MINIREVIEW

Nitric Oxide as the Mediator of the Antiosteoporotic Actions of Estrogen, Statins, and Essential Fatty Acids

UNDURTI N. DAS[1]
EFA Sciences, Norwood, Massachusetts 02062

Estrogen, statins, and essential fatty acids and their metabolites can prevent osteoporosis. However, it is not certain how these three structurally different agents can have the same beneficial action. It is suggested that all three, in addition to their other modes of action in the prevention of osteoporosis, have the ability to augment constitutional (or endothelial) nitric oxide generation, which is known to be beneficial in osteoporosis. If so, it will be interesting to study whether nitric oxide donors and/or nitric oxide precursors can be given together with estrogen, statins, or essential fatty acids to potentiate their benefit in osteoporosis. [Exp Biol Med Vol. 227(2):88–93, 2002]

Key words: nitric oxide; osteoporosis; cytokines; estrogen; statins; essential fatty acids

The skeleton is renewed throughout life as a result of the actions of the bone-resorbing osteoclast and the bone-forming osteoblast. Generally, a balance is maintained between the actions of these two cell types. Osteoporosis, which is characterized by low bone mass and microarchitectural deterioration of bone tissue leading to increased bone fragility, can result in an increased risk of fractures. Postmenopausal bone loss is due to an increase of bone turnover that is estrogen dependent. Several placebo-controlled trials showed that hormone replacement therapy (HRT) prevents bone loss in women in early and late postmenopause (1). Observational studies showed that use of HRT is associated with a 30%-50% reduction of hip, spine, and wrist fractures. Even though estrogens are known to be effective in preventing bone loss in postmenopausal women (1), the exact mechanism(s) by which estrogens influence bone metabolism and increase skeletal strength is not clear. The bone-protective effects of estrogen may involve suppression of inflammatory cytokines that promote osteoclastogenesis and bone resorption, such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and IL-6, and an increase in endothelial nitric oxide (eNO) formation.

Osteoclasts are derived from the monocyte-macrophage lineage under the influence of macrophage-colony-stimulating factor (M-CSF), granulocyte/M-CSF (GM-CSF), and the receptor activator of NF-κB ligand (RANKL), as well as pro-inflammatory cytokines IL-1, IL-6, and TNF (2). RANKL, a TNF-like molecule, and M-CSF are essential for osteoclast differentiation and function. This is supported by the observation that RANKL-deficient mice, who do not have osteoclasts, develop osteopetrosis (2). RANKL is produced by stromal cells, osteoblasts, and lymphoid cells in response to a variety of factors that include vitamin D, parathyroid hormone, and prostaglandin E_2 (PGE_2). Osteoclastogenesis is inhibited by sex steroids, cytokines, γ-interferon (γ-IFN), and certain prostaglandins (PGs). IL-4 secreted by TH_2 lymphocytes modulates macrophage function and regulates the expression of pro-inflammatory cytokines IL-1, TNF-α, and IL-6 (3). IL-4 suppresses bone resorption both *in vitro* and *in vivo* by inhibiting the expression and production of inflammatory cytokines IL-1, TNF, and RANKL. IL-4 can also suppress RANKL-induced osteoclast differentiation by a direct action on monocyte/macrophage precursors (2), which in turn act on the peroxisome proliferator-activated receptor-γ1 (PPAR-γ1). This led to the suggestion that the ability of

This work was supported by Techlead Corporation, Inc.
[1] To whom requests for reprints should be addressed at EFA Sciences LLC, 1420 Providence Highway, Suite 266, Norwood, MA 02062. E-mail: undurti@hotmail.com

Received January 1, 2001.
Accepted October 8, 2001.

1535-3702/02/2272-0088$15.00

PPAR-γ1 to suppress osteoclast differentiation may be responsible for the anti-resorptive effects of thiazolidinedione class of PPAR-γ ligands.

Estrogen and Cytokines

Estrogen has immunomodulatory effects and anti-inflammatory actions. In healthy premenopausal women who underwent oopherectomy, increases in GM-CSF activity was observed as early as 1 week after surgery, whereas elevations in IL-1 and TNFα were detectable 2 weeks after surgery (4). In those who did not receive estrogen replacement therapy, IL-1, TNFα, and GM-CSF reached the highest levels 8 weeks after oopherectomy, and these changes in the cytokine profile were found to be associated with indices of bone resorption (4). On the contrary, those who received estrogen replacement therapy within 4 weeks after oopherectomy showed decreases in the secretion of GM-CSF, IL-1, and TNFα. The cytokine profile did not change in the female controls who underwent simple hysterectomy. This suggests that estrogen suppresses the production of these three cytokines (4) and, thus, prevents osteoporosis. It is also known that estrogen enhances transforming growth factor-β (TGF-β) production (5, 6). TGF-β has antioxidant and anti-TNF actions, it suppresses free radical generation, and it shows anti-inflammatory actions as well (6, 7). TGF-β plays an important role in bone formation, induction, or repair (5). Injection of TGF-β over frontal or parietal bones in neonatal mice or rats and over femur in newborn rats stimulates bone formation (reviewed in Ref. 5). TGF-β can regulate cell proliferation and phenotypic expression in the fracture callus *in vitro* and it can enhance chondrogenesis and osteogenesis *in vivo* (5). When used at physiological concentrations, 17β-estradiol stimulated the production of TGF-β *in vitro* (8). It was also observed that estrogen modulates IL-1 actions on human osteoclasts (9). Isolated human osteoclasts and primary bone marrow-derived osteoclast-like cells expressed both the signaling (IL-1RI) and decoy (IL-1RII) IL-1 receptors, whereas only IL-1RI was detected in osteoblasts (9). IL-1RII/IL-1RI mRNA ratios and release of soluble IL-1RII (sIL-1RII) were found to be lower in osteoclast-like cells derived from women in the late postmenopausal period compared with younger women. Estrogen directly reduced *in vitro* osteoclast-like cell IL-1RI mRNA levels, while it increased IL-1RII mRNA levels and sIL-1RII release. In this study (9), it was noted that estrogen pretreatment significantly inhibited two IL-1 responses, suppressing IL-1-mediated IL-8 mRNA induction and IL-1-promoted osteoclast survival. It is known that IL-8 is released at high levels by human osteoclasts; osteoclast-derived IL-8 inhibits multiple osteoblast bone formative functions (10); IL-8 stimulates osteoclast migration; and IL-8 promotes osteoclast recruitment, development, and bone-resorptive activity (11). Thus, one mechanism by which estrogen exerts bone-protective effects may include a selective modulation of IL-1R isoform levels in osteoclasts, thereby reducing their IL-1 responsiveness and cell survival. Lin *et al.* (12) reported increased expression of the IL-6 receptor in cells of the bone marrow stromal/osteoblastic lineage after loss of sex steroids. This is consistent with the observation that increased IL-6 levels can be detected in bone marrow supernatants from ovariectomized compared with sham-operated mice (13), and in postmenopausal compared with premenopausal women (4). Thus, estrogen suppresses the production of pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) and their actions, and augments the production of TGF-β, events that inhibit bone resorption and prevent osteoporosis. Paradoxically, glucocorticoids, which suppress the production of TNF and other pro-inflammatory cytokines (14), do not prevent osteoporosis. In fact, they induce osteoporosis by suppression of the osteoblast. This indicates that there could be a second messenger involved in osteoporosis through which the actions of cytokines and corticosteroids on bone remodeling are brought about.

Statins and Osteoporosis

Recent studies suggested that 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA reductase) inhibitors or statins have anabolic effects, increase bone formation, and may be useful in the treatment of osteoporosis by augmenting the expression of bone morphogenetic protein-2 (BMP-2) (15). Statins are potent competitive inhibitors of HMG-CoA reductase, which are the rate-limiting enzymes of the mevalonate pathway. Mevalonate is the precursor of cholesterol and a variety of isoprenoid-containing compounds. These isoprenoid precursors are necessary for the post-translational lipid modification (called as prenylation) and, hence, the function of *ras* and other small GTPases (16). Hence, inhibition of the mevalonate pathway can disrupt the function of oncogenic forms of *ras*. It has been suggested that small GTPases, which are prenylated products of the mevalonate pathway, may have a negative control on the expression of BMP-2 and other BMPs. Inhibition of the mevalonate pathway by statins prevents the function of small GTPases and enhances the expression of BMP-2 and other BMPs, leading to increased osteoblast formation and enhanced bone formation. In addition, there is evidence to suggest that statins can cause osteoclast apoptosis and inhibit bone resorption *in vitro* (15). Thus, statins have both an antiresorptive effect, as well as an anabolic action. Statins also inhibit the production of pro-inflammatory cytokines TNFα and IL-6 (17), which have a major role in inducing osteoporosis in postmenopausal women. This is supported by the observation that statins increased bone mineral density and decreased fracture risk (18–21). However, this is not without controversy. Studies performed by Reid *et al.* (22) have not confirmed these findings. In their study, no evidence of a reduced frequency of fracture in patients treated with statins was noted. These negative findings could be due to several reasons: the patients probably were not at high risk of osteoporotic fractures and were not recruited on the basis of fracture history or low bone den-

sity. These negative findings may also be due to differences between the statins in their access to bone cells, differences between individual members of statins in their effect on bone mass, and other metabolic abnormalities, such as insulin resistance, which is known to affect bone density. Edwards *et al.* (18) adjusted their results for the differences in body weight and noted that statins can increase bone mineral density and reduce the risk of fracture, whereas in the other studies, this was not done. Clearly more studies are needed to establish the benefit of statins in reducing fracture risk and increase bone mineral density. Thus, the antiosteoporotic action of statins would originate from their action on HMG-CoA reductase. It is possible that statins would act on HMG-CoA reductase within the osteoblast and osteoclast. Action within osteoblast might involve BMP-2 and other BMPs. The effect of statins on the osteoclast are most likely via suppression of protein isoprenylation.

Essential Fatty Acids (EFAs) and Osteoporosis

EFAs and their metabolites such as γ-linolenic acid (GLA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) were also reported to have beneficial action in osteoporosis (23). Fish oil can prevent nephrocalcinosis in animals by reducing urinary calcium excretion. Wohl *et al.* (24) demonstrated that a diet rich in saturated fat interfered with calcium absorption and decreased cancellous bone strength. EFA-deficient animals develop severe osteoporosis and increased renal and arterial calcification (23, 25), a finding that is similar to the osteoporosis and calcification of arteries and kidneys seen in elderly people. A combination of GLA and EPA decreased calcium excretion, enhanced intestinal calcium absorption, and increased bone calcium content (reviewed in Ref. 23). One mechanism by which these fatty acids prevent osteoporosis may involve inhibition of pro-inflammatory cytokines IL-1, IL-2, and TNFα (reviewed in Ref. 23), which are known to have a major role in osteoporosis.

Similar to statins, EFAs and their metabolites can lower cholesterol, triglyceride, and low-density lipoprotein levels, and they can block HMG-CoA reductase activity (23, 26). Animals with EFA deficiency showed an increase in HMG-CoA reductase activity, which reverted to normalcy following topical application of linoleic acid (LA) (23, 26). Further, both n-3 and n-6 fatty acids showed inhibitory action on HMG-CoA reductase activity (reviewed in Ref. 23). Hence, similar to statins, EFAs and their metabolites also may enhance the expression of BMPs. This could be yet another mechanism by which EFAs are useful in osteoporosis (23). It may be noted here that even though EFAs and their metabolites are thought to be useful in osteoporosis, more definitive studies need to be performed in humans. It is also possible that EFAs have to be given for long periods of time to obtain their possible benefit in osteoporosis.

Estrogen, Statins, EFAs, and NO

Though estrogen, statins, and EFAs and their metabolites are reported to be of benefit in osteoporosis, the exact mechanism(s) by which these three agents can produce their beneficial action is not clear. I suggest that this could be due to their ability to enhance the production of NO.

Arnal *et al.* (27) reported that estrogens induce a receptor-mediated antioxidant effect by suppressing superoxide anion production that enhances the biological activity of eNO. Several other studies have also reported that estrogen can increase NO synthesis, especially that of constitutive or eNO (28–30). Statins activate the protein kinase Akt, leading to increased NO production by the endothelial-type of NO synthase (31). EFAs and their metabolites such as arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid also enhance eNO synthesis. Thus, estrogen, statins, and EFAs, which have beneficial action in osteoporosis, have the ability to augment constitutive or eNO synthesis. Does this mean that NO is mediating the beneficial actions of these three agents in osteoporosis?

NO and Osteoporosis

NO is believed to modulate bone remodeling and bone loss both *in vitro* and *in vivo*. Osteoclasts exhibit substantial NO synthase activity (32). In an *in vitro* study, wherein chicken osteoclasts were cultured for 36 hr on bovine bone slices, nitroprusside, an NO donor, markedly decreased the number of bone pits and the average pit area in comparison with control cultures, whereas a NO synthase inhibitor, N-nitro-L-nitro-L-arginine methyl ester, dramatically increased the number of bone pits and the average bone resorption area per pit (33). These results suggest that endogenous NO production in osteoclast cultures may regulate resorption activity. Further support for the role of NO in osteoporosis is derived from the reported observation that raloxifene, a selective estrogen receptor modulator that is clinically effective for the prevention of postmenopausal osteoporosis, can trigger a rapid and dose-dependent release of NO from endothelial cells (34). This raloxifene-induced NO production was found to be dependent on an estrogen receptor-mediated mechanism because this effect was abolished by estrogen receptor antagonist. These results suggest that eNO (or constitutive NO) has the property to prevent osteoporosis by inhibiting the activity of osteoclasts. Ovariectomy-induced osteopenia can be reversed by NO donor nitroglycerin in rats, probably due to decreased bone resorption and, perhaps, increased bone formation (34, 35). Even corticosteroid-induced bone loss was also prevented by NO donor nitroglycerin in male rats (36). Jamal *et al.* (37) observed that women taking nitrates (which are donors of NO) had greater hipbone mineral density than non-users. It was also noted that nitroglycerin therapy ointment (an NO donor), when applied to the skin once a day (within 4 weeks of undergoing oophorectomy), mimicked estrogen replacement therapy in the prevention of bone loss (38). Nitroglycerin therapy significantly increased serum osteocalcin and bone-specific alkaline phosphatase levels, and was found to be as effective as estrogen in preventing bone loss in surgically induced menopausal women (38).

On the other hand, inflammatory disease is known to be associated with increased production of NO and osteoporosis. Armour *et al.* (39) noted that in an animal model of inflammation-induced osteoporosis, inducible NO (iNO) production was increased in the bone marrow space. Bone mineral density was reduced in inflammation-induced osteoporosis when compared with controls, and this was found to be associated with reduced osteoblast and increased osteoclast numbers (39). Inflammation-induced osteoporosis was reversed by the NO synthase inhibitor, L-N(G)-monomethyl-L-arginine (NMMA). Further, several inflammatory conditions, such as rheumatoid arthritis, osteomyelitis, etc. in which there is increased production of pro-inflammatory cytokines IL-1, TNF, and γ-IFN, are associated with both local and generalized osteoporosis (40). IL-1, TNF, and γ-IFN are potent inducers of iNO. High concentrations of NO (which occurs during the induction of iNO) inhibit osteoblast proliferation and may be responsible for cytokine-induced bone resorption (reviewed in ref. 40).

These results suggest that the effect(s) of NO on osteoblasts and osteoclasts may depend on its local concentrations: at low concentrations (as it occurs with eNO or cNO synthase activity), NO promotes the proliferation of osteoblasts and prevents osteoporosis, whereas high concentrations (as it occurs during the induction of iNO synthase by various pro-inflammatory cytokines) may enhance bone resorption by promoting osteoclast formation and activity and suppressing osteoblast proliferation (40).

Conclusions

It is evident from the preceding discussion that cytokines and NO play an important role in the pathobiology of osteoporosis. The beneficial effect of estrogens, statins, and

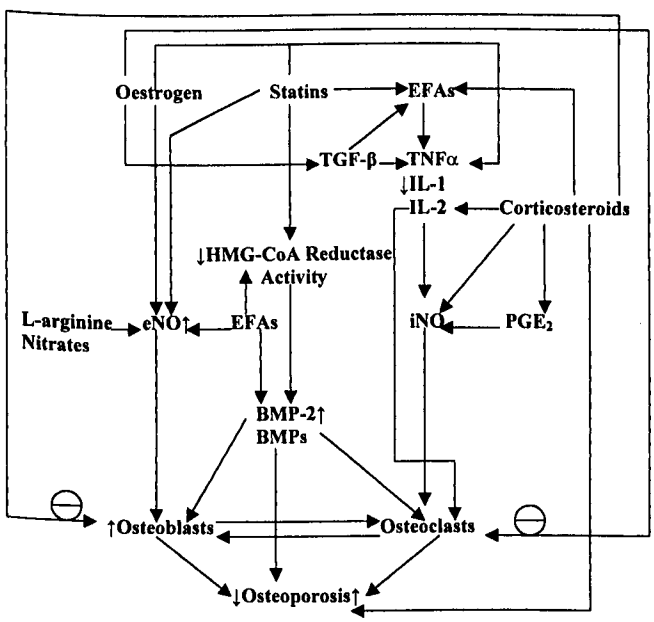

Figure 1. Scheme showing possible interactions between estrogen, statins, EFAs, NO, HMG-CoA reductase activity, BMPs, and osteoporosis. – indicates inhibition of activity.

Table I. Summary of the Actions of Estrogen, Statins, EFAs, and Corticosteroids That Are Relevant to Their Role in Osteoporosis

Oestrogen
Enhances eNO synthesis
Blocks the production of pro-inflammatory cytokines
Enhances TGF-β synthesis
Suppress osteoclast activity
Statins
Increases the production of BMPs probably by their inhibitory action on HMG-CoA reductase
Suppresses the production of pro-inflammatory cytokines
Enhances eNO synthesis through Akt
Augments EFA metabolism
EFAs and their metabolites
Enhances eNO synthesis
Suppresses the production of pro-inflammatory cytokines
Enhances the concentrations of BMPs probably by their inhibitory action on HMG-CoA reductase
EFAs are needed for the action of TGF-β
Corticosteroids
Increases iNO synthesis
Inhibits EFA metabolism
Suppresses the production of pro-inflammatory cytokines
Suppresses osteoblast activity
What is not known or needs to be established
Can estrogen alter the concentrations of BMPs?
Does estrogen modify the metabolism of EFAs?
Is there any interaction between statins and TGFβ?
Are the concentrations of BMPs regulated by EFAs?
How exactly EFAs and their metabolites interact with estrogen?
Can EFAs enhance eNO generation by modulating Akt activity?
What are the pathways by which estrogen, EFAs, and statins act on eNO synthesis? Are they same or different?
Can corticosteroids influence eNO and iNO synthesis in different tissues differently?
Is there a dose-dependent affect on NO on osteoblasts and osteoclasts?

EFAs in osteoporosis can be attributed to their direct action on osteoblasts and osteoclasts, their ability to inhibit the formation of pro-inflammatory cytokines IL-1, IL-2, and TNFα and their ability to enhance the production of eNO (Fig. 1). Thus, estrogen, statins, and EFAs, though structurally unrelated to each other, all three seem to possess similar action(s) on cytokines and NO at the molecular level (see Table 1 for a summary of their actions). Corticosteroids inhibit the osteoblast activity. This may in part be related to their capacity to suppress the formation of pro-inflammatory cytokines and enhance the expression of iNO synthase, and they do not alter the expression of cNO (eNO) synthase. This increase in iNO synthase activity can promote bone resorption as discussed above; however, this action of glucocorticoids is not without controversy. Radomski *et al.* (41) reported that glucocorticoids inhibit the expression of an iNO, but not a cNO, synthase in vascular

endothelial cells. It is possible that the inhibitory action of corticosteroids on iNO or eNO synthesis may vary from tissue to tissue. It is likely that corticosteroids may enhance the expression of iNO in the bone marrow. Further research is needed to verify this possibility.

Corticosteroids are known to interfere with the metabolism of EFAs and PGs: they block the activity of the enzymes, δ-6-desaturase and δ-5-desaturase, which are necessary for EFA metabolism (14, 22), and they inhibit the production of two-series PGs from arachidonic acid (AA) (14). In this context, it is interesting to note the interaction between cytokines, PGE_2, and iNO. Kanematsu *et al.* (42) reported that in osteoblastic MC3T3-E1 cells, IL-1 and TNF-α induced increases in the production of both NO and PGE_2. The increase in NO production was preceded by the expression of iNO synthase mRNA. The temporal profile of PGE_2 production revealed a biphasic pattern: the first small peak (within 3 hr) was caused by *de novo* synthesis of PGE_2 through inducible cyclooxygenase 2 (COX-2) mRNA, while the subsequent progressive accumulation of PGE_2 was mediated through the activation of COX-2 by NO. It was observed that the increase in NO production in response to cytokines was further stimulated by aspirin, a COX inhibitor, and was inhibited by PGE_2. This suggests that PGE_2 has a negative feedback control on NO production. Hence, it is reasonable to suggest that steroid use leads to a sustained increase in iNO production in the absence of a negative feedback control exerted by PGE_2 (see Fig. 1). In addition, EFAs augment eNO synthesis (43) and steroids by interfering with EFA metabolism may block eNO production. This ultimately results in osteoporosis.

The abundance of NO-producing endothelial cells in bone marrow and their proximity to osteoclasts suggests that marrow endothelial cells can have a physiological role in bone metabolism. Postmenopausal osteoporosis is primarily a disease of excessive osteoclastic bone resorption. Estrogen supplementation or adequacy of this hormone enhances eNO production, which in turn inhibits the activity of osteoclasts and thus, protects against osteoporosis. On the other hand, statins enhance bone formation by increasing the formation of osteoblasts with little effect on osteoclasts. Hence, a combination of statins and NO donors or NO precursors may have an added benefit. EFAs prevent bone resorption and enhance bone mineralization or both, and so NO donors may potentiate the beneficial action of EFAs in osteoporosis. EFAs serve as endogenous PPAR-γ ligands (reviewed in Ref. 44). This could be one mechanism by which EFAs can suppress osteoclast differentiation and prevent osteoporosis because PPAR-γ ligands prevent bone loss. The exact mechanism of action of NO on osteoclasts is not known. Cyclic GMP is not involved in its (NO) actions on osteoclasts (45) because dibutyryl or 8-bromo cyclic GMP did not mimic the actions of NO on osteoclasts. If it is true that NO has a major role in osteoporosis, it will be interesting to study whether osteoporosis can be prevented by the oral administration of L-arginine, the precursor of NO, and other drugs that augment eNO synthesis.

1. Clinical Synthesis Panel on HRT. Hormone replacement therapy. Lancet **354:**152–155, 1999.
2. Kong Y-Y, Yoshida HY, Sarosi I, Tan H-L, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature **397:**315–323, 1999.
3. Miossec P, Briolay J, Dechanet J, Wijdenes J, Martinez-Valdez H, Banchereau J. Inhibition of the production of proinflammatory cytokines and immunoglobulins by interleukin-4 in an ex vivo model of rheumatoid synovitis. Arthritis Rheum **35:**874–883, 1992.
4. Pacific R, Brown C, Puscheck E, Frederich E, Slatopolsky E, Maggio D, McCracken R, Avioli LV. Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. Proc Natl Acad Sci U S A **88:**5134–5138, 1991.
5. Finkelman RD, Bell NH, Strong DD, Demers LM, Baylink DJ. Ovariectomy selectively reduces the concentration of transforming growth factor-β in rat bone: implications for estrogen deficiency-associated bone loss. Proc Natl Acad Sci U S A **89:**12190–12193, 1992.
6. Ascroft GS, Dodsworth J, van Boxtel E, Tarnuzzer RW, Horan MA, Schultz GS, Ferguson MW. Estrogen accelerates cutaneous wound healing associated with increase in TGF-β1 levels. Nat Med **3:**1209–1215, 1997.
7. Das UN. Transforming growth factor-β: Is it an endogenous cardioprotector? Med Sci Res **21:**373–375, 1993.
8. Gray TK, Lipes B, Linkhart T, Mohan S, Baylink D. Transforming growth factor-β mediates the estrogen-induced inhibition of UMR106 cell growth. Connect Tissue Res **20:**23–32, 1989.
9. Sunyer T, Lewis J, Collin-Osdoby P, Osdoby P. Estrogen's bone-protective effects may involve differential IL-1 receptor regulation in human osteoclast-like cells. J Clin Invest **103:**1409–1418, 1999.
10. Rothe L, Collin-Osdoby P, Chen Y, Sunyer T, Chaudhary L, Tsay A, Goldring S, Avioli L, Osdoby P. Human osteoclasts and osteoclast-like cells synthesize and release high basal and inflammatory stimulated levels of the potent chmokine IL-8. Endocrinology **139:**4353–4363, 1998.
11. Fuller K, Owens JM, Chambers TJ. Macrophage inflammatory protein-1α and IL-8 stimulate the motility but suppress the resorption of isolated rat osteoclasts. J Immunol **154:**6065–6072, 1995.
12. Lin S-C, Yamate T, Taguchi Y, Borba VZC, Girasole G, O'Brien CA, Bellido T, Abe E, Manolagas SC. Regulation of the gp80 and gp130 subunits of the Il-6 receptor by sex steroids in the murine bone marrow. J Clin Invest **100:**1980–1990, 1997.
13. Miyaura C, Kusano K, Masuzawa T, Chaki O, Onoe Y, Aoyagi M, Sasaki T, Tamura T, Koishihara Y, Ohsugi Y, Suda T. Endogenous bone-resorbing factors in estrogen deficiency: cooperative effects of IL-1 and IL-6. J bone Miner Res **10:**1365–1373, 1995.
14. Das UN. Interaction(s) between essential fatty acids, eicosanoids, cytokines, growth factors and free radicals: relevance to new therapeutic strategies in rheumatoid arthritis and other collagen vascular diseases. Prostaglandins Leukotrienes Essen Fatty Acids **44:**201–210, 1991.
15. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G. Stimulation of bone formation in vitro and in rodents by statins. Science **286:**1946–1949, 1999.
16. Zhang FL, Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. Annu Rev Biochem **65:**241–269, 1996.
17. Rosenson RS, Tangney CC, Casey LC. Inhibition of proinflammatory cytokine production by pravastatin. Lancet **353:**983–984, 1999.
18. Edwards CJ, Hart DJ, Spector TD. Oral statins and increased bone mineral density in postmenopausal women. Lancet **355:**2218–2219, 2000.
19. Chund Y-S, Lee M-D, Lee S-K, Kim H-M, Fitzpatrick LA. HMG-CoA

reductase inhibitors increase BMD in type 2 diabetes mellitus patients. J Clin Endocrinol Metab **85:**1137–1142, 2000.
20. Meier CR, Schleinger RG, Kraenzlin ME, Schlegel B, Jick H. HMG-CoA reductase inhibitors and the risk of fractures. J Am Med Assoc **283:**3205–3210, 2000.
21. Chan KA, Andrade SE, Boles M, Buist DS, Chase GA, Donahue JG, Goodman MJ, Gurwitz JH, LaCroix AZ, Platt R. Inhibitors of hydroxymethylglutaryl-coenzyme A reductase and risk of fracture among older women. Lancet **355:**2185–2188, 2000.
22. Reid IR, Hague W, Emberson J, Baker J, Tonkin A, Hunt D, MacMahon S, Sharpe N. Effect of pravastatin on frequency of fracture in the LIPID study: secondary analysis of a randomized controlled trial. Lancet **357:**509–512, 2001.
23. Das UN. Essential fatty acids and osteoporosis. Nutrition **16:**386–390, 2000.
24. Wohl GR, Loehrke L, Watkins BA, Zernicke RF. Effects of high-fat diet on mature bone mineral content, structure, and mechanical properties. Calcif Tissue Int **63:**74–79, 1998.
25. Kruger MC, Horrobin DF. Calcium metabolism, osteoporosis and essential fatty acids: a review. Prog Lipid Res **36:**131–151, 1997.
26. Proksch E, Feingold KR, Elias PM. Epidermal HMG-CoA reductase activity in essential fatty acid deficiency: barrier requirements rather than eicosanoid generation regulate cholesterol synthesis. J Invest Dermatol **99:**216–220, 1992.
27. Arnal JF, Clamens S, Pechet C, Negre-Salvayre A, Allera C, Girolami J-P, Salvayre R, Bayard F. Ethinylestradiol does not enhance the expression of nitric oxide synthase in bovine endothelial cells but increases the release of bioactive nitric oxide by inhibiting superoxide anion production. Proc Natl Acad Sci U S A **93:**4108–4113, 1996.
28. Hernandez I, Delgado JL, Diaz J, Quesada T, Teruel MJ, Llanos MC, Carbonell LF. 17β-estradiol prevents oxidative stress and decreases blood pressure in ovariectomized rats. Am J Physiol Regul Integr Comp Physiol **279:**R1599–R1605, 2000.
29. Thompson LP, Pinkas G, Weiner CP. Chronic 17β-estradiol replacement increases nitric oxide-mediated vasodilation of guinea pig coronary microcirculation. Circulation **102:**445–451, 2000.
30. Guetta V, Quyyumi AA, Prasad A, Panza JA, Waclawiw M, Cannon R III. The role of nitric oxide in coronary vascular effects of estrogen in postmenopausal women. Circulation **96:**2795–2801, 1997.
31. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa C, Walsh K. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med **6:**1004–1010, 2000.
32. Kasten TP, Collin-Osdoby P, Patel N, Osdoby P, Krukowski M, Misko TP, Settle SL, Currie MG, Nickols GA. Potentiation of osteoclast bone resorption activity by inhibition of nitric oxide synthase. Proc Natl Acad Sci U S A **91:**3569–3573, 1994.
33. Simoncini T, Genazzani AR. Raloxifene acutely stimulates nitric oxide release from human endothelial cells via an activation of endothelial nitric oxide synthase. J Clin Endocrinol Metab **85:**2966–2969, 2000.
34. Wimalawansa SJ. Restoration of ovariectomy-induced osteopenia by nitroglycerine. Calcif Tissue Int **66:**56–60, 2000.
35. Wimalawansa SJ, De Marco G, Gangula P, Yallampalli C. Nitric oxide donor alleviates ovariectomy-induced bone loss. Bone **18:**301–304, 1996.
36. Wimalawansa SJ, Chapa MT, Yallampalli C, Zhang R, Simmons DJ. Prevention of corticosteroid-indcued bone loss with nitric oxide donor nitroglycerin in male rats. Bone **21:**275–280, 1997.
37. Jamal SA, Browner WS, Bauer DC, Cummings SR. Intermittent use of nitrates increases bone mineral density: the study of osteoporotic fractures. J Bone Miner Res **13:**1755–1759, 1998.
38. Wimalawansa SJ. Nitroglycerin therapy is as efficacious as standard estrogen replacement therapy (premarin) in prevention of oophorectomy-induced bone loss: a human pilot clinical study. J Bone Miner Res **15:**2240–2244, 2000.
39. Armour KE, Van'T Hof RJ, Grabowski PS, Reid DM, Ralston SH. Evidence for a pathogenic role of nitric oxide in inflammation-induced osteoporosis. J Bone Miner Res **14:**2137–2142, 1999.
40. Evans DM, Ralston SH. Nitric oxide and bone. J Bone Miner Res **11:**300–305, 1996.
41. Radomski MW, Palmer RMJ, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc Natl Acad Sci U S A **87:**10043–10047, 1990.
42. Kanematsu M, Ikeda K, Yamada Y. Interaction between nitric oxide synthase and cyclooxygenase pathways in osteoblastic MC3T3-E1 cells. J Bone Miner Res **12:**1789–1796, 1997.
43. Mohan IK, Das UN. Effect of L-arginine-nitric oxide system on the metabolism of essential fatty acids in chemical-induced diabetes mellitus. Prostaglandins Leukotreine Essen Fatty Acids **62:**35–46, 2000.
44. Suresh Y, Das UN. Protective action of arachidonic acid against alloxan-induced cytotoxicity and diabetes mellitus. Prostaglandins Leukotreine Essen Fatty Acids **64:**37–52, 2001.
45. MacIntyre I, Zaidi M, Towhidul Alam ASM, Datta HK, Moonga BS, Lidbury PS, Hecker M, Vane JR. Osteoclastic inhibition: an action of nitric oxide not mediated by cyclic GMP. Proc Natl Acad Sci U S A **88:**2936–2940, 1991.