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Research Article

Scoparone attenuates RANKL-induced osteoclastic differentiation through controlling reactive oxygen species production and scavenging

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ABSTRACT

Scoparone, one of the bioactive components of *Artemisia capillaris* Thunb, has various biological properties including immunosuppressive, hepatoprotective, anti-allergic, anti-inflammatory, and antioxidant effects. This study aims at evaluating the anti-osteoporotic effect of scoparone and its underlying mechanism in vitro. Scoparone demonstrated potent cellular antioxidant capacity. It was also found that scoparone inhibited the receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation and suppressed cathepsin K and tartrate-resistant acid phosphatase (TRAP) expression via c-jun N-terminal kinase (JNK)/extracellular signal-regulated kinase (ERK)/p38-mediated c-Fos–nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) signaling pathway. During osteoclast differentiation, the production of general reactive oxygen species (ROS) and superoxide anions was dose-dependently attenuated by scoparone. In addition, scoparone diminished NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 1 (Nox1) expression and activation via the tumor necrosis factor receptor-associated factor 6 (TRAF6)–cSrc–phosphatidylinositol 3-kinase (PI3k) signaling pathway and prevented the disruption of mitochondrial electron transport chain system. Furthermore, scoparone augmented the expression of superoxide dismutase 1 (SOD1) and catalase (CAT). The overall results indicate that the inhibitory effect of scoparone on RANKL-induced osteoclast differentiation is attributed to the suppressive effect on ROS and superoxide anion production by inhibiting Nox1 expression and activation and protecting the mitochondrial electron transport chain system and the scavenging effect of ROS resulting from elevated SOD1 and CAT expression.

Abbreviations: TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of nuclear factor-κB ligand; ROS, reactive oxygen species; Nox1, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 1; SOD 1, superoxide dismutase 1; CAT, catalase; NFATc1, nuclear factor of activated T cells, cytoplasmic 1; TRAF6, tumor necrosis factor receptor-associated factor 6; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; GTP-Rac1, guanosine triphosphate (GTP) bound Rac1

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Introduction

Bone remodeling is an orchestrated process involving osteoclasts and osteoblasts. After mineralized bone is absorbed by osteoclasts and bone matrix is formed by osteoblasts, resorbed lacunae are filled to the original level by osteoblasts [1]. Bone formation relies on osteoblastic proliferation, alkaline phosphatase activity, collagen synthesis, and mineralization, whereas bone resorption is closely related to osteoclast formation and tartrate-resistant acid phosphatase (TRAP) activity [2]. Thus, excessive osteoclastic bone resorption relative to osteoblastic bone formation often results in bone diseases including osteoporosis and bone loss [3,4].

The osteoclast is a large multinucleated cell derived from such mononuclear precursors as monocytes and macrophages [3]. Differentiation of precursors into osteoclasts can be induced by the receptor activator of nuclear factor-κB ligand (RANKL) produced by osteoblasts [3]. The binding of RANKL to their receptors induces small nontoxic amounts of reactive oxygen species (ROS) like various growth factors and cytokines including tumor necrosis factor-α [5]. It has been suggested that NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 1 (Nox1) as well as mitochondria may be involved in ROS production through RANKL-mediated effects during osteoclast differentiation [6,7]. Our previous study reported that ROS production reaches to a maximum level after two days of the osteoclast differentiation period [8]. The low level ROS increase may play a crucial role as a secondary messenger in RANKL-induced signaling pathways for osteoclast differentiation [5]. Accordingly the suppression of ROS production by natural antioxidants such as anthraquinone [2], scopoletin [8], genistein [9], silibinin [10], resveratrol [11], curcumin [12], and sauchinone [13] can attenuate osteoclastic differentiation from precursors [14].

Scoparone, 6,7-dimethoxy coumarin, is one of the bioactive components of *Artemisia capillaris* Thunb which has been used as both an edible herb and a folk medicine in Asian countries due to several therapeutic functions such as hepatoprotective, diuretic, analgesic, and antioxidative activities [15,16]. Recent studies have demonstrated that scoparone has various biological properties including immunosuppressive [17,18], hepatoprotective [19], anti-allergic [20], anti-inflammatory [21], and antioxidant effects [19]. In view of the structure–activity relationship of coumarin derivatives, scoparone appears to show less antioxidant activity compared to other coumarin derivatives because it does not carry any hydroxyl groups which are required for exerting cellular antioxidant activity [22]. In an animal study using rats, the potent hepatoprotective effect of scoparone through preventing ROS generation on carbon tetrachloride-induced hepatic injury was reported [19]. In addition, the oral administration of scoparone induced an increase in superoxide dismutase (SOD) and catalase (CAT) activity in liver tissue [18]. To the best of our knowledge, however, the direct evidence of the suppressive effect of scoparone on osteoclast differentiation through the control of ROS generation and the underlying mechanism have not been reported yet. These characteristics of scoparone as an intracellular antioxidant inspire us to investigate the inhibitory effect of scoparone through the control of ROS production on osteoclast differentiation.

In this study, after the cellular antioxidant capacity of scoparone was evaluated, the inhibitory effect of scoparone on osteoclast differentiation through attenuating osteoclastogenic biomarkers such as cathepsin K and TRAP and ROS level was examined when RAW264.7 macrophages were cultured in RANKL-supplemented medium to multinucleated osteoclasts. In addition, for elucidating the control of ROS level by scoparone, the down-regulating effects on Nox1 activation system, the up-regulating effects on the mitochondrial membrane potential (MMP), and the expression of SOD and CAT as ROS scavengers were investigated.

Materials and methods

Reagents

Scoparone, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), neocuproine, Dulbecco's modified Eagle's medium (DMEM), minimum essential medium alpha medium (α-MEM), fetal bovine serum (FBS), β-glycerophosphate, Triton X-100, Hank's balanced salt solution (HBSS), 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA), dihydroethidium (DHE), rhodamine 123, Alizarin red S, RANKL, leukocyte acid phosphatase assay kit, sodium tartrate, p-nitro-phenylphosphate (PNPP), phosphate buffered saline (PBS, pH 7.4), diphenethyleneiodonium chloride (DPI), phenylmethanesulfonyl fluoride (PMSF), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A bone resorption assay kit was purchased from CosMo Bio Co., Ltd. (Tokyo, Japan). Antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) included Rac1, TRAF6, c-Src, phosphatidylinositol 3-kinase (PI3K), SOD1, CAT, cathepsin, and TRAP. Anti-Nox1 was purchased from Abcam (Cambridge, UK). Anti-SOD1 and p-PI3K were purchased from Cell Signaling Technology (Beverly, MA, USA). HepG2 and RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA).

Cellular antioxidant capacity

Cellular oxidative stress due to ROS generated by AAPH or H_2O_2 was measured spectrofluorometrically using the DCFH-DA method. DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterase to non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. HepG2 cells were first cultured in 96-well plates (5×10^5/ml) with DMEM for 24 h. After the cells were incubated with different concentrations of scoparone dissolved in DMSO for 30 min, the medium was discarded, and the wells were gently washed twice with PBS. HBSS, which is fluorescently stable, was then added to each well instead of normal medium, and AAPH or H_2O_2 was used as an oxidative stress inducer. After the cells were treated with 60 μM AAPH or 1 mM H_2O_2 for 30 min, DCFH-DA was added to the culture plates at a final concentration of 40 μM and incubated for 30 min at 37 °C in the dark. Trolox (10 μM) was used as the positive control. After incubation, the cells were washed with HBSS, and DCF fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Tecan GENios fluorometric plate reader (Salzburg, Austria).

TRAP staining and activity

RAW264.7 cells were seeded in 96-well plates (1×10^4 cells/well) containing DMEM medium plus 10% FBS and the medium was replaced with test samples in a differentiation medium containing 50 ng/ml RANKL. The differentiation medium was changed every 2 days. After 5 days, the cells were fixed in 3.5% formalin for 10 min

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