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Bone Cell Function, Regulation, and Communication: A Role for Nitric Oxide

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Abstract A large array of factors serve as vital communication links between cells and the characterization, regulation, and mechanisms of action of such factors are topics of intense research efforts. Most intercellular messenger molecules which have been described over the years are represented by proteins, small peptides, amino acids or their derivatives, ions, lipid metabolites, or steroids. However, a small uncharged free radical, nitric oxide, has recently garnered much attention as a potent multifunctional signal molecule with widespread actions within and between diverse tissues. Biochemical, molecular, and regulatory studies of the family of enzymes responsible for nitric oxide synthesis, nitric oxide synthases, have established that there are at least three distinct isoforms of this enzyme which are differentially expressed and regulated in various cells or tissues. Modulation of these isoenzyme levels or activities by diverse signals is mediated via transcriptional, translational, and/or post-translational mechanisms, and consequently, alterations in such control may influence normal or pathological processes. Nitric oxide appears to exert pronounced effects on skeletal physiology and its production by various bone cells, elicited target cell responses, modulation by other signalling molecules (e.g., cytokines, hormones, fatty acid derivatives), and chemical interactions with other free radicals (e.g., superoxide anions, hydroxyl radicals) may form one important facet of the many complicated communication pathways controlling bone cell physiology and remodeling. Further cell and molecular studies are needed to address the precise roles that nitric oxide plays in bone development and in the formation and degradation of bone during ordinary bone metabolism. In addition, alterations in the regulation and action of the bone nitric oxide system as a function of certain bone disorders may be manifested by perturbations in bone integrity or mineral homeostasis. In this article, we review the current evidence implicating nitric oxide as an important messenger molecule in bone intercellular communication, speculate on potential roles for this radical in bone biology, and discuss possible future directions for advanced research into the function of nitric oxide in skeletal physiology.

Key words: nitric oxide, bone remodeling, free radicals, osteoclasts, osteoblasts, cytokines

Over the past 10 years, the short-lived reactive radical nitric oxide (NO) has received significant attention relative to its physiological role as a messenger molecule in the cardiovascular, neurologic, and immune systems. This interest is clearly underscored by the proliferation of original papers and review articles related to NO and the NO synthase (NOS) isoenzymes responsible for its biological production. NO is a labile, uncharged, reactive radical that functions as a sensitive mediator of intercellular communication in diverse tissues. The small size and uncharged nature of the molecule permit NO to freely diffuse out of cells that produce NO and into nearby target cells. Iron appears to be a preferred receptor for NO; therefore, NO exerts many of its effects through binding to iron-containing enzymes and consequently modifying their activity. One important example is the binding of NO to the heme moiety (complexed with iron) of guanylyl cyclase in target cells, causing an activation of this enzyme and an ensuing production of guanosine monophosphate (cGMP). This triggers a cascade of phosphorylation events that culminate in the alteration of various cellular processes (e.g., cell proliferation, smooth muscle relaxation, cytotoxicity, neurotransmission). Apart from interacting with guanylyl cyclase, NO displays wide-

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ranging effects on cell physiology (e.g., respiration, intermediary metabolism, ion transport, DNA synthesis, and mutagenesis) via its binding to other heme or iron–sulfur proteins, stimulation of ADP ribosylation and deamination, nitrosylation of proteins, regulation of gene transcription and translation, and by its chemical interactions with oxygen reactive species such as superoxide anions [Stamler et al., 1992; Moncada et al., 1991; Nussler and Billiar, 1993; Schmidt et al., 1993; Moncada and Higgs, 1991]. Thus, this simple molecule participates in more intracellular and intercellular roles than any other known messenger molecule identified to date.

NO levels are commonly elevated during immune activation in response to inflammation or infection, and high levels are associated with various autoimmune disorders and inflammatory diseases. During inflammation or as part of the immune response to infection, cytokines [e.g., interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β)] are produced, which, as part of their action, activate intracellular nuclear transcription factors such as NFκB within the macrophage. This leads to an upregulation in the transcription of NOS, and ultimately, an elevation in NO synthesis. Since NFκB can also be activated by NO itself (and by superoxide), a positive feedback loop may exist in which NO production is amplified [Lowenstein et al., 1994]. If left unchecked, the excessive NO could have pathological consequences. However, other cytokines (e.g., TGF-β, IL-8, IL-10) or modulatory factors (e.g., essential NOS cofactors, substrate) may normally temper such amplification as part of the complex regulation governing NO production and NOS activity [Schini et al., 1992].

NITRIC OXIDE SYNTHASES

The synthesis of nitric oxide (NO) from L-arginine in mammalian tissues is the result of nitric oxide synthase (NOS) enzymes, which vary in their calcium requirement, mode of regulation (inducible or constitutive), kinetics, tissue distribution, structure, and functional roles. At least three different isozymes of NOS have been identified, whose distribution in cells and tissues is quite diverse. These multicatalytic NOS enzymes perform a remarkable variety of complex reactions and have been implicated in a wide range of physiological and pathological processes [Knowles and Moncada, 1994; Lowenstein et al., 1994; Nussler and Billiar, 1993]. Two constitutive isoforms of NOS are known to exist: one associated primarily with endothelial membranes, and the other found in the cytosol of central and peripheral neurons. Low levels of NO are generated for short intervals by the constitutive NOS enzymes upon their stimulation by calcium/calmodulin. Endothelial NOS activity is elevated in response to acetylcholine (ACh), 5-HT, ADP, vasopressin, or estrogen and may be increased by phosphorylation. The NO released by endothelial cells limits blood clotting and functions as a prime regulator of vascular tone (dilation) and blood pressure by activating soluble guanylyl cyclase in platelets and smooth muscle cells, respectively. In the brain, NO acts as a neurotransmitter, produced in response to glutamate-evoked calcium influxes in neurons from which it diffuses to adjacent cells and activates guanylyl cyclase and phosphorylation cascades. A third NOS that is distinct from the brain/endothelial isoenzymes has been identified whose expression is increased in macrophages activated by cytokines or other inflammatory stimuli. Once expressed, this inducible NOS isoform remains continuously active and results in the prolonged synthesis of NO at levels sufficiently high (1,000-fold over constitutive) as to prove cytotoxic to tumor cells and microorganisms. Anti-inflammatory corticosteroids are capable of suppressing the expression of inducible NOS [Moncada and Palmer, 1991]. The various NOS isoenzymes have been purified and cloned from several species, although isolated cDNA clones for all three have only been reported to date for humans [Nussler and Billiar, 1993]. Overproduction of NO may have pathological consequences and appears linked to such disorders as hypertension, stroke, and neurodegenerative diseases (e.g., Huntington's and Alzheimer's). Excessive NO production via inducible NOS has been implicated in the pathogenesis of septic shock, rheumatoid and osteoarthritis, diabetes, periodontal disease, autoimmunity, acquired immunodeficiency syndrome (AIDS), and other inflammatory-mediated diseases [Kiechle and Malinski, 1993; Beckman and Crow, 1993; Moncada and Higgs, 1993]. However, no reports have demonstrated NOS abnormalities in patients with immunodeficiency syndromes.

NOS generates NO from the amino acid L-arginine in a process requiring molecular oxygen that is dependent on NADPH and other cofactors, and which yields L-citrulline. NOS activity can therefore be assayed by quantitating the formation of L-citrulline or by measuring

the longer-lasting oxygenation products of NO [Kiechle and Malinski, 1993]. Modulation of NO levels in biological systems can be achieved either by using agents that spontaneously release NO and activate guanylyl cyclase (e.g., sodium nitroprusside), through competitive inhibition of NOS activity by various arginine analogues, or by transcriptional or translational blockers of NOS. Whereas some arginine analogues inhibit both constitutive and inducible isoforms of NOS (e.g., N-nitro-L-arginine, L-NNA, or its methyl ester, L-NAME), others act more or less selectively in inhibiting the inducible NOS isoform (e.g., L-canavanine or aminoguanidine). In rats treated acutely with L-NAME, a marked vasoconstriction of the blood vessels occurs due to blocking constitutive NOS-mediated basal vasodilation. This leads to a sharp increase in peripheral vascular resistance and systemic blood pressure. NO levels are not only dependent on the availability of L-arginine and essential cofactors for NOS activity but are also governed by the diverse chemical reactivities of NO with iron/sulfur proteins and other free radicals or associated enzymes. Thus, NO can be rapidly eliminated through reaction with superoxide radicals. Additionally, superoxide dismutase removal of superoxides can enhance NO effects. NO binding to iron/sulfur proteins can stabilize and prolong its effects; recent evidence indicates that proteins involved in iron transport and metabolism, as well as NOS itself, are subject to feedback inhibition (transcriptional and translational) by NO [Knowles and Moncada, 1994; Pantopoulos et al., 1994]. Thus, NO binding to iron regulatory protein (IRP) enables it to associate specifically with iron response elements (IRE) in the 5′ or 3′ untranslated regions of proteins involved in cellular iron traffic and either block transcription or stabilize mRNA, respectively. During the past few years, the role of NO and NOS isoenzymes operating in bone cells has become the subject of growing research interest with respect to both normal and pathological bone remodeling. Here we review this research, reflect on its potential significance in regulating skeletal remodeling, and discuss some of the unanswered questions still to be addressed in future research relative to NO in bone.

BONE REMODELING

Bone remodeling follows a coupled cyclical sequence comprised of two arms: the stimulation of resorption in response to bone formation, and the synthesis of new bone as a result of its degradation. Recent progress in understanding the regulation of bone turnover in both normal and disease states has begun to reveal a fascinating but complex dialogue between bone-forming osteoblasts or bone-degrading osteoclasts and other cell types in bone. The interplay between local and systemic bone remodeling regulatory pathways involves complex, overlapping, and multitiered communication networks responsible for balancing the normal remodeling sequence. Redundant regulatory mechanisms have likely evolved to help ensure that minor signaling aberrations are not translated into major remodeling disorders such as those associated with periodontal disease, osteoporosis, and osteoarthritis. Moreover, the alliance between endocrine, autocrine, and paracrine signals orchestrates bone remodeling through effects on a dynamic population of stem cells and cells in various physiological states or stages of development, maturation, and senescence. Thus, the recruitment, differentiation, and function of osteoblasts and osteoclasts are governed by an assortment of systemic regulators of bone metabolism (including parathyroid hormone (PTH), vitamin D_3, and estrogen) and local mediators, including inflammatory cytokines (e.g., IL-1β, IL-6, and TNF), arachidonic acid metabolites (prostaglandins and leukotrienes), free radicals (e.g., superoxide anions, hydrogen peroxide, and NO), and bone matrix components. Osteoblasts and osteoclasts both produce and respond to a variety of these modulators [Skjodt and Russell, 1992]. Other cells within the bone environment, such as vascular endothelial, bone marrow stromal, and immune system cells also serve as the source and/or target of local mediators, including NO.

OSTEOCLASTS

Bone-degrading osteoclasts arise from cells related to the monocyte–macrophage lineage and, although possessing a unique ability to resorb bone, they share various characteristics with macrophages [Gay, 1992; Horton and Helfrich, 1992]. Certain phenotypic traits found associated with both osteoclasts and macrophages may simply reflect their common early lineage or the degradative nature of both mature cell types. However, other attributes shared by these cells may be selectively regulated or otherwise modified in one or the other cell type,

for example, as required to accommodate the specialized regulatory and physiological demands associated with osteoclast physiology, mineral homeostasis, and focal bone remodeling. A major challenge for understanding bone resorption and bone remodeling relates to our need for a comprehensive description of the similarities and subtle differences that distinguish osteoclasts from non-bone-resorbing macrophages, particularly with regard to unique properties defining the osteoclast phenotype. The NO/NOS system may be among those properties differing in certain respects between the macrophage and osteoclast.

NO has been recently implicated in the regulation of osteoclast function. MacIntyre et al. [1991] have reported that NO is a potent inhibitor of bone resorption, also reducing the cell spread area of isolated rat osteoclasts on bone slices. Based on the lack of an effect by either dibutyryl or 8-bromo-cGMP, this group proposed that the mode of action of NO in these cells might be independent of cGMP, unlike that which has been commonly found for most other systems. By contrast, other groups have reported that NO-generating compounds appeared to elevate cGMP both in isolated chicken osteoclasts [Howard, 1985] and in the fetal rat limb organ culture assay accompanied by a reduction of PTH-stimulated bone resorption [Stern and Diamond, 1992]. Similarly, our laboratories have reported that NO increased cGMP levels while inhibiting the resorptive activity of highly purified chicken osteoclasts [Kasten et al., 1994]. Furthermore, infusion of selective NOS inhibitors into normal or ovariectomized rats caused bone loss in vivo, as reflected in the diminished spine and femoral bone mineral densities measured over a 4-week period [Kasten et al., 1994]. These in vivo findings are consistent with the in vitro inhibitory effect of NO on osteoclast bone resorption. A role for local NO regulation of osteoclast activity is also supported by the recent studies of Lowik et al. [1994] using cultured explants of fetal mouse long bones. A combination of cytokines known to upregulate inducible NOS, consisting of TNF-α + IFN-γ + lipopolysaccharide (LPS), elevated NO levels and inhibited osteoclastic resorption monitored by $^{45}Ca^{++}$ release in these organ cultures. The NO generator sodium nitroprusside had a similar inhibitory effect; conversely, the addition of the NOS inhibitor L-NMMA alleviated the inhibition of resorption due to induction of NO. Interestingly, basal resorptive levels were inhibited by sodium nitroprusside but were not influenced by L-NMMA. Therefore, perhaps the cytokine-induced elevation of NO, which inhibits its resorptive activity, serves as a countermeasure for the bone loss also stimulated by inflammatory cytokines. Our preliminary findings suggest that osteoclasts may possess both a constitutive NOS as well as an inducible NOS. If so, osteoclast NO production may be modulated via dual mechanisms: (1) as a function of calcium concentration, which fluctuates during bone resorption and could thereby activate the constitutive NOS isoform, and (2) as a consequence of osteoclast exposure to various factors, including inflammatory cytokines that cause expression of the inducible NOS isoform.

Other levels of regulation for NOS may also be operative in bone. For instance, the half-life of inducible NOS mRNA in mesangial cells appears to be extended as a result of elevated cAMP levels [Kunz et al., 1994]. Since it is well known that the potent osteoclast inhibitor calcitonin acts, in part, by elevating cAMP levels, it is possible that calcitonin inhibition of osteoclast activity may be due to a cAMP-mediated increase in the lifespan of NOS mRNA, and hence NO levels. Estrogen, another inhibitor of bone resorption that acts directly upon osteoclasts (as well as on other bone cells), has been reported to increase Ca^{2+}-dependent NOS activity in a range of tissues and the mRNA levels of endothelial and neuronal NOS isoforms in skeletal muscle [Knowles and Moncada, 1994]. If osteoclasts also contain constitutive NOS, and estrogen upregulated this isoform in these cells, one facet of the estrogen inhibition of osteoclast resorption may involve elevated NO levels. Bone matrix proteins may also regulate NOS, since it has recently been reported that osteopontin, a bone acidic phosphoprotein involved in the attachment, signaling, and regulation of osteoclasts, suppresses the expression of inducible NOS in kidney cells and macrophages [Hwang et al., 1994; Denhardt and Guo, 1993]. It would therefore be of great interest to explore how calcitonin, estrogen, and osteopontin (as well as other matrix proteins) influence the osteoclast NOS system and what effects this may have on the regulation of bone resorption.

Zaidi et al. [1993] have proposed a paracrine influence of endothelial cell-derived NO and interactions of this radical with reactive oxygen species in bone remodeling. Certainly, the physi-

cal proximity of bone vascular endothelial cells to osteoclasts would enable endothelial cell-derived NO to exert potential paracrine effects. In addition, NO may function as an autocrine regulator of osteoclast resorption, since these cells may possess inducible (and perhaps constitutive) NOS. Schmidt et al. [1992] and our laboratories [Kasten et al., 1994] have shown strong staining for an NADPH-dependent diaphorase activity, which is often considered reflective of NOS, in rat or avian osteoclasts of bone tissue sections, as well as in purified avian osteoclasts isolated and cultured on bone slices. Thus, osteoclasts represent a potential contributing source of NO in bone. However, this presents an apparent paradox in that osteoclasts that are actively resorbing bone seem to display substantial NOS (diaphorase) activity, yet NO clearly inhibits osteoclastic bone resorption. Perhaps osteoclasts normally limit their own activity via autoproduction of NO, explaining why the administration of NOS inhibitors to rats in vivo or isolated osteoclasts in vitro stimulates basal osteoclast bone resorption [Kasten et al., 1994]. Alternatively, the NO produced by osteoclasts may primarily serve some other purpose, especially given the vast array of bioactivities reported for this simple molecule. For example, NO can chemically combine with superoxide to form a more highly reactive and longer-lived free radical, peroxynitrite, which might contribute to matrix degradation or may further dismutate under physiological conditions to less reactive free radical species [Freeman, 1994]. Thus, NO may act directly to degrade bone via the generation of free radicals. Immune activated macrophages release peroxynitrite, and this major cytotoxic oxidant of inflammatory cells is highly reactive with metalloproteins (including superoxide dismutase), ion-channel proteins, DNA, and lipids, to name a few of its targets. Since peroxynitrite demonstrates more toxic effects at neutral pH than it does at an acidic pH, we can only speculate as to whether the NO–superoxide interaction within the acidic bone resorption site might serve a favorable or deleterious role. In this context, inhibition of NO levels has repeatedly been found to exacerbate tissue injury in septic shock, whereas the administration of superoxide dismutase, up to a certain point, has proved protective [Freeman, 1994]. These findings have been interpreted by many as providing evidence for a superoxide scavenging role of NO.

NO interactions with superoxide anions have important potential implications for bone physiology for multiple reasons. Formation of peroxynitrite upon chemical combination of NO with superoxide not only relieves some of the cellular burden of superoxide radical-mediated intracellular damage, but at the same time provides another highly reactive radical having somewhat different effects within the cell. In addition, since superoxide anions have been linked to osteoclast bone resorption, their elimination via reaction with NO may impact on osteoclast function [Ries et al., 1992; Garrett et al., 1990]. Osteoclasts are highly active metabolic cells and contain the greatest number of mitochondria (per cell or cell volume) of any cell in the body. Oxygen-derived free radicals represent normal by-products of mitochondrial, microsomal, and arachidonic acid metabolism. Owing to their charged nature, superoxide radicals cannot move freely across organellar and plasma membranes. Osteoclasts have been shown to produce significant levels of superoxides and to experience a reduction in these levels when resorption is inhibited by calcitonin. Furthermore, increased osteoclast formation and bone resorption has been linked to an elevation in the generation of reactive oxygen species in bone, abnormal osteoclast function is reflected in diminished superoxide production in patients with malignant osteopetrosis, and the exogenous addition of superoxide dismutase (SOD) inhibits bone resorption. Superoxide radicals have been detected by nitroblue tetrazolium reduction at the ruffled border region of the osteoclast, which suggests that these anions are produced at the site of resorption and may play some role in bone degradation. A monoclonal antibody (MAb) directed against a novel osteoclast membrane-associated glycoprotein with apparent homologies to manganese SOD blocks osteoclast-mediated bone resorption and the expression of this membrane component is correlated with the acquisition of bone resorptive activity during osteoclast development. Thus, there is sufficient evidence implicating osteoclast-derived superoxides in bone resorption to warrant investigating what effect the interaction of NO and superoxides has on osteoclast biology [Greenwald and Rifkin, 1992].

Superoxides have been proposed to contribute to connective tissue destruction. For example, neutrophil-derived oxygen radicals cause activation of the latent forms of collagenase and gelati-

nase. In addition, peroxides produced during phagocytosis can convert glutathione (GSH) to GSSG and modulation of the GSH/GSSG ratio apparently cycles collagenase through active and inactive states. This mode of regulation is of particular interest, since anti-collagenase antibodies have recently demonstrated that this enzyme is localized to the matrix underlying resorbing osteoclasts [Delaisse et al., 1993]. Superoxides have also been found to engender a rapid and extensive degradation of the polysaccharide hyaluronic acid in a bovine model for rheumatoid arthritis, concomitant with a decrease in the viscosity and lubricating quality of the joint. The addition of exogenous SOD completely protected against superoxide-induced degradation in the synovial fluid. Therefore, there is an intriguing relationship between connective tissue destruction and superoxide levels, and so, one might expect that multiple mechanisms exist to guard against undesirable effects of these reactive radicals while permitting such species to carry out their intended roles. Whether NO collaborates with superoxides in the degradation of bone matrix, especially in light of its unrestricted mobility within and outside of cells, or instead chemically removes superoxides required for resorption and thereby interferes with osteoclast activity is not entirely clear. Perhaps NO is also directionally delivered to the resorption cavity through binding to metalloproteinases destined for secretion. It is intriguing to speculate that the NO and superoxide free radical systems in osteoclasts are coupled and that the regulation and interplay between them provides an additional means of modulating osteoclastic resorptive activity.

In summary, NO production by osteoclasts may serve a complex autocrine role in regulating osteoclast resorptive activity. To date there is little, if any, information on whether modulators of osteoclast activity have a direct influence on the regulation of NO production by the osteoclast. However, it is clear that increased NO inhibits, whereas reduced NOS activity stimulates, osteoclast bone resorptive activity.

OSTEOBLASTS

Osteoblasts differentiate from embryonic mesenchyme or marrow stromal cell populations and their development is characterized by an elevation in the expression of alkaline phosphatase, PTH receptors, type I collagen, and several noncollagenous bone matrix proteins. Osteoblast development occurs following replacement of an avascular cartilage model with vascularized bone and marrow during endochondral ossification, but proceeds in the absence of a cartilage intermediary during intramembraneous ossification. Bone formation by the osteoblast is regulated by a complicated network of systemic and local signals that include vitamin D metabolites, PTH, and local growth factors or cytokines such as IGF-1, TGF-β, and bone morphogenetic proteins [Skjodt and Russell, 1992]. It is possible that NO may also participate in modulating osteoblast-mediated bone formation.

Three recent independent studies have demonstrated that osteoblasts produce NO in response to pro-inflammatory cytokine stimulation. In cultured human osteoblasts obtained from explants of trabecular bone, low basal levels of NO production were markedly increased along with cGMP levels after exposure of the cells to IL-1β in combination with TNF-α and IFN-γ [Ralston et al., 1994]. Induction of NO was inhibited by cyclohexamide, actinomycin D, dexamethasone, or competitive inhibitors of NOS, the latter suggesting a potential autocrine role for NO in osteoblasts. RT-PCR analysis showed that the human osteoblasts expressed the inducible NOS isoform as opposed to the constitutive endothelial or neuronal NOS isoenzymes. Induction of NO was not elicited in these osteoblasts by exposure to the cytokines individually or by treatment with PTH or 1,25-dihydroxyvitamin D_3. Similarly, primary cultures of rat osteoblasts or osteoblast-like rat UMR-106 cells demonstrated an elevated production of NO following their treatment with TNF-α in combination with IFN-γ and lipopolysaccharide, and NO induction was augmented further when IL-1α was also included [Lowik et al., 1994]. Consistent with the reported inhibitory action of TGF-β on NOS in other cell types, TGF-β suppressed the cytokine induction of NO in the UMR-106 cells without affecting their basal NO levels. Since TGF-β exerts dose-dependent effects on both osteoblasts and osteoclasts, either or both of these cells may represent targets for inhibition of NO synthesis. In another study, a mouse osteoblastic cell line designated MC3T3-E1 was also shown to respond to these same inflammatory stimuli by increasing NO production [Damoulis and Hauschka, 1994]. Both UMR-106 and human osteoblasts were found in the above studies to exhibit a decrease in proliferation or viability as NO levels were elevated.

However, since no other osteoblastic phenotypic parameters (e.g., changes in matrix elaboration, collagen production, alkaline phosphatase activity) were examined in these studies, the elevation of cGMP and suppression of proliferation represent the only osteoblast traits altered in association with NO modulation known to date. Thus, perhaps proinflammatory stimuli increase osteoblast-derived NO, in addition to osteoclast NO production, in order to offset the stimulated bone resorption that is also promoted by these inflammatory cytokines. Whether NO depresses osteoblast bone formation in parallel with osteoclast bone resorption, and thereby dampens the entire bone remodeling process, is a subject for future investigation.

Like osteoclasts, osteoblasts both respond to systemic and local regulatory substances as well as produce an array of locally active factors, some of which may have autocrine effects on osteoblasts or may influence osteoclast development and action. One or more of these may interface with the NO/NOS systems of these cells. Various laboratories have reported that membrane-associated or diffusable products from osteoblasts or marrow stromal cells are required for osteoclast cytodifferentiation and activity [Roodman, 1991; Suda et al., 1992]. These products include prostaglandin E_2 (PGE_2) and various peptides, proteins, and other compounds ranging in size from 1,000 to 110,000 kd. There are conflicting reports regarding the effects of hormonal stimulation on the secretion of these factors. Greenfield et al. [1993] recently reported that IL-6 and leukemia inhibitory factor (LIF) mRNA levels are stimulated by PTH in MC3T3-E1 cells. Moreover, a growing number of known cytokines have been shown to be produced by osteoblasts that may act to stimulate osteoclast formation or resorption [Lorenzo, 1991]. These include IL-1, IL-6, TNF-α, granulocyte–macrophage colony-stimulating factor (GM-CSF), M-CSF, TGF-β, and LIF. Recent RT-PCR analysis of normal human and pagetic osteoblast-like cells has demonstrated mRNA for IL-1, IL-6, TGF-β, and IL-8 [Birch et al., 1993]. Depending on the cell isolation and culture conditions, species source, and potential stage of differentiation, various cytokines and levels have been reported. Therefore, the precise role of osteoblast generated factors in osteoclast developmental and functional processes has not been completely resolved. However, it can be speculated that osteoblast-derived cytokines, as well as those released from other bone cells, could modify osteoblast and/or osteoclast NO production and consequently the physiology and function of these bone formative and degradative cells.

Local bone remodeling is also subject to regulation by arachidonic acid metabolites generated via the lipoxygenase, cyclo-oxygenase, or eicosanoic pathways. Many studies have documented potent transitory effects of prostaglandins, leukotrienes, and hydroxyeicosanoic acids on bone physiology, as well as actions of such metabolites on both osteoblasts and osteoclasts. Prostaglandins of the E series (PGE) have been the most widely studied in regard to bone research [Raisz and Martin, 1984]. Bone resorption is stimulated by PGE in cultured fetal rat long bones via a cAMP pathway [Klein and Raisz, 1970], although PGE appears to cause a transitory inhibition of resorption by isolated osteoclasts [Chambers and Ali, 1983]. These apparently inconsistent findings may perhaps be explained by further observations showing that prostaglandins stimulate osteoclast development and that low doses of PGE_2 may actually stimulate bone formation [Collins and Chambers, 1992]. Clearly, prostaglandins can mediate the effects of cytokines in bone [Gallwitz et al., 1991]. Inhibitors of prostaglandin synthesis, such as indomethacin or flurbiprofen, appear to reduce inflammatory-related bone loss, for example in periodontal disease or arthritis [Mohammed et al., 1989; Williams et al., 1992]. Possible sources of prostaglandins in bone include osteoblasts and marrow stromal cells. Several reports have shown that osteoblasts treated with PTH or stimulated by mechanical deformation release small compounds, some of which may be leukotrienes, that can stimulate osteoclasts [Conway et al., 1986]. In addition, stromal cells from a PTH-responsive giant cell tumor of bone have been shown to generate 5-lipoxygenase metabolites, including peptidoleukotrienes and 5-HETE, the latter of which has been shown to stimulate bone resorption [Gallwitz et al., 1991]. Leukotrienes have also been implicated in orthodontic tooth movement, where the administration of the leukotriene inhibitor AA861 decreased tooth movement, despite the presence of elevated prostaglandin levels [Mohammed et al., 1989]. Therefore, prostaglandins and leukotrienes may differentially modulate various facets of bone remodeling. It will be of interest to determine in future research how these lipid

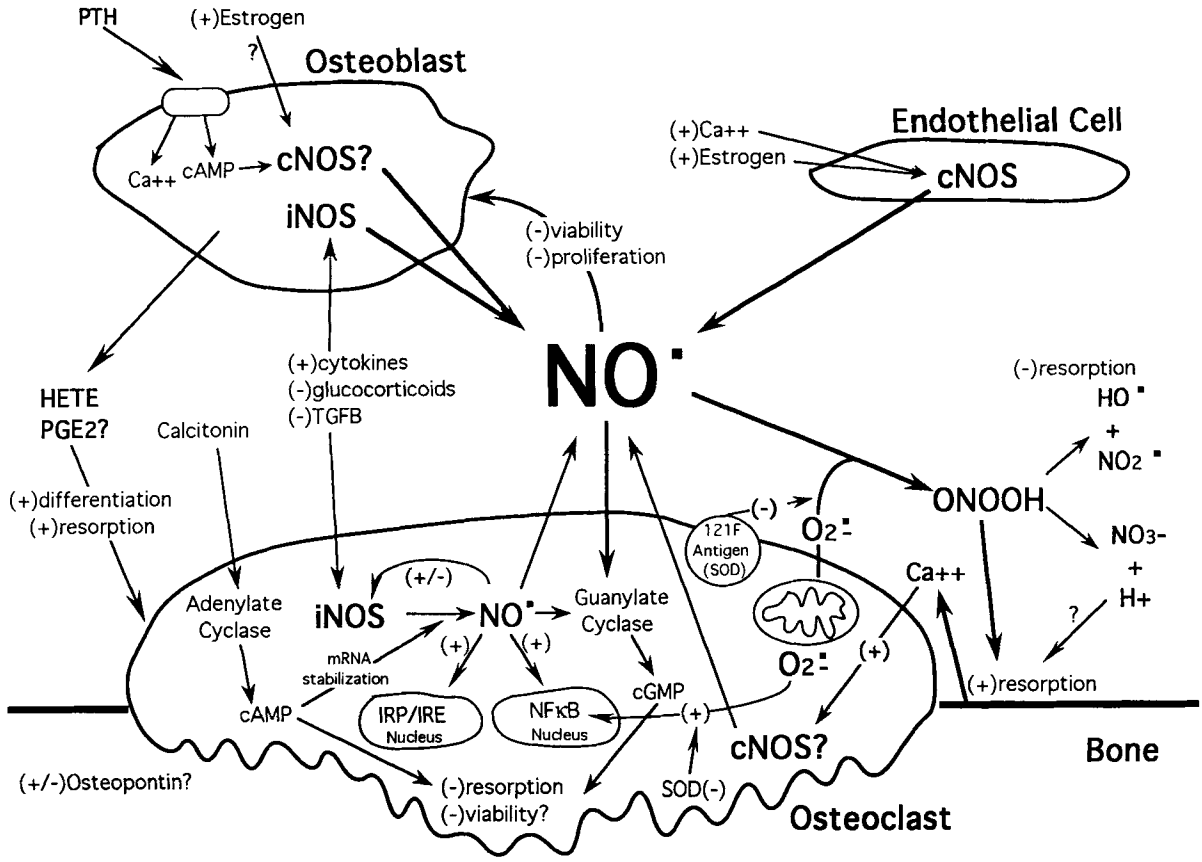

Fig. 1. Production of Nitric Oxide (NO) and reactive radicals by bone cells and the regulation of bone cell function by NO. NO is produced by constitutive (cNOS) and inducible (iNOS) isoforms residing in the membrane and cytosol of bone endothelial cells, osteoblasts, and osteoclasts. NO generally inhibits cell viability and bone resorption activity through generation of cGMP, nuclear transcriptional/translational effects, and interactions with other reactive molecules, such as superoxide (O_2^-). SOD, superoxide dismutase; ONOOH, peroxynitrite: IRP/IRE, iron-regulatory protein/iron response element; NF-κB, a nuclear transcription factor; HETE, hydroxyeicosatetraenoic acid; PGE_2, prostaglandin E_2.

metabolites interface with other bone cell modulators including NO.

Arachidonic acid is converted into prostaglandins via the action of cyclo-oxygenase (COX) isoenzymes. Like NOS, there are both constitutive and inducible isoforms, with the constitutive isoform (COX-1) present in nearly all cells and the inducible isoform (COX-2) subject to upregulation by pro-inflammatory stimuli like those that elevate inducible NOS. Therefore, under inflammatory conditions, both stimulators (prostaglandins) and inhibitors (NO) of bone resorption can be simultaneously produced, and their ratios may contribute to the overall regulation of bone remodeling. Complicating matters further, low levels of NO may activate but high NO concentrations inhibit the induction of COX-2, TGF-β may induce COX but inhibit NOS, and prostaglandins may inhibit NO production [Vane et al., 1994]. Thus, various mechanisms for controlling the COX and NOS systems relative to one another and with respect to other modulators require further investigation.

CHONDROCYTES

In addition to bone loss, inflammation frequently involves localized cartilage erosion, such as that which occurs in the joints of arthritic patients. Synovial fluids drawn from such inflammed joints have been shown to contain elevated concentrations of cytokines, prostaglandins, metalloproteinases, and NO. Stadler et al. [1991] demonstrated that articular chondrocytes respond to inflammatory stimuli like IL-1 and LPS, but not TNF-α, with increased NO production. The combination of IL-1, LPS, and TNF-α led to an initial stimulation in the production of metalloproteinase (gelatinase) and PGE_2

in these chondrocytes, and this induction appeared to be dependent on NO production, since it was prevented when NOS inhibitors were present. In contrast to the short-term effect, prolonged exposure of chondrocytes to elevated NO levels led to a dramatic inhibition of chondrocyte gelatinase and prostaglandin release. Similarly, Palmer et al. [1993] have reported that IL-1, TNF, and LPS upregulated an inducible NOS in human articular chondrocytes, as determined by Northern blot analysis. Since proteolytic enzymes and prostaglandins play such prominent roles in inflammation, the potential modulation of these compounds by NO suggests that it may act as a prime regulator of cytokine-induced chondrocyte activities that lead to excessive matrix degradation in various pathological disorders. Whether or not degradative enzymes associated with osteoblasts (collagenase) or osteoclasts (metalloproteinases) are similarly controlled by NO awaits further analysis.

FUTURE DIRECTIONS

Figure 1 depicts selected possible sources and interplay of the NO system in bone with other regulatory and functional aspects of bone remodeling. Clearly, one challenge for future efforts is to refine our understanding of the cellular and molecular events regulating the NOS isoenzymes present in bone and how NO may function in normal and pathological bone remodeling. No doubt further research will reveal a plethora of roles for this multifunctional signal molecule in the development, physiology, and communication pathways linking osteoblasts, osteoclasts, and other cells within bone.

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