Limonene attenuates methylglyoxal-induced dysfunction in MC3T3-E1 osteoblastic cells

Kwang Sik Suh*, Suk Chon* and Eun Mi Choi

Department of Endocrinology & Metabolism, School of Medicine, Kyung Hee University, Seoul, Republic of Korea

ABSTRACT
Limonene is a common natural terpene with powerful antioxidative properties. This study investigated the effects of limonene, a terpene found in citrus fruits, on the function of the murine pre-osteoblast cell line, MC3T3-E1 cells. The results showed that limonene treatment significantly elevated collagen synthesis, alkaline phosphatase activity, osteocalcin synthesis, and mineralization in osteoblastic cells. Methylglyoxal (MG), a highly reactive dicarbonyl metabolite, is a major precursor of advanced glycation end products, which are involved in the pathogenesis of diabetic osteopathy. We therefore investigated the effects of limonene on MG-induced cytotoxicity. Pre-treatment of MC3T3-E1 cells with limonene prevented MG-induced cell death and apoptosis. Limonene also reduced MG-triggered endoplasmic reticulum (ER) stress, as indicated by decreases in the levels of the ER-localized transmembrane signal transducers ATF-6 and IRE1. Furthermore, limonene treatment significantly reduced MG-induced autophagic activity and reactive oxygen species release. These results suggest that limonene may prevent the development of diabetic osteopathy.

Introduction
Bone is a dynamic organ that is continuously being reshaped and repaired to maintain homeostasis. Osteoporosis is a disease resulting from a decreased renewal capacity of bone, which leads to decreased density and strength, and a tendency for bone to break more easily (Michaëlsson, Melhus, Ferm, Ahlbom, & Pedersen, 2005). Bone undergoes a continuous remodeling process that involves, in sequence, the steps of osteoclast-mediated bone resorption and formation. Bone formation must be accelerated to effectively treat osteoporosis. As osteoblasts are responsible for new bone formation, agents that induce osteoblastic differentiation can increase bone formation (Lane & Kelman, 2003). Osteoblastic differentiation, an important process for proper bone function, confers mechanical rigidity and strength to bone while also maintaining elasticity and flexibility.

Methylglyoxal (MG) is a highly cytotoxic compound, generated as an intermediate of glycolysis during physical glycation in diabetes. It is considered to be a potent precursor of...
advanced glycation end products (AGEs). MG and MG-derived AGEs have been frequently implicated in the development of diabetic complications (Thornalley, 1994). MG toxicity may be involved in diabetes-associated bone loss. Studies investigating the effects of diabetes on osteoporosis have shown that patients with type I diabetes have high rates of bone resorption and turnover and decreased bone mineral density (Kayath, Tavares, Dib, & Vieira, 1998; Nicodemus & Folsom, 2001). Chan, Wu, and Shiao (2007) reported that MG treatment induces apoptotic changes in human osteoblasts. Furthermore, in vivo animal model experiments showed that MG causes bone mineral density loss (Chan et al., 2007).

Several types of foods are known to trigger high plasma MG levels, which constitutes both a nutritional and health concern. Patients with diabetes have higher MG levels than non-diabetic subjects (Khuhawar, Kandhro, & Khand, 2006). Therefore, the development and investigation of AGE inhibitors, particularly natural products with anti-AGE activity, may yield potential therapeutic approaches for delaying and preventing premature aging and diabetic complications.

Limonene (Figure 1) is a major chemical component of oils obtained from citrus fruits, including oranges, lemons and grapefruits (Arruda, Miguel, Yokoyama-Yasunaka, Katzin, & Uliana, 2009; Del Toro-Arreola et al., 2005). Limonene is listed in the Code of Federal Regulations as “generally recognized as safe (GRAS)” for use as a flavoring agent (Sun, 2007). It is commonly used as an additive in perfumes and soaps, and in food products as a flavoring agent (Whysner & Williams, 1996). Limonene has been shown to have antioxidant, antitumorigenic and anti-inflammatory properties (Roberto, Micucci, Sebastian, Graciela, & Anesini, 2010), and to scavenge oxygen free radicals and thereby protect organisms from oxidative damage (Roberto et al., 2010; Tounsi et al., 2011). Rufino et al. (2015) reported that limonene decreased interleukin (IL)-1β-induced NF-κB, JNK and p38 activation and the expression of inflammatory (iNOS) and catabolic (MMP-1 and MMP-13) genes, while increasing the activation of anti-catabolic genes (TIMP-1) and ERK1/2 relative to IL-1β-treated cells in a cellular model of osteoarthritis. Bai, Zheng, Wang, and Liu (2016) reported that limonene effectively protected human lymphatic endothelial cells from H2O2-induced oxidative stress, and that its antioxidant effect was significant, increasing cell survival rate. Based on previous studies, we anticipated that the antioxidant properties of limonene could protect bone from the oxidative stress that induces bone loss, and that this terpene could be beneficial for treating osteoporosis. Thus, we investigated the effects of limonene on osteoblastic differentiation and assessed the protective effects of limonene against MG-induced cytotoxicity in osteoblastic MC3T3-E1 cells.

Figure 1. Structure of limonene.
Materials and methods

Materials

Limonene was purchased from ChromaDex Inc. (Irvine, CA, USA). α-Modified minimal essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other reagents, which were of the highest commercial grade available, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Osteoblastic MC3T3-E1 Subclone 4 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). MC3T3-E1 cells were cultured at 37°C in a 5% CO₂ atmosphere in α-MEM (Gibco). Unless otherwise specified, the media contained 10% heat-inactivated FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were treated at confluence with culture media containing 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid to initiate differentiation. After 3 days (for collagen content and alkaline phosphatase (ALP) activity) or 14 days (for osteocalcin and calcium deposition assay), the cells were incubated with limonene for 3 days.

Collagen content

Collagen content was quantified by Sirius Red-based colorimetric assay. Cultured osteoblasts were washed with phosphate buffered saline (PBS), followed by fixation in Bouin’s fluid for 1 h. After fixation, the fixative was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air-dried and stained with Sirius Red dye reagent for 1 h under mild shaking on a shaker. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove non-bound dye. The stained material was dissolved in 0.1 N NaOH and absorbance was measured at 550 nm. A standard curve was constructed using well-known concentrations of commercial collagen (Sigma-Aldrich).

ALP activity

The cells were lyzed with 0.2% Triton X-100, and the lysate centrifuged at 14,000×g for 5 min. The clear supernatant was used to measure ALP activity, which was determined using an ALP Activity Assay kit (Asan Co., Seoul, South Korea). Protein concentrations were determined using the BioRad Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Osteocalcin measurement

Osteocalcin content in cytosol was measured using a sandwich ELISA Assay Kit (Biomedical Technologies Inc., Stoughton, MA, USA). Two mouse osteocalcin antibodies were employed, each directed toward an end (C- or N-terminus) of the osteocalcin molecule. In this assay, the N-terminal antibody binds to the well, which binds the mouse osteocalcin standard or sample. The biotin labeled C-terminal mouse osteocalcin antibody completes the sandwich. Both carboxylated and decarboxylated mouse osteocalcin are recognized.
Calcium deposition assay

Upon harvesting, cells were fixed in 70% ethanol for 1 h, and then stained with 40 mM Alizarin Red S for 10 min with gentle shaking. To quantify the bound dye, the stain was washed with Dulbecco’s PBS (DPBS) and solubilized with 10% cetylpyridinium chloride by shaking for 15 min. The absorbance of the solubilized stain was measured at 561 nm.

Cell viability

Cells were pre-incubated for 1 h with media containing 0.1% FBS and limonene before treatment with MG for 48 h. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. MTT (20 μl) in PBS, pH 7.4 (5 mg/ml), was added to each well, and the plates were incubated for an additional 2 h. After removal of solutions from the wells, dimethyl sulfoxide was added to dissolve formazan products, and the plates were shaken for 5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm.

Measurement of apoptosis

Apoptosis was assessed with a Cell Death Detection ELISAPLUS Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. The assay is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This allows specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates.

Measurement of cyclophilin B (CypB), ATF-6, and IRE1

The levels of CypB, ATF-6, and IRE1 in cytosol were determined by an ELISA Kit (MyBioSource, Inc., San Diego, CA, USA). The assay was performed according to the instructions provided by the manufacturer.

Autophagy detection assay

An Autophagy Detection Kit (Abcam, Cambridge, UK) was used according to the manufacturer’s protocol, via a fluorescence microplate reader. The 488 nm-excitable green fluorescent detection reagent supplied in the kit becomes brightly fluorescent in vesicles produced during autophagy and has been validated under a wide range of conditions known to modulate autophagy pathways. This live cell analysis kit provides a convenient approach for analysis of the regulation of autophagy at the cellular level.

Measurement of intracellular reactive oxygen species

Formation of intracellular reactive oxygen species (ROS) was measured using 2′,7′-dichlorodihydrofluorescin diacetate (H₂DCFDA). In order to load the cells with the fluorescence dye, the cells were incubated with H₂DCFDA in Hank’s solution at the final concentration of 10 μM for 45 min at 37°C in the dark. Following washing with DPBS, ROS levels were determined by measuring the fluorescent intensity at excitation wavelength 485 nm and emission wavelength 530 nm (Molecular Devices, Sunnyvale, CA, USA).
Statistical analysis

The results are expressed as means ± standard error of the mean. Statistical significance was determined by analysis of variance and subsequent application of Dunnett’s t-test. Significance was set at \( P < .05 \).

Results and discussion

Effects of limonene on the differentiation of osteoblastic MC3T3-E1 cells

Osteoblasts are the bone-forming cells of the skeleton; they synthesize and regulate the deposition and mineralization of the extracellular matrix of bone (Meyer et al., 1999). To evaluate the effects of limonene on osteoblast differentiation markers, we assessed collagen content, ALP activity, osteocalcin release, and calcium deposition (Figure 2). Collagen, an early osteoblastic marker, is the most abundant protein synthesized by active osteoblasts and its expression denotes the onset of osteoblastic differentiation. As shown in Figure 2(A), incubating cells with limonene (0.01–1 \( \mu \)M) increased collagen synthesis. We then examined the activity of ALP, an early-stage osteogenic differentiation marker, in MC3T3-E1 osteoblastic cells. ALP is released into the osteoid to initiate mineral

Figure 2. Effects of limonene on the differentiation of osteoblastic MC3T3-E1 cells. The cells were treated at confluence with culture media containing 10 mM \( \beta \)-glycerophosphate and 50 \( \mu \)g/ml ascorbic acid to initiate differentiation. After six days, collagen content (A) and ALP activity (B) were measured. After 17 days, osteocalcin levels (C) and mineralization (D) were measured. Cells were treated with limonene for the final three days. Data are expressed as a percentage of the controls. The control values for collagen content, ALP activity, osteocalcin, and mineralization were 6.72 ± 0.107 \( \mu \)g/10^6 cells, 0.708 ± 0.006 unit/mg protein, 4.308 ± 0.2 ng/mg protein, and 0.746 ± 0.042 OD/10^6 cells, respectively. \(*P < .05\) compared with controls.
deposition and has been reported to be an essential enzyme for mineralization (Bellows, Aubin, & Heersche, 1991). Culturing cells in the presence of limonene (0.01–1 μM) significantly increased ALP activity (Figure 2(B)). Especially, the ALP activity of treatment with 0.1 μM limonene was higher than 0.01 and 1 μM limonene. When osteocalcin, a late-stage osteogenic differentiation marker, was measured in the cytosol of the cells, limonene (0.1 and 1 μM) significantly increased osteocalcin secretion compared with controls (Figure 2(C)). Matrix mineralization, the final step in osteoblastic differentiation, plays a critical role in maintaining the mechanical integrity of bone tissues. After mineralization, bone possesses mechanical properties for withstanding external forces, providing a hard framework and protecting the vital organs (Fratzl-Zelman, Misof, Klaushofer, & Roschger, 2015). To detect the effects of limonene on mineralization, MC3T3-E1 osteoblastic cells were stained with Alizarin Red S. As shown in Figure 2(D), the increase in mineralization was significant at limonene concentrations of 0.01–1 μM. These results suggest that limonene induces osteogenic differentiation processes from the early to the late phase.

**Effects of limonene on CypB release in MG-treated MC3T3-E1 cells**

Cyclophilins have peptidyl-prolyl isomerase activity and thus act as molecular chaperones, facilitating protein folding, intracellular trafficking, and the maintenance of multiprotein complex stability (Andreeva, Heads, & Green, 1999). Recently, several reports have described the effects of CypB mutants on type I collagen modification and components of the prolyl 3-hydroxylation complex, where CypB-deficient mice present with severe osteogenesis imperfecta-like phenotypes (van Dijk et al., 2009). CypB has been found to play a key regulatory role, as a member of several foldase and chaperone complexes, in processes such as promoting the folding of multiple substrates within the endoplasmic reticulum (ER) (Jansen et al., 2012). Thus, we also investigated whether limonene modulates the production of CypB in MC3T3-E1 osteoblastic cells (Figure 3). When 400 μM MG was added to the cells, CypB production significantly decreased. However, CypB production was significantly increased by pretreatment with limonene at concentrations of 0.01–1 μM. CypB is known to associate with various proteins and regulate their functions. Kim

![Figure 3](image-url)

**Figure 3.** Effects of limonene on cyclophilin B (CypB) release in osteoblastic MC3T3-E1 cells. The cells were treated at confluence with culture media containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid to initiate differentiation. After one week, CypB was measured. Cells were treated with limonene for the last three days. The control value for CypB was 29.74 ± 1.344 ng/mg. *P < .05 compared with controls.
et al. (2008) showed that CypB is protective against an ER stress-inducing drug, blocking Ca\(^2+\) leakage from the ER to the cytosol and ultimately preventing ER stress-induced cell death. Jeong et al. (2014) demonstrated that CypB interacts with the transcription factor CHOP (C/EBP homology protein), one of the best-characterized pro-apoptotic molecules, leading to its p300-mediated ubiquitination and degradation. Subsequently, elevated CypB displays protective effects against hypoxia-induced cell death. CypB may also act as an anti-inflammatory factor and mediate tolerance to cells exposed to pro-inflammatory stimuli (Marcant et al., 2012). CypB has been found to associate with collagen (Smith, Ferreira, Hebert, Norris, & Sauk, 1995). The biosynthesis of procollagen is a complex process that requires several co- and post-translational modifications within the ER (Myllyharju & Kivirikko, 2004). Cabral et al. (2014) demonstrated that CypB is the major peptidyl-prolyl cis–trans isomerase catalyzing the rate-limiting step in collagen folding. CypB facilitates collagen folding directly, but also indirectly regulates collagen hydroxylation, glycosylation, crosslinking, and fibrillogenesis through its interactions with other collagen-modifying enzymes in the ER (Cabral et al., 2014). In our study, limonene increased CypB levels, which suggests that limonene may increase collagen folding in MC3T3-E1 osteoblastic cells.

**Cytoprotective effects of limonene on osteoblastic MC3T3-E1 cells**

MG and MG-derived AGEs are commonly implicated in the development of diabetic complications (Thornalley, 1994). Excess MG formation can induce ROS production and cause oxidative stress-induced cellular damage. To examine the effects of limonene itself on osteoblastic cells, we treated MC3T3-E1 cells with various concentrations of limonene for 48 h and measured their viability. Limonene at concentrations of \( \leq 1 \mu M \) had no effect on the viability of MC3T3-E1 cells (Figure 4(A)). To determine whether limonene had a protective effect against MG-induced cytotoxicity, cells were pre-incubated with limonene for 1 h and then cultured with 400 \( \mu M \) MG for 48 h. As shown in Figure 4 (A), MG (400 \( \mu M \)) treatment induced nearly 50% cell death compared with non-treated control cells, but limonene (0.01–1 \( \mu M \)) inhibited the MG-induced cytotoxicity. Apoptotic cell death was evaluated with a Cell Death Detection ELISA Kit. As shown in Figure 4(B), MG (400 \( \mu M \)) induced apoptosis in MC3T3-E1 cells, but limonene (0.01–1 \( \mu M \)) reduced such effects. Thus, limonene may prevent MG-induced cell death.

**Limonene inhibits MG-induced ER stress in MC3T3-E1 cells**

ER stress is defined as the accumulation of unfolded or misfolded proteins in the ER luminal environment. When the demand for ER function is over capacity, ER stress arises (Rutkowski & Kaufman, 2004). In response to ER stress, the unfolded protein response pathway is activated, to prevent such accumulation in the ER lumen and cope with the stressful conditions (Ron & Walter, 2007). It is now recognized that three ER-localized transmembrane signal transducers, including protein kinase IRE1 (inositol-requiring kinase 1) and the transcription factor ATF6 (activating transcription factor 6), can be activated to initiate stress adaptive responses in the ER (Marciniak & Ron, 2006). In the present study, we investigated the effects of limonene on the activation of ER stress sensors elicited by MG stimulation. Our data show that pre-treatment of MC3T3-E1 cells with limonene (0.01–1 \( \mu M \)) or aminoguanidine (AG, 300 \( \mu M \)), a carbonyl scavenger, followed by co-incubation with MG, significantly reduced the levels of IRE1 and ATF6 (Figure 5). These results
indicate that limonene inhibits the activation of ER stress sensors induced by MG in MC3T3-E1 osteoblastic cells. Thus, the modulation of ER stress signaling pathways by limonene may be important for protecting against MG-induced cellular damage.

**Effects of limonene on autophagy and ROS production in MG-treated MC3T3-E1 cells**

ER stress can induce autophagy to alleviate the burden of unfolded proteins and ER membrane aggregation, thus enabling the cell to avoid apoptosis (Zhu, Dunner, & McConkey, 2010). The autophagic pathway is one of the ways in which cells can degrade unfolded proteins after ER stress. Autophagy constitutes a cytoprotective response that provides a survival advantage to cells undergoing nutrient deprivation or other cellular stress signals. However, in the apoptotic state, autophagy may play different roles, favoring cell survival or death as a function of the cell type, stimulus, and microenvironment (Mariño, Nisos-Santano, Baehrecke, & Kroemer, 2014). Our results show that autophagy increased during incubation with MG, and a remarkable decrease in autophagy was seen in limonene- (0.01–1 μM) or AG-treated cells in the presence of MG (Figure 6). It is suggested that ROS may act as signaling molecules to increase mitochondrial membrane lipid peroxidation and mitochondrial dysfunction, resulting in autophagic cell death (Kirkland,
Figure 5. Limonene inhibited the ER stress induced by MG in MC3T3-E1 cells. Osteoblasts were pre-incubated with limonene (Lim) before treatment with 400 μM MG for 24 h. The control values for (A) ATF4 and (B) IRE1 were 54.72 ± 0.498 ng/mg and 3.095 ± 0.179 ng/mg, respectively. #P < .05, compared with untreated cells; *P < .05, compared with cells treated with MG alone.

Figure 6. Autophagic activity in MC3T3-E1 cells exposed to limonene in the presence of MG. Osteoblasts were pre-incubated with limonene (Lim) or AG before treatment with 400 μM MG for 24 h. Data are expressed as mean relative percentages of fluorescence. #P < .05, compared with untreated cells; *P < .05, compared with cells treated with MG alone.
Adibhatla, Hatcher, & Franklin, 2002; Xue, Fletcher, & Tolkovsky, 1999). ROS reportedly induce autophagy by activating ERK and JNK, in turn inhibiting mTOR signaling by activating AMPK (Cheng et al., 2013; Kang, Livesey, Zeh, Lotze, & Tang, 2011). There is also evidence that ROS mediate strong upregulation of Beclin 1 by activating NF-κB, which is responsible for ROS-induced autophagy (Haar et al., 2014; Zeng et al., 2013). Bai et al. (2016) reported that limonene can effectively prevent the oxidative damage caused by ROS. In our research, ROS levels significantly increased when MC3T3-E1 osteoblastic cells were incubated with MG, but treatment with limonene decreased intracellular ROS levels (Figure 7). Thus, we speculate that MG may induce autophagy by elevating ROS levels in MC3T3-E1 osteoblastic cells, and limonene protects cells by reducing ROS generation, weakening MG-induced autophagy.

Since diabetes and accumulation of AGEs are important consequences of MG toxicity, early treatments to reduce AGE-induced ROS may affect metabolic diseases. From our observations, we expect that the oral ingestion of MG-inhibitory limonene provide a safe and efficacious treatment that may slow the progression of diabetes-induced bone diseases.

In conclusion, limonene, which may stimulate osteoblastic cell function, is a promising compound for the development of disease-modifying drugs for preventing and treating osteoporosis. Moreover, our results revealed that limonene treatment significantly reduced the levels of ER stress and autophagy induced by MG. Therefore, limonene might contribute to the prevention of diabetic osteopathy.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**Figure 7.** Antioxidant effect of limonene on MG-induced oxidative stress in MC3T3-E1 cells. Osteoblasts were pre-incubated with limonene (Lim) before treatment with 400 μM MG for 48 h. The data show changes in levels of ROS, which was measured by DCF fluorescence method. #P < .05, compared with untreated cells; *P < .05, compared with cells treated with MG alone.
Notes on contributors

Kwang Sik Suh had extensive experience and skills for the experiments of endocrinology and metabolism, especially pancreatic beta cells and osteoblast cell culture. Suh received Ph.D. degree from Dongguk University. He serves as research associate for research institute of endocrinology, Kyung Hee University Hospital. He is the author of more than 35 experimental papers.

Suk Chon is associate professor, department of endocrinology and metabolism, Kyung Hee University since 2013. She received a license of medical doctor, board of internal medicine, and subspecial board for endocrinology and metabolism in Korea. Clinical research experience: Clinical research center for type 2 diabetes mellitus, clinicaltrial.gob(NCT01212198), executive investigator (2005–2014), Korea Health Technology R&D project through the Korea Health Industry Development Institute, Second subject principal investigator (Dec, 2014 to present).

Eun Mi Choi is currently research professor of Kyung Hee University in Korea, where she has been since 2010. She received a B.S., M.S., and Ph.D. from Kyung Hee University in 1994, 1996, and 2001, respectively. She has more than 20 years experience in Food Science and is the author of more than 140 scientific publications.

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