

Effect of [6]-Gingerol, a Pungent Ingredient of Ginger, on Osteoblast Response to Extracellular Reducing Sugar

Young-Ho Kim*, Huu Tung Nguyen, Yan Ding, Sang-Heock Park, and Eun-Mi Choi

College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea

Abstract Diabetes is marked by high glucose levels and is associated with decreased bone mass and increased fracture rates. To determine if [6]-gingerol could influence osteoblast dysfunction induced by 2-deoxy-D-ribose (dRib), osteoblastic MC3T3-E1 cells was treated with dRib and [6]-gingerol and markers of osteoblast function and oxidized protein were examined. [6]-Gingerol (10^{-7} M) significantly increased the growth of MC3T3-E1 cells in the presence of 30 mM dRib ($p < 0.05$). [6]-Gingerol (10^{-7} M) caused a significant elevation of alkaline phosphatase (ALP) activity, collagen content, and osteocalcin secretion in the cells. We then examined the effect of [6]-gingerol on the production of osteoprotegerin and protein carbonyl in osteoblasts. Treatment with [6]-gingerol (10^{-9} and 10^{-7} M) increased osteoprotegerin secretion in osteoblastic cells. Moreover, [6]-gingerol (10^{-9} and 10^{-7} M) decreased protein carbonyl contents of osteoblastic MC3T3-E1 cells in the presence of 30 mM dRib. Taken together, these results demonstrate that [6]-gingerol inhibits dRib-induced damage and may be useful in the treatment of diabetes related bone diseases.

Keywords: [6]-gingerol, osteoblast, 2-deoxy-D-ribose, MC3T3-E1 cell

Introduction

Ginger rhizome (*Zingiber officinale*), known commonly as ginger, is consumed worldwide as a spice and a flavoring agent. It has a long history of both culinary and medicinal use (1-4), with many claims of its therapeutic benefits. In particular, it has been used in traditional oriental medicine against symptoms such as inflammation, rheumatic disorders, and gastrointestinal discomforts (5). It contains pungent phenolic substances, aromatic ketones known collectively as gingerols, which are thought to be the most pharmacologically active components. One of the major pungent principles of ginger, [6]-gingerol (1-4'-hydroxy-3'-methoxyphenyl-5-hydroxy-3-decanone), has diverse effects including anticancer (6), antioxidation and anti-inflammation (7), anti-platelet aggregation (8), inhibition of nitric oxide synthesis (9), inhibition of cyclooxygenase-2 (COX-2) activity (10), and protecting neuronal cells from α -amyloid insult (11). [6]-Gingerol has also been found to possess a variety of interesting pharmacological effects, for example, antipyretic, cardiotoxic effects, and inhibition of spontaneous motor activity and prostaglandin biosynthesis (12, 13).

Osteopenia occurs in association with type 1 diabetes, and evidence of reduced osteoblast activity has reported (14). This suggests that hyperglycemia can suppress osteoblast differentiation. Sugars that contain aldehyde groups that are oxidized to carboxylic acids are classified as reducing sugars. Reducing sugars produce reactive oxygen species (ROS) through autooxidation and protein glycosylation (15). Glucose is the least reactive of the reducing sugars (16). Accordingly, long-term exposure to high glucose concentrations is necessary before toxic effects are expressed in cells. 2-Deoxy-D-ribose (dRib) is a

reducing sugar with high reactivity with protein (17). Therefore, we chose a dRib as an agent to induce oxidative damage of osteoblastic MC3T3-E1 cells *in vitro*. To investigate whether [6]-gingerol could reduce dysfunction caused by dRib, a surrogate for glucose, in osteoblastic MC3T3-E1 cells, we examined the effects of [6]-gingerol on the alkaline phosphatase activity, collagen, osteocalcin, osteoprotegerin, and protein carbonyl in osteoblastic MC3T3-E1 cells.

Materials and Methods

Cell culture and materials Murine osteoblastic MC3T3-E1 cells were cultured at 37°C in 5% CO₂ atmosphere in α -modified minimal essential medium (α -MEM; GibcoBRL, Grand Island, NY, USA). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin. When cells reached confluence, cells were subcultured using 0.02% EDTA-0.05% trypsin solution. The cells, seeded in 6 well plate, were treated at 90% confluence with culture medium containing 10 mM β -glycerophosphate, and 50 μ g/mL ascorbic acid to initiate *in vitro* mineralization (18). Medium was changed every 2-3 days. After 6 or 14 days, respectively for the measurement of cell viability, alkaline phosphatase activity, and collagen content, or calcium deposition, osteocalcin and osteoprotegerin secretion, the cells were cultured with medium containing 2-deoxy-D-ribose (30 mM) and hesperetin (10^{-9} - 10^{-5} M) for 2 days. Cell culture reagents and most other biochemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

MTT assay MTT test was used to determine the effects of [6]-gingerol on the proliferation of MC3T3-E1 cells. Cells were seeded at a density of 2×10^4 cells per well in 24-well plates. Proliferation and normal growth were determined 24 hr after [6]-gingerol treatment using a

*Corresponding author: Tel: 82-42-823-6566; Fax: 82-42-821-5933

E-mail: yhk@cnu.kr

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colorimetric assay based on the uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt (MTT) by viable cells. In brief, the MTT solution (0.5 mg/mL) was added to the cells and incubated at 37°C to allow cleavage of the tetrazolium ring by mitochondrial dehydrogenases and formation of blue formazan crystals. After 3 hr, the residual MTT was carefully removed and the crystals were dissolved by incubation with DMSO for 30 min. The plates were shaken for 5 min and the absorbance at 560 nm was measured by spectrophotometry.

Alkaline phosphatase activity At harvest, the medium was removed and the cell monolayer was gently washed twice with phosphate buffered saline (PBS). The cells were lysed with 0.2% Triton X-100 and the lysate was centrifuged at 14,000×g for 5 min. The clear supernatant was used for the measurement of alkaline phosphatase (ALP) activity and protein concentration. ALP activity and protein concentration were determined using an ALP activity assay kit (Somang Co., Korea) and a Bradford (Bio-Rad, Hercules, CA, USA) assay kit, respectively.

Collagen content At harvest, cellular collagen content was measured using Sircol Collagen Assay kit (Biocolor Ltd., Newtownabbey, Northern Ireland). This assay is a quantitative dye-binding method designed for the analysis of collagens extracted from mammalian tissues and cells during *in vitro* culture. The dye reagent binds specifically to the [Gly-X-Y]_n helical structure found in mammalian collagens (type I to V).

Osteocalcin content Osteocalcin content in culture medium was measured using sandwich enzyme-linked immunosorbent assay (ELISA) assay kit (Biomedical Technologies Inc., Stoughton, MA, USA). Two mouse osteocalcin antibodies are employed, each directed toward an end of the (C- or N- terminal) osteocalcin molecule. The N-terminal antibody is bound to the well, which binds the mouse osteocalcin standard or sample. The biotin labeled C-terminal mouse osteocalcin antibody completes the sandwich. This sandwich ELISA kit is specific for intact mouse osteocalcin only. Both carboxylated and decarboxylated mouse osteocalcin are recognized.

Measurement of osteoprotegerin Osteoprotegerin (OPG) acts as a soluble secreted receptor for RANKL that prevents it from binding to and activating RANK (osteoclast differentiation and activation receptor) on the osteoclast surface, thus inhibiting the osteoclast development. OPG content in culture medium was measured using sandwich ELISA assay kit (Biomedical Technologies Inc.).

Protein carbonyl (PCO) The most general indicator of protein oxidation is protein carbonyl content. The Protein Carbonyl Assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) was used to measure the protein carbonyl content in cell lysates. This kit utilizes the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. The amount of protein-hydrazone produced is quantified spectrophotometrically at an absorbance between 360-385 nm. The carbonyl content can then be standardized to protein concentration.

Statistical analysis The results are expressed as mean±SEM (n=6). Statistical analysis was performed using one-way ANOVA ($p<0.05$). The analysis was performed using SAS statistical software.

Results and Discussion

This study was intended to investigate the protective effects of [6]-gingerol against dRib-induced dysfunction and oxidative stress in cultured mouse osteoblasts as an *in vitro* experimental model. MC3T3-E1 cells are pre-osteoblastic cells that undergo osteoblastic differentiation and mineralization when grown in the presence of β -glycerophosphate and L-ascorbic acid. After repeated subcultivations, the MC3T3-E1 cells retain competency for differentiation, which is similar to primary cultures of calvarial osteoblasts (19). Osteoblasts produce ALP, collagen, osteocalcin, which are associated with matrix maturation and mineralization. In the present study, osteoblastic MC3T3-E1 cells at confluence were cultured with differentiation-inducing medium for 14 day and then were incubated in medium containing [6]-gingerol (10^{-9} - 10^{-5} M) in the absence or presence of 30 mM dRib for 2 days. Since treatment with dRib significantly inhibited the differentiation of osteoblastic MC3T3-E1 cells at the concentration of 20-50 mM (data not shown), 30 mM dRib was used for induction of osteoblast dysfunction. As shown in Fig. 1, no toxic effect was observed after incubation with [6]-gingerol (10^{-9} - 10^{-7} M). After treatment of the culture with 10^{-5} M [6]-gingerol, the majority of the cells were found dead. This result indicated that [6]-gingerol alone was not toxic at the concentrations of 10^{-9} - 10^{-7} M. When the cells were treated with [6]-gingerol in the presence of 30 mM dRib, [6]-gingerol (10^{-7} M) increased the growth of MC3T3-E1 cells compared to dRib-treated control, suggesting that [6]-gingerol suppresses the dRib-induced cytotoxicity.

To investigate osteoblast function, ALP activity (Fig. 2), collagen content (Fig. 3), osteocalcin (Fig. 4), and osteoprotegerin (Fig. 5), markers of osteoblast function were determined. Compared with control cells, the presence of

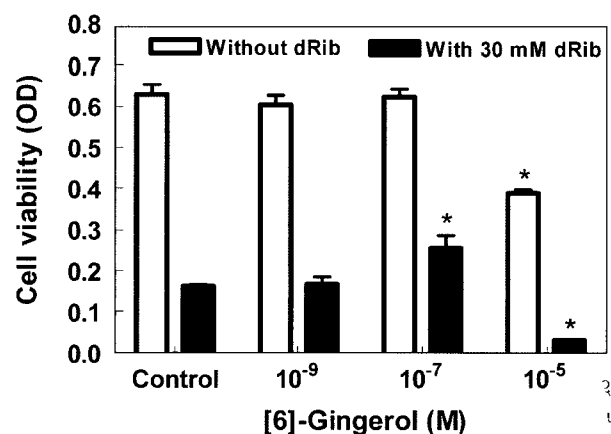


Fig. 1. Effect of [6]-gingerol on dRib-induced cytotoxicity in MC3T3-E1 cells. Data shown are mean±SEM (n=6); * $p<0.05$ vs. control.

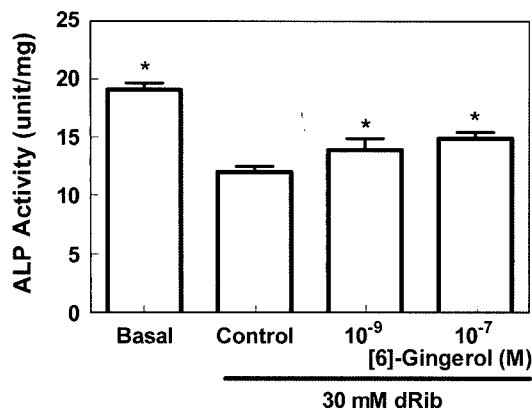


Fig. 2. Effect of [6]-gingerol on the alkaline phosphatase activity of MC3T3-E1 cells in the presence of dRib. Data shown are mean \pm SEM; * p <0.05 vs. control.

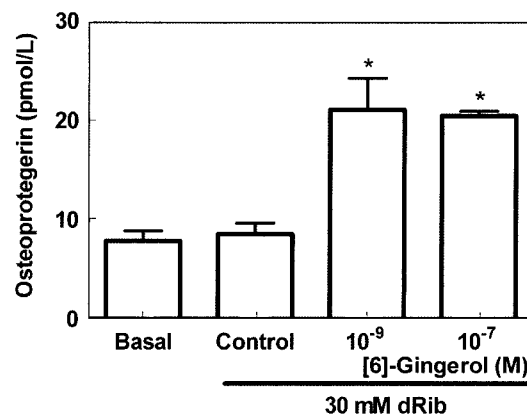


Fig. 5. Effect of [6]-gingerol on the osteoprotegerin secretion of MC3T3-E1 cells in the presence of dRib. Data shown are mean \pm SEM; * p <0.05 vs. control.

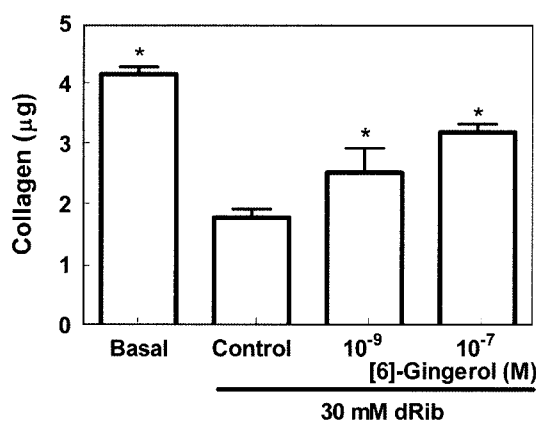


Fig. 3. Effect of [6]-gingerol on the collagen synthesis of MC3T3-E1 cells in the presence of dRib. Data shown are mean \pm SEM; * p <0.05 vs. control.

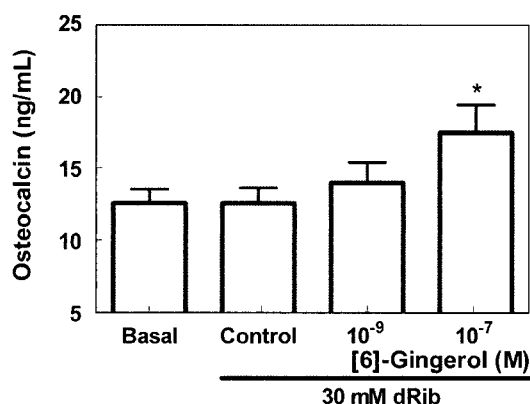


Fig. 4. Effect of [6]-gingerol on the osteocalcin secretion of MC3T3-E1 cells in the presence of dRib. Data shown are mean \pm SEM; * p <0.05 vs. control.

dRib significantly decreased cellular ALP activity and collagen contents. However, the secretion of osteocalcin and osteoprotegerin was not altered by the dRib cultures versus control. When the osteoblasts were treated with [6]-

gingerol (10^{-9} - 10^{-7} M) in the presence of 30 mM dRib, significant increases in ALP activity, collagen content, and osteoprotegerin secretion were observed. Moreover, [6]-gingerol (10^{-7} M) supplementation significantly increased the osteocalcin secretion compared with dRib treated control. In calcified tissue, ALP activity is one of the most frequently used parameters for cell differentiation and for identifying the osteogenic properties. In addition, ALP plays an important role in bone formation, particularly mineralization (20). High levels of ALP activity are seen in both preosteoblasts and osteoblasts *in vivo* and in differentiating osteoblasts *in vitro*. Bone matrix mainly is comprised of type I collagen and a variety of non-collagenous proteins such as osteocalcin (21). Although the exact function of osteocalcin has not been conclusively determined, it is thought to play a critical role in regulating cell differentiation, bone matrix formation, and mineralization. It appears to be expressed during the developmental sequence of osteoblast differentiation (22). Given that osteocalcin is a marker of mature osteoblasts, our result suggests that [6]-gingerol may increase mineralization and ultimately bone mass in diabetes-related bone disease.

This *in vitro* study demonstrated that [6]-gingerol significantly increased cellular OPG production in osteoblastic cells. OPG, a member of the tumor necrosis factor receptor (TNF-R) superfamily (23), acts as a decoy receptor by blocking the interaction of RANKL (receptor activator of NF- κ B ligand), necessary for osteoclast formation and function, with its functional receptor RANK (24), and thereby inhibiting osteoclastogenesis. OPG, which acts as a soluble neutralizing receptor to RANKL, is produced by cells of the osteoblastic lineage and therefore likely to be a major regulator of bone metabolism. The increase in osteoclast number and the associated development of bone disease, osteolytic lesions, and decrease in bone volume were prevented by treatment with recombinant OPG, indicating the importance of OPG *in vivo* (25). The [6]-gingerol-induced up-regulation of OPG suggests that [6]-gingerol may exert its anti-resorptive effects on bone, at least in part, by stimulating OPG expression in osteoblast. An increased content of carbonyl groups is an index of the oxidative modification of amino acids, including the

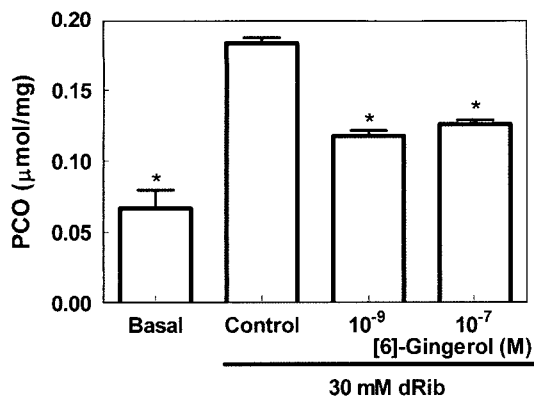


Fig. 6. Effect of [6]-gingerol on the protein carbonyl contents of MC3T3-E1 cells in the presence of dRib. Data shown are mean \pm SEM; * p <0.05 vs. control.

glycation reaction. We measured the carbonyl levels in MC3T3-E1 cells after incubation with dRib and [6]-gingerol. After 2 days of incubation with 30 mM dRib, carbonyl formation was increased; this increase was suppressed by the addition of 10⁻⁹-10⁻⁷ M [6]-gingerol (Fig. 6). This result indicates that [6]-gingerol could decrease the oxidant load of osteoblast by reducing sugar. Under diabetic conditions, the production of several reducing sugars is increased through glycolysis or the polyol pathway, and oxidative stress is provoked. Glucose, glyceraldehyde 3-phosphate, and other simple monosaccharides are prone to transition-metal-catalyzed oxidation via their enediols, generating superoxides, and α -ketoaldehydes. The ketoaldehyde products of monosaccharide autoxidation contribute substantially to the total monosaccharides that are covalently attached to protein during the exposure of proteins to glucose *in vitro* (26). The activities of superoxide dismutase and glutathione peroxidase (GPx) are reported to decrease under diabetic conditions (27). Consequently, several organs are exposed to a large amount of ROS. Matsuoka *et al.* (28) reported that N-acetyl-L-cysteine protected HIT-T15 cells against inhibition of insulin gene expression caused by exposing them for 72 hr to the strong reducing sugar D-ribose. The current study demonstrates that adverse consequences induced by dRib can be partially prevented by including [6]-gingerol in the culture medium. [6]-Gingerdiol has already been found as an antioxidant in ginger (29).

In summary, the data presented here suggest that [6]-gingerol inhibits dRib-induced dysfunction of osteoblastic MC3T3-E1 cells and may exert its anti-resorptive effects on bone, at least in part, by stimulating OPG expression in osteoblasts. Moreover, the results of the present study demonstrate that gingerol can act as a biological anti-oxidant in a cell culture experimental system and protect cells from oxidative stress-induced toxicity, which may promote bone recovery under diabetes related bone diseases conditions.

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