was executed by Alizarin Red S staining. Western blot assay was conducted to determine the expression levels of osteogenic-related markers, including type I collagen, osteopontin, runt-related transcription factor 2, and the 5’ adenosine monophosphate-activated protein kinase (AMPK) signaling pathways related proteins. The key finding was that pterostilbene could attenuate the H$_2$O$_2$-induced cellular apoptosis and oxidative stress. Pterostilbene also promoted osteogenic differentiation in H$_2$O$_2$-treated MC3T3-E1 cells through activation of the AMPK pathway. In conclusion, pterostilbene blocked the H$_2$O$_2$-induced MC3T3-E1 cells dysfunction, indicating its potential to be a promising medication for treating osteoporosis.

Keywords: pterostilbene; osteogenic differentiation; osteoporosis; AMPK pathway; MC3T3-E1

Introduction

Osteoporosis is a systemic bone disease characterized by reduction in mineral density and bone mass and deterioration of skeletal structure. The pathological progress of osteoporosis may be caused by imbalance between bone formation and resorption. Recent studies have reported that production of intracellular reactive oxygen species (ROS) can induce osteoblast apoptosis, thereby promoting the progression of osteoporosis (Liu et al., 2019b).
In addition, overexpression of ROS could significantly attenuate osteogenic differentiation of MC3T3-E1 cells (Zhang et al., 2020). Accumulating evidence suggests that protective autophagy in cells could alleviate the development process of osteoporosis (Li et al., 2017). Furthermore, appropriate activation of autophagy can moderate the apoptosis of MC3T3-E1 cells induced by oxidative stress, and autophagy can also promote osteogenic differentiation in MC3T3-E1 cells (Feng et al., 2020; Li et al., 2019).

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene), a natural component extracted from blueberry, is a dimethylated analog of resveratrol which has greater lipophilicity and a higher potential for cellular uptake with powerful physiological functions and bioavailability. Pterostilbene’s characteristic feature is that as an antioxidant it has suppressive effects on inflammatory response, oxidative stress, and apoptosis in mammalian cells. Moreover, it is also evidenced as an effective anticancer agent in several common malignancies. A previous study has suggested that pterostilbene could inhibit the cell stemness of cervical cancer (Shin et al., 2020). Pterostilbene prevents hepatocyte epithelial–mesenchymal transition in fructose-induced liver fibrosis by suppressing miR-34a/Sirt1/p53 and TGF-β1/Smads signaling pathways (Song et al., 2019). Pterostilbene can also improve myocardial fibrosis by modulating the Pitx2c/miR-15b pathway induced by ROS (Ko et al., 2015). Furthermore, pterostilbene demonstrated neuroprotective effect against astrocyte inflammation and neuronal oxidative stress, thereby alleviating ischemia/reperfusion-induced nerve injury through inhibition of NF-κB pathway (Liu et al., 2019c). Pterostilbene also has potential in orthopedic treatment and might restrain the formation of osteoclasts and reduce bone loss via suppressing the phosphorylation of Mitogen-activated protein kinase (MAPK) pathway (Nikhil et al., 2015). Additionally, numerous studies have demonstrated that pterostilbene could promote autophagy (He et al., 2018; Wang et al., 2020) and activate the 5’ adenosine monophosphate-activated protein kinase (AMPK) pathway by stimulating the osteogenic differentiation of MC3T3-E1 cells (Cheng et al., 2019; Liu et al., 2019a; Yang et al., 2015). The present investigation scrutinized pterostilbene’s therapeutic potency and explored the underlying signaling mechanism on hydrogen peroxide (H₂O₂)-induced cell apoptosis, oxidative stress, and inhibition of osteogenic differentiation.

**Methods**

**Cell culture**

MC3T3-E1 is an osteoblast precursor cell line derived from newborn mouse calvaria. The cells were purchased from the American Type Culture Collection and maintained in α-minimum essential medium containing 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. Cells were harvested when reached approximately 80% confluence to execute the following experiments. MC3T3-E1 cells were treated with H₂O₂ (0–1 mM) and pterostilbene (0.125–2 mM) for 12 h. MC3T3-E1 cells were divided into the following five groups: control, H₂O₂ + 0.25-mM pterostilbene, H₂O₂ + 0.5-mM pterostilbene, and H₂O₂ + 1-mM pterostilbene. All the experiments were repeated thrice.

**MTT assay**

MC3T3-E1 cells were seeded at a concentration of 1 × 10⁴/well on 96-well plates for 24 h. When cells reached 80% confluence, different concentrations of pterostilbene (0.125–2 mM) were added 1 h prior to H₂O₂ treatment. After treatment, cells were incubated with 10 μL of 5 mg/mL 3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA). After incubation for 4 h at 37°C, formazan crystals were solubilized in dimethyl sulfoxide (DMSO). The microplate reader was used to recorded the OD value of each well at 570 nm.

**Flow cytometry**

The flow cytometry Annexin V-FITC/PI apoptosis detection kit (Thermo Fisher Scientific, Inc., USA) was used to determine the apoptosis of MC3T3-E1 cells after exposure to H₂O₂ and pterostilbene. MC3T3-E1 cells were harvested and resuspended at a concentration of 5 ×10⁵ cells/mL. FITC-Annexin V and propidium iodide (PI) were added and kept on ice in the dark for 20 min. Finally, the apoptotic positive cell ratio was quantified by flow cytometry.

**Enzyme-linked immunosorbent assay (ELISA)**

The expression levels of glutathione peroxidase (GPx) and malondialdehyde (MDA) in the supernatants of different groups of MC3T3-E1 cells were determined at room temperature using commercial ELISA kits according to the manufacturer’s protocol (R&D Systems, USA).

**ROS measurement (DCFDA staining)**

MC3T3-E1 cells were seeded at a concentration of 1 × 10⁴ cells/well on 24-well plates, allowed to confluence to 80% overnight, and then treated with H₂O₂ (0–1 mM)
and pterostilbene (0.125–2 mM) for 12 h. After that, the cells were cultured with 2′,7′-dichlorodihydrofluorescein-diacetate (H2DCFDA; Sigma-Aldrich), used as an ROS indicator, to detect intracellular ROS level. The mean fluorescence intensity was determined with an inverted fluorescent microscope using an excitation/emission wavelength of 535 nm.

**Western blot assay**

MC3T3-E1 cells were extracted using lysis buffer, including protease inhibitors, according to manufacturer’s instructions. Protein concentration was quantified by bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich), and 30 μg of protein was separated with 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore Sigma, Burlington, MA, USA). The membranes were incubated overnight at 4°C with primary antibodies against type I collagen (COL1A1, 1:3,000; Cell Signaling Technology, USA), osteopontin (OPN, 1:2,000; Invitrogen, Carlsbad, CA, USA), runt-related transcription factor 2 (Runx2, 1:3,000; Abcam, Cambridge, UK), p-AMPK (1:3,000; Abcam), AMPK (1:8,000; Abcam), β-actin (1:10,000; Invitrogen), and subsequently incubated with secondary antibodies for 1 h. Protein signals were measured using the enhanced chemiluminescence system (Bio-Rad Clarity Western ECL, Hercules, CA, USA) and analyzed using the ImageJ software (http://rsb.info.nih.gov/ij/; Bethesda, MD, USA) (Shu et al., 2020).

**Cellular alkaline phosphatase (ALP) measurement (Alizarin Red S Staining)**

MC3T3-E1 cells were seeded at a concentration of 1 × 10^3/well on 12-well plates to assess the situation of osteogenic differentiation. After exposure to H_2O_2 and pterostilbene, MC3T3-E1 cells were fixed with 4% paraformaldehyde and stained using Alizarin red S dye (Abcam) for 30 min at room temperature. The stained cells were washed again with Dulbecco’s phosphate-buffered saline (DPBS), and images were captured using a light microscope with digital camera.

**Statistics**

All results were performed in triplicate to ensure reproducibility, and analyzed using SPSS-17.0. All data were presented as mean ± SD. One-way ANOVA followed by Bonferroni’s post-hoc test was used to make comparison, and P < 0.05 indicated that the difference was statistically significant.

**Results**

**Effect of pterostilbene on the viability and apoptosis of H_2O_2-treated MC3T3-E1 cells**

In order to determine the effect of H_2O_2 on cell viability, MC3T3-E1 cells were treated with H_2O_2 at different concentrations (0–1 mM) for 12 h, and the viability was measured by MTT assay. The results revealed that H_2O_2 attenuated the cell viability of MC3T3-E1 cells in a dose-dependently manner (Figure 1A). On the other hand, compared to the control group, the cell viability demonstrated no significant difference in MC3T3-E1 cells treated with pterostilbene at different concentrations (0.125–2 mM; Figure 1B). Next, effect of pterostilbene on the viability and apoptosis of H_2O_2-treated MC3T3-E1 cells was evaluated. Results from MTT and Annexin V apoptosis assays indicated that pterostilbene promoted cell viability but suppressed the apoptosis of H_2O_2-treated MC3T3-E1 cells in a dose-dependent manner (Figures 1C and D). These data suggested that pterostilbene had potential for attenuating the apoptosis of H_2O_2-induced MC3T3-E1 cells.

**Effect of pterostilbene on oxidative stress in H_2O_2-treated MC3T3-E1 cells**

In order to determine whether pterostilbene had protective effect on H_2O_2-induced oxidative stress, the levels of GPx and MDA were evaluated in MC3T3-E1 cells. After treatment with pterostilbene (0.25, 0.5, and 1.0 mM) and H_2O_2 (1 mM) the MDA level was remarkably enhanced in the H_2O_2 treatment group but pterostilbene suppressed the simulative effects of H_2O_2 on MDA level in a dose-dependent manner (Figure 2A). The level of GPx was significantly reduced after H_2O_2 treatment. Conversely, co-treatment of H_2O_2 with pterostilbene could significantly reverse the suppressive effects of H_2O_2 on GPx level in a dose-dependent manner (Figure 2B). Moreover, the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) staining was conducted to evaluate ROS production in MC3T3-E1 cells. The level of ROS in H_2O_2-treated group had increased significantly whereas ROS production was suppressed after co-treatment with different concentrations of pterostilbene (Figure 2C). These results indicated that pterostilbene produced significant reduction in the H_2O_2-induced oxidative stress in MC3T3-E1 cells.

**Effect of pterostilbene on osteogenic differentiation in H_2O_2-treated MC3T3-E1 cells**

In order to understand the effects of pterostilbene on the osteogenic differentiation of H_2O_2-treated MC3T3-E1 cells, MC3T3-E1 cells were exposed to...
different concentrations of pterostilbene after treating with 1-mM H\textsubscript{2}O\textsubscript{2}. The osteogenic-related markers, including COL1A1, OPN, and Runx2, were determined by Western blot assay. In this study, H\textsubscript{2}O\textsubscript{2} treatment suppressed the levels of osteogenic-related proteins. As shown in Figure 3A, the expression levels of COL1A1, OPN, and Runx2 in the co-treated H\textsubscript{2}O\textsubscript{2} + pterostilbene groups were significantly higher than in the H\textsubscript{2}O\textsubscript{2} group. To further determine whether pterostilbene had protective effect on osteogenic differentiation, we examined the activity of ALP, which is a biochemical marker of osteoblasts, after exposure to pterostilbene and H\textsubscript{2}O\textsubscript{2}. The ALP activity was visualized by ALP enzyme histochemistry, where 1-mM pterostilbene generated the highest improvement in ALP activity compared to the H\textsubscript{2}O\textsubscript{2} group (Figure 3B). These findings strongly suggested that pterostilbene could protect against the H\textsubscript{2}O\textsubscript{2}-induced inhibition of osteogenic differentiation.

Effect of pterostilbene on the activation of AMPK signaling pathway

The AMPK signaling pathway is reported to be involved in osteogenic differentiation. Therefore, the molecular mechanisms underlying the relationship between AMPK signaling pathway and pterostilbene-induced osteogenic differentiation in MC3T3-E1 cells was explored. Compared to the control group, the phosphorylation level of AMPK was evidently reduced in the cells exposed to 1-mM H\textsubscript{2}O\textsubscript{2}. Conversely, Western blot analysis revealed that pre-incubation with pterostilbene improved the phosphorylation level of AMPK and reversed the H\textsubscript{2}O\textsubscript{2}-induced effect in a dose-dependent manner (Figure 4). Taken together, these results indicated that pterostilbene could reverse the inhibited osteogenic differentiation of MC3T3-E1 cells caused by H\textsubscript{2}O\textsubscript{2} through the activation of AMPK pathway.

Discussion

The key finding of this study was that pterostilbene has potential in attenuating the H\textsubscript{2}O\textsubscript{2}-induced cell apoptosis and oxidative stress. Pterostilbene could promote the osteogenic differentiation of inhibited H\textsubscript{2}O\textsubscript{2}-treated MC3T3-E1 cells through the activation of AMPK pathway. Osteoporosis is one of the common degenerative skeletal diseases that causes millions of bone fractures yearly (Poursaemii et al., 2018). It is commonly encountered in elderly population and postmenopausal women. The treatment of osteoporosis makes the world to spend billions of dollars every year, including drug intervention and hospitalization costs. Osteoporosis is characterized by destruction of bone microstructure and bone density reduction, and is caused by imbalance between bone resorption and formation (Bouxsein, 2003; Choksi et al., 2018). The current limited understanding of the potential mechanisms of osteoporosis obstructs the development of therapeutic approach to osteoporosis. Although there are multiple treatment methodologies, people are more concerned about the adverse and long-term effects...
Pterostilbene promotes the osteogenic differentiation by AMPK pathway

Figure 2. Effect of pterostilbene on H$_2$O$_2$-induced oxidative stress in MC3T3-E1 cells. (A) The level of MDA was measured by MDA ELISA kit. (B) The level of GPx was measured by GPx ELISA kit. (C) The ROS production was detected by DCFH-DA staining and observed under fluorescence microscopy. Data were presented as mean ± SD. *P < 0.05 compared with the H$_2$O$_2$ group, **P < 0.01 compared with the H$_2$O$_2$ group; ##P < 0.01 compared with the control group.

of currently available palliative drugs (Chan et al., 2016). Currently, many factors are known to induce osteoporosis, and the molecular basis of pathological progress may be having diversity under different conditions of osteoporosis. Especially, close coordination between transcription network and signaling pathways in the process of bone development at the genetic level is the hot topic of recent research in osteoporosis. Pterostilbene, as an antioxidant, has gained increasing attention because of its functions in the potential prevention and treatment of chronic diseases, including cancer, atherosclerosis, coronary heart disease, diabetes, and neurological degeneration (McCormack and McFadden, 2012, 2013). Previous studies have suggested that ROS concentration beyond a certain level could disturb the oxidant–antioxidant balance and lead to many inflammatory diseases, such as osteoporosis (Abdollahi et al., 2005; Schröder, 2015). ROS could induce cell damage through DNA destruction, and stimulation of some signaling pathways trigger the osteoclastic bone resorption. Recently, pterostilbene has been established as a promising medication to alleviate the inflammation-induced degeneration of cartilage, since it could inhibit the expression of inflammatory mediators, including inducible nitric oxide synthase (iNOS), nitric oxide (NO), and intracellular ROS production, in chondrocytes (Pan et al., 2008; Xue et al., 2017). The present study rigorously investigated the cellular mechanisms responsible for the protective effect of pterostilbene on osteogenic differentiation. Therefore, it explored the role of pterostilbene in the inhibition of ROS production and apoptosis in osteoblast induced by H$_2$O$_2$. The results revealed that cell viabilities increased after treatment with H$_2$O$_2$ and caused the inactivation of AMPK signaling pathway. However, pterostilbene suppressed the H$_2$O$_2$-induced cell apoptosis and oxidative stress, and improved the survival rate of MC3T3-E1.
Figure 3. Effect of pterostilbene on osteogenic differentiation in MC3T3-E1 cells. (A) The protein expression levels of COL1A1, OPN, and Runx2 were analyzed with Western blot assay. (B) MC3T3-E1 cells were incubated at different concentrations of pterostilbene and fixed for ALP staining. Data were presented as mean ± SD. *P < 0.05 compared with the H$_2$O$_2$ group, **P < 0.01 compared with the H$_2$O$_2$ group; ##P < 0.01 compared with the control group.

Figure 4. Pterostilbene attenuated the inhibitory effects of H$_2$O$_2$ on the AMPK signaling pathway. MC3T3-E1 cells were treated with or without different concentrations of pterostilbene (0.25, 0.5, and 1.0 mM) before exposure to H$_2$O$_2$. The expression levels of p-AMPK and AMPK were measured by Western blot analysis. Quantitative analysis of p-AMPK and AMPK was manifested. Data were presented as mean ± SD. *P < 0.01 compared with the H$_2$O$_2$ group; **P < 0.01 compared with the control group.
cells. These results were consistent with other investigations, demonstrating that pterostilbene could attenuate herbicide-induced oxidative damage to the growth and metastatic activity of B16 melanoma (Ko et al., 2015). Together with these foundations, it was concluded that pterostilbene could promote cell viability by reducing oxidative stress.

In order to clarify the protective effect of pterostilbene and its downstream signaling pathway on osteogenic differentiation after $\text{H}_2\text{O}_2$ exposure, this study investigated the expression of osteogenic-related biomarkers and molecular mechanisms of AMPK signaling pathway. The mechanisms mediating the protective effects of pterostilbene included multiple signaling pathways such as extracellular regulated kinase, AMPK, and nuclear factor kappa B (NF-kB) pathways, among which the activation of AMPK pathway was established as a promising approach (Lin et al., 2012; Xue et al., 2017). Previous studies have established that AMPK signaling pathway is related to the modulation of cellular oxidative stress (Auciello et al., 2014; Wu and Wei, 2012). Oxidative stress could trigger the activation of AMPK, which stimulates nuclear factor erythroid 2-related factor 2 (Nrf2) and its downstream antioxidant enzymes (Zimmermann et al., 2015). It has been reported that AMPK activation regulates cellular energy content by promoting generation of adenosine triphosphate (ATP) through upregulation of mitochondrial biogenesis, which is a requisite of oxidative stress inhibition. These results indicated that the expression levels of COL1A1, OPN, and Runx2 in the co-treatment $\text{H}_2\text{O}_2$ + pterostilbene groups were obviously higher than in the $\text{H}_2\text{O}_2$ group. In addition, the co-treatment of $\text{H}_2\text{O}_2$ + pterostilbene generated the highest ALP activity compared to the $\text{H}_2\text{O}_2$ group. It was also determined that pre-incubation with pterostilbene improved the phosphorylation level of AMPK and reversed the $\text{H}_2\text{O}_2$-induced injury.

**Conclusion**

In conclusion, results of the present study indicated that pterostilbene led to a significant reduction in the $\text{H}_2\text{O}_2$-induced apoptosis and oxidative stress of MC3T3-E1 cells. It was also revealed that pterostilbene induced AMPK signaling pathway activation to exert antioxidant effect and inhibit ROS production after $\text{H}_2\text{O}_2$ exposure. Importantly, pterostilbene blocked the $\text{H}_2\text{O}_2$-induced MC3T3-E1 cells dysfunction, indicating that pterostilbene could be a potential medication for treating osteoporosis. Although additional studies are required to confirm the efficacy of pterostilbene in the treatment of osteoporosis in vivo, these results indicated that pterostilbene could be a potential therapeutic target for disorders associated with bone loss.

**Competing Interests**

The authors stated that there were no conflicts of interest to disclose.

**Author Contributions**

Haiyan Li and Jiang Guo designed and conducted the study. Dong Zhang supervised the data collection, and analyzed and interpreted the data. Jihong Huang and Xuejia Li prepared the manuscript for publication and reviewed the final manuscript. All authors read and approved the final manuscript.

**References**


