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Limonene promotes osteoblast differentiation and 2-deoxy-D-glucose uptake through p38MAPK and Akt signaling pathways in C2C12 skeletal muscle cells

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ABSTRACT

Background: Limonene is a cyclic monoterpene (CTL) found in citrus fruits and many plant kingdoms. It has attracted attention as potential molecule due to its diverse biological activities. However, molecular mechanism involved in the osteogenic induction of CTL in C2C12 skeletal muscle cells remain unclear.

Purpose: Skeletal development maintains the bone homeostasis through bone remodeling process. It coordinated between the osteoblast and osteoblast process. Osteoporosis is one of the most common bone diseases caused by a systemic reduction in bone mass. Recent osteoporosis treatment is based on the use of anti-resorptive and bone forming drugs. However, long term use of these drugs is associated with serious side effects and strategies on the discovery of lead compounds from natural products for osteoblast differentiation are urgently needed. Therefore, we planned to find out the role of CTL on osteoblast differentiation and glucose uptake in C2C12 cells and its effect on signaling pathways.

Methods: Cell proliferation, alkaline phosphatase (ALP) activity, calcium deposition, genes, and proteins associated with osteoblast activation and glucose utilization were analysed.

Results: CTL did not affect the cell viability. CTL significantly increased ALP activity, calcium depositions and the expression of osteogenic specific genes such as Myogenin, Myogenic differentiation 1 (MyoD), ALP, Run-related transcription factor 2(RUNX2), osteocalcin (OCN). In addition, CTL induced the mRNA expression of bone morphogenetic proteins (BMP-2 BMP-4 BMP-6 BMP-7 BMP-9). CTL treatment enhanced 2-Deoxy-D-glucose (2DG) uptake. Moreover, CTL stimulated the activation of p38 mitogen activated protein kinase (p38MAPK), Protein kinase B (Akt), Extracellular signal related kinase (ERKs) by increasing phosphorylation. CTL treatment abolished p38 inhibitor (SB203580) mediated inhibition of osteoblast differentiation, but no effect was noted by ERKs specific inhibitor (PD98059).

Conclusion: These results suggest that limonene induces osteoblast differentiation and glucose uptake through activating p38MAPK and Akt signaling pathways, confirming the molecular basis of the osteoblast differentiation by limonene in C2C12 skeletal muscle cells.

Introduction

Skeletal muscle development is strictly regulated process involving formation of mesodermal into myoblast, following the differentiation and fusion, finally changed into multinucleated myotubes. Body constitutes 40% skeletal muscle cells and plays many roles in locomotion and whole body metabolism. It is a major part of the energy production in human and utilizes the more than 70% of glucose and maintains the

Abbreviations: CTL, Limonene is a cyclic monoterpene; ALP, alkaline phosphatase; MyoD, Myogenic differentiation 1; RUNX2, Run-related transcription factor 2; OCN, osteocalcin; BMP, bone morphogenetic proteins; 2DG, 2-Deoxy-D-glucose; p38MAPK, p38 mitogen activated protein kinase; Akt, Protein kinase B; ERKs, Extracellular signal related kinase; MRFs, Myogenic regulatory factors; bHLH, basic helix-loop-helix; Myf5, myogenic factors-5; DMEM, Dulbecco modified Eagle medium; FBS, fetal bovine serum; OPN, osteopontin; RDA, Rural development adiministration

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lipid homeostasis. Myogenic regulatory factors (MRFs) play an important role in muscle fusion during differentiation (Dedieu et al., 2002; Doherty et al., 2011). Among these factors, basic helix-loop-helix (bHLH) transcription factors, myogenin, myogenic differentiation −1 (MyoD), myogenic factors-5(Myf5) and myogenic regulator factor −4 are plays critical role in muscle formation. Skeletal muscle cells maintain the bone homeostasis through bone remodeling process. It coordinated between the osteoblast-mediated bone formation and osteoclast-mediated bone resorption (Harada and Rodan, 2003). Osteoporosis is one of the most common bone diseases caused by a systemic reduction in bone mass (Ross et al., 1990). Current osteoporosis treatment is based on the use of anti-resorptive and bone forming drugs. However, long term use of these drugs is associated with serious side effects (Yu et al., 2013). Therefore, effective treatment strategies without side effects are urgently needed for activation of osteoblast differentiation. Many researchers have studied the osteoblast enhancing effects of lead compounds derived from natural products (Jang et al., 2007; Kim et al., 2011; Kim et al., 2015; Kim and Kim, 2010).

Monoterpenes are the major constituents of plant kingdom and it possess different biological functions including antimicrobial, allelepathic, herbivore deterring, pollinator attracting, antioxidative, antipalogistic, anti-tumor, anti-viral, and antinoceptive properties, antiobesity, anti-inflammatory, and immunomodulatory effects (Alkhateeb and Bonen, 2010; Jing et al., 2013; Kamatou et al., 2013; Tan et al., 2016; Victor Antony Santiago et al., 2012) . In addition, it has been reported to enhance glucose uptake in 3T3-L1 adipocytes (Tan et al., 2016). However, the mechanism of cyclic terpene limonene (Fig. 1) in skeletal muscle cell differentiation is poorly understood. Because of there are no reports on the molecular mechanism of limonene in osteoblast differentiation. In this study, we aimed to determine the effect of (R)-(+)-limonene on osteogenic differentiation and glucose utilization in C2C12 cells. In addition, the molecular mechanism involved in the effects of limonene was explored in this study.

Methods and materials

Cell culture and chemicals

The C2C12 cell line was obtained from the American Type Culture Collection [Rockwille, MD, USA]. Dulbecco modified Eagle medium [DMEM] and fetal bovine serum (FBS) were procured from Gibco-BRL [Gaithersburg, MD, USA]. Kits for mRNA extraction, iScript cDNA synthesis kit and qPCR were purchased from Bio-Rad [Bio-Rad, USA]. Limonene was obtained from sigma Aldrich USA. SB203580 and PD98059 inhibitors were purchased from cell signaling technology (USA, MA).Antibodies used in the study was obtained from cell signaling technology and Santa Cruz Biotechnology, USA.

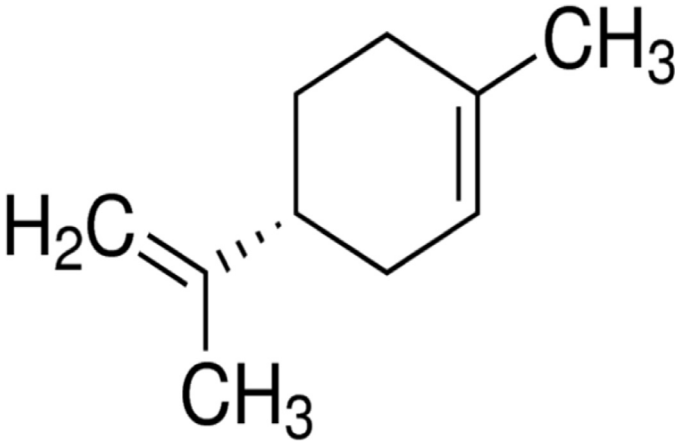

Fig. 1. Molecular structure of cyclic terpene Limonene (CTL).

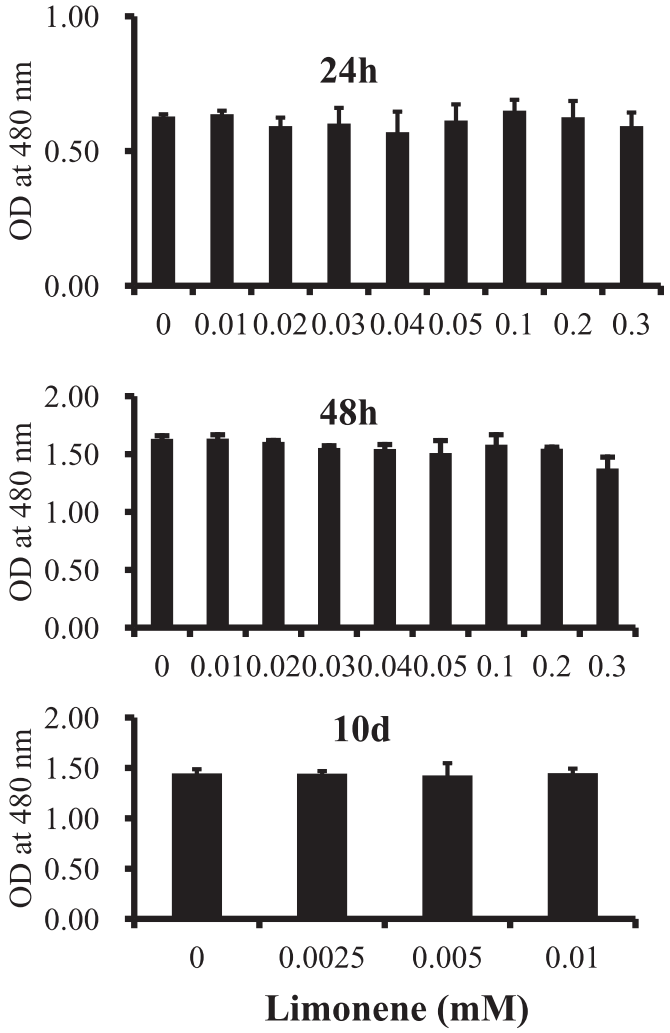

Fig. 2. Cell viability study of CTL using Ez-cytox reagent. 1×10^4 cells were seeded in 96 well plates and treated with different concentration of CTL. Plates were incubated at 37 °C with 5% CO_2 for 24 h, 48 h and 10 days. Cell viability was assayed with Ez-cytox reagent.

Cell viability study

Ez-cytox assay kit (iTSBiO, Korea) was used to determine cell viability effects of limonene. Briefly, C2C12 cells (ATCC, USA) were seeded into 96-well cell culture plates at density of 1^4 cells/well and incubated at 37 °C with 5% CO_2 for 24 h. These cells were then treated with different concentrations of cyclic terpene limonene (CTL) and incubated for 24 h, 48 h, and 10 days. Ten microliters of WST reagent were added to each well and incubated at 37 °C with 5% CO_2 for 1–2 h. Absorbance (color intensity) of each well was measured at wavelength of 450 nm using a spectra count ELISA plate reader [Packard Instrument Co., USA].

C2C12 skeletal muscle differentiation

C2C12 skeletal muscle cells were seeded into 6-well cell culture plates at density of 5 × 104 cells/well. Cells were cultured in 10% FBS in DMEM (ATCC30-2002) medium and incubated at 37 °C with 5% CO_2. Growth medium was replaced by differentiation medium every 24 h with different concentration of CTL in the presence of 2% horse serum after cells reached 80 − 90% confluence (Ilavenil et al., 2016).