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Immunology 2001 **103** 255–261 **REVIEW ARTICLE**

Nitric oxide and bone

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SUMMARY

Nitric oxide (NO) is a free radical which has important effects on bone cell function. The endothelial isoform of nitric oxide synthase (eNOS) is widely expressed in bone on a constitutive basis, whereas inducible NOS is only expressed in response to inflammatory stimuli. It is currently unclear whether neuronal NOS is expressed by bone cells. Pro-inflammatory cytokines such as IL-1 and TNF cause activation of the iNOS pathway in bone cells and NO derived from this pathway potentiates cytokine and inflammation induced bone loss. These actions of NO are relevant to the pathogenesis of osteoporosis in inflammatory diseases such as rheumatoid arthritis, which are characterized by increased NO production and cytokine activation. Interferon gamma is a particularly potent stimulator of NO production when combined with other cytokines, causing very high concentrations of NO to be produced. These high levels of NO inhibit bone resorption and formation and may act to suppress bone turnover in severe inflammation. The eNOS isoform seems to play a key role in regulating osteoblast activity and bone formation since eNOS knockout mice have osteoporosis due to defective bone formation. Other studies have indicated that the NO derived from the eNOS pathway acts as a mediator of the effects of oestrogen in bone. eNOS also mediates the effects of mechanical loading on the skeleton where it acts along with prostaglandins, to promote bone formation and suppress bone resorption. Pharmacological NO donors have been shown to increase bone mass in experimental animals and preliminary evidence suggests that these agents may also influence bone turnover in man. These data indicate that the L-arginine/NO pathway represents a novel target for therapeutic intervention in the prevention and treatment of bone diseases.

INTRODUCTION

Nitric oxide (NO) is a free radical involved in the regulation of many physiological processes, such as vascular relaxation,[1] neurotransmission, platelet aggregation and in immune regulation.[2] Over recent years it has become apparent that NO has important effects on bone cell function and here we review the role of the L-arginine–NO pathway as a regulator of bone remodelling in health and disease.

Bone is a complex tissue composed of several cell types which is continuously undergoing a process of renewal and repair termed 'bone remodelling' (Fig. 1). The two major cell types responsible for bone remodelling are osteoclasts, which resorb bone, and osteoblasts, which form new bone. Osteoclasts are multinucleated cells of haemopoietic origin, which differentiate from precursors in the monocyte/macrophage lineage, in response to co-ordinated expression of lineage-specific regulatory molecules including c-fos, M-CSF RANK (receptor activator of NFkB), RANK ligand (RANKL) and osteoprotegerin (OPG).[3] Osteoblasts, on the other hand, are cells of mesenchymal origin which differentiate from bone marrow stromal cells in response to activation of the transcription factor Cbfa1.[4] During the bone remodelling cycle, old or damaged bone is removed by osteoclasts, by the secretion of acid and proteolytic enzymes onto the bone surface. Subsequently the osteoclasts migrate away from the area of bone undergoing resorption and undergo apoptosis. They are replaced by osteoblasts, which lay down new bone matrix in the form of osteoid. Later, the osteoid becomes calcified to form mature bone. During bone formation, some osteoblasts become embedded within the bone matrix, and differentiate into osteocytes, a third cell type unique to bone. Osteocytes interconnect with one another and with cells on the bone surface through a series of long cytoplasmic processes that run through cannaliculi in the bone matrix. It is thought that osteocytes act as sensors of mechanical stress in the skeleton, by detecting and responding to changes in fluid flow

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Abbreviations: iCa^{++}, intracellular calcium; NADP, nicotinamide adenine dinucleotide phosphate; NOS, nitric oxide synthase; ecNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS

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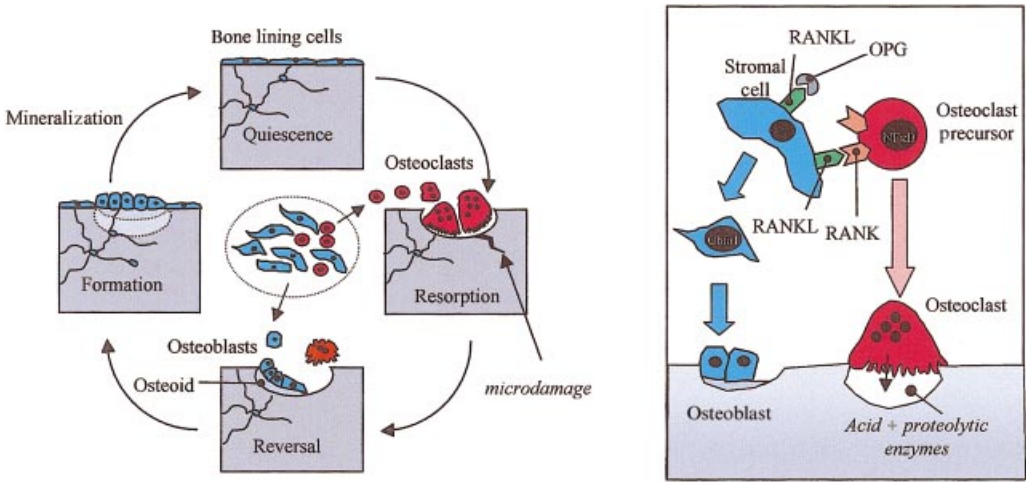

Figure 1. The bone remodelling cycle. The bone remodelling cycle of resorption, reversal and bone formation is responsible for renewal and repair of old and damaged bone (main panel). Osteoclasts differentiate from haemopoetic precursors in the monocyte lineage in response to activation of RANK, by its ligand RANKL, expressed on stromal cells (inset). This interaction is blocked by OPG which acts as a decoy receptor for RANKL. Osteoblasts differentiate from mesenchymal precursors in bone marrow in response to activation of the osteoblast-specific transcription factor Cbfa1.

which run through cannaliculi in bone. Bone remodelling is regulated by several systemic hormones, such as parathyroid hormone (PTH), 1,25 dihydroxyvitamin D3, sex hormones and calcitonin, as well as by local factors such as NO, prostaglandins, growth factors and cytokines. It is currently believed that many of the factors which regulate bone remodelling do so by influencing local expression of RANK, RANKL and OPG,[5–7] which together form a paracrine system that plays an essential role in regulating osteoclast differentiation and function (Fig. 1, inset).[3,8]

NITRIC OXIDE SYNTHESIS AND REGULATION

NO is generated by the nitric oxide synthase enzymes (NOS) from molecular oxygen and the terminal guanidino nitrogen of the amino acid L-arginine, yielding L-citrulline as a coproduct.[9] This reaction can be inhibited by substituted arginine analogues such as L-N^G-monomethyl arginine (L-NMMA) and L-nitro-arginine-methyl ester (L-NAME). NO can also be generated nonenzymatically from nitrite in the acid environment of the stomach[10] and pharmacologically by compounds such as organic nitrates (e.g. nitro-glycerine) and sodium nitroprusside,[9] which are used clinically as vasodilators (Fig. 2).

NO can be measured by chemiluminescence[9] and by the use of special NO-sensitive electrodes,[11] but since the half-life of NO is in the order of seconds, these direct measurements are difficult. NO rapidly reacts with oxygen to yield the stable metabolites nitrate (NO_3) and nitrite (NO_2), and NO production can be more easily assessed indirectly by measuring accumulation of NO_3 and NO_2 using the Griess reaction.[9] The amount of accumulated NO_3 and NO_2 does not always reflect the amount of NOS enzyme present, as enzyme activity may be limited by availability of its cofactors. To measure the amount of NOS enzyme, the conversion of radiolabelled L-arginine to L-citrulline is measured using an *in vitro* assay.[9]

Three isoforms of NOS have been identified so far; a neuronal form (nNOS or NOS1),[12] an endothelial form (eNOS or NOS3)[13] and an inducible form (iNOS or NOS2).[14] Both

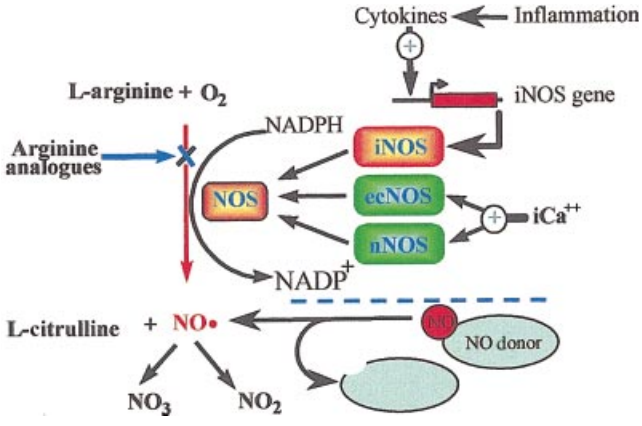

Figure 2. The L-arginine–nitric oxide pathway. NO is synthesized from L-arginine and molecular oxygen by the NOS group of enzymes. In inflammation, cytokines such as IL-1 and TNF are upregulated. The cytokines induce expression of the inducible NOS (iNOS) gene, leading to synthesis of the iNOS enzyme, which produces nitric oxide (NO) from L-arginine. NO can also be produced by pharmacological NO donors such as SNAP. NO is a very reactive molecule and rapidly reacts with oxygen to form nitrite and nitrate.

eNOS and nNOS are constitutively expressed at low levels in their tissues of origin and their activity is mainly regulated by changes in free intracellular Ca^{++} concentration ($[iCa^{++}]$).[9] However, it has recently been shown that eNOS can also be activated by certain cytokines through the IP3/akt pathway. The protein kinase akt phosphorylates the eNOS protein at serine 1177 and this activates the enzyme.[15] Little is known of the factors that regulate nNOS expression at a transcriptional level, but it has been shown that the eNOS gene promoter contains several oestrogen response elements and shear-stress responsive elements.[16] Consistent with this, increases in eNOS mRNA have been found in endothelial cells exposed to shear stress[17] or oestrogen.[18]

Regulation of iNOS mainly takes place at the level of gene transcription. Transcription of the iNOS gene is activated by pro-inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor alfa (TNFα), gamma interferon (γIFN) and endotoxin, whereas glucocorticoids and the anti-inflammatory cytokines IL-4, IL-10 and TGFβ are inhibitory.[2] Sequence analysis of the human iNOS promoter has shown substantial differences from the murine promoter.[19] These differences are probably responsible for differences in the ease with which cytokines can induce NO production in different species.[20] Recently Sunyer *et al*.[21] have shown that chicken osteoclasts can be induced to express iNOS, but that this requires elevated [iCa^{++}] and activation of PKC, rather than stimulation with cytokines.

Different cell types differ in their ability to produce NO after cytokine stimulation. Human primary osteoblast and hepatocyte cultures require combinations of two or three cytokines for a significant induction of NO synthesis,[22,23] whereas human chondrocytes can be induced by single cytokines such as IL-1 and TNF to produce NO.[24,25] In all cell types, however, combinations of cytokines are generally more potent inducers of NO than single cytokines.[22,24,26]

The iNOS pathway is thought to be capable of generating much larger quantities of NO (nanomolar range) over a more prolonged time frame than the constitutive NOS (cNOS) enzymes (picomolar concentrations).[2,27] However, because iNOS activity depends on transcription, response to external stimuli is not as rapid as that of cNOS (hours rather than seconds).

MECHANISMS OF NO ACTION

Nitric oxide is a highly reactive molecule, and because of this has many potential molecular targets. In vascular smooth muscle and platelets, NO binds to the haem moiety of soluble guanylyl cyclase (sGC)[9] thus increasing the enzyme activity. The resulting elevation in cGMP levels causes activation of a cascade of cGMP dependent protein kinases, which mediate smooth muscle relaxation, and inhibition of platelet adhesion. Interestingly, the conformational changes induced by binding of NO to the haem moiety of the NOS enzymes inhibit enzyme activity, thereby providing an autoregulatory feedback loop by which increased NO levels limit NO production.[28]

Other biological effects of NO are mediated by its ability to react with sulphydryl residues and iron-sulphur centres in proteins. The effect on iron-sulphur centres is thought to be responsible for the inhibitory effect of high NO concentrations on cell growth via interaction with mitochondrial aconitase, a critical enzyme in the Krebs cycle.[9]

Recently, Landers *et al*.[29–31] have shown that the small GTPase $p21^{ras}$ is another molecular target for NO. NO nitrosylates the cysteine residue 118 (Cys118) of $p21^{ras}$, resulting in activation of GTPase activity and stimulation of the map kinase and NFkB signal transduction pathways.

NO can also react with oxygen-derived free radicals such as superoxide anions to form highly reactive molecules such as the peroxynitrite anion and the hydroxyl radical.[9] Production of such toxic moieties may contribute to the tissue damage that is a characteristic feature of an inflammatory response, by inducing lipid peroxidation.[32]

The molecular targets for NO action in bone cells are poorly understood. Initially it was thought that the inhibitory effects of NO on osteoclastic bone resorption were mediated by a cGMP independent mechanism,[26,33] but evidence has recently been presented to suggest that cGMP dependent pathways may also be involved.[34]

EXPRESSION OF NOS ISOFORMS IN BONE

Recent data from several groups have shown that ecNOS is widely expressed on a constitutive basis in bone marrow stromal cells, osteoblasts, osteocytes and osteoclasts.[35–37]

Two studies have failed to detect nNOS expression in cultured bone derived cells and in bone[36,38] although expression of nNOS mRNA has been detected in bone by the reverse transcription-polymerase chain reaction (RT-PCR).[36] A more recent study using a different antibody has, however, shown evidence of nNOS protein expression in bone lining cells and in osteocytes.[39]

Expression of iNOS has been observed in foetal bone,[40] suggesting a role in skeletal development, but iNOS does not appear to be expressed constitutively in normal adult bone.[36,39] Expression of iNOS can be induced in osteoblasts and bone marrow cells *in vitro* by stimulation with pro-inflammatory cytokines and/or endotoxin however.[22,41–43] It is currently unclear whether osteoclasts are able to express iNOS. Our own studies have failed to detect iNOS protein or mRNA in cultured osteoclasts[36] and studies in bone marrow cocultures show that the majority of cytokine-induced NO derives from osteoblasts.[43] Nonetheless, weak iNOS expression has been detected by other workers in osteoclasts *in vivo*[39] and in preosteoclastic cell lines *in vitro*.[35]

EFFECTS OF NO ON BONE RESORPTION

There is good evidence to suggest that NO has biphasic effects on osteoclastic bone resorption. Low concentrations of NO have been shown to potentiate IL-1 induced bone resorption, based on the observation that NOS inhibitors inhibit IL-1 induced bone resorption *in vitro*.[42] Constitutive production of NO within osteoclasts has been suggested to be essential for normal osteoclast function, based on the observation that NOS inhibitors inhibit activity and motility of isolated osteoclasts.[35] Nonetheless, examination of bones from animals with iNOS or eNOS deficiency has shown no major defect in bone resorption under physiological conditions, which suggests that these isoforms are not essential for osteoclast formation or activity. Accumulating evidence suggest that the iNOS pathway plays an important role in cytokine and inflammation induced bone loss however. Inflammation induced osteoporosis has been shown to be mediated in part by activation of the iNOS pathway[44] and recent studies have shown that activation of the iNOS pathway is essential for IL-1-stimulated bone resorption, both *in vivo* and *in vitro*.[45] The mechanism by which iNOS promotes IL-1 induced bone resorption has been investigated in cocultures of normal osteoblasts with iNOS-KO osteoclast precursors. These studies have shown that IL-1 primarily acts on osteoblasts to increase NO synthesis by activation of the iNOS pathway and that this in turn promotes nuclear translocation of the transcription factor NFκB in osteoclast progenitors. Whilst osteoclast progenitors from iNOS deficient

animals also show NFkB activation in response to IL-1, the response is transient, implying that NO has a key role to play in sustaining NFkB activation in osteoclast precursors.[45]

High concentrations of NO inhibit osteoclast formation and activity. Experiments using cell and organ cultures have shown that IFNγ in combination with IL-1 and/or TNFα strongly induces iNOS expression, leading to very high levels of NO that inhibit bone resorption.[35,41,42,46] These high NO concentrations have been shown to be responsible for the inhibitory effect of IFNγ on IL-1 and TNFα stimulated bone resorption.[41,42] The inhibitory effect of high levels of NO appear to be due to the inhibition of both osteoclast formation and activity[47] and NO-induced apoptosis of osteoclast progenitors.[47]

A possible mechanism for the inhibition of osteoclast activity by NO is the modification of cathepsin K. Cathepsin K is highly expressed in osteoclasts and plays a key role in the bone resorption mechanism, since it degrades bone collagen. NO and several NO donors have been shown to inhibit the activity of purified cathepsin K, although the modification of the protein does not seem to involve nitrosylation, but rather oxidation of thiol moieties.[48]

EFFECTS OF NO ON BONE FORMATION

Nitric oxide appears to have biphasic effects on osteoblast activity. Studies *in vitro* have indicated that the small amounts of NO which are produced constitutively by osteoblasts may act as an autocrine stimulator of osteoblast growth and cytokine production.[49] Whilst some investigators have shown that slow release NO donors stimulate osteoblast growth and differentiation *in vitro*,[34,50,51] other workers reported that NO donors and NOS inhibitors had little effect on osteoblast growth or differentiation, except at high concentrations where inhibitory effects were observed.[22,37] The most compelling evidence supporting a role for NO in osteoblast function derives from studies in eNOS knockout animals. Two groups of investigators have reported major defects in bone formation and osteoblast activity both *in vivo* and *in vitro* in eNOS deficient animals and a reduced anabolic response to exogenous oestrogen. The molecular mechanisms responsible for this remain to be defined, but indicate the existence of an important interaction between eNOS and the molecular pathways involved in osteoblast differentiation and function.[50,52,53]

High concentrations of NO, such as those observed after stimulation with pro-inflammatory cytokines, have potent inhibitory effects on osteoblast growth and differentiation, however.[22,34,54] Recent evidence suggests that this may be partly due to pro-apoptotic effects of NO on osteoblasts,[55,56] and that these effects are mediated in part by cGMP.[34] This observation may explain the inhibitory effects of pro-inflammatory cytokines on bone formation and the reduced bone formation which has been detected in conditions associated with cytokine activation[57,58] and in the animal model of inflammation-mediated osteopenia.[44,59,60]

OESTROGEN, NO AND BONE

Oestrogen stimulates eNOS activity and mRNA levels in endothelial cells[18] and osteoblasts,[61] raising the possibility that NO derived from the eNOS pathway plays a role in mediating the effects of sex hormones in bone. In keeping with this hypothesis, Wimalawansa *et al*.[62,63] have shown that NOS inhibitors abolished the protective effect of oestrogen on bone in ovariectomised rats, whereas in the same studies, the NO donor nitro-glycerine was almost as effective as oestrogen in preventing ovariectomy induced bone loss.[62,63] Furthermore, studies by Armour *et al*. showed that the anabolic effects of oestrogen on bone formation were blunted in eNOS-knockout mice.[52] Taken together, these findings indicate that both the anti bone resorptive and anabolic effects of oestrogen on bone may be mediated in part by NO.

NO AND MECHANICAL STRESS

Several groups have shown that NO synthesis can be induced in osteoblasts and osteocytes by mechanical strain and shear stress. These stimuli cause rapid but transient increases in the production of NO and prostaglandins by bone-derived cells and organ cultures.[64–67] The high concentrations of NO induced by these stimuli may augment bone gain by inhibiting the bone resorbing actions of the prostaglandins.[26] The fact that osteocytes and osteoblasts under noninflammatory conditions only express the ecNOS isoform, taken together with the rapid induction of NO by shear stress and mechanical strain synthesis points to a role for the ecNOS isoform rather than iNOS.[65,66] It has also been reported that osteocytes show a greater increase in NO-production as the result of mechanical loading than osteoblasts, supporting the hypothesis that osteocytes are the principal sensors and effectors of mechanical stress in bone.

NO AND INFLAMMATORY DISEASE

Nitrate levels are elevated in urine and serum of patients suffering from rheumatoid arthritis (RA),[68,69] and even higher levels have been detected in the synovial fluid,[70] suggesting that the inflamed joint is the source of the NO. Furthermore, NO levels are higher in patients with active disease, than in those with quiescent disease,[71] and NO levels fall with prednisolone treatment,[69] indicating an association between disease status and NO production.

Considerable amounts of nitrosylated protein have been detected in the synovial fluid of RA patients, but not in osteoarthritis (OA) patients.[72,73] It has been suggested that nitrosylation may be an important cause of joint damage in RA and the effects of several anti-inflammatory drugs on nitrosylation levels have recently been studied.[74]

The iNOS enzyme is readily detected in synovium and cartilage of RA patients, is expressed at lower levels in OA but is virtually absent from normal synovium and cartilage.[75–77] Synovial cells and chondrocytes are able to produce high levels of NO upon cytokine stimulation *in vitro*,[24,75,78] and the iNOS expression is associated with apoptosis of synovial cells and chondrocytes.[76,79,80] One mechanism by which NO can induce apoptosis is by upregulating expression of the tumour suppressor gene p53[81] and p53 overexpression has been observed in the synovial lining cells in RA.[82] In vitro, NO has been shown to be a potent stimulator of chondrocyte apoptosis[83] and this may contribute to the cartilage damage observed in RA. Furthermore, cytokine induced NO stimulates

the production of matrix metallo-proteinases in chondrocytes,[71] which may lead to further destruction of the cartilage, and it has been shown that the protective effects of IL-4 are at least in part mediated by suppression of NO synthesis.[84]

As mentioned earlier, NO potentiates cytokine induced bone resorption[42] and high levels can also inhibit bone formation.[22,34,42,42,54] Therefore, NO produced within the inflamed joint may contribute to the peri-articular bone loss observed in RA. In an experimental model of inflammation induced bone loss, NOS-inhibitors reduced the bone loss observed.[44] Further experiments using iNOS-knockout mice indicate that iNOS is the isoform involved in the inflammatory bone loss.[60] Histomorphometric analysis of bones from normal animals with inflammation induced bone loss showed a profound depression of bone formation[44] and evidence of osteoblast apoptosis. These changes were not observed in iNOS knockout animals, which suggests that iNOS activation may contribute to inflammation induced osteoporosis by suppressing bone formation and promoting osteoblast apoptosis (K. J. Armour *et al*. unpublished results).

It has been shown that NOS inhibitors can reduce the severity of arthritis in animal models of arthritis.[85–88] Whilst this suggests that NOS inhibitors may have an important clinical use in the treatment of inflammatory disease in humans, no human studies have been published so far.

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