The Role of Polyunsaturated ω -3 Fatty Acid Eicosapentaenoic Acid–Derived Resolvin E1 (RvE1) in Bone Preservation

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ABSTRACT: Resolvin E1 (RvE1) is a recently discovered lipid-derived mediator that is endogenously synthesized from the polyunsaturated fatty acid eicosapentaenoic acid. RvE1 is locally generated in response to inflammation where it enhances the resolution phase of inflammation by diminishing neutrophil chemotaxis and by enhancing nonphlogistic macrophage-directed clearance of apoptotic neutrophils. RvE1 was also found to be effective in preventing and restoring bone loss in the inflammatory bone disease periodontitis. This review examines experimental evidence on RvE1's actions in bone. Current data indicate that in addition to anti-inflammatory actions, RvE1 also directly acts on bone cells and promotes bone preservation.

KEY WORDS: lipid mediator, bone resorption, inflammation, osteoclast, periodontal disease

ABBREVIATIONS: AA, arachidonic acid; ASA, acetylsalicylic acid; BLT, leukotriene B₄ receptor; ChemR23, chemerin receptor 23; COX, cyclooxygenase; DC-STAMP, dendritic cell-specific transmembrane protein; EPA, eicosapentaenoic acid; IL, interleukin; LO, lipoxygenase; LT, leukotriene; LX, lipoxin; NFATc1, nuclear factor of activated T cells, cytoplasmic 1; OPG, osteoprotegerin; PGE₂, prostaglandin E₂; PUFA, polyunsaturated fatty acid; RANKL, receptor activator of nuclear factor κ B ligand; RvD1, Resolvin D1; RvE1, Resolvin E1; TNF α , tumor necrosis factor α .

I. INTRODUCTION

Multitasking is the rule rather than the exception in biological systems, and bone is no exception. In addition to providing a motile skeleton and safe enclosure for vital organs, bone serves as a calcium source for many cellular processes and also houses hematopoietic cells in its marrow. Bone metabolism encompasses a wide variety of processes, including bone growth and development, bone remodeling to renew and accommodate to changing loads, and maintenance of circulating calcium, as well as pathological processes such as osteoporosis, inflammatory bone diseases, and bone tumors. Molecular regulation of bone metabolism is multilayered as well and includes central hormonal actors regulated by serum calcium levels such as parathyroid hormone and calcitonin, as well

as local regulators such as calcitriol, prostaglandin E₂ (PGE₂), cytokines, 1,25-dihydroxyvitamin D₃, corticosteroids, estrogens, transforming growth factors, and bone morphogenic proteins, among others. As seen in other biological systems, the same molecule may perform multiple functions.

II. BONE REMODELING

With growth and various physical activities, the forces acting upon bones will change. Bone can adapt remarkably to these changing demands, as demonstrated in space travel in which as much as 21% of bone mineral density is lost in weight-bearing bones during long-duration space flights.¹ Similarly, the absence of biting forces after tooth extraction leads to marked resorption of the alveolar bone.²

There is a continuously ongoing process of bone remodeling in which osteoclasts resorb bone and thus form resorption lacunae, which in turn are filled by osteoblasts with new bone matrix that is subsequently mineralized.³ A portion of osteoblasts will be entrapped in lacunae, the newly formed bone where they become osteocytes. Osteocytes extend distinct dendritic processes through a canalicular system in the bone, enabling them to connect with each other. This cellular network is proposed to play a cardinal role in sensing physiological stresses (e.g., body weight and exercise) on bone.⁴ Extracellular and intracellular fluid is forced along the canalicular system as the bone slightly deforms under pressure, and this fluid flow is detected by primary cilia extending from osteocytes.⁵ *In vitro* models identified the lipid mediator PGE₂ and nitric oxide as molecular mediators of osteocyte mechanotransduction.⁶ PGE₂ acts synergistically with receptor activator of nuclear factor κ B ligand (RANKL) to stimulate osteoclast differentiation and to initiate bone remodeling.⁷ Bone stress also leads to osteocyte apoptosis, which in turn induces RANKL expression from neighboring osteoblasts.

Thus, the bone remodeling cycle begins: Osteoclasts resorb bone tissue, followed by osteoblast activation that deposits bone matrix in the resorption lacuna. Although many cellular and molecular actors of the bone remodeling cycle are well characterized, the molecular coupling mechanism linking bone formation to recent osteoclast activity is not clear. However, in health, bone resorption is in balance with bone formation and the structure of newly formed bone corresponds to current physical loads.

III. PATHOLOGICAL BONE LOSS

The balance of bone resorption and bone formation can be upset by systemic conditions such as osteoporosis, hyperparathyroidism, and weightlessness as well as local inflammatory conditions such as rheumatoid arthritis and periodontitis. The most common form of osteoporosis is estrogen deficiency-induced osteoporosis. Estrogen stimulates osteoprotegerin (OPG), which in turn inhibits osteoclast differentiation by acting as a decoy receptor of RANKL, thus leading

to decreased osteoclast differentiation, osteoclast survival, and preservation of bone.⁸ Inversely, menopause or ovariectomy will decrease OPG and promote osteoclast differentiation, survival, and consequently bone resorption. Estrogen is also anti-inflammatory; thus, postmenopausal estrogen deficiency promotes osteoclast activation and bone resorption by providing a proinflammatory milieu as well.⁸ Interleukin (IL)-7 is a key cytokine in postmenopausal osteoporosis because it stimulates RANKL expression by T cells and inhibits osteoblasts.⁹ T cells also produce tumor necrosis factor α (TNF α), which in turn increases osteoclast sensitivity to RANKL.¹⁰

Rheumatoid arthritis is another example of inflammatory bone resorption in which plasma cells, macrophages, and T cells accumulate in the synovial membrane of affected joints, prominently in the proximal interphalangeal joints. T cells produce soluble and cell membrane-attached RANKL, inducing osteoclast differentiation and survival, leading to subchondral bone resorption in the joint surfaces.¹¹

Periodontal disease is a bacterially induced bone disease of the jaw, affecting approximately one-half of the adult US population.¹² Chronic inflammation of periodontal soft tissues leads to release of collagenases such as matrix metalloproteinase-8 by neutrophil polymorphonucleocytes, leading to gingival soft tissue destruction. Antigen receptor-activated T cells directly express RANKL, leading to osteoclast activation and alveolar bone resorption.¹³ T cells also express the proinflammatory cytokines TNF α and IL-1, which in turn induce RANKL expression in osteoblasts and bone marrow stromal cells, thus providing an additional indirect pathway to osteoclast activation and bone resorption.¹⁴ Reducing bacterial load with proper oral hygiene and periodontal therapy arrests inflammation and bone loss, with limited spontaneous regeneration.

IV. CELLULAR MECHANISMS OF BONE LOSS

Osteoclasts are considered the main, if not the only, cell type capable of bone resorption. Osteoclasts develop from colony-forming unit macrophages, the same myelomonocytic progenitor cells that give rise

to macrophages and dendritic cells.¹⁵ A subclass of peripheral blood monocytes can also differentiate to form bone-resorbing cells; thus, these cells are considered circulating osteoclast precursors, which respond to osteoclast-specific factors and differentiate into true osteoclasts.¹⁶ These osteoclast-specific factors include macrophage colony-stimulating factor and RANKL as well as OPG, the endogenous decoy receptor for RANKL. Macrophage colony-stimulating factor appears to be a permissive factor necessary in the early cell fate determination, whereas RANKL is the final executor of many physiological regulators of bone resorption (e.g., parathyroid hormone, calcitonin, and calcitriol) and is an obligatory factor for osteoclast differentiation and survival.¹⁵ The prominence of this paracrine pathway in regulating osteoclast differentiation has been demonstrated by several investigators in a series of knockout experiments.^{17–19} Complete absence of osteoclasts was found in mutant mice lacking RANKL or the RANKL receptor RANK, and severe osteoporosis and increased osteoclast formation were demonstrated in mice mutant in OPG.^{17–19}

Proinflammatory molecules play a significant but primarily indirect role in osteoclast regulation, as they act through modulating RANKL and OPG. TNF α , IL-1, IL-6, IL-11, and IL-17 act on osteoblasts and bone marrow stromal cells to increase RANKL and/or decrease OPG.^{14,15} Prostaglandins and transforming growth factor β also lack receptors on osteoclasts and act through RANKL/OPG mediation. Conversely, anti-inflammatory cytokines IL-4, IL-10, and interferon- γ inhibit bone resorption via decreasing RANKL or increasing OPG.¹⁴ Thus, the RANKL/OPG ratio appears to be the main direct determinant of osteoclast differentiation and survival, and is thus the main molecular factor in regulating bone resorption.

V. LIPID MEDIATORS OF BONE METABOLISM

A. Prostaglandins

PGE₂ is arguably the best characterized lipid mediator of bone metabolism. PGE₂ is synthesized from the

ω -6 polyunsaturated fatty acid (PUFA) arachidonic acid (AA) by cyclooxygenase (COX) and prostaglandin E synthase.²⁰ PGE₂ is a key molecule in acute inflammatory reaction, and is responsible for mediating cardinal inflammatory symptoms of fever, pain, and swelling.²¹ In bone, PGE₂ is primarily synthesized by osteoblasts and acts in an autocrine manner on osteoblast PGE₂ receptors.²² Early evidence for the role of prostaglandins in bone metabolism came from tumor studies in which fibrosarcoma-induced hypercalcemia was prevented by the prostaglandin-synthase inhibitor indomethacin.²³ In experimental conditions in which both osteoblasts and osteoclasts are present, PGE₂ induces bone resorption by inducing RANKL production in osteoblasts and thus inducing osteoclast differentiation.²⁴ However, PGE₂ can also induce bone formation in isolated osteoblast cultures.²⁵ Thus, in effect, PGE₂ accelerates bone turnover. Fluid shear stress in osteocytes induces PGE₂ synthesis, which contributes to the anabolic effect of mechanical loading.²⁶ Endogenous levels of COX-2 and PGE₂ are upregulated in experimental bone fracture,^{27,28} and fracture healing is delayed in COX-2-deficient mice²⁹ and after nonsteroidal anti-inflammatory drug treatment.²⁷ These findings are consistent with the notion that PGE₂ accelerates bone turnover. PGE₂ is strongly implicated in bone destruction in rheumatoid arthritis. PGE₂ is produced in the synovial fluid of patients with rheumatoid arthritis, and bone-resorbing activity of synovial culture supernatants can be inhibited by indomethacin.³⁰ Elevated PGE₂ production is associated with chronic osteomyelitis, failing joint prostheses, and periodontal disease.³¹ In periodontal disease, PGE₂ and leukotriene (LT) B₄ levels in gingival crevicular fluid are increased³² and alveolar bone loss is decreased in PGE₂ receptor-deficient mice.³³ Moreover, nonsteroidal anti-inflammatory drugs have been successfully used to suppress alveolar bone loss in human periodontal disease.³⁴

B. Leukotrienes

LTs are proinflammatory mediators generated by 5-lipoxygenase (LO) from AA. The initial discovery of 5-LO derivatives of AA that stimulate osteoclast

development and bone resorption led to the identification of LTs as potent osteoclast-regulating mediators.³⁵ Osteoclasts were shown to possess high-affinity binding sites for LTB₄ that stimulate osteoclast fusion, tartrate-resistant acid phosphatase expression, and bone resorption.^{36,37} Moreover, LTB₄ and LTD₄ inhibit bone formation, thus contributing to inflammation-induced bone loss.³⁸ Genetic disruption of the LTB₄ receptor (BLT) subtypes 1 and 2 in mice confers protection against collagen-induced arthritis, indicating that LTB₄ is critical in the development of inflammatory joint disease.³⁹

C. Eicosapentaenoic Acid

Health benefits of increased dietary intake of ω -3 PUFA were first suggested in studies of cardiovascular morbidity in Greenland Eskimos, in which a diet rich in eicosapentaenoic acid (EPA) was correlated with a low incidence of myocardial infarction.⁴⁰ Prospective cohort studies demonstrated that EPA suppresses arrhythmias and decreases cardiovascular death.^{41,42} EPA lowers plasma triglycerides, suppresses platelet aggregation, and inhibits inflammation.⁴³ However, a broad review of randomized controlled trials and cohort studies failed to confirm significant benefits of ω -3 PUFA on overall mortality or cardiovascular events in humans.⁴⁴ High fish consumption and EPA intake is associated with bone protection, as measured by hip bone mineral density in the Framingham Osteoporosis Study.⁴⁵ Protection against bone loss by fish oil food supplementation and inhibition of RANKL expression was found in ovariectomized mice.⁴⁶ On the other hand, an EPA-supplemented diet in rats exacerbated ovariectomy-induced bone loss.⁴⁷ Available *in vitro* data are similarly controversial, as EPA was found to inhibit osteoclast differentiation at micromolar doses (20–80 μ M)^{46,48} but stimulated osteoclast differentiation at lower micromolar and nanomolar doses (10–100 nM).^{49,50} Orchard et al. found contrasting results in a recent systematic review of randomized controlled trials on postmenopausal women, with four studies reporting favorable outcomes and five studies reporting no difference in bone density between ω -3 PUFA-supplemented groups and control groups.⁵¹ A possible

explanation for these controversial findings may be that various EPA metabolites are generated under different conditions and these metabolites, and not EPA itself, may thus be responsible for the observed biological actions.

VI. LIPID MEDIATORS OF INFLAMMATION RESOLUTION

Resolution of inflammation is considered a passive process that follows the elimination of microbial invaders and tissue injury. However, the identification of novel mediators that are spatially and temporally generated in inflammatory exudates in the resolution phase and actively resolve inflammation has established that resolution is an active process.^{52–54}

A. Lipoxins

Lipoxins (LXs) were the first family of lipid mediators identified as proresolution mediators.⁵⁴ LXA₄ and LXB₄ are generated from AA via transcellular routes utilizing 5-LO and 15-LO.⁵⁵ Acetylsalicylic acid (ASA)-triggered LXs arise from ASA-acetylated COX-2 and leukocyte 5-LO, giving rise to 15-epi-LXA₄ and 15-epi-LXB₄.⁵⁴ The anti-inflammatory and proresolving actions of LX were extensively studied and reviewed elsewhere.^{55,56} The first observation suggesting that LX may have bone-protective actions in addition to inflammation resolution was made in a rabbit model of periodontal disease.⁵⁷ Periodontal disease in healthy rabbits was induced by tying a silk ligature in the sulcus of the second mandibular premolars and by topically applying the human periodontal pathogen *Porphyromonas gingivalis* (strain A7436, 10⁹ CFU) directly to the ligated tooth. This treatment results in periodontal inflammation and alveolar bone loss in 6 weeks. Topical treatment with 6 mg of the LX analog ASA-triggered LXA₄ three times a week prevented the development of inflammation and diminished alveolar bone loss.⁵⁷ To dissect bone-specific actions of LX, isolated newborn calvaria were treated with polymethylmethacrylate to induce inflammation and were treated with LXA₄ (1–100 nM). *Ex vivo*

measurement of calcium release indicated that LXA₄ significantly diminished inflammatory bone loss.⁵⁸ Although this observation confirms bone-specific action on the organ level, it does not indicate whether bone cells are directly affected by LXs or whether traditional targets (e.g., leukocytes present in the marrow) mediate its actions.

B. Resolvins

Resolvin E1 (RvE1) is a member of the E series resolvins and is biosynthesized from EPA.⁵⁹ RvE1 was first identified *in vivo* in the resolution phase of inflammation from peritoneal exudates.⁵⁴ RvE1 is produced by hypoxia-activated human endothelial cells that convert EPA to 18*R*-hydroxy-EPA via ASA-acetylated COX-2 as well as via a second route involving p450-like reactions.⁵⁴ Once formed, 18*R*-hydroxy-EPA is rapidly transformed by activated leukocytes to a 5(6)-epoxide-18*R*-hydroxy containing an intermediate in which 5-LO carries out consecutive steps. Physiologically relevant concentrations of resolvins are detectable in the blood following ω -3 supplementation.⁶⁰ RvE1 complete stereochemistry is established (5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid). RvE1 is a stereoselective agonist that interacts with at least two identified G protein-coupled receptors: chemerin receptor-23 (chemR23)⁶¹ and BLT1.^{61,62} BLT1 is expressed on neutrophils, whereas chemR23 is expressed on monocytes, macrophages, dendritic cells, and, to a lesser extent, in neutrophils and CD4⁺ T lymphocytes.^{61,63} Among bone cells, bone marrow stromal cells and osteoblasts express chemR23,^{64,65} whereas osteoclasts express the BLT1 receptor.⁴⁹ RvE1 is endogenously synthesized in murine bone marrow, as determined with liquid chromatography and tandem mass spectroscopy.⁴⁷ EPA supplementation increases bone marrow RvE1, indicating that endogenous RvE1 synthesis is substrate limited.⁴⁷ Transcellular biosynthesis of RvE1 in bone was proposed because primary osteoclast cultures generate the RvE1 precursor 18*R*-hydroxy-EPA in the presence of EPA and ASA, whereas coincubation of osteoclasts with zymosan-activated neutrophils under the same conditions result in RvE1 synthesis.⁴⁹ In

this scenario, osteoclasts provide the ASA-acetylated COX-2, whereas neutrophils are the source of 5-LO in the RvE1 synthetic pathway.⁴⁹

VII. ACTIONS OF RESOLVINS IN BONE

The first *in vivo* evidence for RvE1's bone-protective action was demonstrated in periodontal bone loss.⁵⁷ Periodontal disease was induced in New Zealand white rabbits by applying a silk ligature and the periodontal pathogen *P. gingivalis* to the second mandibular premolar, as described above. RvE1 (4 mg) was administered topically three times per week, and 6 weeks later alveolar bone loss was evaluated. RvE1 treatment significantly inhibited alveolar bone loss, as evidenced by morphometric analysis and radiography. Histologic analysis revealed a diminished number of tartrate-resistant acid phosphatase-positive osteoclasts around the alveolar bone treated with RvE1. Soft tissue inflammation was also decreased in RvE1-treated gingiva; thus, the question of whether RvE1 directly affects osteoclasts, or modulates bone metabolism via inflammatory mediators, remained open.

A direct comparison of RvE1's actions with those of structurally related lipids PGE₂ and LTB₄ was performed in the same rabbit periodontitis model.⁶⁶ In this series of experiments, lipid mediator treatment was initiated in an established periodontal lesion, following the removal of the silk ligature and cessation of *P. gingivalis* administration. Whereas PGE₂ and LTB₄ further worsened alveolar bone loss, RvE1 treatment resulted in bone regeneration, along with regeneration of the periodontal ligament and cementum. RvE1 treatment significantly decreased tartrate-resistant acid phosphatase-positive osteoclast counts attached to alveolar bone, and histologic evidence of marked osteoblast activity was found.

These *in vivo* data raised the question of whether RvE1 acts directly on osteoclasts and other bone cells, or indirectly, through inhibiting proinflammatory and proresorptive mediators such as PGE₂ or IL-1. *In vitro* studies using murine bone marrow-derived primary osteoclasts showed that RvE1 indeed directly acts on osteoclasts. Nanomolar doses (3–30 nM) of RvE1 administered to primary osteoclast

FIG. 1: Dual mechanism of action of RvE1 on osteoclast differentiation. RvE1 directly inhibits osteoclast maturation by binding to its cell surface receptor BLT1 and attenuating NFATc1 binding to the promoter region of the DC-STAMP gene. RvE1 also enhances OPG production by osteoblasts. OPG acts as decoy receptor for the principal osteoclast differentiating molecule RANKL, thus RvE1 interferes with not only osteoclast fusion but also with osteoclast precursor development and osteoclast survival. OB, osteoblast; OC, osteoclast.

cultures markedly decreased the number and size of differentiated osteoclasts induced by macrophage colony-stimulating factor and RANKL.⁴⁹ *In vitro* resorption pit formation was correspondingly decreased, and a specific RvE1 cell surface receptor (BLT1) was identified as the mediator of RvE1's actions on osteoclasts. Similarly to RvE1, resolvin D1 (RvD1), an LO-derivative of the 22-carbon long ω -3 PUFA docosahexaenoic acid, is also a strong inhibitor of osteoclastogenesis.⁵⁰ These observations show that RvE1 and RvD1 directly inhibit osteoclast differentiation and that the bone-protective actions

of RvE1 are distinct from those on inflammation resolution. The cellular mechanism of RvE1-mediated inhibition of osteoclast differentiation was determined with time-course studies and with intravital microscopy showing delayed fusion of osteoclast precursors.⁶⁷ Expression of the essential osteoclast fusion protein dendritic cell-specific transmembrane protein (DC-STAMP) is markedly downregulated by RvE1, due to defective binding of the osteoclast-specific transcription factor nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) to the DC-STAMP promoter (Fig. 1).⁶⁷

TABLE 1: Actions of RvE1 in bone

Function	Experimental model	Resolvin dose	Route of administration	Reference
Decreased alveolar bone loss	Silk ligature and <i>P. gingivalis</i> -induced periodontal disease in rabbits	4 mg/tooth 3 times/wk for 6 wk	Topical, intraoral	Hasturk et al. ⁵⁷
Periodontal bone regeneration	Silk ligature and <i>P. gingivalis</i> -induced periodontal disease in rabbits	4 mg/tooth 3 times/wk for 6 wk	Topical, intraoral	Hasturk et al. ⁶⁶
Inhibition of osteoclast differentiation and bone resorption	<i>In vitro</i> murine osteoclast growth on dentin disc	1–10 ng/mL	In culture media	Herrera et al. ⁴⁹
Inhibition of osteoclastogenesis	<i>In vitro</i> murine osteoclast growth	10 μ M	In culture media	Yuan et al. ⁵⁰
Acceleration of craniotomy defect healing	Uniform craniotomy in mouse	100 ng/d for 2 wk	Supracalvarial injection	Gao et al. ⁶⁴
Rescue of IL-6 suppression of OPG	Mouse neonatal calvarial culture	1–100 nM	In culture media	Gao et al. ⁶⁴
Inhibition of osteoclast fusion and DC-STAMP expression	<i>In vitro</i> murine osteoclast culture	10 nM	In culture media	Zhu et al. ⁶⁷

Because *in vivo* studies suggested enhancement of bone regeneration by RvE1 in periodontal disease, bone-forming actions of RvE1 were also explored *in vivo* and *in vitro*. The healing rate of a small circular craniotomy defect was measured in mice in which RvE1 (100 ng) was injected subcutaneously over the craniotomy site every other day for 2 weeks. The bone healing rate was significantly accelerated under RvE1 treatment.⁶⁴ This observation prompted experiments examining direct catabolic effects of RvE1 on osteoblasts. However, RvE1 treatment of cultured osteoblasts failed to alter *in vitro* bone nodule formation or the levels of the osteoblast differentiation markers alkaline phosphatase, bone

sialoprotein, and runt-related transcription factor 2, suggesting that RvE1 does not directly regulate bone growth.⁶⁴ By contrast, measurements of RANKL and OPG in osteoblast cultures revealed that RvE1 rescues OPG expression suppressed by proinflammatory mediators, thus decreasing the RANKL/OPG ratio and favoring bone anabolism.⁶⁴ These findings indicate that although RvE1 acts directly on osteoblasts, it does not directly modulate bone formation but it regulates the RANKL/OPG ratio, thus contributing to osteoclast regulation in addition to its direct actions on the osteoclast cell surface BLT1 receptor. RvE1's actions on bone are summarized in Table 1.

VIII. CONCLUSIONS AND FUTURE DIRECTIONS

RvE1, a molecular derivative of EPA, has specific bone-preserving actions in addition to its well-established inflammation-resolving function. RvE1 acts on the BLT1 receptor on osteoclasts and inhibits osteoclast maturation by interfering with the expression of the osteoclast fusion protein DC-STAMP. In addition, RvE1 stimulates OPG release, thus antagonizing proresorptive actions of the osteoclast-stimulating RANKL. Although *in vivo* RvE1 is anabolic in bone, direct actions on bone formation have not been established. RvE1 prevents alveolar bone loss in periodontal disease and promotes periodontal bone regeneration. Potential actions of RvE1 on other inflammatory bone disease such as rheumatoid arthritis and osteoporosis are yet to be elucidated. However, RvE1 and other resolvins are promising molecular templates for the development of pharmaceutical agents with both anti-inflammatory and bone-preserving actions.

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