Journal of Cellular Biochemistry 56:367–373 (1994)

Osteoclast Radicals

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Abstract In biological research, new ideas arise and quickly spread to encompass the entire field. Thus, the evolution of molecular biology has significantly changed our methods of approaching our research. A similar far-reaching finding has been the advent of radical reactions into biology. Although radical chemistry has been utilized for many technological advances that affect our daily lives, the appreciation of this same process within our cells has opened an unexplored arena for research enquiry. As cellular messengers, radical molecules seem whimsically designed: they are evanescent, rapidly and apparently indiscriminately reactive, and barely detectable by most biological methods. Yet, our initial probing of these reactive agents in cells and organisms has led us to postulate a virtually undescribed system of communication within and among cells which may have significant effects in multiple organs. In bone, radical reactants have been attributed with an important role in the control of bone resorption. © 1994 Wiley-Liss, Inc.

Key words: osteoclasts, superoxide, nitric acid, radicals

RADICALS IN BIOLOGY

Radical reactions in chemistry have been studied extensively and form the basis for modern polymer chemistry which has created our contemporary plastic environment. Biological radicals are latecomers to the scene, having been recognized in conjunction with radiation therapy for life-threatening malignancies, and in the normal defensive response of neutrophils to invading organisms. The short half-life and extreme lability of important biological radicals have complicated investigation of radical chemistry in biological milieu. Radicals, by definition, are compounds in which there is a reactive electron. The radical can be charged, like superoxide, $O_2^{\cdot -}$ or neutral, like nitric oxide, NO. Products of radicals with other molecules, for example, the reaction of superoxide with water to form hydrogen peroxide;

$$1/2 O_2^{\cdot -} + H_2O \rightarrow H_2O_2$$

or the reaction of nitric oxide with superoxide to form peroxynitrite;

$$NO + O_2^{\cdot -} \rightarrow ONOO^-$$

Received July 20, 1994; accepted July 26, 1994.

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may result in reactive peroxides. Both the original radicals and the peroxide adducts are capable of propagating radical reactions. Radical reactions include generation of new radicals, but are predominately effective in linking organic or biological molecules to each other. These chains of molecules are the basis of nylon, and polyethylene, but in biological systems, chaining reactions result in the disruption of protein, RNA, and DNA conformational structure, and are an effective method of cell killing.

RADICAL INTERACTIONS IN OSTEOCLASTS

Osteoclasts, members of the monocyte-macrophage lineage, may employ radical metabolites similar to other phagocytes [1–6]. In particular, toxic radicals which are utilized by the white cell for the destruction of foreign organisms, may be important agents of bone destruction. Secretion of lysosomes containing multiple proteolytic enzymes, excretion of noxious gases including superoxide and nitric oxide, and generation of acid may be common features of osteoclasts and other monocyte-macrophages.

Catabolism of bone requires both the dissolution of the mineral phase and the concomitant destruction of the complex protein matrix. How does the cell handle these powerful destructive forces without being destroyed? This is of particular relevance to the osteoclast, which overlays the resorption pit containing multiple toxic

compounds. In addition to the metabolic and substrate requirements for synthesizing radicals, the osteoclast must utilize metabolic energy for self protection. As the resources are limited, their regulation is critical to cell function. What is known of the synthesis, degradation, and metabolic control of these radicals with respect to osteoclast radical generation? Are there new approaches to investigating the regulation and deployment of toxic radicals by the osteoclast?

Some have proposed that osteoclastic resorption is modulated by oxygen radicals and nitric oxide [2–4,6–8]. The ability of osteoclasts to produce these autocoids has not been extensively explored. To examine the mechanism of action of these potentially powerful modulators of bone resorption, it is helpful to outline the relationship of these cellular products to each other and to the energy metabolism of the cell. For this analysis, we will rely on what is known about the white cell, as direct information on the osteoclast is limited.

SUPEROXIDE AND RELATED OXYGEN RADICALS

In neutrophils and macrophages, superoxide is generated by the action of NADPH-oxidase on molecular oxygen [9]. NADPH-oxidase is a multiple subunit enzyme, reported to be identical to cytochrome b_{559}, which is assembled at the cell membrane in response to chemical stimuli, e.g., phorbol myristate acetate [10–13]. Oxygen consumption increases abruptly over seconds to minutes. Superoxide is produced and rapidly reacts or is transmutated into hydrogen peroxide by superoxide dismutase (SOD). The reaction of oxygen radicals with other molecules is augmented in the presence of iron and chloride ions. The hydrogen peroxide formed is a powerful oxidizing agent which reacts with cell membranes or interstitial proteins. If the hydrogen peroxide is not completely consumed in these oxidation reactions, it is subsequently detoxified by catalase into water and diatomic oxygen (Fig. 1). Oxygen radicals are also generated as part of the regular function of mitochondria [14]. Usually, these byproduct radicals are degraded by SOD and catalase as part of the cell defenses against radical damage. The presence of reduced thiols, and in particular, glutathione, is considered to be a protective device used by the cell to trap oxygen radicals. In white blood cells, the function of superoxide is presumably to inactivate foreign organisms by causing oxidative damage to membranes and other substituents of the invading organism. Genetic lack of superoxide production is associated with an immune deficiency in which the subjects are particularly prone to bacterial infection [15]. Although other cells generate superoxide [16,17], the appearance of the radical moieties in other cell types is generally less rapid and the characteristics of the oxidases may differ from those found in the white blood cell. In osteoclasts, superoxide has been suggested to act by activating latent collagenases to facilitate bone resorption [18]. Byproducts of superoxide, such as hydrogen peroxide and hypochloric acid, are also powerful oxidizers or can generate organic radicals which result in defective lipids, proteins, RNA, and DNA [19,20]. Defective proteins are rapidly targeted for degradation by the cell, probably by protease recognition. In the case of osteoclasts, matrix removal could be aided by the oxidation of matrix proteins which would trigger this particular group of rapid degradative proteases. Thus, the production of oxygen radicals by the osteoclast and release of these compounds into the resorption lacunae could be an additional process which augments bone resorption. While oxygen radical metabolism seems directed toward increasing bone resorption, some authors have recently suggested that nitric oxide may inhibit osteoclastic activity [1,2,6,7].

NITRIC OXIDE (NO)

Nitric oxide is a neutral gas which contains an unpaired electron. The half-life for this radical moiety in biological systems has been estimated at 2–30 s [21]. Nitric oxide in neutrophils and macrophages is synthesized from the guanidino nitrogen atom of L-arginine by an inducible nitric oxide synthase (NOS; EC 1.14.23) [22]. The oxygen atom is contributed by diatomic oxygen. The NO is split from a ω-hydroxy-L-arginine intermediate (see Fig. 2) to give citrulline containing an ureido oxygen also derived from diatomic oxygen [23]. NADPH is required for the synthase reaction. Also necessary are FAD, FMN, and tetrahydrobiopterin. NO-synthase is a cytochrome, with an absorption maximum of 447 nm, which is indicative of a P-450 type hemoprotein and appears to be the first example of a cytoplasmic P-450 cytochrome, though some workers have found a particulate location of this enzyme as well [24,25]. Unlike the constitutive synthase found in vascular en-

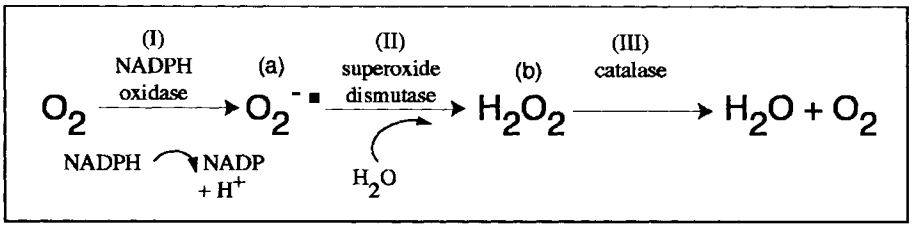

Fig. 1. Oxygen radicals. Superoxide (a) is generated by NADPH-oxidase (I) and then is dismutated to hydrogen peroxide (b) by superoxide dismutase (II). Hydrogen peroxide is converted by catalase (III) to diatomic oxygen and water.

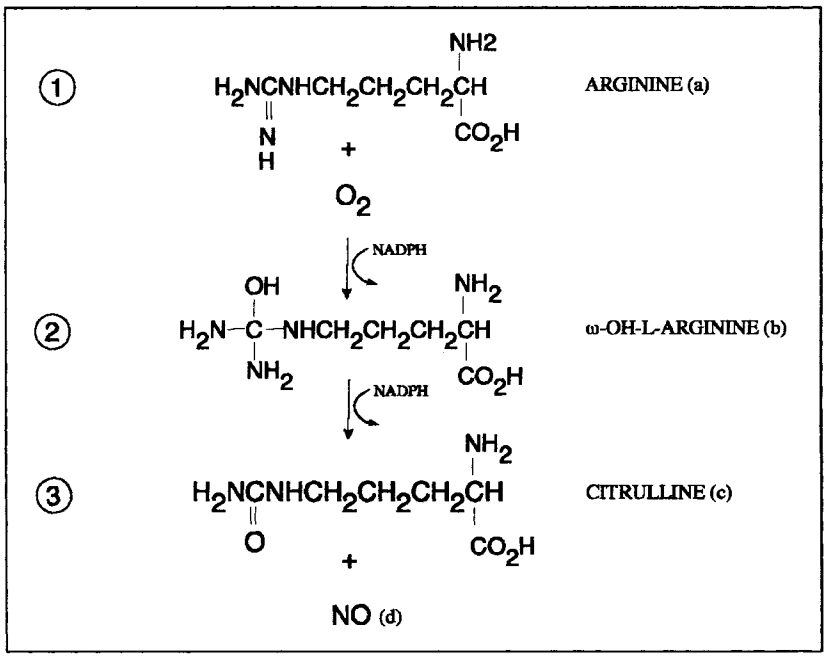

Fig. 2. Nitric oxide synthesis. Nitric oxide is generated by a two-step process from arginine (a). The first reaction forms an intermediate ω-OH-L arginine (b) which is converted to citrulline (c) and nitric oxide (d) NO-synthase is a p-450 type hemeprotein and iron is implicated in its function.

dothelial cells, which is calcium and calmodulin dependent, the white blood cell synthase is calcium independent. Inhibition of nitric oxide synthase in white blood cells has been associated with inefficient chemotaxis, decreases in DNA synthesis and cell proliferation and increased adhesion of leukocytes to vascular walls [27]. In osteoclasts on bone, in vitro, inhibition of NO-synthase was accompanied by increases in pit number and pit area [6]. In addition, osteoclasts which were exposed to exogenous NO demonstrated retraction of the osteoclast cell body and a decreased area of resorption [1].

CELLULAR REDUCTIVE RESERVE

Cellular reserves of reducing equivalents maintain the integrity of the cell exposed to radical or oxidative stress. Reducing equivalents are proportional to the concentration of reduced glutathione and NADPH within the cell. NADPH comprises the initial defense against oxidative cell damage. Glutathione provides a secondary reserve for NADPH regeneration if significant oxidative stress occurs. The generation of both superoxide and nitric oxide require oxidation of NADPH molecules, reducing the available cellular reserves. Both of these enzymes contain cytochromes which utilize NADPH. For NADPH-oxidase, diatomic oxygen is reduced by the addition of an electron from NADPH and the resultant production of NADP and H^+ as the oxidized species. Nitric oxide synthase requires 3 equivalents each of NADPH and O_2 [24]. The first 2 equivalents are utilized to carry out suc-

cessive hydroxylations and yield an intermediate, ω-hydroxy-L-arginine [24]. The third step of the process which leads to the formation of the final products is still controversial, and may involve oxidation of NADPH via an Fe(III) peroxide and a heme molecule which carries the second oxygen from diatomic oxygen to the carboxyl site of citrulline [23]. (The first oxygen from diatomic oxygen is used to form the known intermediate, ω-hydroxy-L-arginine.) Products from both enzymes are dependent on adequate stores of NADPH, which is mainly regenerated in the cell by the metabolism of glucose via the pentose phosphate shunt. The relative level of pentose shunt activity to glycolysis in the cell is measured by the ratio of 1 and 6 terminal carbons from labeled, metabolized glucose which are detected in evolved CO_2. In white blood cells, during the respiratory burst of superoxide production, most of the glucose utilized by the cell is metabolized through the pentose shunt pathway [26]. Thus, production of radicals in the osteoclast depends on adequate stores of NADPH and is tied to the overall oxidative status of the cell.

INTERRELATION OF SUPEROXIDE AND NITRIC OXIDE

In white blood cells, superoxide and nitric oxide radical reactions are interrelated. For example, inhibition of nitric oxide synthase is accompanied by increases in superoxide, whereas the addition of excess L-arginine, the substrate for NO-synthase, decreases detectable superoxide concentrations [27–29]. These effects may be mediated by direct chemical reaction between superoxide and nitric oxide which leads to the formation of new radical compounds at rates approaching the diffusion coefficient of nitric oxide [30–31]. This rapid reaction rate suggests that superoxide generation may limit nitric oxide action as a paracrine regulatory substance. Conversely, nitric oxide may degrade superoxide, by reacting to form peroxynitrite, $OONO^-$ [30]. The biochemistry of superoxide and nitric oxide illustrate multiple interactions, therefore, understanding the role of these systems in bone resorption is complex. A first approach might probe the effect of decreasing the production of either of these radicals. This may be effected by either inhibiting the enzyme product or by observing cells in which the enzyme is lacking.

Although one can argue that inhibition of an enzyme is not an ideal method of elucidating the role of an enzyme product, for small molecules like superoxide and nitric oxide, direct measurement is difficult. Thus, most of our information on these compounds has been obtained either indirectly or by chemical reactions outside the biological milieu. A human phenotype lacking white cell superoxide production is known (chronic granulomatous disease), but no bone disease has been reported [15]. At present, genetic defects of white cell nitric oxide production have not been described. A cell phenotype lacking one of these radicals might display one end of the spectrum of the interaction of these radicals in biological systems and could contribute greatly to our understanding of radical biology. However, the physiological range of radical interaction would not be accessible to these abnormal phenotypes. Varying polymorphisms, with gradations of enzyme activity for both enzymes, would provide an optimal system for investigating the interaction of these two radicals during bone resorption. However, to evaluate an intermediate phenotype for either oxygen radical or nitric oxide production, quantitative assessment of radical production is mandatory. After characterization of the polymorphisms by relative deficiency of superoxide or nitric oxide production, functional analysis of osteoclastic bone resorption would follow.

SPECIFIC METHODS FOR DETERMINING OXYGEN RADICALS AND NITRIC OXIDE

Experiments might be designed to measure the generation of these radicals during osteoclastic bone resorption. There are several difficulties to be overcome in order to employ this approach. First, there are other cells present in the bone which have been shown to produce either oxygen radicals, nitric oxide, or both radicals; among these are endothelial cells [7], osteoblasts [8], chondrocytes [16], and macrophages. Since osteoclasts are known to respond to signals from other cells, some authors have argued that radical generation by non-osteoclastic cells in the bone milieu regulate bone resorption [7,8]. Thus, an investigation of the role of radicals in bone resorption would need to differentiate between radicals of autocrine and paracrine origin. A second difficulty associated with assessing radi-

cal production by osteoclasts is related to the uncertainties in current methodology used to measure oxygen radicals and nitric oxide concentrations. Nitrotetrazolium blue or NADPH diaphorase assays may indicate radical presence, but both are difficult to quantitate and neither readily allows differential measurements of superoxide and nitric oxide as the electron donor [32]. The currently recommended method for nitric oxide determination, which uses oxyhemoglobin to bind NO, requires the inclusion of superoxide dismutase and catalase to destroy oxygen radicals which would react with the nitric oxide or with the oxyhemoglobin itself [33]. Use of spin trapping compounds, with analysis by electron spin resonance (ESR), differentiates between superoxide and nitric oxide because nitric oxide initially has a prohibitively slow on rate [35]. Presence of nitric oxide in the sample, however, can decrease the efficiency of trapping of other radicals, as NO will react with and destroy the adduct-spin-trap complex [36]. It is noteworthy that a porphyrinic-based microsensor has been employed as a method of detection of nitric oxide at biological concentrations, and is capable of measuring nitric oxide evolved from a single cell [35]. Interactions of the radicals, however, are more difficult to assess. One of the confounding factors in detection of either superoxide or nitric oxide is the presence of Fe(III) in biological samples. Fe(III) is present in cellular cytochromes and is a primary target for either radical. Reaction of a radical with Fe(III) will reduce the iron to Fe(II) and decrease the quantity of the radical seen by any detection device [33,36]. The reduction of Fe(III) cytochrome c is used as an assay for superoxide [37]. Specific methodology to probe the generation of oxygen radicals and nitric oxide simultaneously should be able to detect the small differences in either molecular weight or other chemical or physical properties of superoxide and nitric oxide. These criteria would be satisfied by either infrared spectroscopy or mass spectrometry. As far as infrared spectroscopy is concerned, the resolution of the individual radical peaks given the background of the biological milieu would be the most difficult part of this methodology. Mass spectrometry would be an interesting, potential method capable of resolving the two radicals by molecular weight. The preparation of the biological milieu for the mass spectrometer inlet would need to be minimal in order to make determinations during radical production. Some reports have utilized spin trapping compounds in the mass spectrometer for these types of experiments.

In addition to identifying each of the radical species, the ideal methods would be sensitive enough to detect the low levels of radicals which would be expected in a biological system. These quantities are small compared to chemical generating systems employing, for example, pulse radiolysis [37]. The most sensitive would probably be the porphyrinic microsensor, which can detect nitric oxide from a single cell, but neither superoxide nor hydrogen peroxide are detectable by this method. Infrared spectroscopy sensitivity would be dependent on the size of the extinction coefficient of the individual radical, whereas ESR is limited to μM detection of superoxide or other oxygen radicals. New techniques of 2D ESR may have increased resolution both spatially and by signal intensity, but may be limited by microwave heat radiation of the biological sample [38]. Mass spectrometry would have a detection sensitivity between that of the microsensor and infrared spectroscopy and could be coupled to the radical generating chamber by a membrane interface [39].

In summary, recent investigations of oxygen radicals and nitric oxide have focused on possible roles of these reactive cellular messengers in osteoclastic bone resorption. Preliminary studies have suggested an effect of oxygen radicals on activation of bone destruction, while inhibition of nitric oxide synthesis also increases bone resorption. Are these small evanescent molecules a current example of the yin and yang concept of biological control in osteoclasts and perhaps other cells? Are they a product of the osteoclast itself or of other cells in the bone milieu? The metabolic interrelationship of these molecules in vivo as well as their extreme lability dictates an approach to this investigation which will encompass physical measurements of generated radicals as well as more standard methods of biological investigation. These physical measurements will undoubtedly require further development of existing technology, which at present is predominately adapted for analysis of these radicals in concentrations and environments which are more suitable to chemical than to biological milieu.

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