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Original Contribution

Nrf2 deficiency induces oxidative stress and promotes RANKL-induced osteoclast differentiation

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ABSTRACT

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that regulates the expression of a variety of antioxidant and detoxification genes through an antioxidant-response element. Nrf2 has been shown to protect several types of cells against the acute and chronic injury that accompanies oxidative stress, but its role in osteoclasts remains unclear. In this study, we investigated the role of Nrf2 in osteoclast (OC) differentiation, a process in which reactive oxygen species (ROS) are generated and then participate, using Nrf2-knockout mice. Receptor activator of nuclear factor κB ligand (RANKL)-induced OC differentiation, actin ring formation, and osteoclastic bone resorption were substantially promoted in Nrf2-deficient OC precursor cells compared to wild-type cells. Under both unstimulated and RANKL-stimulated conditions, Nrf2 loss led to an increase in the intracellular ROS level and the oxidized-to-reduced glutathione ratio and a defect in the production of numerous antioxidant enzymes and glutathione. Moreover, pretreatment with *N*-acetylcysteine or diphenyleneiodonium significantly reduced the OC differentiation and decreased the intracellular ROS level in both Nrf2-deficient and wild-type cells. Pretreatment with sulforaphane and curcumin also inhibited the OC differentiation by activating Nrf2 in part. Nrf2 deficiency promoted the RANKL-induced activation of mitogen-activated protein kinases, including c-Jun N-terminal kinase, extracellular signal-regulated kinase, and p38; the induction of c-Fos; and the consequent induction of nuclear factor of activated T cells, cytoplasmic 1, a pivotal determinant of OC differentiation. Our results suggest that Nrf2 probably inhibits RANKL-induced OC differentiation by regulating the cellular redox status by controlling the expression of oxidative response genes, findings that might form the basis of a new strategy for treating inflammatory bone diseases.

Although bone resorption is essential for cellular calcium homeostasis and the metabolic remodeling of bone, excessive bone resorption is a major pathophysiological factor in such chronic inflammatory diseases as osteoporosis, rheumatoid arthritis, and periodontitis [1–3]. The cell type responsible for bone resorption is the osteoclast (OC)[1], a large multinucleated cell derived from the hematopoietic lineage through the cooperative action of macrophage colony-stimulating factor (M-CSF) [4] and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) [5], which is also known as tumor necrosis factor (TNF)-related activation-induced cytokine [6], OC differentiation factor [7], and osteoprotegerin ligand [8]. Therefore, the control of the formation and function of OCs has significant therapeutic implications [9].

RANKL is an essential cytokine for OC differentiation that is expressed in osteoblasts, activated T cells, and stromal cells [10]. Upon the binding of RANKL to its receptor RANK, TNF receptor-associated factor (TRAF) family proteins, such as TRAF6, are recruited to the activated RANK, leading to the activation of NF-κB and mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK), and p38 [11,12], and to the expression of c-Fos. NF-κB then induces the initial expression of nuclear factor of activated T cells, cytoplasmic 1 (NFATc1). As a component of the activator protein-1 (AP-1) transcription factor [13], c-Fos is involved in the robust

Abbreviations: OC, osteoclast; M-CSF, macrophage colony-stimulating factor; NF-κB, nuclear factor-κB; RANKL, receptor activator of NF-κB ligand; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; NFATc1, nuclear factor of activated T cells, cytoplasmic 1; AP-1, activator protein-1; TRAP, tartrate-resistant acid phosphatase; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; Nox, NADPH oxidase; NAC, *N*-acetylcysteine; DPI, diphenyleneiodonium; Nrf2, nuclear factor-erythroid 2-related factor 2; ARE, antioxidant response element; Keap1, Kelch-like ECH-associated protein 1; GSH, glutathione; NQO1, NAD(P)H:quinone oxidoreductase 1; SOD, superoxide dismutase; Prx, peroxiredoxin; Trx, thioredoxin; TrxR, Trx reductase; Srx, sulfiredoxin; BMM, bone marrow-derived macrophage; MEM, minimal essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GSSG, oxidized GSH; $TRAP^+$ MNC, TRAP-positive multinucleated cell; MKP, MAPK phosphatase

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induction of NFATc1, which, in turn, induces the expression of such osteoclastic genes as tartrate-resistant acid phosphatase (TRAP) and cathepsin K in cooperation with AP-1, PU.1, and microphthalmia-associated transcription factor [11,12].

Many studies have shown that reactive oxygen species (ROS) regulate the formation and function of OCs, with the formation and bone resorption of OCs being stimulated by ROS [14–16]. Moreover, ROS are produced in activated OCs via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) and are thought to play a role in the process of bone resorption [17,18]. Recently, it was demonstrated that the RANKL-meditated stimulation of OC precursor cells transiently generates ROS through a signaling cascade involving TRAF6, Rac1, and Nox1 [19]. In addition, a variety of antioxidants, including *N*-acetylcysteine (NAC), ascorbate, lycopene, α-lipoic acid, coenzyme Q10, and curcumin, and such Nox inhibitors as diphenyleneiodonium (DPI), were found to inhibit the formation and bone resorption of OCs by suppressing ROS generation [19–24].

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a redox-sensitive basic leucine zipper transcription factor that regulates the expression of many antioxidant and phase II detoxifying enzymes by binding to a *cis*-acting enhancer sequence termed the antioxidant response element (ARE) [25–28]. Under normal conditions, Kelch-like ECH-associated protein 1 (Keap1), a cytoplasmic cysteine-rich protein, sequesters Nrf2 in the cytoplasm through the interaction of its double glycine-rich domains with a hydrophilic region in the Neh2 domain of Nrf2 [29]. Oxidative or electrophilic stress results in the oxidation or covalent modification of the cysteine residues of Keap1, which leads to the release of Nrf2 from Keap1, the escape of Nrf2 from proteasomal degradation, and Nrf2 nuclear translocation [30]. In the nucleus, Nrf2 associates with small Maf proteins, forming heterodimers that bind to AREs to induce the expression of genes encoding phase II detoxification or antioxidant enzymes, such as glutathione (GSH) *S*-transferase, NAD(P)H:quinone oxidoreductase 1 (NQO1), γ-glutamylcysteine synthetase, heme oxygenase-1, superoxide dismutase (SOD) 2, GSH reductase 1, GSH peroxidases 2 and 3, peroxiredoxins (Prx′s) I and VI, thioredoxin (Trx) 1, Trx reductase (TrxR) 1, and sulfiredoxin (Srx) [25,31–38]. Many studies have shown that Nrf2-knockout mice exhibit a severe sensitivity to a variety of acute and chronic chemical and environmental stresses, leading to oxidative damage in multiple target organs, including the liver, lungs, stomach, and brain [39].

The central role of ROS in the formation and function of OCs and the fact that Nrf2 is a redox-sensitive transcription factor that responds to a variety of oxidative stresses led us to hypothesize that Nrf2 may play a role in the differentiation and function of OCs. Therefore, we investigated the effects of Nrf2 deficiency on RANKL-induced OC differentiation, bone resorption, and signaling transduction using OC precursors derived from Nrf2-knockout mice. Nrf2 deficiency led to the promotion of RANKL-induced OC differentiation, bone resorption, and MAPK activation, most probably owing to a higher level of oxidative stress that was, in part, caused by the aberrant production of antioxidant enzymes and GSH. These results might provide a rationale for using noncytotoxic Nrf2 inducers to treat inflammatory bone diseases.

Materials and methods

Reagents and antibodies

The human recombinant RANKL and M-CSF proteins were purchased from PeproTech (Rocky Hill, NJ, USA) and R&D Systems (Minneapolis, MN, USA), respectively. An antibody specific for Srx has been described previously [40]. Antibodies specific for Prx, Trx, TrxR, and SOD isoforms were purchased from Young In Frontier (Seoul, Korea). A rabbit polyclonal antibody specific for β-actin was purchased from Abcam (Cambridge, MA, USA). Rabbit polyclonal antibodies specific for phosphorylated (phospho)-IκBα, phospho-JNK, phospho-ERK, and phospho-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibodies specific for IκBα, JNK1, p38, c-Fos, and Nrf2, and a mouse monoclonal antibody specific for NFATc1, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NAC, DPI, sulforaphane, curcumin, SP600125, and MG132 were purchased from Sigma–Aldrich (St. Louis, MO, USA); 5-(and-6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was from Molecular Probes (Invitrogen, Carlsbad, CA, USA).

Preparation of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMMs) were prepared as OC precursor cells from the femurs and tibiae of 4- to 8-week-old male mice, as described below. The bone marrow cells were flushed from the bone marrow cavity using α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were harvested via centrifugation at 1000 rpm at room temperature, and the cell pellets were then resuspended in the same medium. After incubation at 37 °C for 1 day, nonadherent cells were harvested and incubated in Gey′s solution for 10 min to remove the red blood cells. After clarification via centrifugation, the cells were cultured in α-MEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml recombinant human M-CSF. After 3 days, the adherent cells were used as OC precursor cells for osteoclastogenesis.

OC differentiation and TRAP staining

BMMs were loaded into a 48-well plate and treated with 100 ng/ml RANKL in α-MEM supplemented with 10% FBS and 20 ng/ml M-CSF for 3–5 days. After OC differentiation, the cells were washed twice with 1× phosphate-buffered saline (PBS), fixed for 10 min with 4% paraformaldehyde, and stained for TRAP using a leukocyte acid phosphatase cytochemistry kit (Sigma–Aldrich) according to the manufacturer′s instructions. The TRAP-positive multinucleated cells ($TRAP^+$ MNCs) containing three or more nuclei were counted as mature OCs using a light microscope.

Actin-ring staining

BMMs were fixed with 3.7% formaldehyde solution in PBS, permeabilized with 0.1% Triton X-100, and incubated with Alexa Fluor 488–phalloidin (Invitrogen) for 20 min. After being washed with PBS, the cells were incubated with 4′,6-diamidino-2-phenylindole (Roche, Mannheim, Germany) for 2 min and then photographed under a fluorescence microscope.

Bone resorption assay

BMMs were plated onto dentine disks (Immunodiagnostic Systems, Scottsdale, AZ, USA) and treated with 20 ng/ml M-CSF and 100 ng/ml RANKL for 7 days. The cells were completely removed from the dentine disks via abrasion with a cotton tip, and the dentine disks were stained with hematoxylin. Photographs of the resorption pits were obtained under a light microscope at 40× magnification, and the areas were measured using Image-Pro Plus 4.5 software (Media Cybernetics, Rockville, MD, USA).