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β-Carotene suppresses osteoclastogenesis and bone resorption by suppressing NF-κB signaling pathway

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ABSTRACT

Aims: β-Carotene is a natural anti-oxidant, which has been used for treatment of cancer and cardiovascular diseases. Recently, the ameliorating function of β-carotene in osteoporosis has been implicated. However, the precise mechanism of β-carotene in prevention and treatment of osteoporosis is largely unknown. In the present study, we aimed to elucidate how β-carotene affects osteoclast formation and bone resorption.
Main methods: Bone marrow-derived monocytes/-macrophages (BMM) were exposed to 0.05, 0.1, 0.2, 0.4 and 0.6 μM β-carotene, followed by evaluation of cell viability, lactate dehydrogenase (LDH) release, receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclastogenesis and resorption pits formation. Key factors in nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) pathways were evaluated with western blot after BMM cells were exposed to RANKL and β-carotene. The effects of β-carotene in nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), c-Fos and cathepsin K (CTSK) expression were also evaluated.
Key findings: β-Carotene significantly inhibited BMM viability and promoted LDH release at concentrations of 0.4 and 0.6 μM. A decrease in RANKL-induced osteoclastogenesis and resorption was also observed after β-carotene treatment. β-Carotene attenuated the NF-κB pathway activation by RANKL, with no effect on MAPK pathway. β-Carotene suppressed the upregulation of NFATc1 and c-Fos by RANKL.
Significance: We clarified the anti-osteoclastogenic role of β-carotene, which is mediated by NF-κB signaling.

1. Introduction

Bone osteoporosis is a devastating disease characterized by low bone density, bone fragility and susceptibility to fracture. Women, especially postmenopausal women, are extremely vulnerable to osteoporosis. Osteoporosis results from an imbalance in bone remolding, which are mediated by two types of cells: osteoclasts are bone-absorbing multinucleated cells generated from hematopoietic mononuclear precursor cells, the over-activation of which significantly contributes to the aberrant bone resorption; osteoblasts are bone-forming cells. Reduction in bone mass is a result of increased osteoclast activity and decreased osteoblast activity [1]. The formation of osteoclasts, also called osteoclastogenesis, is a multi-stage process regulated by a number of genetic, humoral, and mechanical factors [2]. Macrophage colony-stimulating factor (M-CSF) [3] and receptor activator of nuclear factor kappa B ligand (RANKL) [4] are two putativepromoters of osteoclastogenesis. These factors are culminated in the activation of c-Fos and nuclear factor of activated T cell c1 (NFATc1) to facilitate osteoclast differentiation. Efforts have been devoted to the development of drugs that target these factors to suppress osteoclastogenesis.

The link between nutrient intake and bone health has been implicated in numerous studies [5,6]. Fruits and vegetables are rich source of nutrients, including magnesium, potassium, vitamins, calcium, etc., which are modifiable protective factors of bones [7]. Emerging evidences indicate that carotenoid intake is associated with a reduced risk of osteoporosis [8–10]. Carotenoids are putative anti-oxidants that have been applied for cardiovascular care [11], cancer inhibition [12], etc. It has been realized that oxidative stress is also a significant contributor to the activation of osteoclastogenesis and bone resorption [13,14]. Therefore, it is likely that carotenoids exert ameliorating effect in bone osteoporosis by acting as anti-oxidants. In spite of reports that implicated the role of carotenoids in suppressing osteoclastogenesis [15], the precise mechanism of action of carotenoids has not been clarified.

Our unpublished study indicate that β-carotene is enriched in pepino (*Solanum muricatum Ait.*), a fruit that was demonstrated to promote

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bone repairing in an animal model. Herein, we set force to study the effect of β-carotene in the proliferation and differentiation of osteoclasts, as well as its impact on resorption pits formation in vitro. As NF-κB and MAPK pathways are closely associated with osteoporosis and oxidative stress, we further investigated if β-carotene inhibited osteoclastogenesis through regulating NF-κB and MAPK pathways. It was found that β-carotene exerted inhibitory effect on osteoclastogenesis and resorption pits formation and this effect was mediated by NF-κB pathway. The findings in this study validated the protective role of β-carotene in osteoporosis and provided basis for further application of β-carotene as a medication for osteoporosis.

2. Material and methods

2.1. Isolation of bone marrow-derived monocytes/-macrophages

Isolation of bone marrow-derived monocytes/-macrophages (BMM) was performed according to a previously reported protocol [16]. Briefly, after sacrificing the Balb/c mice (The Jackson Laboratory, Bar Harbor, Maine, USA), abdomen and hind legs of the mice were sterilized. An incision in the midline of the abdomen was made, followed by clipping to expose the hind legs. Muscle tissue on the bone was then removed using scissors. Bones were crushed in a mortar. The Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin and 20 mM HEPES was used to suspend the bone marrow cells. The bone marrow cells were then cultured in a humidified incubator with 5% CO_2 at 37 °C, during which differentiation of bone marrow cells generated BMMs that were adherent to the plate. Once sufficient BMMs were generated, the adherent cells were collected for further assays.

2.2. Cell viability assay

BMM cells were seeded in 6-well plates and cultured to approximately 80% confluency. β-Carotene (Sigma, St Louis, MO) was dissolved in tetrahydrofuran (Sigma) to a stock solution of 10 mM, then it was diluted with the culture medium to concentrations of 0.05, 0.1, 0.2, 0.4 and 0.6 μM followed by incubation for 72 h. Then medium was replaced with medium containing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and was cultured for another 4 h. The cell viability was determined by measuring absorbance at 490 nm using a plate reader. Lactate dehydrogenase (LDH)-release assay was also perform to evaluate the cell damage from β-carotene. The supernatant of culture medium (50 μL) after 72 h incubation with β-carotene was transferred to a 96-well plate. Then, 50 μL reaction mixture (Pierce LDH Cytotoxicity Assay Kit, WI, USA) were added and incubated at room temperature for 30 min, followed by adding 50 μL of stopping solution. Absorption at 490 and 680 were measured to evaluate the LDH content in the medium.

2.3. Osteoclastogenesis assay

BMM cell seeded in 96-well plates were induced with RANKL (200 nM) for 48 h before adding β-carotene. After 72 h, medium was removed and cells were fixed with 4% paraformaldehyde (PFA), stained for tartrate-resistant acid phosphate (TRAP) activity and counterstained with hematoxylin. The stained cells were then photographed using an inverted microscope equipped with a digital camera. Four random fields were recorded and the $TRAP^+$ areas were measured using ImageJ (NIH, Bethesda, MD, USA).

2.4. Resorption pit assay

BMM cells were plated at the density of 250,000 cells/well in 96-well plates previously equipped with bone slices (Sigma) and allowed to adhere to plate overnight. RANKL was added to the medium and after 48 h, β-carotene was added and incubated for another 12 h. Cells were then washed twice with PBS and detached from bone slices by ultrasonication in 250 μL of 70% isopropanol for 20 min. Resorption pit formation was visualized by toluidine blue (1%). The slices were photographed and the area/number of resorption was counted.

2.5. Western blot

Cells seeded in 6-well plates were first lysed using Radioimmunoprecipitation assay (RIPA) buffer (Abcam, Cambridge, MA, USA) according to manufacturer's protocols. For western blot analysis of cytoplasmic and nuclear components of the cells separately, the NE-PER nuclear and cytoplasmic extraction kit (catalog no. 78835, Thermo Scientific, Cambridge, MA, USA) was used. In brief, cells were suspended in cytoplasmic extraction reagent I, followed by incubation on ice for 10 min. Ice-cold buffer cytoplasmic extraction reagent II was then added. After vigorous vortex, centrifugation (16,000 × *g*) at 4 °C for 5 min was performed to obtain the nuclear pellets. The protein concentration was determined using a Bio-Rad protein assay kit. Protein content of cell lysates was quantified using the bicinchoninic acid (BCA) assay (Pierce, WI, USA). Protein was loaded into precast gels, resolved using electrophoresis and transferred to nitrocellulose membranes. Rabbit antibodies against p65, IκBα, p-IκBα, ERK, JNK, pJNK, p38, NATFc1, c-Fos and CTSK were purchased from Cell Signaling (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies were subsequently used, followed by adding bioluminescence substrates for visualization of protein bands. Band intensities were quantified by ImageJ.

2.6. Luciferase transcriptional reporter assay

To generate stable NAFTc1-luc-expressing BMM cells, the lenti-plasmid with luciferase cDNA was constructed, and transfected with packaging plasmids using the lentiviral packing kit (OriGene, Rockville, MD, USA) into HEK 293T cells for virus packaging. The BMM cells were infected with the packaged viruses and selected by puromycin for 48 h. BMM cells stably expressing NAFTc1-luc reporter were treated with RANKL, with or without β-carotene. At the end of treatment, cells were harvested and measured for luciferase activity using Luciferase Assay System (Promega, Madison, WI, USA). All measurements were performed in triplicates. Luciferase activities were normalized to the cell number.

2.7. Statistical analysis

All experiments were repeated three times unless otherwise stated. Data were analyzed using one-way ANOVA analysis followed by a Tukey's post hoc test (SPSS, Chicago, IL). The differences with a P value of <0.05 were considered statistically significant.

3. Results

3.1. β-Carotene attenuates osteoclastogenesis in vitro

To evaluate the effects of β-carotene on osteoclastogenesis, we first exposed BMM cells to a range of concentrations of β-carotene and evaluated the cell viability and LDH release. As shown in Fig. 1a, the viability of BMM was decreased with increasing β-carotene concentrations. This inhibition on viability became significant when β-carotene concentration reached 0.4 μM ($p < 0.05$ and $p < 0.01$ for 0.4 μM and 0.6 μM, respectively). Consistently, a significant higher LDH release was seen in presence of 0.4 μM and 0.6 μM β-carotene (Fig. 1b), suggesting that β-carotene exerted cytotoxic effects by inducing damage to BMM cell membrane.

In osteoclastogenesis assay, BMM cells were first activated by RANKL for 48 h, followed by incubation with various concentrations of β-