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Nitric Oxide and Cyclic GMP Functions in Bone

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Abstract

Nitric oxide plays a central role in the regulation of skeletal homeostasis. In cells of the osteoblastic lineage, NO is generated in response to mechanical stimulation and estrogen exposure. Via activation of soluble guanylyl cyclase (sGC) and cGMP-dependent protein kinases (PKGs), NO enhances proliferation, differentiation, and survival of bone-forming cells in the osteoblastic lineage. NO also regulates the differentiation and activity of bone-resorbing osteoclasts; here the effects are largely inhibitory and partly cGMP-independent. We review the skeletal phenotypes of mice deficient in NO synthases, sGC, or PKGs, and the effects of NO and cGMP on bone formation and resorption. We examine the roles of NO and cGMP in bone adaptation to mechanical stimulation. Finally, we discuss preclinical and clinical data showing that NO donors and NO-independent sGC activators may protect against estrogen deficiency-induced bone loss. sGC represents an attractive target for the treatment of osteoporosis.

Keywords

Nitric oxide; cGMP; protein kinase G; bone; osteoporosis; osteoblasts; osteoclasts

Introduction

The appendicular skeleton and vertebrae develop via a process called endochondral ossification, where mesenchymal cells differentiate into chondroblasts to form a cartilaginous “template” that is replaced by osteoblasts producing mineralizing matrix (1). After birth, endochondral ossification continues in the growth plates, where it governs longitudinal bone growth. Throughout the organismal lifespan, mineralized bone undergoes constant remodeling to maintain skeletal homeostasis and strength: osteoclasts initiate the remodeling cycle by resorbing mineralized matrix, while osteoblasts form new matrix (Fig. 1). An imbalance in bone remodeling –caused by excess bone resorption or decreased bone formation relative to resorption– results in a loss of bone mass and quality, leading to osteoporosis and an increased risk of bone fractures (2).

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Bone-resorbing osteoclasts are multi-nucleated cells derived from hematopoietic precursors of the monocyte/macrophage lineage, and their differentiation and function is controlled by cytokines such as receptor of activated nuclear factor kappa-B ligand (RANKL)[1] and its antagonist osteoprotegerin (OPG) (2). Bone-forming osteoblasts differentiate from bone marrow stromal cells in response to Wnts and other growth factors; they secrete extracellular matrix proteins to produce osteoid, which becomes calcified to form mature bone (2). During this process, some osteoblasts differentiate into osteocytes, which are completely embedded in mineralized matrix, but interconnected via cytoplasmic processes extending through micro-canaliculi. Osteocytes are long-lived and highly active cells, which sense mechanical stress and produce regulatory peptides, including sclerostin, a potent inhibitor of Wnt signaling (3). Bone remodeling is controlled by systemic hormones, such as estrogens and parathyroid hormone, by locally-produced factors, such as RANKL/OPG and Wnts/sclerostin, and by small signaling molecules such as NO and prostaglandins (2,3). Nitric oxide in bone has been the subject of several reviews in the past (4-7); here we concentrate on NO functions in bone that are mediated via activation of soluble guanylyl cyclase (sGC) and production of cGMP.

Expression of NO synthases and regulation of NO synthesis in bone cells

All three NO synthase (NOS) forms have been identified in bone and in isolated osteoblasts and osteoclasts by RT-PCR and immunohistochemistry (8-15). NOS-1 and -3 are constitutively expressed in primary osteoblasts from humans and rodents, with NOS activity stimulated by increases in intracellular calcium concentrations. Mechanical stimulation significantly increases NO production in osteoblasts and osteocyte-like cells, and this increase is blocked by calcium chelation, but the source(s) of mechanically-stimulated NO synthesis in osteoblastic cells remain controversial (11,16-19). Estrogens rapidly stimulate NOS-3 activation via a membrane-bound estrogen receptor; this requires an increase in intracellular calcium and Akt phosphorylation of NOS-3 in endothelial cells, with similar mechanisms operative in osteoblasts and osteocytes (20-24). In addition, estrogen treatment increases NOS-3 mRNA expression over 24 h, likely via nuclear estrogen receptor binding to the NOS-3 promoter (25). Thyroid hormone treatment of osteoblasts increases NO production via a membrane-bound thyroid receptor; thyroid hormone-induced NO synthesis is coupled to an increase in intracellular calcium concentrations and is abolished in NOS-3-deficient osteoblasts (26).

NOS-2 mRNA expression is regulated primarily at the transcriptional level, and is induced by inflammatory cytokines such as TNF-α, interferon-γ, and interleukin-1; NOS-2 expression in osteoblasts is also up-regulated by mechanical stimulation (9,10). NOS-2 expression in differentiating osteoclasts is induced by RANKL, and exerts a negative feedback to restrict differentiation (13) (described below).

[1]The following abbreviations were used: BFR, bone formation rate; BMD, bone mineral density; BMSC, bone marrow stromal cell; BV/TV, bone volume fraction; CNP, C-type natriuretic peptide, GC-B, guanylyl cyclase-B; GSK, glycogen synthase kinase; MAR, mineral apposition rate; NO, nitric oxide; NOS, NO synthase; OB, osteoblast; OC, osteoclast; OPG, osteoprotegerin; OVX, ovariectomy; PKG, cGMP-dependent protein kinase; RANKL, receptor of activated nuclear factor-KB ligand; sGC, soluble guanylyl cyclase.

The bone phenotype of NOS-deficient mice

NOS-1 knockout mice.

NOS-1-deficient mice have an osteosclerotic phenotype with high bone mass, at least partly due to decreased osteoclastic bone resorption (27). NOS-1-deficient mice demonstrate increased trabecular and cortical bone mineral density, and histomorphometry shows decreased osteoclast and osteoblast numbers, with reduced bone remodeling, reflected in low mineral apposition and bone formation rates (27). Reduced osteoclast number and activity in NOS-1-deficient mice was confirmed in an *in vivo* model of inflammation-induced bone resorption (28). Unexpectedly, more osteoclasts are formed from NOS-1-deficient compared to wild type bone marrow in the presence of RANKL and M-CSF *in vitro*, but the NOS-1-deficient osteoclasts are abnormally large and have reduced bone resorptive capacity (27,28) (Table 1). The high bone mass, low bone turnover phenotype of globally NOS-1-deficient mice may not be solely explained by defects of NOS-1 deficient bone cells, but indirect effects, e.g. hormonal changes due to NOS-1 deficiency in the nervous system, may contribute.

NOS-2 knockout mice.

NOS-2-deficient mice do not have obvious bone abnormalities, with normal femur lengths, trabecular bone volume fraction, bone formation rate, and osteoclast surface (29). However, NOS-2-deficient mice show altered responses to mechanical loading (discussed below, and Table 1).

NOS-3 knockout mice.

Different strains of NOS-3-deficient mice have been generated with some differences in bone phenotypes, perhaps due to different genetic backgrounds (30-35). NOS-3-deficient mice show defects in endochondral bone formation causing abnormal pre-natal and post-natal bone development, including fetal growth restriction, limb malformations, reduced longitudinal bone growth with hypocellular growth plates, and increased perinatal fatality (30-32). Young (6-9 week old) NOS-3−/− mice demonstrate marked retardation in post-natal bone formation, not only reduced longitudinal growth —a function of chondroblast growth and differentiation in growth plates— but also reduced bone volumes associated with defects in osteoblast maturation and activity (32,33). These NOS-3−/− mice show markedly reduced osteoblast numbers and profound defects in bone formation and mineral apposition rates, with decreased trabecular bone volume and cortical thickness compared to wild type mice (32,33). Osteoclasts appear to be unaffected by NOS-3 deficiency (32,33). Bone densitometry scanning and micro-CT analysis show reduced femoral and spinal bone mineral density in 8 week-old NOS-3-deficient compared to wild type mice, with some investigators reporting that these abnormalities persisted at 20 weeks (33), whereas others found that the differences diminished and normalized by 12-18 weeks (32,35,36). Altered responses of NOS-3-deficient mice to fluid shear stress or estrogen treatment after ovariectomy are discussed below (and Table 1).

In vitro, NOS-3-deficient osteoblasts proliferate slower and differentiate less well compared to wild type cells; both defects can be restored by the addition of exogenous NO

(32,34). NOS-3-deficient osteoblasts form less mineralized nodules, show reduced alkaline phosphatase activity, and express lower amounts of runx-2 (a master transcription factor for osteoblast lineage cells) and osteocalcin (an extracellular matrix protein important for mineralization) (32,33,36).

Cyclic GMP synthesis in bone cells

NO-stimulated cGMP synthesis and/or sGC expression have been documented in (pre)osteoblasts and osteoclasts (19,37-40). Transcripts for the common, heme-containing β1 subunit of sGC are easily detectable in bone, but the distribution of sGC α1 and α2 subunits has not been determined.

Cyclic GMP is also generated by receptor guanylyl cyclases in bone, especially by the C-type natriuretic peptide (CNP)-activated GC-B receptor (41-43). GC-B is expressed in chondroblasts, osteoblasts, and osteocytes, and has important functions in endochondral ossification – with deficiencies in CNP or GC-B leading to dwarfism, whereas over-expression or activating mutations in the receptor lead to skeletal over-growth in humans and rodents (43-47). This system has been the subject of previous reviews and is not further discussed here (43,48).

Mice deficient in sGC and mice with NO-unresponsive sGC

Mice with a global deletion of the β1 subunit of sGC, or “Apo-sGC” mice with a H105F point mutation in the β1 subunit —resulting in a heme-free, NO-unresponsive enzyme—are viable but have a reduced lifespan (49,50). These mice display gastrointestinal abnormalities and growth retardation, which is not entirely explained by malnutrition or malabsorption (49,50). However, the skeletal phenotype of these mice has not been examined in detail.

Cyclic GMP targets in bone: PKG1 and 2 and Phosphodiesterases

The main cGMP effector proteins in bone are cGMP-dependent protein kinases (PKG1 and PKG2). The largely cytoplasmic PKG1 has two isoforms (PKG1α and 1β) which are generated by differential splicing of the first exon and differ only in the N-terminal ~100 amino acids; this unique N-terminal domain mediates dimerization and docking to specific target proteins (51). (Pre)osteoblasts and osteocytes express both PKG1 isoforms as well as PKG2, with all three enzymes displaying different functions (19,24,52). For example, the pro-proliferative effects of cGMP in osteoblasts require PKG2 activation of Src and Erk-1/2, whereas the anti-apoptotic effects of cGMP in osteocytes are mediated by PKG1α and PKG2 via distinct mechanisms discussed below (Fig. 3) (19,24,52). Osteoclasts express primarily PKG1α and 1β (53,54).

Phosphodiesterases, which degrade cAMP and/or cGMP and can be allosterically regulated by cGMP binding, have not been studied extensively in bone cells (51). Osteoblasts contain multiple phosphodiesterases, but the cGMP-regulated PDE-5 was not detected (55).

PKG1- or PKG2-deficient mice

Mice globally deficient in PKG1 have a short life span due to severe intestinal dysfunction, and their growth retardation is primarily due to malnutrition (56). PKG1 knockout mice with smooth muscle-specific expression of either PKG1α or 1β have a much improved life expectancy, but their skeletal phenotype was not reported (57).

Mice globally deficient in PKG2 are dwarfs due to severe defects in endochondral ossification (58). PKG2 deficiency also causes dwarfism in rats, cattle, and humans, indicating a central function of PKG2 in longitudinal bone growth across multiple species (59-61). The skeletal phenotype of PKG2 knockout mice mimics that of CNP- or GC-B-deficient mice, and PKG2 acts downstream of CNP in chondroblasts (58, 62). Cartilage-specific CNP transgene expression rescues the dwarfism phenotype of CNP-deficient mice, but has no effect on the skeletal phenotype of PKG2−/− mice (62). In addition to the CNP/GC-B pathway, the NOS-3/sGC pathway likely contributes to PKG2 activation in growth plate chondroblasts, since NOS-3-deficient mice show at least transient growth retardation in long bones (described above) (30-32). To examine the functions of PKG1 and PKG2 in adult skeletal homeostasis, we are presently characterizing osteoblast-specific PKG1- and PKG2-knockout mice.

In vitro effects of NO and cGMP in cells of osteoblastic lineage

Nitric oxide has biphasic effects on (pre)osteoblasts and osteocytes *in vitro*. Low NO concentrations promote proliferation, differentiation, and survival, whereas high NO concentrations have opposite effects (37,38,63). The positive effects of low NO concentrations on osteoblast proliferation are mediated by sGC and PKG2, because they are mimicked by cell-permeable cGMP analogs and prevented by pharmacological inhibition of sGC or PKG, or siRNA-mediated knock-down of PKG2 (37,52,64). The anti-apoptotic effects of low NO concentrations in bone marrow stromal cells and osteocytes are also mediated by cGMP, and require both PKG1 and PKG2; they are reduced by siRNA-mediated knock-down of PKG2 or PKG1α (24,37,65). PKG2 exerts its anti-apoptotic and pro-proliferative functions in osteoblasts via activation of Src, Erk-1/2 and Akt; the latter two kinases directly phosphorylate and inactivate the pro-apoptotic protein BAD (24). Akt also phosphorylates and inactivates glycogen synthase kinase-3β, which leads to stabilization and nuclear translocation of β-catenin and activation of Wnt-pathway genes (Fig. 3, below) (37,66). Wnt signaling plays an essential role in (pre)osteoblast differentiation, proliferation, and survival and drives bone formation *in vivo* (2).

Low doses of NO donors enhance osteogenic differentiation of bone marrow stromal cells and (pre)osteoblasts *in vitro*, by stimulating mRNA expression of osteoblastic genes, e.g. alkaline phosphatase, osteocalcin, and collagen-1, and increasing bone matrix synthesis and mineralization (38,63,67,68). Again, these NO effects are blocked by sGC inhibitors and mimicked by cell-permeable cGMP analogs or NO-independent stimulators of sGC, suggesting that NO works via cGMP (42,63,64,69). The osteoblast differentiation-promoting effects of NO/cGMP likely involve stimulation of Wnt/β-catenin pathway activity (see above), and increased expression and activity of the osteoblast master transcription factor

Runx2, and of Fos-related proteins which cooperate with Runx2 (19,36,68,70,71). Thus, NO activation of sGC exerts positive effects on cells of the osteoblastic lineage at all stages of differentiation, from immature bone marrow stromal cells (BMSCs) to mature osteocytes (Fig. 2).

In vitro effects of NO and cGMP on osteoclasts

Similar to its actions in the osteoblastic lineage, low concentrations of NO may promote, while higher concentrations inhibit osteoclast differentiation and survival (27,37,72). *In vivo*, NOS-1 appears to be required for normal osteoclast differentiation and/or survival, since NOS-1-deficient mice have decreased osteoclast numbers, and *in vitro*, NOS-1-deficient bone marrow monocytes produced poorly-functioning osteoclasts, as described above (Table 1) (27,28). The effects of pharmacological NOS inhibition on osteoclast differentiation *in vitro* are varied and perhaps depend on the time of addition and the NOS isoform targeted (13,14,73-75). RANKL induces NOS-2 expression in osteoclast precursors, and the high NO concentrations produced by NOS-2 inhibit osteoclastic differentiation in a cGMP-independent fashion (13). Correspondingly, osteoclast precursors isolated from NOS-2-deficient mice show enhanced RANKL-induced differentiation and bone pit formation *in vitro* (Table 1) (13). NOS-2 induction by interleukin-1 and interferon-γ also suppresses osteoclast formation, activity and survival (14,74-76).

NO donors, NO-independent sGC activators (YC-1), and cell-permeable cGMP analogs inhibit osteoclast differentiation and bone pit formation *in vitro* (37,40,77). YC-1 induces osteoclast apoptosis via caspases-3 and -8 activation (77). NO induces detachment of mature osteoclasts from bone and down-regulates their acid secretion, thereby inhibiting bone resorption (40,78-80). These effects appear to be mediated by endogenous NO production and require cGMP and PKG, since NOS or PKG inhibition increase attachment and acid secretion (40,79,80). NO stimulates osteoclast motility and detachment via PKG1 phosphorylation of the vasodilator-stimulated protein (VASP) and the inositol-1,4,5-triphosphate receptor-associated protein (IRAG), events that lead to cytoskeletal rearrangement and calcium activation of the proteinase μ-calpain, respectively (53,54,81). Thus, the effects of NO and cGMP on osteoclast differentiation and function *in vitro* are largely inhibitory.

In vivo effects of NO and cGMP on bone formation and resorption

The low bone volume/low bone formation phenotype of NOS-3-deficient mice suggests that NOS-3-derived NO is important for osteoblast differentiation and function *in vivo*, as described above (Table 1) (32,33). Administration of non-isoform-specific NOS inhibitors, such as L-NAME or aminoguanidine, to adult rats causes a significant decrease in bone formation and bone mineral density, again suggesting that basal NO production is required for skeletal homeostasis (82-84).

Consistent with a positive role of NO in bone formation, administration of the NO donor nitrosyl-cobinamide (NO-Cbi) to adult mice increases mineral apposition and bone formation rates, as well as trabecular bone volume fraction and bone mineral density (37).

NO-Cbi-treated mice demonstrate increased osteoblast numbers and osteocalcin mRNA expression, while osteoclast numbers and osteoclast-specific gene expression (cathepsin-K and tartrate-resistant acid phosphatase) are decreased. The negative effect of NO-Cbi on osteoclasts may be partly due to reduced RANKL production in mice treated with the NO donor (37).

Administration of sGC-stimulating agents to young rats –at high doses that cause profound hypotension– increases bone formation and resorption with increased numbers of osteoblasts and osteoclasts, leading to a striking increase in bone turnover within a short period of time (7 d), which is slowly reversible (after 35 d) (85). In contrast, administration of the heme-independent sGC activator cinaciguat to adult mice –at a low dose that did not significantly lower systolic blood pressure– increases osteoblast number, mineral apposition and bone formation rates, without significantly affecting osteoclast numbers (69). Thus, NO –via activation of sGC– increases osteoblastic bone formation in rodents, but the effect on osteoclasts may require higher doses and depend on the age of the animals.

NO/cGMP functions in bone adaptation to mechanical stimulation

Mechanical stimulation is a primary determinant of bone growth and remodeling, with fluid flow through the bone canalicular system generating shear stress that stimulates osteoblasts and osteocytes and enhances their anabolic activity (3,6,86-89). Bone marrow stromal cells, osteoblasts, and osteocyte-like cells all respond to fluid shear stress *in vitro* with increasing proliferation and survival (6,88). These anabolic responses require increased NO production, which occurs acutely from calcium-mediated NOS-3 activation, and longer-term from increased NOS-2 and NOS-3 mRNA expression (6,10,19,52,90). We found that the NO/cGMP/PKG2 signaling pathway activates Src in mechanically stimulated osteoblasts to initiate Erk-1/2 activation and a proliferative response (Fig. 3) (52). Fluid shear stress triggers the recruitment of PKG2, Src, and SHP-1 into an integrin β3-containing membrane complex (a “mechanosome”, which translates mechanical stimuli into biochemical responses), and PKG2 directly phosphorylates and stimulates the phosphatase SHP-1 to de-phosphorylate Src on an inhibitory site (52). The anti-apoptotic effects of fluid shear stress in osteoblasts and osteocytes require Akt activation, which occurs through two parallel pathways: one via calcium activating NOS and NO/cGMP/PKG2 activating Src, the other via calcium-dependent activation of focal adhesion kinase and Src, independent of NO (66,90).

Unloading of bone causes rapid bone loss due to reduced bone formation and increased resorption (88). In contrast, (re)loading of bone stimulates osteogenesis via increased Wnt signaling (due to suppression of sclerostin) and inhibits bone resorption via suppression of RANKL signaling (due to upregulation of the RANKL antagonist OPG) (3,88). (Re)loading of bone increases NOS-2, NOS-3, and *c-fos* mRNA expression in osteoblasts and osteocytes, and NOS inhibitors block mechanically-induced bone formation and *c-fos* mRNA induction (10,29,82,83,91-95).

In a murine hind limb suspension model, bone loss occurs within 7-14 d and is reversed by reloading; bone loss in this model can be prevented by increasing interstitial fluid flow

via venous ligation (35). Mice deficient in NOS-2 or NOS-3 experience the same bone loss in unloaded limbs as wild type mice, but NOS-3-deficient animals are not protected by venous ligation, and NOS-2-deficient animals fail to increase bone formation after reloading (29,35). While a role of cGMP/PKG signaling downstream of NO in osteoblast/osteocyte mechano-transduction has been established *in vitro* (52,66), the role of cGMP/PKG *in vivo* awaits experiments in osteoblast-specific PKG2 knockout mice.

NO/cGMP effects in estrogen-deficient rodents

Ovariectomy-induced estrogen deficiency in rodents causes marked bone loss with associated changes in bone architecture and turnover that recapitulate those observed in post-menopausal women, including increased bone resorption, an inadequate bone formation response, and enhanced osteocyte apoptosis (96). Estrogens normally limit osteoclast survival by transcriptionally upregulating Fas ligand via nuclear estrogen receptor (independent of NO) (97,98). In contrast, the beneficial effects of estrogens on cells of the osteoblastic lineage are largely mediated via a membrane-bound estrogen receptor-α, which couples to NOS-3 (20-22,99,100). Estrogens promote the survival of osteoblasts and osteocytes via NO/cGMP-dependent phosphorylation and inactivation of BAD (directly, by PKG1, and indirectly, by PKG2 stimulation of Erk and Akt, as described above) (23,24). NO also mediates estrogen stimulation of (pre)osteoblast proliferation and differentiation (23,32).

Data in rodents are consistent with a requirement for NOS-3 activation downstream of the membrane-bound estrogen receptor for at least some of the bone-protective effects of estrogens: (i) bone formation induced by high doses of estrogens *in vivo*, in intact mice or in ovariectomized rats, is blocked by administration of the NOS inhibitor L-NAME (101,102); (ii) NOS-3 knockout mice loose more bone after ovariectomy than wild type mice, and their response to exogenous estrogens is blunted (12,33); and (iii) NO-generating agents can at least partly substitute for estrogens' bone protective effects *in vivo* (37,102,103). In fact, treating ovariectomized rats with NO-generating nitrates (e.g., nitroglycerin) preserves bone mineral density and improves mechanical bone properties to a similar degree as estrogen replacement (102,103). On the other hand, in ovariectomized mice with a point mutation which abolishes membrane localization of the estrogen receptor the estrogen's bone-protective effects are partly abolished, suggesting an important role of the NOS-3-coupled estrogen membrane receptor in bone (99,100).

While organic nitrates are the only NO donors currently FDA-approved for long-term use in humans, their clinical benefits are limited by development of tolerance and induction of oxidative stress (104-107). Nitrates require enzymatic activation to release NO, and during the activation, reactive oxygen species are generated (106,108). Novel NO donors circumvent this problem; for example, nitrosyl-cobinamide (NO-Cbi) releases NO directly, without biotransformation or generation of reactive oxygen species (109). NO-Cbi increases trabecular bone mass in both intact and ovariectomized mice, at a dose that does not significantly affect systolic blood pressure (37). NO-Cbi increases intracellular cGMP concentrations, Wnt/β-catenin signaling, proliferation, and osteoblastic gene expression in murine primary osteoblasts, and protects cells from apoptosis (37). Correspondingly,

in intact and ovariectomized mice, NO-Cbi increases serum cGMP concentrations, bone formation, and osteoblastic gene expression (37). In ovariectomized mice, NO-Cbi prevents estrogen deficiency-induced osteocyte apoptosis (37). NO-Cbi also reduces osteoclast numbers in intact mice and prevents the ovariectomy-induced increase in osteoclasts, in part due to a reduction in the RANKL/OPG gene expression ratio, and in part due to direct inhibition of osteoclast differentiation, observed *in vitro* in the presence of excess RANKL (37). Although the positive NO effects in osteoblasts are mediated by cGMP/PKG, the osteoclast-inhibitory effects are partly cGMP-independent (37).

Ovariectomized mice have lower serum cGMP concentrations compared to sham-operated animals, suggesting that estrogen deficiency causes a state of systemic NO/cGMP deficiency due to defective NOS-3 activation in endothelial cells (20,21,37,69). NO's bone-protective effects appear to be largely mediated by cGMP, because treating ovariectomized mice with the NO-independent sGC activator cinaciguat improves trabecular bone microarchitecture and enhances osteocyte survival, with effect sizes similar to those obtained with estrogen replacement therapy (69). However, compared to 17β-estradiol or NO-Cbi, which completely prevent the ovariectomy-induced increase in osteoclast number, cinaciguat has a lesser effect on osteoclasts (37,69). On the other hand, the NO-independent sGC activator YC-1 effectively prevents the increase in bone resorption and improves bone architecture in ovariectomized rats (77).

Thus, NO donors and sGC activators show anabolic effects in pre-clinical models of estrogen-deficiency osteoporosis.

NO effects on bone in human studies

Similar to rodents, estrogens also appear to induce NO production in humans. Plasma concentrations of stable nitric oxide derivatives (nitrites and nitrates) correlate positively with estrogen concentrations in humans, and are decreased in post-menopausal women and increased with estrogen supplementation (110-112). Several epidemiological studies have reported a bone-protective effect of NO-generating organic nitrates (nitroglycerin and isosorbide mononitrate), which are clinically used for treatment of heart failure and coronary insufficiency (113-115). Two case control studies compared nitrate use in individuals who sustained a fracture to that in age- and sex-matched controls without a fracture, and found a 10-15% reduction in fracture risk in nitrate users after adjusting for confounding factors (113,114). The greatest benefit appeared to be among patients using low doses of short-acting nitrates on an intermittent basis (113,115).

A positive effect of nitrates on bone, at doses lower than those used for vasodilation, has also been demonstrated in several independent, prospective, randomized clinical trials: (i) nitroglycerin was as effective as estrogen replacement in preventing bone loss in young women after ovariectomy (116); (ii) in post-menopausal women with established osteoporosis, subjects randomized to isosorbide mononitrate treatment showed similar improvement in bone mineral density as subjects who received a bisphosphonate (a proven anti-resorptive therapy for osteoporosis) (117); and (iii) in healthy post-menopausal women, isosorbide mononitrate treatment for 12 weeks increased a bone formation marker (bone-

specific alkaline phosphatase) and decreased a bone resorption marker (N-telopeptide) compared to placebo (118). However, a three-year trial in early post-menopausal women failed to show an effect of once-daily nitroglycerin ointment on bone mineral density, albeit with poor treatment adherence (119). A second trial testing transdermal nitroglycerin versus placebo in post-menopausal women was completed by a different group, but the report was retracted.

Thus, while there is a substantial amount of data *in vitro* and *in vivo* indicating bone-anabolic effects of NO via activation of sGC and PKG, more work is required to test the clinical usefulness of this approach. Organic nitrates are clearly suboptimal NO donors because of tolerance development and oxidative stress (104,106). Novel classes of NO donors are under clinical development (37,107). However, estrogen-deficiency and aging cause excess oxidative stress, which may affect NO bioavailability and render sGC insensitive to NO (107,120). Therefore, cinaciguat and newer, NO-independent sGC activators may be more ideally suited to restore NO/cGMP signaling in post-menopausal and aging bone, and may represent a new paradigm for the treatment of osteoporosis (69,121).

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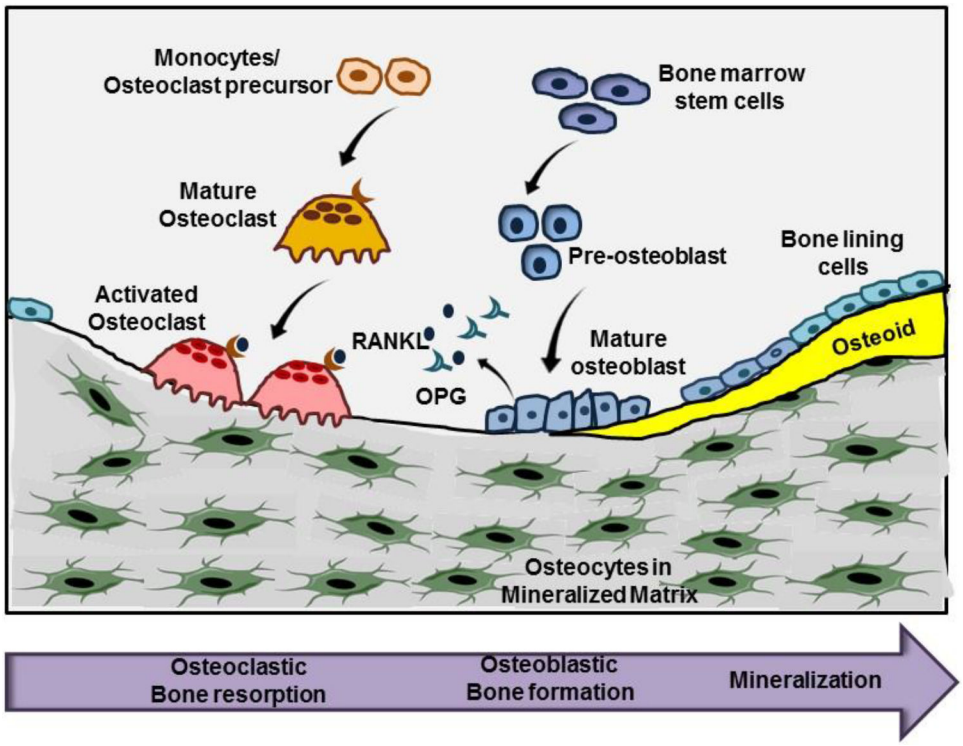

Fig. 1: Bone remodeling by osteoblasts and osteoclasts:

Osteoclasts differentiate from hematopoietic precursors under the influence of RANKL, which is secreted by osteoblasts together with its antagonist OPG. Osteoclasts resorb calcified bone matrix and recruit osteoblasts to fill the defect with new bone. Osteoblasts differentiate from mesenchymal stem cells and secrete extracellular matrix to form osteoid. Osteoid calcifies to form mature bone and surrounds mature osteocytes.

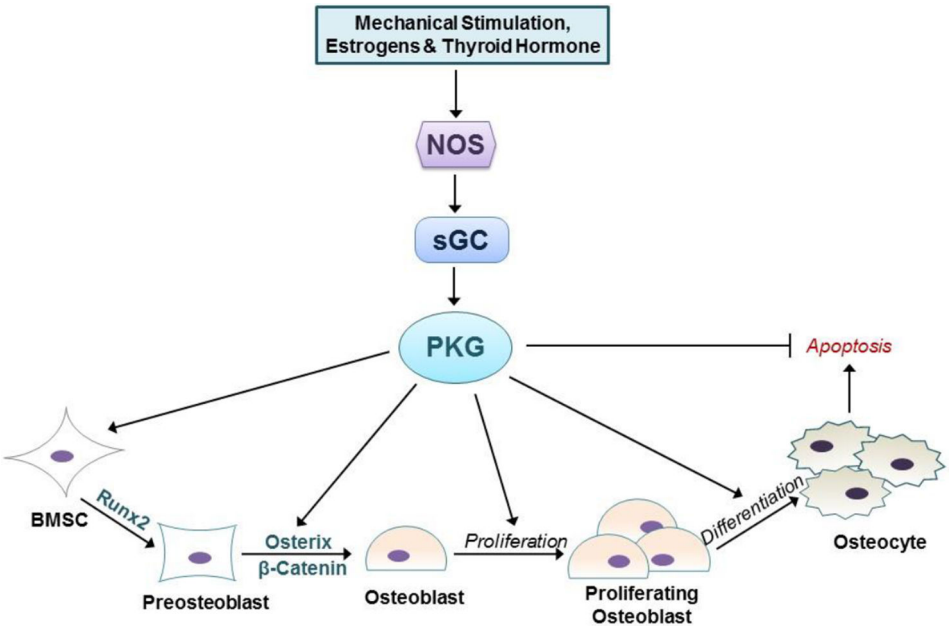

Fig. 2: NO/cGMP regulation of osteoblastic cells.

NO is synthesized in response to mechanical stimulation, or treatment with estrogen and thyroid hormones. NO-stimulated cGMP synthesis results in PKG activation. PKG regulates all stages of osteoblastic cells, promoting proliferation and differentiation, and inhibiting apoptosis.

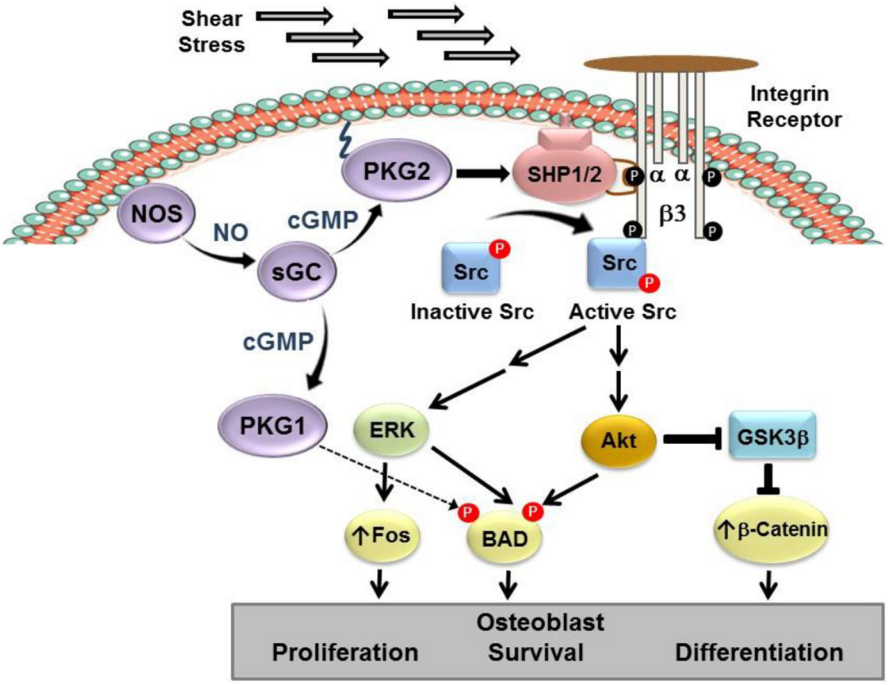

Fig. 3: NO/cGMP signaling in fluid shear-stressed osteoblasts.

Shear stress activates NOS via increased calcium. Membrane-bound PKG2 activates integrin-bound Src to stimulate Erk and Akt, which regulate osteoblast proliferation, survival, and differentiation via BAD phosphorylation and increased *fos*- and *β*-catenin-controlled genes. PKG1α directly phosphorylates and inactivates BAD to promote survival.

Table 1:

Bone Phenotypes of NO Synthase-deficient mice

Global Knockout	Bone Phenotype	Histomorphometry	*In vitro* Studies	References
NOS-1 −/−	Osteosclerosis, increased BMD	Increased BV/TV, trabecular and cortical thickness, OC number Decreased OB number, MAR & BFR	Increased OC size & number, decreased resorption	27,28
NOS-2 −/−	No obvious bone phenotype at baseline, Decreased reloading-induced osteogenesis	Normal BV/TV, BFR, OC number	OC: enhanced RANKL-induced differentiation	13,29
NOS-3 −/−	Reduced bone length & bone volumes, reduced BMD (only up to 8-10 weeks of age?) Decreased osteogenesis in response to fluid shear (venous ligation) More bone loss after OVX and blunted response to estrogens	Reduced BV/TV, trabecular number, BFR & MAR, OB number (normal OC number)	Defects in chondrocyte and OB proliferation, defects in OB differentiation	12,30-36