Platelet-Activating Factor Production in the Spinal Cord of Experimental Allergic Encephalomyelitis Mice via the Group IVA Cytosolic Phospholipase A_2-Lyso-PAFAT Axis[1]

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Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) plays a critical role in inflammatory disorders including experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis (MS). Although PAF accumulation in the spinal cord (SC) of EAE mice and cerebrospinal fluid of MS patients has been reported, little is known about the metabolic processing of PAF in these diseases. In this study, we demonstrate that the activities of phospholipase A_2 (PLA_2) and acetyl-CoA:lyso-PAF acetyltransferase (LysoPAFAT) are elevated in the SC of EAE mice on a C57BL/6 genetic background compared with those of naive mice and correlate with disease severity. Correspondingly, levels of groups IVA, IVB, and IVF cytosolic PLA_2s, group V secretory PLA_2, and LysoPAFAT transcripts are up-regulated in the SC of EAE mice. PAF acetylhydrolase activity is unchanged during the disease course. In addition, we show that LysoPAFAT mRNA and protein are predominantly expressed in microglia. Considering the substrate specificity and involvement of PAF production, group IVA cytosolic PLA_2 is likely to be responsible for the increased PLA_2 activity. These data suggest that PAF accumulation in the SC of EAE mice is profoundly dependent on the group IVA cytosolic PLA_2/LysoPAFAT axis present in the infiltrating macrophages and activated microglia. *The Journal of Immunology*, **2008, 181: 5008–5014.**

Platelet-activating factor (PAF[3]; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a potent proinflammatory lipid mediator (1), is believed to be synthesized via two distinct pathways, the de novo and remodeling pathways (Ref. 2 and see Fig. 1). The latter pathway is primarily involved in the synthesis of PAF by stimulated inflammatory cells such as murine peritoneal cells (3, 4) and human granulocytes (5). The initiation of the remodeling pathway requires membrane phospholipid hydrolysis by phospholipase A_2s (PLA_2; EC 3.1.1.4) that supply lyso-PAF, a precursor of PAF. Acetyl-CoA:lyso-PAF acetyltransferase (LysoPAFAT; EC 2.3.1.67) converts lyso-PAF into PAF. PAF activates the PAF receptor (PAFR), a member of the superfamily of G protein-coupled receptors (6), and elicits a variety of biological responses (1). PAF is rapidly degraded by PAF acetylhydrolases (PAF-AH; EC 3.1.1.47) that cleave the acetyl group at the *sn*-2 position to reform lyso-PAF (7).

PLA_2 are classified into three groups: group VI calcium-independent PLA_2s (iPLA_2s), secretory PLA_2s (sPLA_2s), and group IV cytosolic PLA_2s (cPLA_2) (8). Group IVA cPLA_2 preferentially liberates arachidonic acid from 2-arachidonoyl-phospholipids (8, 9). The released arachidonic acids are in turn converted into PGs and leukotrienes via the arachidonic acid cascade (10). It is thought that group VI iPLA_2 and some types of sPLA_2s have the potential to initiate the arachidonic acid cascade, even though these enzymes lack significant substrate specificity (8). Group IVA cPLA_2 is also essential for producing PAF, since PAF synthesis is significantly diminished in calcium ionophore-stimulated macrophages derived from group IVA cPLA_2-deficient mice as compared with those from wild-type mice (11). Recently, our group has successfully overcome the long-standing challenges of cloning and identifying LysoPAFAT (12), a critical enzyme that produces PAF. We termed the enzyme LsoPAFAT/LPCAT2 (lysophosphatidylcholine acyltransferase 2) (12). We have demonstrated that murine macrophages and neutrophils express LysoPAFAT/LPCAT2 mRNA and possess a LysoPAFAT activity (3, 12). Furthermore, LysoPAFAT/LPCAT2 mRNA is induced by the ligands for TLRs 4 and 9 in murine macrophages (12). These results imply that LysoPAFAT plays a crucial role in the enhanced PAF production in inflammatory disorders.

Multiple sclerosis (MS) is considered to be a $CD4^+$ T cell-mediated autoimmune disease and is characterized by inflammation and demyelination in the CNS (13). The mechanism of MS,

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[3] Abbreviations used in this paper: PAF, platelet-activating factor; PLA_2, phospholipase A_2; LysoPAFAT, acetyl-CoA:lyso-PAF acetyltransferase; LysoPAFAT/LPCAT2, LysoPAFAT/lysophosphatidylcholine acyltransferase 2; lyso-PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAFR, PAF receptor; PAF-AH, PAF acetylhydrolase; PC, phosphatidylcholine; iPLA_2, calcium-independent PLA_2; sPLA_2, secretory PLA_2; cPLA_2, cytosolic PLA_2; MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; SC, spinal cord; ESI-MS/MS, electrospray ionization-tandem mass spectrometry; LPCAT1, lysophosphatidylcholine acyltransferase 1; APMSF, amidinophenylmethanesulfonyl fluoride; GFAP, glial fibrillary acidic protein.

however, remains obscure because of limited access to the CNS at various phases of MS. An animal model, experimental allergic encephalomyelitis (EAE), is indispensable for a better understanding of MS pathology (14). Howat et al. (15) suggested an involvement of PAF in EAE for the first time. We have found that PAFR-KO mice immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (MOG_{35-55}) show less severe symptoms than wild-type mice (16). Group IVA $cPLA_2$ deficiency protects mice from EAE pathology (17). We also have reported that there is a correlation between the PAF level in the spinal cord (SC) and EAE symptoms (16), which is consistent with PAF levels in the cerebrospinal fluid of relapsing-remitting MS patients (18). In the SC of EAE mice, PAF seems to exist in the nanomolar range, which is adequate to provoke biological responses through the PAFR (6). Moreover, the level of PAFR transcript is up-regulated in MS lesions (19) and the CNS of EAE-induced SJL and C57BL/6 mice (16, 20). The elevated levels of both PAF and PAFR transcripts probably worsen the MS/EAE pathology. EAE, as an animal model of MS, is useful for understanding the roles of PAF in MS (14), since studies on PAF in MS lesions are in accordance with those in EAE lesions (16, 18–20). However, the metabolic processing of PAF and involvement of LysoPAFAT/LPCAT2 in EAE pathology are largely unknown. In the present study, we have induced EAE in C57BL/6 mice with the MOG_{35-55} peptide and revealed that PAF accumulation in SCs of EAE mice is dependent on the up-regulation of the expression and activities of both group IVA $cPLA_2$ and LysoPAFAT. This is the first report suggesting the involvement of LysoPAFAT/LPCAT2 in the disease models.

Materials and Methods

Induction of EAE

EAE was induced in 8-wk-old C57BL/6 female mice. The maintenance of the facility and the use of animals were in full compliance with the University of Tokyo Ethics Committee for Animal Experiments. MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK), corresponding to the fragment of mouse MOG from aa 35–55, was synthesized by Sigma-Aldrich. Mice were immunized s.c. in the flank with 300 μg of MOG_{35-55} peptide in 0.1 ml of PBS and 0.1 ml of CFA containing 0.4 mg of *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories) on days 0 and 7 and injected i.p. with 250 ng of pertussis toxin (List Biological Laboratories) on days 0 and 2. Mice were scored as follows: 0, no sign; 0.5, mild loss of tail tone; 1.0, complete loss of tail tone; 1.5, mildly impaired righting reflex; 2.0, abnormal gait and/or impaired righting reflex; 2.5, hind limb paresis; 3.0, hind limb paralysis; 3.5, hind limb paralysis with hind body paresis; 4.0, hind and fore limb paralysis; 4.5, moribund; and 5.0, death. To understand the EAE pathology, we divided the disease course into induction, acute, and chronic phases in accordance with the clinical symptoms as previously described (Ref. 16 and Fig. 2*A*).

Quantification of PAF

PAF and eicosanoid levels were estimated simultaneously as previously described (21, 22). The results of the eicosanoid levels will be published elsewhere (Y. Kihara, S. Ishii, Y. Kita, S. Uematsu, S. Akira, and T. Shimizu, unpublished data). SCs of naive mice and EAE mice were removed on days 12, 19, and 32, frozen immediately with liquid nitrogen, and stored at −80°C until use. The frozen tissues (~100 mg) were powdered with an SK-100 mill (Tokken), and lipids were extracted for 60 min at 4°C with methanol containing deuterium-labeled 16:0 PAF (Cayman Chemical) as an internal standard. The extracts were loaded onto Oasis HLB cartridges (30 mg; Waters) preloaded with methanol and 0.03% (v/v) formic acid/H_2O. The cartridges were washed with 0.03% formic acid/H_2O, 15% (v/v) ethanol, and petroleum ether. Lipids were extracted with 100% methanol and PAF levels were quantified by reversed-phase HPLC electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) as described previously (21, 22).

Quantitative real-time PCR

On days 11–12, 18–19, and 30–31, naive and EAE mice were anesthetized with urethane (1.5 g/kg of body; Sigma-Aldrich) and intracardially perfused with 10 ml of ice-cold PBS. The SCs were removed and total RNA was isolated using an RNeasy Mini Kit (Qiagen). The purity and integrity of total RNA were determined by the absorbance at $A_{260/280}$ and gel electrophoresis, respectively. One microgram of total RNA was reverse-transcribed using SuperScript II (Invitrogen Life Technologies) according to the manufacturer's instructions. The RT^2 Profiler PCR Array System for PLA_2 (groups IVA, IVB, IVC, IVD, IVE, and IVF $cPLA_2$s, groups V and X $sPLA_2$s, and group VI $iPLA_2$) was purchased from SuperArray, and quantitative RT-PCR for these PLA_2 mRNAs was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems). The relative abundance of PLA_2 mRNA levels in EAE mice compared with naive mice was calculated by the comparative cycle threshold method using hypoxanthine phosphoribosyltransferase as a normalization control. Quantification of LysoPAFAT, lysophosphatidylcholine acyltransferase 1 (LPCAT1), and β-actin mRNA levels was performed with LightCycler FastStart DNA Master SYBR Green I (Roche) as previously described (12, 23). Results were quantified by using standard curves derived from SCs in the acute phase of EAE.

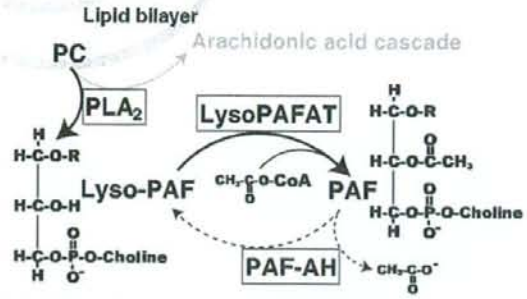

FIGURE 1. PAF production in the remodeling pathway (bold arrow) and degradation pathway (dotted arrow).

Sample preparation for enzyme assays and Western blotting

The SCs of naive mice and EAE mice on days 12, 19, and 34 were removed following perfusion, frozen immediately with liquid nitrogen, and stored at −80°C until use. The tissues (~100 mg) were homogenized with a Physcotoron homogenizer (Microtec) in 500 μl of buffer A (100 mM Tris-HCl (pH 7.4) containing 10.26% sucrose, 20 μM amidinophenylmethanesulfonyl fluoride (APMSF), 5 mM 2-ME, and 1 × Complete Protease Inhibitor Mixture (Roche)). The homogenate was centrifuged at 9,000 × g for 10 min at 4°C and the resulting supernatant was centrifuged at 100,000 × g for 60 min at 4°C. The pellet was resuspended in buffer B (20 mM Tris-HCl (pH 7.4) containing 20 μM APMSF, 5 mM 2-ME, and EDTA-free 1 × Complete Protease Inhibitor Mixture) and stored at −80°C until use. Protein concentrations were determined by the Bradford method using a protein assay solution (Bio-Rad) and BSA (fraction V, fatty acid-free; Sigma-Aldrich) as a standard.

PLA_2 assay

PLA_2 activity was measured by Dole's method with some modifications (24). Briefly, 5 μg of protein (100,000 × g supernatant) was incubated at 37°C for 30 min in a total volume of 0.25 ml of assay buffer (100 mM HEPES-NaOH (pH 7.4) 1 mg/ml BSA, 4 mM $CaCl_2$, and 1 mM DTT) containing mixed micelles (4 μM Triton X-100 and 2 μM 1-palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylcholine (PC) (1.961 GBq/mmol, GE Healthcare BioSciences). The reaction was terminated by adding 1.25 ml of Dole's reagent (isopropanol:*n*-heptane:sulfuric acid, 78:20:2), followed by the sequential addition of 0.75 ml of *n*-heptane and 0.5 ml of water. After centrifugation, an aliquot (0.8 ml) of the upper layer was mixed with 120–150 mg of silica gel, which had been preincubated with 0.75 ml of *n*-heptane. The radioactivity of an aliquot (0.8 ml) was estimated using an LS6500 liquid scintillation counter (Beckman Coulter) in the presence of 1 ml of Microscinti-0 (PerkinElmer).

LysoPAFAT assay

LysoPAFAT activity was measured according to the method of Kume et al. (4, 12, 25), with some modifications. Briefly, 5 μg of protein (100,000 ×

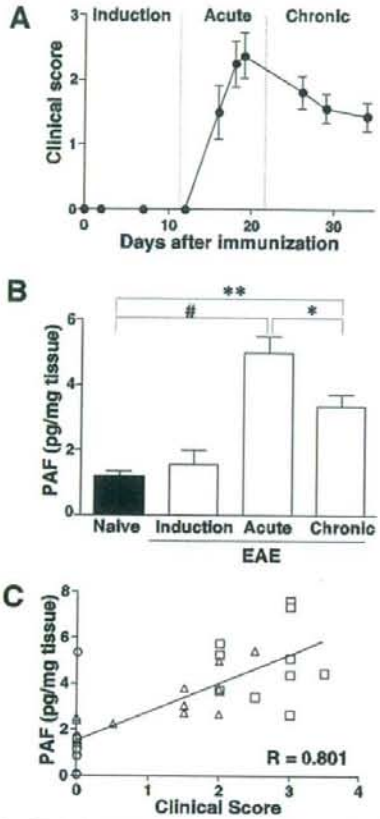

FIGURE 2. Clinical course and PAF levels during EAE. *A*, C57BL/6 female mice were immunized with the MOG_{35-55} peptide. Data are the mean clinical scores ± SEM of eight animals. *B*, PAF levels were determined in SCs of naive mice and EAE mice in the induction, acute, and chronic phases (n = 10 animals). Data represent means ± SEM. #, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$ by ANOVA with the Tukey-Kramer test. *C*, PAF levels of naive (●) and EAE mice in the induction (○), acute (□), and chronic (△) phases are positively correlated with the clinical scores ($p < 0.0001$ by the Spearman rank correlation test). Each data point represents the result from a single animal.

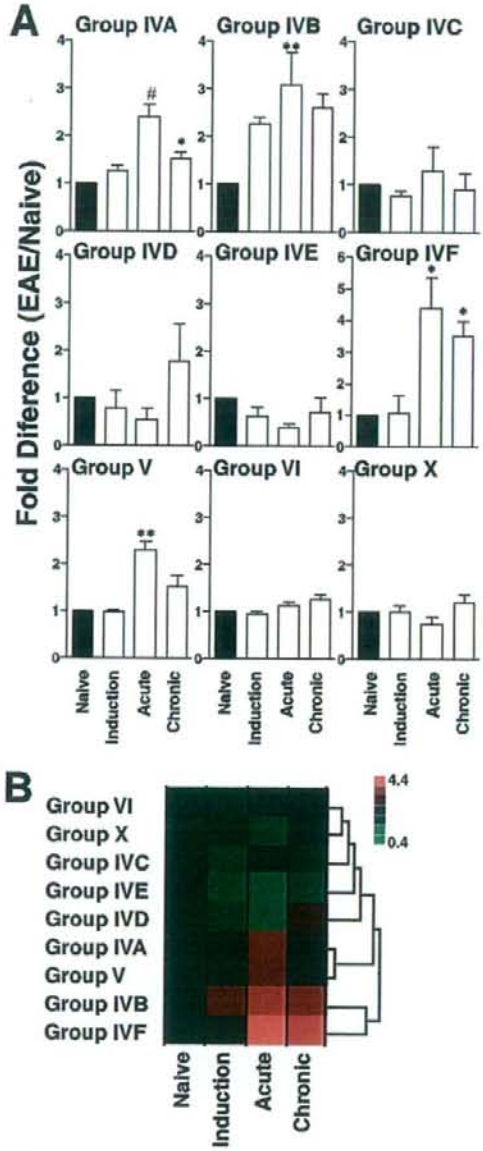

FIGURE 3. PLA_2 mRNA expression in SCs of naive and EAE mice. *A*, Expression of PLA_2 transcripts was quantified by real-time PCR in SCs of naive mice and EAE mice in the induction, acute, and chronic phases (n = 6, 5, 6, and 5 animals, respectively). The relative abundance of PLA_2 mRNA levels in EAE mice compared with naive mice is shown. Data represent means ± SEM. #, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$ compared with naive mice by the Kruskal-Wallis test with Dunn's post hoc test. *B*, The relationships among PLA_2 mRNA levels were evaluated by cluster analysis using JMP6 software (Hulinks). The relative expression levels shown in *A* are divided into seven parts and colored from red to green.

g pellet) was incubated at 37°C for 10 min in a total volume of 0.1 ml of reaction mixture (buffer B containing 2 mM $CaCl_2$, 1 mg/ml PC (Sigma-Aldrich), and 100 μM [^{3}H]acetyl-CoA (1.11 GBq/mmol; GE Healthcare BioSciences)) with or without 20 μM lyso-PAF (Cayman Chemical). Subsequently, 122 μl of ice-cold methanol was added to terminate the reaction. The product was bound to 6 mg of C8 resin (Millipore), washed eight times with 55% (v/v) methanol in 20 mM Tris-HCl (pH 7.4), and eluted with 100% methanol. After drying at 50°C for 2 h, the radioactivity was determined using a TopCount microplate scintillation counter (PerkinElmer) in the presence of 200 μl of Microscinti-0. LysoPAFAT activity was calculated by subtracting the radioactivity obtained without lyso-PAF from that obtained with lyso-PAF.

PAF-AH assay

PAF-AH activity was evaluated under the same conditions as reported previously, with minor modifications (26, 27). Briefly, 10 μg of protein (100,000 × g supernatant) was incubated at 37 °C for 30 min in a total volume of 0.25 ml of assay buffer (50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 5 mM 2-ME, and 100 μM [*acetyl*-^{3}H]PAF (85 MBq/mmol; PerkinElmer)). The reaction was stopped by adding 2.5 ml of chloroform/methanol (4:1, v/v), followed by 0.25 ml of water. The radioactivity of an aliquot (0.6 ml) of each water phase was measured with 2 ml of the liquid scintillation mixture, Atomlight (PerkinElmer), to determine the amount of acetyl groups liberated from PAF.

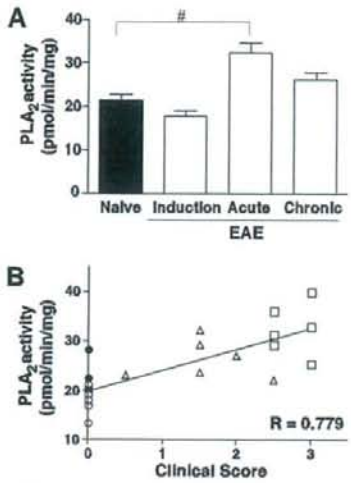

FIGURE 4. PLA_2 activity in SCs of naive and EAE mice. *A*, PLA_2 activity in SCs of naive mice and EAE mice in the induction, acute, and chronic phases ($n = 6$ animals) was measured using mixed micelles containing 1-palmitoyl-2-[^{14}C]arachidonoyl-PC and Triton X-100 in the presence of Ca^{2+} and DTT. Data represent means ± SEM. #, $p < 0.001$ by ANOVA with the Tukey-Kramer test. *B*, PLA_2 activity in naive (●) and EAE mice in the induction (○), acute (□), and chronic (△) phases is positively correlated with the clinical score ($p < 0.0001$ by the Spearman rank correlation test). Each data point represents the result from a single animal.

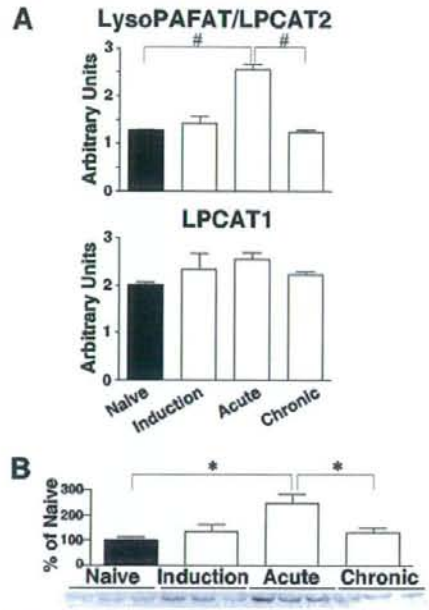

FIGURE 5. LysoPAFAT/LPCAT2 expression in SCs of naive and EAE mice. Expression levels of LysoPAFAT/LPCAT2 and LPCAT1 mRNAs (*A*) and LysoPAFAT/LPCAT2 proteins (*B*) were quantified by real-time PCR and Western blotting with densitometry, respectively, in SCs of naive mice and EAE mice in the induction, acute, and chronic phases ($n = 6$ animals). A representative blot from two independent experiments is shown for LysoPAFAT ($n = 3$ animals). Data represent means ± SEM. #, $p < 0.001$ and *, $p < 0.05$ by ANOVA with the Tukey-Kramer test.

Western blotting

Ten micrograms of protein was resolved by 10% SDS-PAGE and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare Bio-Sciences). The membrane was blocked with 5% skim milk and incubated with anti-LysoPAFAT antiserum (Immuno-Biological Laboratories). After washing, the membranes were incubated with HRP-linked anti-rabbit IgG (GE Healthcare BioSciences), washed, and then exposed to the Western blotting detection reagents (GE Healthcare BioSciences). The membranes were scanned with a LAS-4000 luminescent image analyzer (Fuji film).

Primary culture

Primary young cortical neurons were prepared from C57BL/6 mouse brains on embryonic day 13 as previously described (28, 29). Primary astrocytes and microglia were obtained from cerebral hemispheres of C57BL/6 mouse brains on postnatal day 1, as previously described, with minor modifications (28–30). Briefly, after a 14-day culture period, astrocytes were purified by two passages. Microglia was prepared as a floating cell suspension and transferred to culture dishes. Unattached cells were removed before isolating total RNA. The purities of astrocytes and microglia were estimated to be >90% and >99%, respectively, by immunostaining for glial fibrillary acidic protein (GFAP) and Iba1. Total RNA (1 μg) was reverse-transcribed as described above.

$CD4^+$ and $CD8^+$ T cells were obtained from spleens of C57BL/6 mice using a MACS magnetic cell separation system (Miltenyi Biotec). The purities of $CD4^+$ and $CD8^+$ T cells were estimated to be >90% by flow cytometry (Beckman Coulter). T cells were stimulated with or without anti-CD3ε Ab (BD Biosciences) for 24 h, followed by reverse transcription of total RNA (100 ng) as described above.

Statistical analysis

Results are expressed as means ± SEM. Data were analyzed statistically by means of ANOVA with the Tukey-Kramer post hoc test, the Kruskal-Wallis test with Dunn's post hoc test, or the Spearman rank correlation test as appropriate, using GraphPad PRISM software. Values of $p < 0.05$ were considered to be statistically significant. Cluster analysis was performed using JMP6 software (Hulinks).

Results

Elevation of PAF levels in SCs of EAE mice

C57BL/6 female mice were immunized with MOG_{35-55} and clinical symptoms were monitored (Fig. 2*A*). All mice developed EAE and the mean maximal clinical score was 2.6 ± 0.16 ($n = 8$ animals). To confirm our previous report, PAF levels in SCs were measured by HPLC-ESI-MS/MS. The SCs were collected from naive mice and immunized mice in the induction, acute, and chronic phases of EAE (Fig. 2*A*). PAF levels were significantly elevated in the acute phase (Fig. 2*B*) and positively correlated with the clinical score ($p < 0.0001$; Fig. 2*C*). Thus, the fluctuation in PAF levels during the disease course was reproduced (16). These results demonstrate that the metabolism of PAF (Fig. 1) in the SC was perturbed by the pathogenesis of EAE. Therefore, we determined the enzymes that synthesize and degrade PAF using EAE mice.

Up-regulation of PLA_2 mRNA expression and activity in SCs of EAE mice

The various PLA_2 (groups IVA, IVB, IVC, IVD, IVE, and IVF $cPLA_2$s, groups V and X $sPLA_2$s, and group VI $iPLA_2$) mRNA levels were determined by quantitative RT-PCR to elucidate the effects of PLA_2s on PAF production. Group IVA $cPLA_2$ and group V $sPLA_2$ mRNA levels were elevated in the acute phase of EAE and decreased in the chronic phase to a level that was still higher than that in naive mice (Fig. 3*A*). The relationships among the mRNA levels were evaluated by cluster analysis that distinguished group IVA $cPLA_2$ and group V $sPLA_2$ from other PLA_2s (Fig. 3*B*). In addition, the poorly characterized groups IVB and IVF

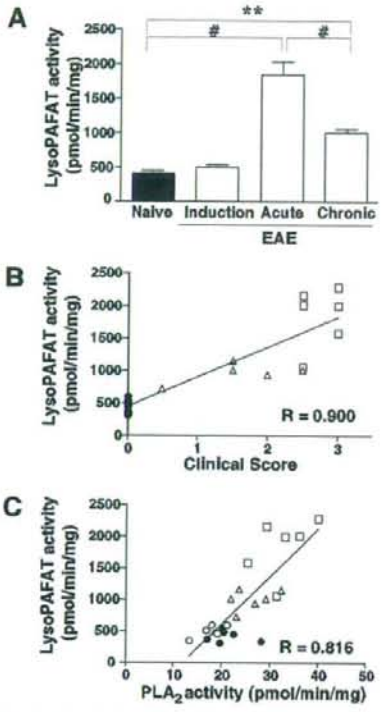

FIGURE 6. LysoPAFAT activity in SCs of naive and EAE mice. *A*, LysoPAFAT activity in SCs of naive mice and EAE mice in the induction, acute, and chronic phases ($n = 6$ animals) was measured as described in *Materials and Methods*. Data represent means ± SEM. #, $p < 0.001$ and **, $p < 0.01$ by ANOVA with the Tukey-Kramer test. *B* and *C*, LysoPAFAT activity in SCs of naive (●) and EAE mice in the induction (○), acute (□), and chronic (△) phases is positively correlated with the clinical score (*B*; $p < 0.0001$ by the Spearman rank correlation test) and PLA_2 activity (*C*; $p < 0.0001$). Each data point represents the results from a single animal.

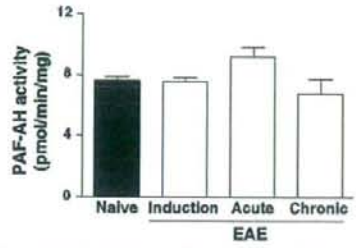

FIGURE 7. PAF-AH activity in SCs of naive and EAE mice. PAF-AH activity in SCs of naive mice and EAE mice in the induction, acute, and chronic phases ($n = 6$ animals) was measured as described in *Materials and Methods*. Data represent means ± SEM.

cPLA$_2$s were up-regulated and clustered together (Fig. 3, *A* and *B*). PLA_2 activity was measured using 1-palmitoyl-2-[^{14}C]arachidonoyl-PC as a substrate with Ca^{2+} and DTT. The enzyme activity increased with the progression of EAE pathology ($p < 0.001$; Fig. 4*A*) and correlated significantly with the clinical score ($p < 0.0001$; Fig. 4B). These results suggest that PAF accumulation in SCs of EAE mice may be due to an up-regulation of PLA_2 and lysoPAFAT (see below).

Enhancement of LysoPAFAT/LPCAT2 expression and activity in SCs of EAE mice

To examine the involvement of LysoPAFAT/LPCAT2, expression levels of the transcripts and proteins were examined in SCs of naive and EAE mice by quantitative RT-PCR and Western blotting, respectively. LysoPAFAT/LPCAT2 transcripts and proteins were elevated in the acute phase and then declined in the chronic phase of EAE (Fig. 5). In contrast, mRNA expression level of the homologous enzyme LPCAT1 was unaltered during the disease course (Fig. 5). In agreement with these observations, the enzyme activities in the acute and chronic phases were higher than those of naive mice ($p < 0.001$; Fig. 6*A*). We found a significantly positive correlation between the clinical score and the LysoPAFAT activity ($p < 0.0001$; Fig. 6*B*). Furthermore, LysoPAFAT activity was positively correlated with PLA_2 activity ($p < 0.001$; Fig. 6*C*). These results suggest that PAF accumulation in SCs of EAE mice is caused by the enhancement of LysoPAFAT/LPCAT2 expression and the corresponding increase in LysoPAFAT activity.

Unaltered basal PAF-AH activity in SCs of EAE mice

We investigated whether PAF-AH affected the accumulation of PAF in SCs of EAE mice. Although PAF-AH activity appeared to be slightly increased in the acute phase of EAE, the enzyme activity did not change significantly during the disease course (Fig. 7). PAF-AH activity did not correlate with the clinical score, PLA_2 activity, or LysoPAFAT activity (data not shown). Thus, PAF accumulation in SCs of EAE mice may be independent of the PAF degradation system.

LysoPAFAT/LPCAT2 expression in primary cultured murine microglia and astrocytes

We previously demonstrated LysoPAFAT/LPCAT2 mRNA expression in murine brain, macrophages, and neutrophils (12). Its expression was determined by RT-PCR and Western blotting in primary cultured murine neurons, astrocytes, microglia (Fig. 8), $CD4^+$ T cells, and $CD8^+$ T cells (data not shown). We found that LysoPAFAT/LPCAT2 mRNA was expressed in microglia and astrocytes, but not in neurons (Fig. 8*A*). The levels of LysoPAFAT/LPCAT2 transcripts were very low in both T cell subsets, with or without anti-CD3ε Ab stimulation for 24 h (data not shown). LysoPAFAT/LPCAT2 protein expression was observed in microglia, but not in astrocytes (Fig. 8*B*). These results suggest that PAF may

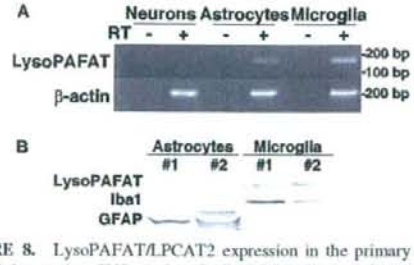

FIGURE 8. LysoPAFAT/LPCAT2 expression in the primary cultured cells of the murine CNS. *A*, LysoPAFAT/LPCAT2 and β-actin mRNA expression in primary cultured neurons, astrocytes, and microglia was determined by RT-PCR. The expected PCR products for LysoPAFAT and β-actin were 167 and 197 bp, respectively. *B*, Expression of LysoPAFAT/LPCAT2, Iba1, and GFAP in primary cultured astrocytes and microglia was determined by Western blotting. Each lane represents cells purified from an individual experiment.

be produced by activated microglia and infiltrating macrophages in SCs of EAE mice.

Discussion

In the present study, we have assessed the metabolic processing of PAF in SCs of naive and EAE mice to explain the enhanced PAF production in EAE mice. In general, accumulation of PAF can be accounted for by the up-regulation of the production system in the remodeling pathway and/or the down-regulation of the degradation system. We have demonstrated that the PAF production system is increased and the degradation system is unchanged in SCs during EAE.

The PAF production system in the remodeling pathway consists of two steps. The first step is production of the PAF precursor lyso-PAF by PLA_2s that hydrolyze the *sn*-2 acyl chain of PC (9) (Fig. 1). Several lines of evidence have suggested that group IVA $cPLA_2$, groups IIA and V $sPLA_2$s, and group VI $iPLA_2$ mRNAs are expressed in the SC of the naive rat (31, 32). In agreement with these studies, we have found that SCs of C57BL/6 mice express these PLA_2 mRNAs, with the exception of group IIA $sPLA_2$ (Fig. 3), which is absent in this mouse strain (33). Because the groups IVA, IVB, and IVF $cPLA_2$s and group V $sPLA_2$ mRNA levels are elevated in the acute phase of EAE (Fig. 3), lipid mediators produced by these four PLA_2s presumably participate in the pathogenesis of EAE. Although little is known about the functions of groups IVB and IVF $cPLA_2$s, it is generally accepted that group IVA $cPLA_2$ and group V $sPLA_2$ stimulate the arachidonic acid cascade (33). Indeed, hierarchical cluster analysis demonstrates the functional analogy of these PLA_2s in EAE pathology (Fig. 3*B*). EAE is not completely ameliorated in PAFR-KO mice (16), whereas group IVA $cPLA_2$ deficiency or treatments with PLA_2 inhibitors protect mice from the EAE pathology (17, 34). Eicosanoid levels were quantified simultaneously and we found that PGE_2 levels were dramatically changed during the disease course (Y. Kihara, S. Ishii, Y. Kita, S. Uematsu, S. Akira, and T. Shimizu, unpublished data). These data suggest that, not only PAF, but also eicosanoids downstream of PLA_2 are critical for the EAE pathology. We have demonstrated the elevation of PLA_2 activity in the acute phase of EAE using 1-palmitoyl-2-[^{14}C]arachidonoyl-PC as a substrate in the presence of Ca^{2+} and DTT (Fig. 4). Since this assay condition is optimized for group IV $cPLA_2$s (35), the elevated PLA_2 activity in the acute phase of EAE may be derived from groups IVA, IVB, and/or IVF $cPLA_2$s. However, groups IVB and IVF $cPLA_2$s have lower PLA_2 activity than group IVA $cPLA_2$ under the present assay conditions (24, 36, 37). Thus, group IVA $cPLA_2$ may be deeply involved in the up-regulation of PLA_2 activity in SCs of EAE mice. Additionally, group IVA $cPLA_2$ is essential for producing PAF, since PAF synthesis is significantly diminished in calcium ionophore-stimulated group IVA $cPLA_2$-deficient macrophages (11). These results suggest that the PAF precursor lyso-PAF is supplied primarily by group IVA $cPLA_2$ during EAE. Because Cunningham et al. (38) reported that $sPLA_2$ activity was up-regulated in urine of EAE rats and MS patients, it may play roles in the EAE pathology. In addition, Bernatchez et al. (39) reported that group V $sPLA_2$ is involved in the PAF production in endothelial cells. Further studies are needed to clarify the roles of group V $sPLA_2$ in EAE lesions.

The second step of the PAF production system is acetylation of lyso-PAF to form PAF by the action of LysoPAFAT, which is critical for the stimulus-dependent formation of PAF (2–4, 12). We have previously shown that LysoPAFAT/LPCAT2 mRNA is expressed in brain, macrophages, and neutrophils (12). Likewise, we have demonstrated the constitutive expression and activity of LysoPAFAT in the SC of naive mice (Figs. 5 and 6). Since LysoPAFAT/LPCAT2 expression is mainly detected in primary cultured microglia by RT-PCR and Western blotting (Fig. 8), microglia may contribute to the production of PAF in the CNS of naive mice for maintaining brain homeostasis (Fig. 9, *left*). A number of inflammatory cells, such as T cells and macrophages, infiltrate the CNS through the broken blood-brain barrier in EAE mice. Furthermore, microglia and astrocytes are activated by cytokines produced by the infiltrating cells (40, 41). The expression and activity of LysoPAFAT were significantly elevated in SCs of EAE mice as compared with those of naive mice (Figs. 5 and 6). Because LysoPAFAT/LPCAT2 is an inducible protein, its expression might be strongly up-regulated in infiltrating macrophages and activated microglia (Fig. 9, *right*). We also have shown that LysoPAFAT activity is correlated with PLA_2 activity (Fig. 6*C*). Kalyvas and David (34) have reported that group IVA $cPLA_2$ is expressed in $CD11b^+$ cells from mice with severe symptoms of EAE. Hence, group IVA $cPLA_2$ and LysoPAFAT appear to be coexpressed in the same cells, such as macrophages/microglia, and to function coordinately in PAF synthesis. In contrast, LysoPAFAT/LPCAT2 mRNA was undetected in T cells stimulation with or without anti-CD3ε Ab for 24 h (data not shown). These results are in accord with previous reports demonstrating that LysoPAFAT activity is present in macrophages (4, 12), but not in T cells (42). The results are also consistent with our previous report that PAF plays a dominant role in the chronic phase of EAE through the activation of macrophages/microglia (16). Taken together, LysoPAFAT induced in macrophages/microglia plays a crucial role in PAF production in EAE pathology (Fig. 9, *right*).

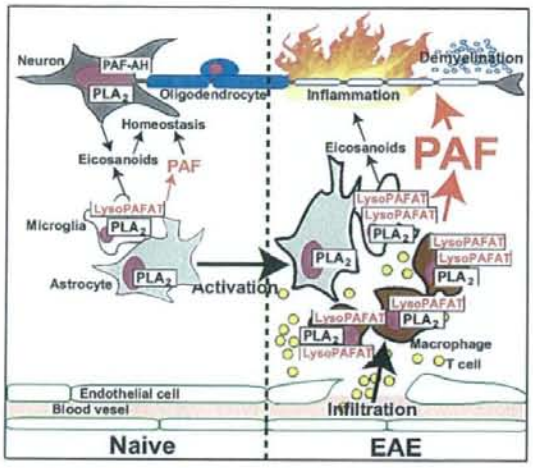

FIGURE 9. Models for PAF production in the CNS of naive mice and EAE mice. *Left*, In the CNS of naive mice, constant levels of PAF produced by microglia and astrocytes may contributes to the maintenance of CNS homeostasis. *Right*, In the CNS of EAE mice, the blood-brain barrier has been broken and inflammatory cells, such as T cells and macrophages, have infiltrated the CNS. LysoPAFAT is induced in activated microglia. Thus, robust PAF production is probably dependent on both LysoPAFAT and group IVA $cPLA_2$ coexpressed in activated macrophages and microglia.

We have measured PAF-AH activity in SCs of naive and EAE mice (Fig. 7) and found that PAF-AH activity is unchanged during the disease course of EAE. Thus, PAF may accumulate in SCs of EAE mice independently of the PAF degradation system.

Our results show that the enzyme activities in the remodeling pathway of PAF synthesis are elevated in SCs of EAE mice due to

up-regulation of group IVA $cPLA_2$ and LysoPAFAT/LPCAT2 present in macrophages and microglia (Fig. 9). Development of LysoPAFAT inhibitors may be therapeutically beneficial for the treatment of MS.

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Disclosures

The authors have no financial conflict of interest.

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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-

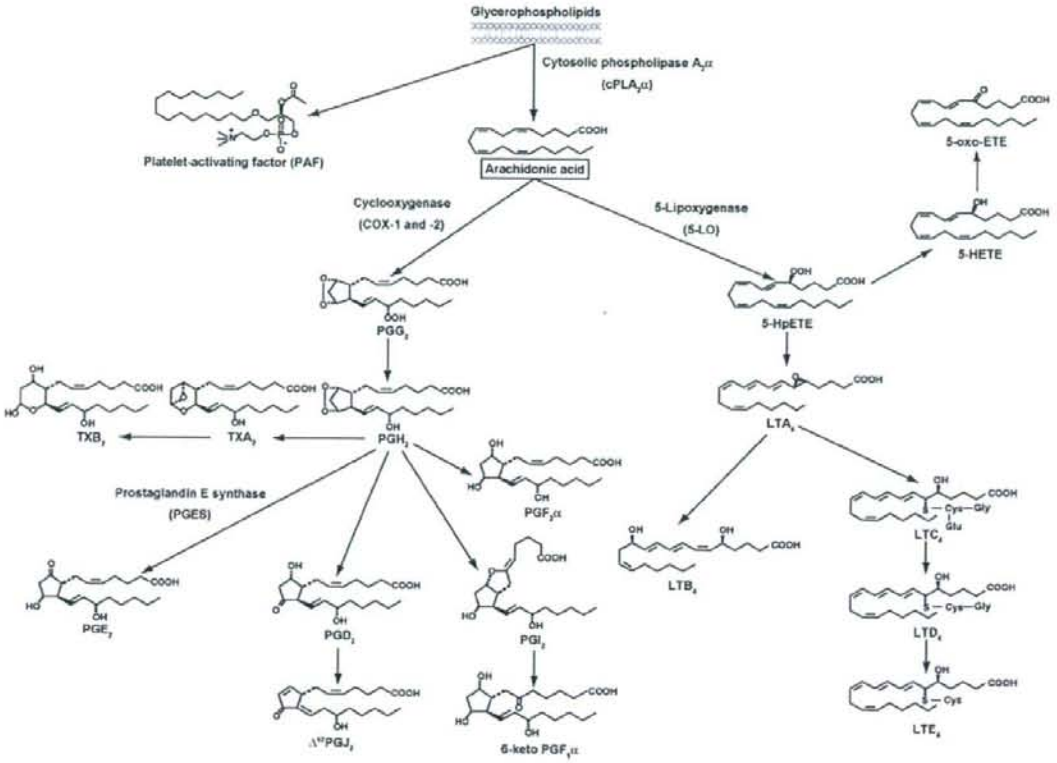

Fig. 1. Metabolic pathways for arachidonic acid. PLA_2 enzymes, particularly $cPLA_2\alpha$, release arachidonic acid from the *sn*-2 position of glycerophospholipids in biomembranes. Arachidonic acid is metabolized to prostanoids and LTs by COX and 5-LO, respectively. PAF is derived from the glycerophospholipids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

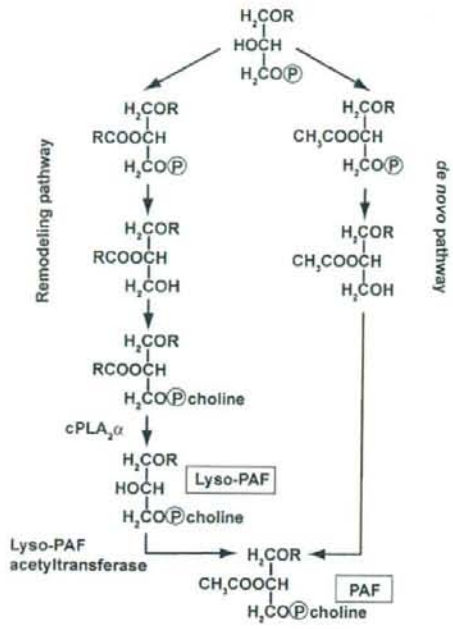

Fig. 2. Synthetic pathways of PAF. PAF is synthesized via two distinct pathways, the *de novo* and remodeling pathways. Lyso-PAF acetyltransferase catalyzes the final reaction for PAF synthesis in the remodeling pathway.

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 1α,25-$(OH)_2$ 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-α 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-α 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 $\approx$ 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-α and IL-1β 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.

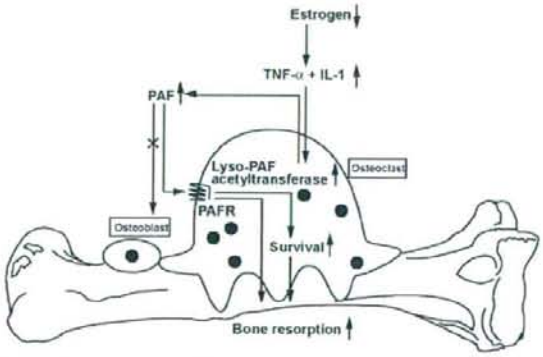

Fig. 3. A schematic model for the function of PAF in bone resorption. Postmenopausal estrogen deficiency increases the levels of TNF-α and IL-1. Subsequently, these cytokines raise PAF production in osteoclasts through the activation of lyso-PAF acetyltransferase. PAF activates PAFR on osteoclasts in 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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