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Review

# The roles of prostanoids, leukotrienes, and platelet-activating factor in bone metabolism and disease

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**Abstract**

The production of a variety of lipid mediators is enhanced in bone-resorptive diseases such as osteoporosis, rheumatoid arthritis, osteoarthritis, and periodontitis. Prostaglandin $E_2$ ($PGE_2$) is one of the most notable lipid mediators of bone remodeling, and has been linked clinically to many bone-resorptive diseases. *In vitro* studies with bone cell cultures have demonstrated that the bone-resorptive activity of $PGE_2$, which is mediated by receptor activator of NF-κB ligand (RANKL), is key for the induction of osteoclast formation. Furthermore, interleukin (IL)-1- and IL-6-stimulated bone resorption involves $PGE_2$ production. In addition to its bone-resorptive effects, $PGE_2$ promotes bone formation *in vitro* by stimulating osteoblastic proliferation and differentiation. The multifaceted nature of $PGE_2$ makes it difficult to discern its role during bone remodeling. Leukotrienes (LTs), and particularly $LTB_4$, have also been implicated in bone remodeling and disease—specifically in rheumatoid arthritis. Moreover, recent studies from our laboratory have shown that platelet-activating factor (PAF) receptor-deficient mice develop only mild osteoporosis. Osteoclast survival in these mice is shortened and osteoclastic bone resorption is impaired. This review article focuses on these families of lipids and their function during bone metabolism and disease.


*Keywords:* $PGE_2$; Osteoblast; Osteoclast; Osteoporosis; PAF

*Abbreviations:* ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif; ALP, alkaline phosphatase; BLT, LTB4 receptor; BMP, bone morphogenetic protein; CAIA, collagen antibody-induced arthritis; cAMP, cyclic AMP; CIA, collagen-induced arthritis; COX, cyclooxygenase; cPGES, cytosolic PGES; $cPLA_2$, cytosolic $PLA_2$; CRTH2, chemoattractant receptor-homologous molecule expressed on TH2 cells; CysLT, cysteinyl LT receptor; DHA, docosahexaenoic acid; DP, $PGD_2$ receptor; EP, $PGE_2$ receptor; EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FLAP, 5-LO-activating protein; GPCR, G protein-coupled receptor; Gs, stimulatory G protein; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-HpETE, 5-hydroperoxyeicosatetraenoic acid; IL, interleukin; IL-6R, IL-6 receptor; IP, $PGI_2$ receptor; 5-LO, 5-lipoxygenase; LPA, lysophosphatidic acid; LPS, lipopolysaccharide; LT, leukotriene; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; MMPs, matrix metalloproteinases; mPGES, membrane-associated PGES; NSAID, nonsteroidal anti-inflammatory drug; OPG, osteoprotegerin; OXE, oxoeicosanoid receptor; 5-oxo-ETE, 5-oxo-eicosatetraenoic acid; PAF, platelet-activating factor; PAFR, PAF receptor; PAFR-KO, PAFR-knockout; PG, prostaglandin; PGES, PGE synthase; $PGI_2$, prostaglandin $I_2$; PKA, protein kinase A; $PLA_2$, phospholipase $A_2$; PPAR, peroxisome proliferator-activated receptor; PTH, parathyroid hormone; RANKL, receptor activator of nuclear factor-jB ligand; sIL-6R, soluble IL-6R; $sPLA_2$, secretory $PLA_2$; TNF, tumor necrosis factor; TX, thromboxane; VEGF, vascular endothelial growth factor.

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**Contents**



## 1. Introduction

Many lipids serve as signaling molecules, including prostanoids (prostaglandins (PGs) and thromboxanes (TXs)), leukotrienes (LTs), platelet-activating factor (PAF), sphingosine 1-phosphate, lysophosphatidic acid (LPA), and endocannabinoids. Several of these are produced by the hydrolytic action of phospholipase $A_2$ ($PLA_2$) enzymes on membrane glycerophospholipids (Fig. 1). To date, four distinct groups of $PLA_2$ enzymes have been identified [1,2]: a low molecular weight (14–17 kDa) secretory $PLA_2$ ($sPLA_2$) group, a high molecular weight cytosolic $PLA_2$ ($cPLA_2$) group, which includes the 85-kDa calcium-sensitive $cPLA_2\alpha$, a calcium-independent $PLA_2$ ($iPLA_2$) group, and a PAF acetylhydrolase group. Most of these $PLA_2$ isozymes release polyunsaturated fatty acids from the *sn*-2 position of glycerophospholipids. Among fatty acids, arachidonic acid is the most important molecule, because it is metabolized to prostanoids and LTs (Fig. 1). Among $PLA_2$ enzymes, $cPLA_2\alpha$ plays a dominant role in arachidonic acid release owing to two distinct characteristics: the tightly regulated activation by submicromolar calcium and phosphorylation in response to extracellular stimuli and the substrate preference for arachidonic acid-containing phospholipids over the others [3]. Cyclooxygenases (COX-1 [4–6] and COX-2 [7]) catalyze the cyclooxygenation of arachidonic acid to $PGG_2$ followed by the hydroperoxidation of $PGG_2$ to $PGH_2$. COX-1 is constitutively expressed and believed to maintain homeostatic conditions, while COX-2 is encoded by a stress-responding gene and responsible for the production of high levels of prostanoids during inflammation [8]. $PGH_2$ can then be converted into $PGE_2$ through the action of PGE synthase (PGES). Several PGES isozymes have been identified including membrane-associated PGES-1 (mPGES-1), whose expression is induced by proinflammatory stimuli [9,10], mPGES-2, and cytosolic PGES (cPGES) that are expressed constitutively [11,12]. The conversion of $PGH_2$ to the other bioactive products, including $PGD_2$, $PGF_2\alpha$, $PGI_2$ (prostacyclin), and $TxA_2$, via specific synthases is also biologically important [13].

In contrast to COX enzymes, 5-lipoxygenase (5-LO) is required for LT biosynthesis [14,15]. In conjunction with 5-LO-activating protein (FLAP) [16], 5-LO converts arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HpETE), which is then dehydrated to $LTA_4$ by the same enzyme [17,18]. 5-HpETE can also converted by peroxidase(s) to 5-hydroxyeicosatetraenoic acid (5-HETE), a precursor of 5-oxo-eicosatetraenoic acid (5-oxo-ETE) [19]. $LTA_4$ is hydrolyzed into bioactive $LTB_4$ by $LTA_4$ hydrolase [20,21] or is converted into $LTC_4$ by $LTC_4$ synthase [22]. $LTC_4$ is sequentially metabolized to $LTD_4$ and then to $LTE_4$ [23]. $LTC_4$, $LTD_4$, and $LTE_4$ are bioactive, and comprise cysteinyl leukotrienes, because they contain a cysteine residue.

PAF is synthesized by either the *de novo* and remodeling pathway (Fig. 2) [24,25]. The remodeling pathway is regu-

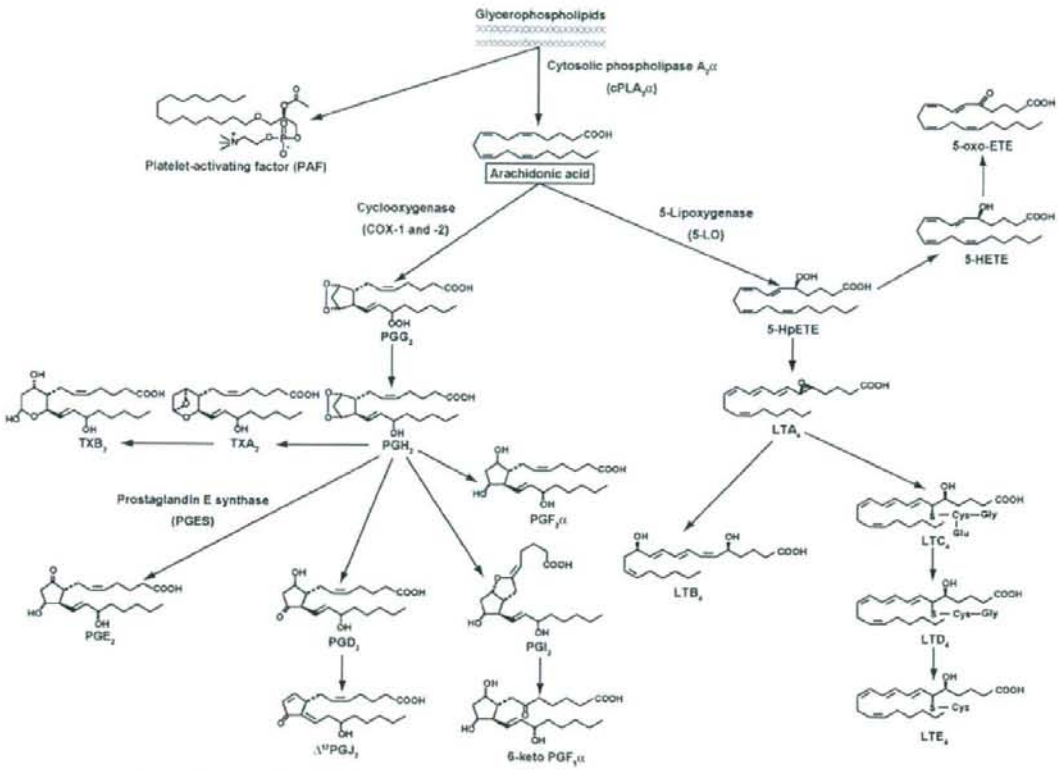


Fig. 1. Metabolic pathways for arachidonic acid. $PLA_2$ enzymes, particularly $cPLA_2\alpha$, release arachidonic acid from the *sn*-2 position of glycerophospholipids in biomembranes. Arachidonic acid is metabolized to prostanoids and LTs by COX and 5-LO, respectively. PAF is derived from the glycerophospholipids remaining after the release of arachidonic acid by $cPLA_2\alpha$. For the details of PAF synthesis reactions, see "the remodeling pathway" in Fig. 2.

lated by extracellular stimuli and is responsible for the bulk of the PAF synthesis under inflammatory conditions. Stimulus-coupled PAF biosynthesis is initiated by the activation of $PLA_2$ enzymes that hydrolyze 1-*O*-alkyl-phosphatidylcholine in biomembranes to 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF) [24,25]. Like prostanoids and LTs, the action of $cPLA_2\alpha$ is important for this hydrolysis reaction [26,27]. Although lyso-PAF is biologically inactive, it becomes acetylated to form PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) by acetyl-CoA:lyso-PAF acetyltransferase, which we have recently cloned [28]. The lipid mediators described above exert their bioactivities on a variety of cells through their specific receptors, which are mostly G protein-coupled receptors (GPCRs) [13,29,30].

Bone is a complex living tissue that has both protective and supportive functions while actively participating in calcium homeostasis. Bone tissues continually alter their internal structure by removing old bone and replacing it with newly formed bone, *i.e.*, bone remodeling, in which osteoblasts and osteoclasts are key players [31]. Osteoblasts arise from local osteoprogenitor cells and are responsible for the bone-matrix production. In addition, osteoblasts are also required for osteoclast formation. Osteoclasts, originating from hematopoietic tissues, are multinucleated cells that resorb bone. These two cell types participate in bone remodeling under the control of many systemic hormones and local regulators including parathyroid hormone (PTH) [32], 1α,25-dihydroxyvitamin $D_3$ (1α,25-$(OH)_2$ vitamin $D_3$) [33], glucocorticoids [34] and estrogen [35]. Fibroblast growth factor (FGF) [36], bone morphogenetic proteins [36], insulin-like growth factor [36], platelet-derived growth factor [36], and several cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 [37] are also known to regulate bone metabolism.

Many studies have examined the physiological and pathological effects of prostanoids on bone [38]. The bone-resorptive action of $PGE_2$ was first reported *in vitro* more than 30 years ago in a rat organ culture system that included both osteoblasts and osteoclasts [39]. Since then, the effects of $PGE_2$ on osteoblasts and osteoclasts have been revealed by both *in vitro* and *in vivo* studies. Currently, $PGE_2$ is recognized as one of the most important local regulators of bone metabolism. Therefore, we will focus this review article on the role of $PGE_2$ in this process. However, other prostanoids and LTs may also play a significant role in bone metabolism and their function will

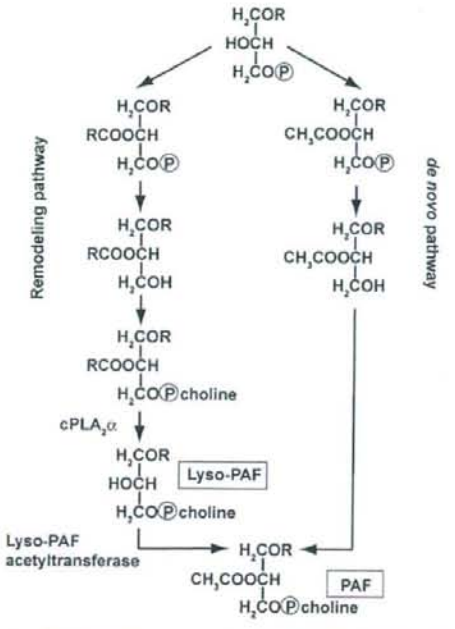


Fig. 2. Synthetic pathways of PAF. PAF is synthesized via two distinct pathways, the *de novo* and remodeling pathways. Lyso-PAF acetyltransferase catalyzes the final reaction for PAF synthesis in the remodeling pathway.

be explored here. Finally, we will highlight the lipid mediator PAF, which we have recently identified as playing a significant role during bone metabolism [40].

## 2. Role of prostanoids in bone metabolism and disease

### 2.1. $PGE_2$

#### 2.1.1. *In vitro actions of $PGE_2$ on bone metabolism*

*2.1.1.1. $PGE_2$ in osteoblasts: production and effects.* Within bone, $PGE_2$ is primarily produced by osteoblasts. More importantly, $PGE_2$ acts in an autocrine fashion on osteoblasts to form osteoclasts *in vitro*, leading to bone resorption [38]. Some of these *in vitro* experiments utilize the organ cultures of bones such as fetal/neonatal calvariae and limb bones [41–43]. The other experiments use the cocultures of primary osteoblasts and osteoclast precursor cells, which are derived from calvaria and either bone marrow or spleen, respectively [44,45].

A large number of cytokines, growth factors, and hormones are known to enhance $PGE_2$ production by affecting $cPLA_2\alpha$-, COX-2- and/or mPGES-1 in osteoblasts (Table 1). Many of these molecules such as IL-1 and IL-6 potentiate bone resorption [37], whereas other cytokines such as IL-4 [46,47] and IL-13 [47] have been reported to inhibit bone resorption by suppressing COX-2-dependent $PGE_2$ production in mouse osteoblasts. Throughout these studies, the role

Table 1
Molecules that enhance $cPLA_2\alpha$, COX-2, and/or mPGES-1-dependent $PGE_2$ production in osteoblasts

| Effector | Class | Affected enzyme(s) | Reference |
|---|---|---|---|
| IL-1α | Cytokine | COX-2 | [288,289] |
| | | COX-2 and mPGES-1 | [180] |
| IL-6 | Cytokine | COX-2 | [90] |
| TNF-α | Cytokine | $cPLA_2\alpha$ and COX-2 | [46] |
| PDGF | Growth factor | $cPLA_2\alpha$ | [289] |
| Basic FGF | Growth factor | COX-2 | [290] |
| BMP-2 | Growth factor | COX-2 | [291] |
| PTH | Hormone | COX-2 | [288] |
| 1α,25-$(OH)_2$ Vitamin D3 | Hormone | COX-2 | [45] |
| LPS | Pathogen | COX-2 and mPGES-1 | [180] |
| $PGE_2$ | Lipid | COX-2 | [288] |
| | | $cPLA_2\alpha$ and COX-2 | [100] |

of $cPLA_2\alpha$/COX-2/mPGES-1-mediated $PGE_2$ production by osteoblasts in bone resorption is clear.

In addition to bone resorption activity through the osteoblast-mediated osteoclastic differentiation, $PGE_2$ displays bone-forming activity in osteoblast monocultures. For example, $PGE_2$ stimulates the formation of mineralized bone nodules (lumps of extracellular mineralization *in vitro* that mimic calcification *in vivo*) and the activity of alkaline phosphatase (ALP; a differentiation maker of osteoblasts) in primary rat calvarial osteoblasts [48,49] and mouse osteoblastic MC3T3-E1 cells [50]. Increasing the extracellular calcium concentration in primary mouse osteoblasts can induce COX-2 expression and $PGE_2$ production through the protein kinase A (PKA) and extracellular signal-regulated kinase (ERK) signaling pathways [51,52]. Consequently, the produced $PGE_2$ stimulates osteoblastic differentiation [52]. Bone cells sense interstitial fluid shear stress upon mechanical loading of bone through fluid flow [53], which is an important mechanism for the anabolic effect of mechanical loading. Runx2/Cbfa1, a transcriptional factor required for osteoblastic differentiation (see Section 2.1.1.4 for detail), induces COX-2 expression in response to fluid sheer stress in the cells [54]. This transcriptional factor is phosphorylated and activated by the ERK pathway [55,56]. Thus, it is consistent that the PKA and ERK signaling pathways in mouse osteoblastic MC3T3-E1 cells becomes activated by fluid shear stress, resulting in increases of COX-2 expression and $PGE_2$ release [57–59]. The produced $PGE_2$ accounts, at least in part, for the anabolic effect of mechanical loading [60,61].

*2.1.1.2. $PGE_2$ receptors in osteoblasts.* There have been four $PGE_2$ receptors identified (EP1–EP4) [13]. By evaluating the functional effects of EP subtype-specific ligands, the presence of EP1, EP2, and EP4 receptors have been shown in mouse osteoblastic MC3T3-E1 cells [62]. EP1 and EP4 transcripts have been detected in MC3T3-E1 cells by

Northern blot [62], while reverse transcriptase–polymerase chain reaction analysis revealed that mouse osteoblasts isolated from calvariae expressed transcripts of all four receptors with the rank order being EP4 > EP1 > EP2 > EP3 [63]. In human osteoblasts, only EP3 and EP4 were observed immunohistochemically [64].

Signaling through EP receptors activates two major intracellular pathways, the cyclic AMP (cAMP)-dependent pathway and the intracellular calcium-dependent pathway. The EP2 and EP4 receptors are known to mediate the stimulation of adenylate cyclase [65]. The binding of $PGE_2$ to EP2 and EP4 receptors induces COX-2 mRNA transcription in primary mouse osteoblasts through cAMP-dependent activation of PKA [66]. Stimulation of EP4 (and somewhat EP2) by specific agonists increases the activity of mouse calvarial osteoblasts and induces the transcription of receptor activator of NF-κB ligand (RANKL; see Section 2.1.1.3 for detail) and the subsequent osteoclast formation [43]. Analysis of the four EP receptor-deficient mice revealed that $PGE_2$ stimulates bone resorption in the cultured calvariae through the EP4-cAMP signaling pathway [63]. EP4 expression on mouse osteoblasts is required for osteoclast formation stimulated by lipopolysaccharide (LPS) and proinflammatory cytokines such as IL-1α, TNF-α and basic FGF in the coculture of primary mouse osteoblasts and bone marrow cells [67]. However, EP2 has also been reported to be indispensable in $PGE_2$-induced cAMP formation in primary mouse osteoblasts [66,68], which partially contributed to an increase in RANKL mRNA expression in cultured calvariae [68] and primary osteoblasts [69]. $PGE_2$-stimulated osteoclast formation in cultures of calvarial osteoblastic cells and spleen cells was reduced by about 90%, when osteoblasts were derived from EP2-deficient mice [70].

In addition to its role in osteoclast formation, EP4 has also been implicated in osteoblast differentiation. $PGE_2$ has been shown to stimulate differentiation of mouse calvarial osteoblasts by activating EP4 [71]. In mouse osteoblastic MC3T3-E1 cells, EP4 and possibly EP2 mediate differentiation of the cells [50,62].

EP1 mediates the intracellular calcium influx [65]. $PGE_2$ signaling through the EP1 receptor is important for many aspects of bone metabolism. Through this pathway, $PGE_2$ induces its own production, a process called $PGE_2$ autoamplification (see Section 2.1.1.3 for detail) in MC3T3-E1 cells [72]. Mineralized bone nodules also develop in response to EP1 signaling in primary rat osteoblasts [73], suggesting a role of EP1 in osteoblastic differentiation. These cells also increase their production of fibronectin, which is important during the early stages of bone formation [74].

We are unaware of any reports of EP3-regulated osteoblast function.

*2.1.1.3. Production of bone-resorbing factors by $PGE_2$ in osteoblasts.*

*2.1.1.3.1. RANKL.* Various systemic hormones (such as $1\alpha,25\text{-}(OH)_2$ vitamin $D_3$ and PTH), growth factors and cytokines increase the ability of osteoclasts to break down bone through osteoblasts [33,75]. Such externally regulated osteoclastic bone resorption is primarily dependent on the cell surface interaction between RANKL on osteoblasts and RANK on osteoclasts [76,77]. Osteoclast formation can be inhibited by osteoprotegerin (OPG), a decoy receptor that binds to RANKL and prevents its interaction with RANK [78]. $PGE_2$ can stimulate RANKL production [43,70] and inhibit OPG production [79,80] in osteoblasts in a cAMP-dependent manner.

*2.1.1.3.2. IL-6.* IL-6 has been reported to stimulate bone resorption [81,82]. $PGE_2$ induces the production of IL-6 in mouse and rat osteoblasts [83–85] through EP1 and EP2 signaling [83]. $PGE_2$ activates the IL-6 promoter through the cAMP-PKA dependent pathway [85]. Therefore, $PGE_2$ may enhance osteoclast formation through the production of IL-6 in osteoblasts.

IL-6 signaling is mediated by membrane bound IL-6 receptor (IL-6R) or soluble IL-6R (sIL-6R) that lacks transmembrane and cytoplasmic regions of IL-6R [86]. Because mouse primary osteoblasts express low levels of membrane bound IL-6R mRNA, IL-6 treatment alone cannot induce osteoclast formation in the mouse coculture of bone marrow cells and osteoblasts [87]. However, the coadministration of sIL-6R and IL-6 triggers osteoclast formation. In another report, simultaneous application of IL-6 and sIL-6R to mouse cultured calvariae enhanced bone resorption with the increased expression of RANKL mRNA and protein [88]. Since sIL-6R is present in human sera [89], it is possible that the sIL-6R/IL-6 complexes enable osteoblasts to promote osteoclast formation *in vivo*.

Contrary to the $PGE_2$-stimulated IL-6 production, IL-6 can also increase COX-2 expression and $PGE_2$ production in MC3T3-E1 cells [90]. Thus, there appears to exist a synergistic interaction between IL-6 and $PGE_2$ during osteoclast formation *in vivo*.

*2.1.1.3.3. IL-1.* The two forms of IL-1, IL-1α and IL-1β, are also potent bone-resorbing cytokines [91,92]. IL-1β mediates $PGE_2$-stimulated mouse osteoclast formation by osteoblasts [93]. Indeed, $PGE_2$ induces IL-1β gene expression and protein production in mouse osteoblasts through the cAMP-PKA pathway [93,94]. Conversely, IL-1β can also stimulate $PGE_2$ production in mouse primary osteoblasts cocultured with bone marrow cells [80]. This is consistent with other reports that IL-1 induces osteoclast formation by a mechanism involving $PGE_2$ in mice and rats [95,96]. Suppression of the OPG production in osteoblasts by the autocrine $PGE_2$ is one of the critical mechanisms of IL-1β-induced osteoclast formation [80,96]. Taken together, an osteoblastic positive feedback loop composed of $PGE_2$ and IL-1 appears to regulate osteoclast formation and bone resorption through a RANKL-dependent mechanism. This synergism for osteoclast formation is similar to that between $PGE_2$ and IL-6. However, Jimi et al. reported a RANKL-independent mechanism by which IL-1 directly affects the differentiation and function of osteoclasts [97,98].

*2.1.1.3.4. $PGE_2$.* $PGE_2$ can amplify its own production in mouse osteoblastic MC3T3-E1 cells [72,99,100]. This phenomenon, termed "autoamplification", is accompanied by an increase in $cPLA_2\alpha$ and COX-2 protein levels. The activation of EP2 and EP4 receptors results in COX-2 mRNA transcription in primary mouse osteoblasts [66]. Another study using specific EP agonists demonstrated that the EP1 receptor was responsible for $PGE_2$ autoamplification in MC3T3-E1 cells [72]. This autoamplification system is thought to be essential for maintaining $PGE_2$ production and prolonging the effects of short-lived $PGE_2$ during bone-resorptive disorders, such as long-term immobilization and bone inflammation.

*2.1.1.3.5. Proteinase.* It has been proposed that osteoblast-secreted proteases can control the access of osteoclasts to the bone surface [101]. Mouse osteoblastic MC3T3-E1 cells secrete collagenase to degrade collagen [102], a crucial step in initiating bone remodeling [103]. $PGE_2$-stimulated bone resorption is accompanied by the induction of two matrix metalloproteinases (MMPs), MMP-2 and MMP-13, in mouse calvarial cultures [63]. Furthermore, Kim et al. reported that $PGE_2$ enhanced the mRNA expression of MMP-1, an interstitial collagenase, in mouse primary osteoblasts [104]. One group of metalloproteinases, a disintegrin and metalloproteinase (ADAM), has both metalloproteinase (proteolytic) and disintegrin (adhesion) domains [105]. $PGE_2$ has been shown to stimulate the expression of a new member of the ADAM family, a disintegrin and metalloproteinase with thrombospondin motif-1 (ADAMTS-1) in osteoblast-enriched femoral metaphyseal region of male rats injected with PTH [106].

*2.1.1.4. Production of bone-forming factors by $PGE_2$ in osteoblasts*

*2.1.1.4.1. Runx2/Cbfa1.* Runx2/Cbfa1 is a transcription factor essential for osteoblastic differentiation [107]. $PGE_2$ induces the expression of Runx2/Cbfa1 through EP4 receptor activation in mouse osteoblasts, resulting in the enhanced formation of mineralized nodules [108].

*2.1.1.4.2. Bone morphogenetic protein.* The bone-forming effects of $PGE_2$ are likely mediated in part by other molecules. Bone morphogenetic proteins (BMPs) are crucial in skeletal development and repair [109]. They stimulate mouse osteoblast formation from mesenchymal progenitors and osteoblastic differentiation by increasing Runx2/Cbfa1 expression [110]. $PGE_2$ induces BMP-2 mRNA expression by binding to the EP4 receptor in human mesenchymal stem cells that are capable of differentiating into osteoblasts [111].

*2.1.1.4.3. Extracellular matrix.* Adhesive interactions between osteoblasts and extracellular matrix components, including type I collagen, fibronectin and bone sialoprotein, are important for osteoblast survival, proliferation, and differentiation [112]. $PGE_2$ enhances collagen synthesis in mouse osteoblasts [113]. Fibronectin is a heterodimeric bone-matrix glycoprotein that promotes the survival of differentiated osteoblasts [114]. Like collagen, fibronectin production is stimulated by $PGE_2$ in rat osteoblasts [74]. Bone sialoprotein is a highly sulfated, phosphorylated, and glycosylated protein that can bind to hydroxyapatite and mediate cell attachment through an RGD sequence. Bone sialoprotein has a potent role in the initiation of bone mineralization. $PGE_2$ also stimulates the bone sialoprotein mRNA transcription in rat osteoblasts [115].

*2.1.1.5. Effects of $PGE_2$ on osteoclasts.* In addition to the indirect effects of $PGE_2$ on osteoclastic differentiation through osteoblasts, $PGE_2$ exerts direct effects on both immature osteoclast precursor cells and mature osteoclasts.

*2.1.1.5.1. Osteoclast differentiation.* $PGE_2$ enhances the differentiation of mouse bone marrow-derived macrophages into osteoclasts synergistically with RANKL [116]. Kobayashi et al. reported that mouse bone marrow-derived macrophages express EP1, EP2, and EP4 [117]. Osteoclastic differentiation of RAW 264.7 cells was also induced by RANKL treatment and $PGE_2$ stimulated the differentiation even further through the EP2/EP4 receptors [118]. $PGE_2$ also enhanced macrophage colony-stimulating factor (M-CSF)/RANKL-induced osteoclast formation in mouse macrophage cultures [119]. In contrast to mouse osteoclast formation, the direct effect of $PGE_2$ on human osteoclasts is controversial. Lader et al. reported that $PGE_2$ stimulates osteoclast formation in human bone marrow cell cultures treated with M-CSF, TNF-$\alpha$ and IL-1 [120], whereas Chenu et al. showed that $PGE_2$ inhibits osteoclast formation in human bone marrow cell cultures treated with $1\alpha,25\text{-}(OH)_2$ vitamin $D_3$ [121]. Because of the lack of receptors for $1\alpha,25\text{-}(OH)_2$ vitamin $D_3$ in osteoclasts [122], this hormone is not expected to have direct effects on these cells. In the report of Chenu et al., therefore, osteoblast-like cells in the bone marrow cultures may mediate the effects of $1\alpha,25\text{-}(OH)_2$ vitamin $D_3$ on osteoclasts. Meanwhile, M-CSF, TNF-$\alpha$ and IL-1 could stimulate osteoclasts directly under the similar experimental conditions of Lader et al. [120,123]. Thus, it is possible that the different extracellular stimuli resulted in different expression profiles of EP receptor subtypes in osteoclasts. This may account for the apparent discrepancy regarding the effect of $PGE_2$ between these two reports.

In addition to bone marrow cell cultures, $PGE_2$ has been demonstrated to inhibit RANKL/M-CSF-induced osteoclast formation in human peripheral blood mononuclear/$CD14^+$ cell cultures [119,124]. This suggests that $PGE_2$ may stimulate the production of an unknown inhibitory factor(s) for osteoclast formation in human $CD14^+$ cells. This inhibitor production appeared to involve the EP2/EP4-cAMP-PKA signaling pathway [119].

The EP2 and EP4 receptors are down-regulated during the differentiation of mouse bone marrow-derived macrophages into osteoclasts [117]. Treatment of EP4-transfected osteoclasts with $PGE_2$ inhibited the formation of both actin rings (an actin-rich large ring-like structure around the periphery of osteoclasts) and resorption pits (dentin holes formed by osteoclasts). This suggests that the loss

of EP2/EP4 signaling during osteoclast formation enabled mature osteoclasts to escape the inhibitory effects of $PGE_2$ on bone resorption. It is notable that calcitonin, a bone resorption-inhibiting hormone, impairs the formation of actin rings and resorption pits in a cAMP-PKA dependent manner [125]. The function of EP1 in mature osteoclasts remains unknown.

*2.1.1.5.2. Mature osteoclasts.* $PGE_2$ inhibits bone resorption by isolated mature rabbit osteoclasts by activating adenylate cyclase through the EP4 receptor [126]. Both EP3 and EP4 receptors have been detected immunologically in mature human osteoclasts [64,127]. As described above, the expression of EP4 receptor is suppressed in mouse osteoclasts formed *in vitro* so that the cells escape the inhibitory effects of $PGE_2$ on bone resorption [117]. Therefore, it is possible that authentic osteoclasts express EP4 and decrease their function in response to $PGE_2$. Indeed, the activation of EP4 inhibits actin ring formation in human mature osteoclasts [127]. Meanwhile, EP3 stimulation increases the number of lamellipodium-harboring osteoclasts [127]. Because lamellipodia are structures responsible for cell movement, EP3 may have a role in the motility of mature osteoclasts.

*2.1.2. In vivo actions of $PGE_2$*

Investigation of the four EP receptor knockout mice has elucidated the actions of $PGE_2$ under various physiological and pathological conditions [65]. In addition, highly selective agonists and antagonists for the $PGE_2$ receptors have been developed [65]. The use of these experimental tools is paramount for understanding the complicated function of $PGE_2$ during bone metabolism and disease.

*2.1.2.1. Bone abnormalities in EP-deficient mice under physiological conditions.* Although the skeletons of EP4-deficient mice (at 4–5 months of age) are normal, an imbalance in bone remodeling is observed in male mice at 15–16 months of age [128]. These mice suffer from a deficiency in bone formation due to a defect in osteoblast formation. EP4-deficient mice also exhibit reduced structural strength and trabecular bone volume, despite having normal body weight and bone size [129]. EP2-deficient mice have abnormally weak bones, whereas EP1 receptor knockouts appear normal [130].

*2.1.2.2. Pharmacological effects of $PGE_2$ on bone metabolism.* As mentioned earlier (Section 2.1.1), $PGE_2$ plays an important role in bone metabolism *in vitro*. However, the number of *in vivo* studies that focus on the pharmacological effects of $PGE_2$ on bone metabolism is few. The anabolic properties of $PGE_2$ have been analyzed by systemically administrating $PGE_2$ to rats [131,132]. Another study demonstrated that exogenously administered $PGE_2$ increases bone formation in response to mechanical loading of the tibia [61]. However, bones from EP4-deficient mice were unresponsive to exogenously administrated $PGE_2$ [108]. Furthermore, the EP4 agonist ONO-4819 prevented bone loss and restored bone mass and strength in rats subjected to ovariectomy and immobilization [108]. The EP2 receptor selective agonist CP-533,536 also stimulated local bone formation at trabecular, endocortical, and periosteal surfaces in rats [133]. In contrast to this anabolic effect, EP2 was also shown to mediate $PGE_2$-stimulated hypercalcemia in a study of EP2-deficient mice [68].

*2.1.2.3. Role of $PGE_2$ in bone disease.* Consistent with the anabolic effects of exogenous $PGE_2$, endogenous $PGE_2$ may participate in the recovery from osteoporosis and bone fractures. However, endogenous $PGE_2$ has also been implicated in several bone-resorptive inflammatory disorders.

*2.1.2.3.1. Bone fracture.* Fracture healing is a complicated process that includes the proliferation and differentiation of mesenchymal stem cells into chondrocytes and osteoblasts. Healing is complete when mature lamellar bone is formed after woven-bone bridges the bone gap [134]. Endogenous levels of $PGE_2$ and COX-2 are increased locally after fracture in experimental animals [135,136]. Several studies have shown that the COX inhibitors, *i.e.*, nonsteroidal anti-inflammatory drugs (NSAIDs), delay bone repair [137,138]. In addition, fracture healing is delayed in COX-2-deficient mice compared with COX-1-deficient and wild-type mice [139]. In this study, osteoblast formation was impaired and Runx2/Cbfa1 expression was reduced in bone marrow stromal cell cultures from COX-2-deficient mice [139]. The addition of $PGE_2$ rescued the defects observed in COX-2-deficient cells. $PGE_2$ improves fracture healing through EP4 receptor signaling in mice [128]. In the absence of EP4 receptor, aged mice suffer from decreased bone mass and an impaired ability to heal from fractures. The EP2 receptor selective agonist CP-533,536 is able to improve the healing process in rats and dogs, suggesting that the EP2 receptor contributes to the anabolic activity of bone in response to $PGE_2$ as well [133,140].

An optimal mechanical stress at the fracture site is essential to achieve prompt and complete healing of a fracture [141]. $PGE_2$ and COX-2 promote bone formation in response to mechanical loading [60,61]. Taken together, $PGE_2$ has bone-forming activity during bone repair.

*2.1.2.3.2. Osteoporosis.* Osteoporosis is a skeletal disorder characterized by weakened bone strength, which increases the risk for fracture. The equilibrium between bone resorption and formation is maintained in young, healthy women. However, in patients suffering from postmenopausal osteoporosis, this equilibrium is shifted towards resorption due to an acute decrease in serum estrogen level after cessation of ovarian function [142].

Administration of $PGE_2$ can suppress bone loss in rats that have undergone ovariectomy or orchidectomy [143,144]. Following EP4 receptor activation, bone formation is stimulated and bone loss is prevented in ovariectomized rats [108]. Consistent with EP4-mediated osteoblastic differentiation of bone marrow cells *in vitro* [145] (see Section 2.1.1.2), the density of osteoblasts lining

the bone surface increases in response to an EP4-specific agonist in these rats [108]. Moreover, another EP4-specific agonist restored bone mass and strength in ovariectomized rats that had established osteopenia [146]. Postmenopausal osteoporosis in women is managed by supplemental estrogen, estrogen-like agents, bisphosphonates, and other drugs [147]. The adverse side effects of $PGE_2$ such as diarrhea, lethargy, and flushing have prevented its therapeutic use for bone-resorptive diseases [140]. Therefore, EP4 agonists may be better therapeutics for postmenopausal osteoporosis [146]. However, it is noteworthy that NSAIDs increase the bone mineral density in osteoporotic animals [148,149] and humans [150–152], suggesting that endogenous $PGE_2$ has the potential to stimulate bone resorption. Provided that EP receptor(s) other than EP4 has strong bone-resorptive activity so that endogenous $PGE_2$ eventually stimulates bone resorption in osteoporosis, it is reasonable that NSAIDs suppress this disease. Despite the anti-osteoporotic effects of EP4, there are conflicting reports that show that EP4 is involved in bone resorption *in vitro* [43,63,153]. Unlike bone-resorptive inflammatory disorders mentioned below, these *in vitro* experiments using calvaria and bone marrow cultures seem inappropriate for the analysis of osteoporotic bone loss, although the reasons remain to be clarified.

*2.1.2.3.3. Rheumatoid arthritis.* Rheumatoid arthritis is characterized by chronic joint inflammation with infiltration of autoreactive T cells and macrophages [154]. T cells activate synovial macrophages that release multiple cytokines, resulting in the amplification of synovial inflammation and the destruction of cartilage and bone. Macrophage-derived cytokines such as IL-1β and TNF-α induce COX-2 expression in human articular chondrocytes and synovial fibroblasts [155]. IL-17, which is produced by activated $CD4^+$ T cells, stimulates COX-2-dependent $PGE_2$ synthesis in mouse primary osteoblasts from the synovial tissues [156]. The expression of mPGES-1 in synovial fibroblasts of rheumatoid arthritis patients rises in response to TNF-α and IL-1β [157]. Anti-TNF-α therapy can suppress mPGES-1 expression and $PGE_2$ production in synovial tissues from patients with rheumatoid arthritis [158]. It is possible that the $PGE_2$ present in the synovial fluids is involved in joint destruction. As mentioned previously (Section 2.1.1.2), EP2 and EP4 induce RANKL expression in osteoblasts [43]. The resulting osteoclasts that develop are likely to promote joint destruction.

Collagen-induced arthritis (CIA) and collagen antibody-induced arthritis (CAIA) are the widely used animal models of rheumatoid arthritis [159]. The lesions of CAIA are milder and its symptoms last for a shorter duration than CIA [160]. Mice deficient in $cPLA_2\alpha$ and COX-2 are resistant to CIA [161] and CAIA [162], respectively. In both arthritis models, mPGES-1 knockout mice displayed significant reduction in the disease severity compared with wild-type controls [163,164]. Significant suppression of CIA is achieved in mice by the simultaneous inhibition of EP2 and EP4 receptors [160], while the severity of CAIA is reduced in mice lacking EP4 receptor [165]. The EP receptor(s) critical for joint inflammation appears to vary depending on the protocol for arthritis model.

*2.1.2.3.4. Osteoarthritis.* Osteoarthritis, the most common arthritic disease, causes a loss of articular cartilage (surface of joints) due to matrix degradation and the hypertrophic bone changes, including the formation of osteophytes (bony spurs) and thickening of the subchondral plate (bone tissues lining under articular cartilage) [166]. Multiple factors such as age, genetic background, hormone levels, and physical stress are considered to contribute to this slowly developing disease. During cartilage degradation in osteoarthritis, $PGE_2$ and the proinflammatory cytokines TNF-α and IL-1 are produced by synovial membrane cells that are mainly composed of fibroblast-like and macrophage-like synoviocyte populations [167,168]. Endogenous $PGE_2$ regulates the production of IL-6, M-CSF, and vascular endothelial growth factor (VEGF) by IL-1β-stimulated human fibroblast-like synoviocytes through the EP2 and EP4 receptors [169]. Osteoarthritic osteoblasts produce more $PGE_2$ than normal osteoblasts [170] and osteoarthritic cartilage has more apoptotic chondrocytes than normal one [171]. Exogenous $PGE_2$ can sensitize human osteoarthritic chondrocytes to cell death induced by nitric oxide [172], which also contributes to the progression of osteoarthritis. These results suggest that the symptoms of osteoarthritis may worsen as a consequence of $PGE_2$ action in articular cartilage.

*2.1.2.3.5. Periodontitis.* Periodontitis is a chronic inflammatory disease characterized by gingival inflammation that leads to periodontal attachment loss, alveolar bone resorption and ultimately tooth loss [173]. This disease is caused by the gram-negative bacterial species *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* that grow on tooth surfaces and in subgingival sites [174]. LPS is the potent stimulator of gingival fibroblasts and macrophages and induces the production of the bone-resorptive cytokines TNF-α and IL-1 [175,176]. IL-1 induces $PGE_2$ production and COX-2 mRNA expression in fibroblastic connective tissue cells in periodontal ligaments [177]. Additionally, LPS directly up-regulates COX-2 expression in periodontal ligament fibroblasts, cementoblasts (cementum-producing cells around dentin), and osteoblasts [178]. In fact, the $PGE_2$ concentration in gingival crevicular fluid is elevated in periodontitis patients [179]. Increased production of $PGE_2$ due to COX-2 up-regulation may be correlated with alveolar bone resorption. Moreover, LPS-induced bone resorption is impaired in $cPLA_2\alpha$-, mPGES-1, and EP4-deficient mice [180–182]. Although $PGE_2$ may not be the sole factor influencing alveolar bone destruction, NSAIDs are effective in suppressing alveolar bone loss in an animal experimental model of periodontitis [183] and in human periodontitis [184]. While some reports suggest that $PGE_2$ stimulates bone formation in periodontal tissues [185,186], stronger evidence supports its role in bone destruction in periodontitis.

### 2.2. Effects of other prostanoids on bone metabolism and disease

Chick primary osteoblasts have been shown to produce $PGI_2$, $PGF_2\alpha$, $PGD_2$, and $TxA_2$ as well as $PGE_2$ [187]. In contrast to the large number of studies investigating the role of $PGE_2$ during bone metabolism, the effects of these prostanoids on bone remodeling are less defined.

#### 2.2.1. $PGI_2$

$PGI_2$ is chemically labile at room temperature with a $T_{1/2}$ of seconds to minutes [188]. It has been implicated in bone resorption in rat long-bone cultures [189]. However, in recent years, a bone-forming effect of $PGI_2$ has been revealed in a study of the mechanical loading effect on bone [190]; in MC3T3-E1 cells, $PGI_2$ mediated the hydrostatic pressure-elicited expression of c-Fos, a potent regulator of osteoblastic proliferation and differentiation [190]. Mechanical loading enhances bone formation [191] and arachidonic acid metabolism, especially the production of $PGE_2$ and $PGI_2$, in bone [192]. Another study showed that $PGI_2$ directly inhibits osteoclastic activity, while it activates osteoclasts in the presence of osteoblasts [193]. Taken together, like $PGE_2$, $PGI_2$ appears to display bidirectional effects on bone metabolism according to the experimental conditions through the stimulatory G protein (Gs)-coupled $PGI_2$ receptor (IP). It is unknown if $PGI_2$ promotes the production of RANKL by osteoblasts. Meanwhile, when activating the peroxisome proliferator-activated receptor (PPAR)β/δ, $PGI_2$ appears to inhibit osteoblastic proliferation [194].

$PGI_2$ is more abundant than $PGE_2$ in the synovial fluid of rheumatoid arthritis patients [195]. By analyzing IP-deficient mice, $PGI_2$-IP receptor signaling was found to be important for joint inflammation in CIA [160]. In conjunction with IL-1β, $PGI_2$ stimulates IL-6 production by activating synovial fibroblasts in an autocrine manner [160]. IL-6 is involved in the pathogenesis of rheumatoid arthritis such as B cell maturation and osteoclast formation [196,197]. Therefore, $PGI_2$ may be an important mediator of this bone disease. Again, this profile for $PGI_2$ is reminiscent of that for $PGE_2$ mediated through the EP2 and EP4 receptors [160] (see Section 2.1.2.3).

#### 2.2.2. $PGF_2\alpha$

$PGF_2\alpha$ stimulates the proliferation of osteoblasts and suppresses ALP activity *in vitro* [198]. Although less potent than $PGE_2$, $PGF_2\alpha$ exhibits anabolic effects in ovariectomized rats by supporting osteoblast recruitment and activity [199]. Activation of the Gq-coupled $PGF_2\alpha$ receptor (FP) in osteoblasts results in the phosphorylation of ERK/mitogen-activated protein kinase (MAPK) and subsequent synthesis of VEGF [200], and basic FGF [201] synthesis. Both growth factors are known to be potent activators of bone formation [202,203]. In mouse osteoblastic MC3T3-E1 cells, transactivation of the EGF receptor accounted for the $PGF_2\alpha$-induced phosphorylation of ERK/MAPK [204]. However, $PGF_2\alpha$-induced Na-dependent phosphate transport, which plays an important role in mineralization, occurs independently of ERK/MAPK activation [205].

#### 2.2.3. $PGD_2$

$PGD_2$ stimulates calcification and IL-6 synthesis in mouse osteoblastic MC3T3-E1 cells and human osteoblasts, respectively [206,207]. Administration of $PGD_2$ not only prevents the ovariectomy-induced decrease in bone mineral density, but also improves the bone mineral density of sham-operated rats [208]. In human primary osteoblasts, $PGD_2$ activates the Gs-coupled $PGD_2$ receptor (DP) and decreases osteoprotegerin production [209]. Interestingly, in the same cells, $PGD_2$ decreases RANKL production upon binding to another $PGD_2$ receptor, chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2), which couples to Gi/o [209]. Thus, $PGD_2$ appears to have both bone-resorptive and -forming activities through two different receptors DP and CRTH2, respectively. The latter activity may be responsible for the anabolic effects of $PGD_2$ on bone *in vivo*.

Two inflammatory cytokines (TNF-α and IL-1) and three regulators of bone formation (PTH, VEGF, and insulin-like growth factor-I) strongly stimulate the production of $PGD_2$ in human primary osteoblasts [209]. $PGD_2$ has been implicated in the control of osteoblast function and bone anabolism. Mechanical loading by strain application increases the mRNA expression of PGD synthase and PPARγ-1 in MC3T3-E1 cells [210]. Indeed, mechanical loading enhances the production of $PGD_2$, and its metabolite $\Delta^{12}PGJ_2$, the natural ligand for PPARγ-1 [210]. In addition, stretching of osteoblasts induces bone nodule formation and the activation of PGD synthase. Thus, the $\Delta^{12}PGJ_2$-PPARγ-1 pathway may have a significant influence on bone formation upon mechanical loading.

#### 2.2.4. $PGE_1$

$PGE_1$ is synthesized from dihomo-γ-linolenic acid by the sequential catalyses of COX and PGES. $PGE_1$ stimulates p38 MAP kinase through PKA activation, which results in the increased ALP activity in MC3T3-E1 cells [211]. It also enhances the production of VEGF through a cAMP-PKA dependent pathway in osteoblastic RCT-3 and MC3T3E-1 cells [212,213]. $PGE_1$ has an affinity to EP receptors with the same rank order as $PGE_2$ (EP3 ≈ EP4 > EP2 > EP1) [13]. Furthermore, $PGE_1$ has a similar affinity for EP1 and IP. Among these receptors, EP2, EP4 and IP couple to Gs.

#### 2.2.5. $TxA_2$

Although $TxA_2$ is a powerful promoter of platelet aggregation and smooth muscle contraction, it is also highly unstable ($T_{1/2}$ = approx. 30 s at 37 °C) and is rapidly hydrated to the more stable metabolite, $TxB_2$ [214]. Because of its unstable nature, little is known about the contribution of $TxA_2$ to bone metabolism. Using the chemically stable

analogue of $TxA_2$, STA2, a role for $TxA_2$ in bone resorption and osteoclast formation was predicted in mice [215]. As for osteoblasts, STA2 stimulates the proliferation of mouse MC3T3-E1 cells through the activation of protein kinase C [216].

## 3. Role of LTs in bone metabolism and disease

### 3.1. 5-LO pathway

Cysteinyl LTs ($LTC_4$, $LTD_4$, and $LTE_4$) and $LTB_4$ are derived from the 5-LO pathway of arachidonic acid metabolism (Fig. 1). LTs are inflammatory mediators that signal through GPCRs. Our laboratory has cloned two distinct $LTB_4$ receptors (BLT1 and BLT2) [217,218]. BLT1 is a high affinity receptor that mediates adenylate cyclase inhibition and calcium influx by coupling with Gi/o and Gq/11 proteins [219]. BLT2 transduces similar intracellular signals but has a lower affinity to $LTB_4$ [219]. Currently, no pharmacological reagents in clinical use antagonize either of the $LTB_4$ receptors, although they are under development. Like $LTB_4$, cysteinyl LTs function through two receptor subtypes (CysLT1 and CysLT2) [220]. CysLT1 binds $LTD_4$ with a more than 100-fold higher affinity than $LTC_4$ [221], whereas CysLT2 binds to $LTC_4$ and $LTD_4$ equally [222]. Both receptors induce calcium influx probably by coupling with Gq/11 proteins [220]. It has also been proposed that Gi/o protein-dependent signaling occurs through these receptors. The CysLT1 selective antagonists montelukast, pranlukast, and zafirlukast are currently being used as treatments for bronchial asthma [220]. No selective CysLT2 antagonists are available.

Products of the 5-LO pathway besides LTs contain 5-HpETE, 5-HETE, and 5-oxo-ETE. All of them are ligands for a Gi protein-coupled oxoeicosanoid receptor (OXE) with a rank order potency of 5-oxo-ETE $\gg$ 5-HpETE > 5-HETE [223]. The role of these 5-LO products in bone metabolism is still obscure, although there have been only a few reports indicating the negative regulation of bone formation by 5-HETE *in vitro* [224,225].

### 3.2. In vitro actions of LTs

Compared with prostanoids, little is known about the biological effects of LTs on bone. In 1998, the bone-resorptive effects of $LTB_4$, $LTC_4$, $LTD_4$ and $LTE_4$ were initially reported with a mouse calvarial organ culture system [226]. Thus, LTs may suppress bone formation by modulating the function of osteoblasts and/or osteoclasts. Further observations made in each of these cell types are as follows.

#### 3.2.1. Osteoclasts

$LTB_4$ increases osteoclastic bone resorption *in vitro* in organ cultures of neonatal mouse calvariae [227]. $LTB_4$ also increases the formation of resorption pits by isolated neonatal rat osteoclasts [227]. In this assay, $LTB_4$ has greater potency than $LTD_4$, although it is unknown whether this difference in potency is due to increased stability or intrinsic biological activity. Radioligand binding assays revealed the presence of $LTB_4$ receptors in avian osteoclasts that increases the bone resorption activity in response to $LTB_4$ [228]. $LTB_4$ promotes osteoclast formation from human peripheral blood mononuclear cells, most likely in a RANKL-independent fashion [229]. $LTC_4$, $LTD_4$, and $LTE_4$ stimulate avian osteoclasts to resorb bone *in vitro* [224,230] and there are fewer osteoclasts in culture following treatment with cysteinyl LT antagonists [224,230]. These results suggest that both $LTB_4$ and cysteinyl LTs directly affect bone resorption by increasing the number and/or activity of osteoclasts. However, over the past year, there are only a limited number of publications that show definitive biochemical data on the mRNA/protein expression and intracellular signaling of LT receptors in osteoclasts.

#### 3.2.2. Osteoblasts

Human primary osteoblasts can synthesize $LTB_4$ [231]. In rat primary osteoblasts, $LTB_4$, but not $LTD_4$, reduces the mineralized nodule formation and ALP activity [225]. Bone formation in mouse calvaria organ cultures is also suppressed by $LTB_4$ [225]. $LTB_4$ partially inhibits the proliferation of rat primary osteoblasts [232]. However, definitive biochemical analyses of $LTB_4$–BLT interactions in osteoblasts have not been done.

### 3.3. In vivo actions of LTs

Local administration of $LTB_4$ to mouse calvarial bone increases osteoclastic bone resorption *in vivo*, likely due to an increase in the osteoclast formation and the activation of mature osteoclasts [227]. Several reports have demonstrated that $LTB_4$ production is stimulated in inflammatory bone-resorptive diseases such as rheumatoid arthritis [233–235], osteoarthritis [236,237] and periodontitis [179,238]. The levels of $LTB_4$ are higher in the synovial fluid from rheumatoid arthritis patients than from osteoarthritis patients [235]. In addition, $LTB_4$ levels are significantly correlated with the number of cells and the concentrations of rheumatoid factor and immune complexes that exist in the synovial fluid from rheumatoid arthritis patients [235]. $LTB_4$ receptor antagonists have also been effective therapeutics for rheumatoid arthritis [239–241]. Recently, through the analysis of BLT1- and BLT1/BLT2-double deficient mice, it was found that BLT1 plays a critical role in mouse models of inflammatory arthritis [242–244]. Neutrophils primarily produce and respond to $LTB_4$ in rheumatoid arthritis [242–244], although the roles of osteoblasts and osteoclasts are not known.

The role of LTs in osteoarthritis is not well understood. $LTB_4$ is elevated in the synovial fluid from osteoarthritis patients [236] and can stimulate TNF-$\alpha$ and IL-1$\beta$ production in synovial cells [245]. $LTB_4$ and $LTC_4$ are produced in synovial tissues, but not in the chondrocytes

of osteoarthritis patients [246]. In addition to NSAIDs, 5-LO inhibitors may also be useful for the treatment of osteoarthritis. Licofelone, a new anti-inflammatory drug with a dual 5-LO and COX inhibitory activity, prevented abnormal subchondral bone cell metabolism in experimental dog osteoarthritis [247].

## 4. Role of PAF in bone metabolism and disease

The term PAF was first used to describe the factor(s) able to aggregate and activate platelets [248]. Since then, the pleiotropic and potent biological effects of PAF have been revealed, including its role in airway constriction, hypotension, and vascular hyperpermeability [25,249–251]. However, the biological role of PAF in bone metabolism and disease was unclear.

All the pharmacological effects of PAF are considered to be caused by activating a single G protein-coupled PAF receptor (PAFR), which was cloned in our laboratory in 1991 [252]. PAFR couples to both Gq/11 and Gi/o proteins that initiate distinct signals [250]. By analyzing PAFR-knockout (PAFR-KO) mice, we have shown the involvement of PAF signaling in various diseases such as allergy [253,254], inflammation [255–259], and infection [260–262]. Our more recent analyses of these mice has revealed its role as an aggravating factor for postmenopausal osteoporosis, one of the most common bone-resorptive diseases [40].

### *4.1. PAF in osteoclasts: production and effects*

#### *4.1.1. PAF synthesis in osteoclasts*

In the remodeling pathway of PAF synthesis, PAF is derived from the glycerophospholipids remaining after the release of fatty acids by $PLA_2$ enzymes, especially $cPAL_2\alpha$ (Fig. 2). We revealed that primary osteoclasts derived from either the spleen or bone marrow expressed more $cPLA_2\alpha$ than primary osteoblasts in mice [40]. Lyso-PAF acetyltransferase catalyzes the final reaction for PAF synthesis (Fig. 2) [24,25]. We measured the activity of this enzyme in cultured mouse bone cells to determine in which cell lineages PAF synthesis occur [40]. Osteoclasts displayed high lyso-PAF acetyltransferase activity that rose significantly following treatment with TNF-α and IL-1β. In contrast, osteoblasts had significantly lower PAF synthesis activity than osteoclasts, even after cytokine-stimulation. Therefore, osteoclasts seem to be a main source for PAF in bone in response to extracellular stimuli.

Estrogen is thought to inhibit bone resorption by suppressing the production of TNF-α and IL-1 [263] and by inducing apoptosis of osteoclasts [264,265]. Estrogen withdrawal in women after surgical ovariectomy or natural menopause is linked to an amplified production of these cytokines [266]. TNF binding protein and IL-1 receptor antagonist have been shown to suppress the bone loss resulting from ovariectomy in mice [267,268]. Because TNF-α and IL-1β stimulate lyso-PAF acetyltransferase activity in osteoclasts *in vitro*, it is possible that ovariectomy up-regulates PAF production in osteoclasts.

The contribution of *de novo* pathway to PAF synthesis in osteoclasts remains to be clarified.

#### *4.1.2. PAF receptor expression in osteoclasts*

We investigated which cells in bone tissue express PAFR [40]. Osteoclasts derived from RAW 264.7 cells and primary mouse osteoclasts had significant amounts of PAFR mRNA. In contrast, mouse osteoblasts expressed much lower levels of PAFR mRNA even after stimulation with TNF-α and IL-1β simultaneously. PAF raised the intracellular calcium level in mouse osteoclasts from wild-type mice, but not from PAFR-KO mice. Furthermore, PAF application to osteoblasts neither elevated intracellular calcium nor affected the expression level of RANKL mRNA. Thus, PAF most likely modulates osteoclasts in an autocrine/paracrine manner independent of osteoblasts.

#### *4.1.3. Effects of PAF on osteoclasts*

Purified mature osteoclasts promptly undergo apoptosis under some experimental conditions [269]. We found that PAF promotes the survival of osteoclasts and the PAFR antagonist WEB 2086 blocks these prosurvival effects [40]. PAF was ineffective on osteoclasts from PAFR-KO mice. IL-1β also enhanced the survival of osteoclasts. Furthermore, their calcium resorptive activity was increased by IL-1β. It is interesting that these positive effects of IL-1β were significantly suppressed by WEB 2086-treatment and genetic PAFR-deficiency [40]. This suggests that PAF is a part of the mechanism by which IL-1β influences osteoblasts.

The survival of mature osteoclasts is affected by several factors including RANKL, IL-1, TNF-α, and M-CSF [270]. Several intracellular signaling molecules such as ERK/MAPK [271,272], NF-κB [98], and phosphatidylinositol-3 kinase [272,273] have been revealed to be relevant to the survival of osteoclasts. PAF also can activate these signaling molecules in several cell lines [250]. Although the downstream pathway that controls cell survival in osteoclasts remains to be resolved, PAF appears to affect resorption by increasing osteoclast life span.

To support the *in vitro* data that show that PAF promotes osteoclastic bone resorption, organ culture experiments were conducted with calvarial bones [40]. Under our experimental conditions, spontaneous osteoclastic bone resorption was observed as pits formed on the calvariae. The degree of pit formation increased after IL-1β treatment. In line with the experiments with cultured osteoclasts, WEB 2086-treatment and genetic PAFR-deficiency prevented the bone resorption on the cultured calvariae. Thus, we confirmed that the PAF–PAFR system mediates, at least in part, the IL-1β-stimulated osteoclastic bone resorption.

Earlier work has shown that rabbit osteoclasts respond to PAF *in vitro* by evoking calcium influx, morphological changes (retraction and re-spreading of pseudopods), and

enhanced osteoclastic resorption [274,275]. Since then, *in vitro* action of PAF in bone metabolism has not been reported until we elucidated that PAF is an autocrine/paracrine activator of osteoclasts as described above [40].

### 4.2. PAF and osteoporosis

The autocrine/paracrine action of PAF on osteoclasts makes it a good target for anti-osteoporosis agents without affecting bone-forming osteoblasts. To investigate the role of PAF/PAFR system in osteoporosis, the impact of ovariectomy on bone mineral density was measured in PAFR-KO and wild-type control mice. It is well known that ovariectomy results in reduced bone mineral density associated with loss of estrogen [276]. The bone mineral density, detected by dual photon X-ray absorptiometry, was significantly low four weeks after ovariectomy in wild-type mice. In contrast, PAFR-KO mice were tolerant to ovariectomy. Histomorphometric analyses on the metaphyseal region of the tibial bones from these mice demonstrated that the volume, number, and density of trabecular bone were minimally affected by ovariectomy in PAFR-KO mice, but were significantly reduced in ovariectomized wild-type mice. Ovariectomy-induced osteoporosis is due to an increase in bone turnover and an imbalance between bone formation and resorption [277]. Both the osteoid thickness (the index of osteoblastic activity) and the osteoclast surface (the index of osteoclastic activity) were nearly normal in ovariectomized PAFR-KO mice.

In summary, we propose a model for the function of PAF in bone resorption (Fig. 3). Deficiencies in estrogen production after ovarian dysfunction raise the levels of TNF-α and IL-1 in the blood and bone marrow [266,278]. These cytokines elevate PAF production in osteoclasts through the activation of lyso-PAF acetyltransferase. PAF then activates PAFR on osteoclasts in an autocrine/paracrine manner, where it can affect cell survival and bone-resorptive activity. Osteoclasts are affected by various systemic hormones, growth factors, and cytokines secondarily to the osteoblast activation [33,75]. It is of note that only a few factors are known to act directly on osteoclasts. Osteoclast bone-resorbing activities can be inhibited directly by calcitonin, a 32-amino acid polypeptide secreted by thyroid C-cells [279]. They can also be stimulated by RANKL. Considering the modes of action of calcitonin and RANKL, PAF may act uniquely on osteoclasts in an autocrine/paracrine manner.

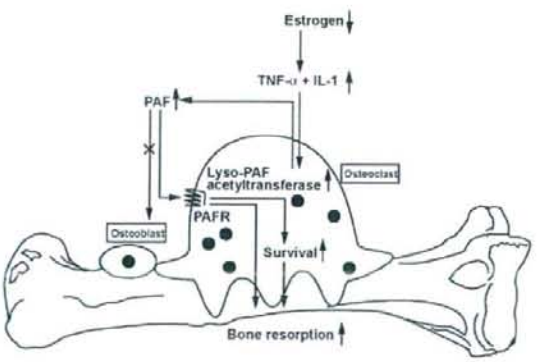


Fig. 3. A schematic model for the function of PAF in bone resorption. Postmenopausal estrogen deficiency increases the levels of TNF-α and IL-1. Subsequently, these cytokines raise PAF production in osteoclasts through the activation of lyso-PAF acetyltransferase. PAF activates PAFR on osteoclasts in an autocrine/paracrine manner and exert positive effects on cell survival and bone-resorptive activity.

### 4.3. PAF and other bone diseases

PAF has also been detected in inflamed human gingival tissue [280] and in arthritic joint fluids of rabbits with acute antigen-induced arthritis [281]. Furthermore, the PAFR antagonist BN 50730 prevented chronic arthritis from developing in mice [282]. However, definitive studies demonstrating a role for PAF in these diseases are still necessary.

## 5. Conclusions

This review has provided an overview of the roles of many lipid mediators in bone metabolism and disease. Understanding the roles of prostanoids, LTs, and PAF in bone metabolism will hopefully result in the development of effective therapies that can treat or even prevent intractable bone-resorptive diseases. Considerable progress has been made over the last several decades in defining the function of $PGE_2$ and the inter- and intracellular signaling pathways that it regulates. Consequently, both the bone-resorbing and bone-forming functions of $PGE_2$ have been revealed. This dual function of $PGE_2$ may be due to the existence of four specific receptors for $PGE_2$, each of which signals through distinct pathways, its ability to target both osteoblasts and osteoclasts, and/or its indirect actions via other molecules in addition to direct ones on target cells. Considering that $PGE_2$ is synthesized through a multistep reaction that is regulated by extracellular stimuli, it is not surprising that $PGE_2$-mediated bone metabolism is complicated. It will be important to further clarify the oftentimes contradicting roles of $PGE_2$ in bone physiology under normal and disease states.

There are relatively few studies on the effects of other prostanoids and LTs on bone metabolism and disease. However, it is critical for clinical applications to determine which of these lipid mediators contribute to bone physiology and pathology. Moreover, phospholipid mediators other than PAF such as sphingosine-1-phosphate and lysophosphatidic acid, which we do not refer to in this review, may prove relevant to bone metabolism by detailed studies. Once role of a given lipid mediator is determined, elucidating the cellular and molecular mechanisms by which it affects bone remodeling is required. In this context, we finally mention that dietary fish oil rich in $n-3$

polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), prevents bone loss in osteoporosis and rheumatoid arthritis [283,284]. Partial replacement of arachidonic acid in cell membranes by EPA and DHA could conceivably lead to decreased production of arachidonic acid-derived prostanoids and LTs, through several mechanisms [284,285]. Incorporation of these $n-3$ fatty acids into phospholipids also affects the mobility and distribution of proteins in membranes, which also could account for the attenuation of immune-cell responses by EPA and DHA [284,285]. Like arachidonic acid, EPA is oxygenated by 5-LO and COX *in vitro* to give rise to less biologically potent eicosanoids, while docosahexaenoic acid is an active substrate for other types of LO, *i.e.*, leukocyte-type 12-LO and 15-LO [285,286]. It has also been proposed that alternative pathways for metabolism of EPA and DHA lead to production of "anti-inflammatory" lipid mediators [287]. Therefore, it is possible that both these $n-3$ fatty acids *per se* and their metabolites exert protective effects against osteoporosis and rheumatoid arthritis. However, key molecule(s) and mechanism(s) still remain elusive.

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