Life Sciences 94 (2014) 45–53

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Involvement of MAPK signaling pathway in the osteogenic gene expressions of Cervi Pantotrichum Cornu in MG-63 human osteoblast-like cells

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ARTICLE INFO

Article history:
Received 4 July 2013
Accepted 1 November 2013

Keywords:
Cervi Pantotrichum Cornu
Osteoblast
MG-63
MAPK
ERK1/2
JNK1/2
p38
ELK1
cJUN
COL1A1
ALPL
BGLAP
SPP1

ABSTRACT

Aims: The purposes of this study were to determine whether Cervi Pantotrichum Cornu (CPC) has osteogenic activities in human osteoblastic MG-63 cells and to investigate the underlying molecular mechanism.
Main methods: The effects of CPC on alkaline phosphatase activity, collagen synthesis, and calcium deposits were measured. The *COL1A1*, *ALPL*, *BGLAP*, and *SPP1* expressions were measured by real-time PCR. Phosphorylated MAP kinases (ERK1/2, JNK1/2, p38, ELK1, and cJUN) were studied by western blot analysis. The involvement of MAPK pathway in osteogenic gene expressions was determined by using each selective MAPK inhibitor (PD98059, SP600125, and SB203580).
Key findings: CPC increased alkaline phosphatase activity, collagen synthesis, and calcium deposits. CPC activated ERK1/2, JNK1/2, p38, and ELK1 phosphorylation except cJUN. CPC increased the *COL1A1*, *ALPL*, *BGLAP*, and *SPP1* gene expressions. The elevated *COL1A1* and *BGLAP* expressions were inhibited by PD98059, SP600125 or SB203580. The elevated *ALPL* expression was blocked by SB203580. The elevated *SPP1* expression was inhibited by SP600125 or SB203580. CPC increased *COL1A1* and *BGLAP* expressions via ERK1/2, JNK1/2, and p38 MAPKs pathways and *SPP1* expression via JNK1/2 and p38 pathways. p38 pathway is needed for *ALPL* expression.
Significance: These results imply that MAPK signaling pathway is an indispensable factor for bone matrix genes expression of CPC in MG-63 human osteoblast-like cells.

Introduction

Cervi Pantotrichum Cornu (CPC) is the young pilose antler of a male *Cervus nippon* Temminck or *Cervus elaphus* Linnaeus, and is renowned as a bone-strengthening drug in many Asian countries. It is widely used in traditional medicinal practices to promote virility, replenish vital essence and blood, and strengthen the tendons and bones (Bensky et al., 2004).

The osteoblasts serve the function of synthesizing the extracellular matrix (ECM) of bone, regulating calcium deposition and mineralization, and responding to mechanical stimuli. Accordingly, osteoblasts are believed to play a pivotal role in strengthening bone (Salgado et al., 2004).

Recent studies have also demonstrated that mechanotransduction in bone cells involves the sequential activations (via phosphorylation cascade) of various intracellular signaling molecules, including mitogen-activated protein kinases (MAPKs) (Greenblatt et al., 2013; He et al., 2012; Mahalingam et al., 2013; Thouverey and Caverzasio, 2012), phosphoinositide 3-kinase (PI3k)/Akt (Danciu et al., 2003), and protein kinases B and C (Biggs et al., 1999; Geng et al., 2001). As a result, mechanical signals can activate transcription factors such as activator protein-1 (AP-1) (Peverali et al., 2001), bone-specific transcriptional regulator (Cbfa1) (Franceschi, 1999; Wu et al., 2012), and NF-kB (Granet et al., 2001) to modulate the expression of genes that regulate different physiological functions (Wu et al., 2006).

There are several studies about the effects of CPC. Ahn and Shim studied the effects of CPC on an aged ovariectomized rat model of postmenopausal osteoporosis in 1998 (Ahn and Shim, 1998) and Lee et al. showed the stimulating effects of fermented CPC on osteoblastic differentiation and mineralization (Lee et al., 2011). However, merely identifying the drug's fragmentary effects is not sufficient. The evaluation of

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http://dx.doi.org/10.1016/j.lfs.2013.11.002

the action mechanisms of a medicine is also important to verify the efficacy of the drug.

In the present study, we tried to identify the MAPKs signaling pathway among numerous mechanisms (extracellular-signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2) and p38). The expression of osteogenic genes including collagen, type I, alpha 1 (*COL1A1*), alkaline phosphatase (*ALPL*), bone gamma-carboxyglutamic acid protein (*BGLAP*, osteocalcin), and secreted phosphoprotein 1 (*SPP1*, osteopontin) were measured by real-time PCR. The signaling pathways of osteogenic mRNAs expressions were verified by using PD98059 (selective inhibitor of ERK1/2 and ELK1), SP600125 and BI-78D3 (selective inhibitors of JNK1/2), and SB203580 (selective inhibitor of p38).

Materials and methods

Materials

The CPC was obtained from Hmax Co. (Jecheon, Chungbuk, Korea) and it had been authenticated by Prof. Bu (the department of herbal pharmacology, Kyung-Hee University). 100 g of CPC was extracted in 2,000 ml of distilled water (DW) at 100 °C for 3 h, filtered through filter paper, and concentrated by rotary evaporator and then freeze-dried. The yield of freeze-dried CPC was calculated to be 29.9%. Anti-JNK, anti-p-JNK, anti-ERK, anti-p-ERK, anti-cJUN, anti-p-cJUN, anti-p-ELK-1, anti-beta actin (ACTB), and secondary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent and mitogen-activated protein kinases (MAPK) inhibitors (PD98059, SP600125, BI-78D3, and SB203580) were purchased from Sigma-Aldrich (New York, NY, USA).

Cell culture and treatment

Human osteoblast-like cells (MG-63) were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured according to the recommendation of the supplier. MG-63 cells were maintained using Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand island, NY, USA) containing 10% v/v fetal bovine serum (heat-inactivated, Invitrogen, Grand island, NY, USA) and 100 U/ml penicillin/streptomycin (Sigma-Aldrich, New York, NY, USA) in an incubator with a humidified atmosphere of 95% air and 5% CO_2, at 37 °C.

MTT assay

Cell proliferations and cytotoxicity were measured with the MTT assay. MG-63 cells were seeded in a 96-well plate in a density of 1.0×10^4 cells/well and incubated at 37 °C. The following day, various concentrations (0 (PBS only), 5, 10, 25, and 50 μg/ml) of CPC were treated and incubated for 24 h. The MTT was added and incubated for 4 h. The formazan crystals that formed in the actively metabolizing cells were extracted with 200 μl of dimethyl sulfoxide (DMSO, Sigma-Aldrich, New York, NY, USA) and the absorbance at 595 nm was measured by a Synergy 2 microplate reader (BioTek Inc., Winooski, VT, USA).

Alkaline phosphatase assay

Alkaline phosphatase (ALP) activities were measured by ALP assay. In brief, MG-63 cells were seeded in a 96-well plate in a density of 1.0×10^4 cells/well and incubated at 37 °C. The following day, various concentrations (0 (PBS only), 5, 10, 25, and 50 μg/ml) of CPC were treated and incubated for 12 h. The cells were then rinsed with saline and recovered by ALP lysis buffer containing 1 mM $MgCl_2$ and 0.05% Triton X-100. The cell lysates were transferred to a new 96-well plate, and 100 μl of 22.4 mM p-nitrophenyl phosphate in 2 M diethanolamine with 1 mM $MgCl_2$ (pH 9.8) was added. The samples were incubated in 37 °C for 30 min and the absorbance of samples was measured at 405 nm.

Collagen assay

Collagen synthesis was measured using picro-sirius red method (Puchtler et al., 1973). MG-63 cells were seeded in 12-well plates in a density of 1.0×10^4 cells/well and incubated at 37 °C. The next day, the medium was removed and washed with phosphate buffered saline (PBS). Then 2 ml of osteogenic medium (Dulbecco's Modified Eagel Medium with 10 mM beta-glycerophosphate, 5 nM dexamethasone, and 50 μg/ml ascorbic acid) was added to each well. Then 0 (PBS only), 10, and 50 μg/ml of CPC were treated and incubated for 7 days. The 10 and 50 μg/ml concentrations were chosen since the minimum effect and plateau had been gained under those two concentrations respectively in cell proliferation and ALP activity assay results. The osteogenic media with CPCs were changed every 2-3 days. After 7 days, the mediums were discarded and the cells were fixed with Bouin's fluid (8.3% formaldehyde and 4.8% acetic acid in saturated aqueous picric acid) for 1 h. Then the fixing solution was removed and the cells were stained with 0.1% sirius red (Direct Red 80, Sigma-Aldrich, New York, NY, USA) in a saturated aqueous solution of picric acid. After 30 min, the dye solution was removed and the cells were washed with 0.1 M HCl. Then, 0.5 M NaOH was added to dissolve the stained dye. The solutions were transferred to a 96-well plate and the absorbance was measured at 540 nm.

Calcium assay

The formation of calcium phosphate was determined by using the alizarin red-S assay (Puchtler et al., 1969). MG-63 cells were seeded and incubated, as described above in the collagen assay process. The 0 (PBS only), 10, and 50 μg/ml of CPC were incubated in MG-63 cells for 7 days. After 7 days, MG-63 cells were washed three times with PBS and fixed with 10 % formaldehyde for 15 min. Fixed cells were washed three times with DW and stained with 2% of alizarin red-S (pH 4.2, Sigma-Aldrich, New York, NY, USA) for 20 min at room temperature. Each well was then washed with DW and observed using an optical microscope. Stained alizarin red-S was extracted with 10% acetic acid and the amount of calcium deposition was quantified at 405 nm.

Western blot analysis

MG-63 cells were seeded in 100-mm culture dishes (5.0×10^5) and incubated in a humidified atmosphere at 37 °C containing 5% CO_2 for 24 h. The next day, the medium was changed to DMEM without FBS. After 24 h, the cells were treated with CPC (50 μg/ml) and incubated for various time points (0, 0.25, 0.5, 1, 2, 4, and 8 h). Proteins were extracted with PRO-PREP Protein Extraction solution (Intron biotechnology, Seongnam, Kyungi, Korea). The cells were washed twice with PBS and 300 μl of PRO-PREP solutions containing phosphatase inhibitor cocktail 2 and 3 (Sigma-Aldrich, New York, NY, USA) were added, and then incubated in -20 °C for 20 min. The cells were homogenized by pipetting and centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatant was transferred to a fresh tube. Protein concentrations were measured using DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). Five microliters of standards and protein samples were transferred to 96-well plate and 25 μl of alkaline copper tartrate solution contains Reagent S. Then 200 μl of dilute Folin Reagent was added and incubated. After 15 min, the protein concentrations were measured at 750 nm using an ELISA reader (Synergy2, Biotek, Winooski, VT, USA). Each protein was denatured with 5× sample buffer and boiled for 5 min. Each protein was then fractionated by electrophoresis through a 10% SDS polyacrylamide gel at 100 V for 2 h, and the proteins were transferred onto PVDF membranes at 100 V for 60 min. Each membrane was blocked with TBST buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% BSA for 1 h and then incubated with primary antibodies (anti-JNK, anti-p-JNK, anti-ERK, anti-p-ERK, anti-cJUN, anti-p-cJUN, anti-p-ELK-1, and anti-ACTB antibodies raised in rabbit) in TBST