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

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

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

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

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

2.2. Effects of other prostanoids on bone metabolism and disease

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

2.2.1. PGI_2

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

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

2.2.2. $PGF_2\alpha$

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

2.2.3. PGD_2

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

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

2.2.4. PGE_1

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

2.2.5. TxA_2

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

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

3. Role of LTs in bone metabolism and disease

3.1. 5-LO pathway

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

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

3.2. In vitro actions of LTs

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

3.2.1. Osteoclasts

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

3.2.2. Osteoblasts

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

3.3. In vivo actions of LTs

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

The role of LTs in osteoarthritis is not well understood. LTB_4 is elevated in the synovial fluid from osteoarthritis patients [236] and can stimulate TNF-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.

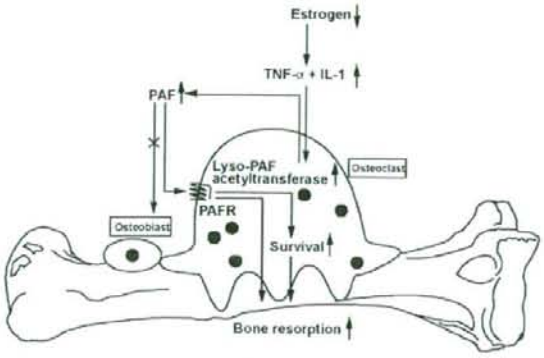

Fig. 3. A schematic model for the function of PAF in bone resorption. Postmenopausal estrogen deficiency increases the levels of TNF-α and IL-1. Subsequently, these cytokines raise PAF production in osteoclasts through the activation of lyso-PAF acetyltransferase. PAF activates PAFR on osteoclasts in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

Acknowledgements

This work was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, Culture and Technology of Japan (to H.H., T.T., S.I., and T.S.), a grant to the Respiratory Failure Research Group from the Ministry of Health, Labour and Welfare, Japan, grants-in-aid for Comprehensive Research on Aging and Health from the Ministry of Health, Labour and Welfare, Japan, and grants from the ONO Medical Research Foundation and the Takeda Science Foundation (to S.I.).

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The American Journal of Pathology, Vol. 172, No. 3, March 2008

DOI: 10.2353/ajpath.2008.070834

Cardiovascular, Pulmonary and Renal Pathology

Endothelial Cysteinyl Leukotriene 2 Receptor Expression Mediates Myocardial Ischemia-Reperfusion Injury

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Cysteinyl leukotrienes (CysLTs) have been implicated as inflammatory mediators of cardiovascular disease. Three distinct CysLT receptor subtypes transduce the actions of CysLTs but the role of the endothelial $CysLT_2$ receptor ($CysLT_2R$) in cardiac function is unknown. Here, we investigated the role of $CysLT_2R$ in myocardial ischemia-reperfusion (I/R) injury using transgenic (tg) mice overexpressing human $CysLT_2R$ in vascular endothelium and nontransgenic (ntg) littermates. Infarction size in tg mice increased 114% compared with ntg mice 48 hours after I/R; this increase was blocked by the CysLT receptor antagonist BAY-u9773. Injection of ^{125}I-albumin into the systemic circulation revealed significantly enhanced extravasation of the label in tg mice, indicating increased leakage of the coronary endothelium, combined with increased incidence of hemorrhage and cardiomyocyte apoptosis. Expression of proinflammatory genes such as Egr-1, VCAM-1, and ICAM was significantly increased in tg mice relative to ntg controls. Echocardiographic assessment 2 weeks after I/R revealed decreased anterior wall thickness in tg mice. Furthermore, the postreperfusion time constant τ of isovolumic relaxation was significantly increased in tg animals, indicating diastolic dysfunction. These results reveal that endothelium-targeted overexpression of $CysLT_2R$ aggravates myocardial I/R injury by increasing endothelial permeability and exacerbating inflammatory gene expression, leading to accelerated left ventricular remodeling, induction of peri-infarct zone cellular apoptosis, and impaired cardiac performance. *(Am J Pathol 2008, 172:592–602; DOI: 10.2353/ajpath.2008.070834)*

Myocardial infarction results from severe impairment of the coronary blood supply usually provoked by thrombotic or other acute alterations of coronary atherosclerotic plaque.[1] It remains the chief cause of death in North America and Europe.[2] With loss of oxygen supply, apoptosis and necrosis of cardiac myocytes in the ischemic area ensues leading to decreased cardiac performance.[1] Rapid reperfusion is essential to limit the extent of myocardial necrosis.[3] However, the consequences of reperfusion are complex and include various deleterious effects collectively referred to as ischemia-reperfusion (I/R) injury.[1] The intense inflammatory response after reperfusion plays a central role not only in promoting tissue injury, but also in repair after infarction.[4] The inflammatory process characterizing early and late reperfusion is an important aspect of the changes leading to tissue damage.[4] Increased vascular permeability and expression of adhesion molecules initiates the inflammatory reaction, and alterations of endothelial function are pivotal in the development of reperfusion damage.[4,5]

Cysteinyl leukotrienes (CysLTs), leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4), and leukotriene E_4 (LTE_4), are well established inflammatory agents that mediate bronchial and vascular smooth muscle constriction and enhance vascular permeability.[6] CysLTs are implicated in inflammatory conditions such as asthma and more recently in cardiovascular disease.[7–9] CysLTs mediate their actions via G protein-coupled receptor (GPCR) proteins, cysteinyl leukotriene 1 receptor ($CysLT_1R$), cysteinyl leukotriene 2 receptor ($CysLT_2R$), and a recently deorphanized GPCR known as

Supported by the Canadian Institutes of Health Research (grants MOP 68930 to C.D.F. and MOP 79506 to L.G.M.), the Heart and Stroke Foundation of Ontario (grant NA 5779 to L.G.M.), and the Pharmacological Society of Canada (Merck Frosst postdoctoral fellowship to S.R.H.).

This publication is dedicated to the honor of Luis G. Melo who passed away suddenly on September 26, 2007 after a brief and courageous battle with pancreatic cancer.

Accepted for publication November 20, 2007.

C.D.F. and L.G.M. hold Canada Research Chairs. C.D.F. is a career investigator of the Heart and Stroke Foundation of Ontario.

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GPR17.[8,10] The $CysLT_2R$ gene is expressed in human heart and coronary vessels, also within the cardiac Purkinje system, as well as in human coronary smooth muscle cells and umbilical vein endothelial cells.[8,11–14] $CysLT_2R$ expression in mouse heart appears to be more restricted with diffuse expression within endothelial cells.[15] We generated previously transgenic (tg) mice overexpressing the human $CysLT_2R$ in vascular endothelium to characterize the role of this receptor in vascular function.[16]

The involvement of CysLTs and their receptors in inflammation and fibrosis has been confirmed in various animal and human studies.[17] Several studies reported enhanced edema and neutrophil infiltration after myocardial I/R concomitant with elevation of CysLTs.[18,19] These eicosanoids are detected as increased urinary LTE_4 levels in patients after admission for suspected acute myocardial infarction and unstable angina.[20] Moreover, the expression of $CysLT_1R$ and $CysLT_2R$ is increased in organs that are prone to ischemic damage and $CysLT_1R$ antagonism exerts anti-inflammatory effects on cerebral and renal I/R injury.[21–23] Few studies have investigated CysLTs and their receptors in acute myocardial infarction and specifically the role of $CysLT_2R$ in myocardial I/R injury has not been established. Here, we report that endothelium-targeted overexpression of $CysLT_2R$ aggravates myocardial I/R injury by increasing endothelial permeability and exacerbating inflammatory gene expression, leading to accelerated left ventricular (LV) remodeling and impaired cardiac performance.

Materials and Methods

Animals

The generation of EC-$CysLT_2R$ transgenic mice has been described previously.[16] These mice express seven copies of the human $CysLT_2R$ gene under control of the Tie2 promoter/enhancer, integrated in a gene-sparse region of chromosome 6. Hemizygous mice were continuously backcrossed with C57BL/6 mice to obtain equal numbers of transgenic and wild-type littermates. 5-Lipoxygenase-deficient ($5LO^{-/-}$) mice, developed in our laboratory previously,[24] were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were backcrossed for more than nine generations to the C57BL/6 background. The $5LO^{-/-}$ mice show absence of 5-lipoxygenase mRNA, protein, and leukotriene synthesis in inflammatory cells. $CysLT_2R$-deficient LacZ mice were generated by standard gene targeting procedures using C57BL/6 embryonic stem cells (S. Ishii, unpublished data) and embryos heterozygous for the genetic modification were transferred from Japan, revived at Queen's University, and littermates of heterozygous offspring (all on a C57BL/6 genetic background) were used in these studies.

Mouse Model of Myocardial I/R and Drug Treatment

Mice (8 to 12 weeks) underwent coronary artery occlusion or sham surgery as previously described.[25] Briefly, mice were anesthetized with sodium pentobarbital (45 mg/kg) intraperitoneally, intubated, and ventilated with a rodent ventilator (Harvard Apparatus, St. Laurent, Canada). A midsternal thoracotomy was performed at the fourth intercostal space to expose the anterior surface of the heart. The proximal left anterior descending artery (LAD) was identified and a 6-0 silk Ethilon suture was placed around the artery and surrounding myocardium just below the atrioventricular border. Regional ischemia was induced for 30 minutes by tightening the suture against a small piece of PE-10 tubing placed on top of the LAD. Ischemia was confirmed by the discoloration of the myocardium. Sham-operated animals served as surgical controls and were subjected to the same surgical procedures as the experimental animals, with the exception that the LAD was not ligated. At the end of ischemia, the ligature was loosened and reperfusion was achieved. The lungs were reinflated and the muscle and skin layers were closed separately. The animals were weaned from the ventilator, extubated, and allowed to recover under a heat lamp before being returned to their cages. For animals receiving drug treatment, Bay-u9773 (0.25 mg/kg; Biomol Research Products, Plymouth Meeting, PA) was diluted in 1× phosphate-buffered saline (PBS) and injected intraperitoneally 4 hours before surgery, and 2, 8, and 16 hours after reperfusion. Surgical procedures and treatment regimens were approved by the University Animal Care Committee at Queen's University and adhered to the guidelines of the Canadian Council of Animal Care and the Guiding Principles in the Care and Use of Animals of the American Physiological Society.

Morphometric Evaluation of Risk Area and Infarction Size

Forty-eight hours after reperfusion, mice were euthanized by an intraperitoneal pentobarbital overdose. The 48-hour time point was selected because it is commonly used to assess early inflammatory events (eg, leukocyte infiltration, vascular leakage). The heart was exposed and the original suture was religated. The heart was then perfused retrogradely with 100 to 200 μl of 2% Evans blue dye in PBS (pH 7.4) to delineate the nonischemic area. The heart was excised and rinsed in ice-cold PBS and the LV, including the interventricular septum, was sectioned into four or five slices of similar thickness perpendicular to the long axis of the heart. The slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Chemicals, St. Louis, MO) at 37°C for 15 minutes to demarcate viable and necrotic tissue. The thickness of each slice was measured using calipers. The slices were photographed on both sides with a digital camera (Canon Corp., Tokyo, Japan). The infarct area (pale white), the area at risk (area excluding Evans Blue), and the total left ventricular area were traced and calculated for both sides of each slice using Image software (National Institutes of Health, Bethesda, MD). The areas for each slice were multiplied by the thickness of the slice to obtain a measure of volume. The cumulative volume for all sections for each heart was used for comparisons. The size

of LV at risk was calculated as the ratio of the LV volume excluding Evans blue dye to the total LV volume. Infarct size was calculated as the ratio of the infarct volume to the volume of the risk area as previously described.[26] Animals with infarct volume in the 35 to 70% range of total LV volume were used as inclusion criteria in the study.[27] Only one mouse was excluded based on these criteria.

Lactate Dehydrogenase (LDH) and Creatine Kinase (CK) Activity in Plasma

Biochemical analysis of myocardial injury was performed in heparinized arterial blood collected at termination of the experiment. Plasma LDH and CK were measured using an automated clinical analyzer at the Kingston General Hospital using clinical grade reagents.

Vascular Permeability Assay in Cardiac Tissue

Forty-eight hours after reperfusion, mice (8 to 12 weeks) were anesthetized by an intraperitoneal injection of pentobarbital (45 mg/kg). ^{125}I-albumin (10^6 cpm, 1.44 mCi/mg; MP Biomedicals, Inc., Mississauga, Canada) was injected into the right external jugular vein via a PE-10 catheter. Twenty minutes after injection, the mice were euthanized, and blood was obtained as above and weighed. Exsanguination and removal of excess ^{125}I-albumin proceeded via the right atrium. A 23-gauge needle was inserted into the apex of the left ventricle and the mouse was perfused retrogradely at 40 mmHg with 5.85 ml/100 g of 0.9% NaCl containing 100 U/ml heparin as described previously.[28] The LAD was then religated and Evans blue dye was perfused as above to delineate the risk area, which was then dissected from the remaining myocardial tissue, weighed, and placed in individual tubes. The radioactivity in the blood, nonrisk area, and risk area were counted separately using a gamma counter (Beckman Instruments, Irvine, CA). The permeability index of the different regions was calculated as the radioactivity per g of wet tissue divided by the radioactivity in 1 g of blood.[29] Sham-surgery controls were subjected to the same manipulations, with the exception that the ligature was not tied.

RNA Extraction and Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated from the risk area of the left ventricle 3 hours after reperfusion using Trizol reagent (Sigma). Total RNA was reverse-transcribed to cDNA using the Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For detection of mouse gene expression, quantitative real-time PCR was performed using a 7500 thermal cycler with TaqMan Universal PCR master mix and TaqMan gene expression assays (Egr-1, VCAM-1, and ICAM-1; Applied Biosystems, Foster City, CA) or with SYBR Green PCR master mix ($CysLT_1R$, and $CysLT_2R$) as described.[16] GAPDH was used as a control housekeeping gene. Data are calculated by the $2^{-\Delta\Delta CT}$ method and are presented as fold induction of transcripts for target genes normalized to GAPDH, with respect to the sham controls.[30]

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling (TUNEL) Staining

TUNEL assays were performed on LV samples with the CardioTACS *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD) as described by Takahashi and colleagues[31] with some modification. The hearts were arrested in diastole with 0.2 N KCl 48 hours after reperfusion and perfused with 3.7% neutralized formaldehyde solution. The heart was then excised, postfixed in the same fixative for another 12 hours, then cut into three sections corresponding approximately to the apex, mid-papillary, and base. The slices were embedded in paraffin, cut into 5-μm sections, and transferred to silicon-coated slides. High-power fields (12 to 20 at $\times$400 magnification) were obtained at the different levels to measure the number of TUNEL-positive cardiomyocyte nuclei in the peri-infarct border and uninfarcted remote zones, respectively. Only nuclei that were clearly located in cardiomyocytes were scored. The number of TUNEL-positive cardiomyocyte nuclei was divided by the total number of nuclei to determine the ratio of TUNEL-positive nuclei.

Immunohistochemical Staining

To determine the numbers of infiltrating leukocytes, formalin-fixed, paraffin-embedded 4-μm sections were mounted on silicon-coated slides and treated with 3% H_2O_2 to block endogenous peroxidase. The sections were incubated for 1 hour at room temperature with rat polyclonal anti-mouse CD45 antibody (PharMingen, San Diego, CA) at a dilution of 1:50. The sections were then incubated with biotinylated rabbit IgG (Vector Laboratories, Burlingame, CA), and CD45 immunoreactivity was visualized using diaminobenzidine substrate. The number of leukocytes in the boundary area was counted in 10 random high-power fields, and the average number in each group was calculated.[32] X-gal staining to determine endogenous $CysLT_2R$ expression based on the LacZ reporter gene was performed essentially as described.[33]

Echocardiography

Mice (8 to 12 weeks) underwent transthoracic echocardiography 1 day before and 2 weeks after acute I/R using a Phillips (Andover, MA) Sonos 5500 equipped with a 15-6L (15-6 MHz) intraoperative linear array transducer essentially as previously described.[34] The 2-week time point was chosen as one of the earliest time points to clearly define remodeling responses in rodents.[34] Briefly, in preparation for echocardiography, animals were lightly

anesthetized by halothane using a nose cone, shaved, and positioned on a heated pad in a recumbent position. Measurements were performed at the midpapillary level from well aligned M-mode images from the parasternal short-axis view. LVd (LV diastolic diameter), PWd (end-diastolic posterior wall thickness), and IVSd (interventricular septum thickness) were determined. The relative wall thickness for each level of the LV was calculated as (PWd + IVSd)/LVd. For each parameter, an average of five cardiac cycles was used for calculations.

Hemodynamic Measurements

Two weeks after acute I/R injury, mice were anesthetized with isoflurane (2%) in medical grade oxygen. The animals were then intubated and ventilated using a pressure controlled respirator (Kent Scientific Corp., Litchfield, CN) at a tidal volume of 200 μl and a frequency of 130 strokes/minute. Body temperature was monitored with a rectal thermometer and maintained at 37°C with the aid of a heat lamp. A midsternal thoracotomy was performed as above to expose the heart. The right jugular vein was cannulated for drug administration. A 1.4F ultra-miniature Millar catheter (SPR 839; Millar Instruments, Houston, TX) was placed into the left ventricle through the apex to record LV pressure. After recording steady-state LV pressures, mice were given an intravenous administration of the synthetic catecholamine dobutamine (10 ng/g body weight) to investigate the functional integrity of adrenergic signaling in the heart. The peak hemodynamic response was recorded using a data acquisition system (PVAN, Millar Instruments). The PVAN software was used for off-line calculation of LV peak systolic pressure, LV end-diastolic pressure, LV peak-positive developed pressure (dP/dt_{max}), LV peak-negative developed pressure (dP/dt_{min}), LV pressure at peak positive developed pressure ($P@dP/dt_{max}$), heart rate, and tau (τ) as described.[35] For calculation of hemodynamic parameters, a minimum of 50 consecutive cardiac cycles were used.

Statistical Analysis

All data are expressed as mean ± SEM. One-way analysis of variance followed by Student-Neuman-Keuls *t*-test were used to compare differences in risk area, infarct size, myocardial enzyme activities, and endothelial permeability, as well as differences in inflammatory gene expression and cardiomyocyte apoptosis. Unpaired *t*-test was used to compare differences in neutrophil infiltration and echocardiographic and functional parameters between tg and ntg mice. Paired *t*-test was used to compare before and after I/R changes in echocardiographic parameters and LV functional responses to dobutamine in the same animals. A P value <0.05 was considered to indicate statistical significance.

Results

CysLT Receptor Expression in Mouse Hearts

The expression of both native murine CysLT$_1$R and CysLT$_2$R was examined in hearts by real-time quantitative PCR as previously done in mouse ear tissue.[16] Gene expression for both CysLT receptors was low in noninfarcted ntg hearts and in infarcted hearts 3 hours after I/R injury (Figure 1). However, 48 hours after I/R injury CysLT$_1$R expression had increased 7.5-fold whereas CysLT$_2$R expression increased 3.5-fold. The human CysLT$_2$R transgene, using specific primers that can distinguish between species, could only be detected in tg mice. Using a second independent technique, we were also able to document elevation of CysLT$_2$R expression after 48 hours of I/R. Thus, using a novel mouse strain in which the *Cysltr2* gene is deleted and replaced with a LacZ reporter gene under control of the *Cysltr2* gene regulatory elements (S. Ishii et al, unpublished data) we were able to demonstrate sparse blue X-gal staining in noninfarcted ventricular tissue and 3 hours after I/R injury (Figure 1, B and C), consistent with the pattern observed previously by *in situ*

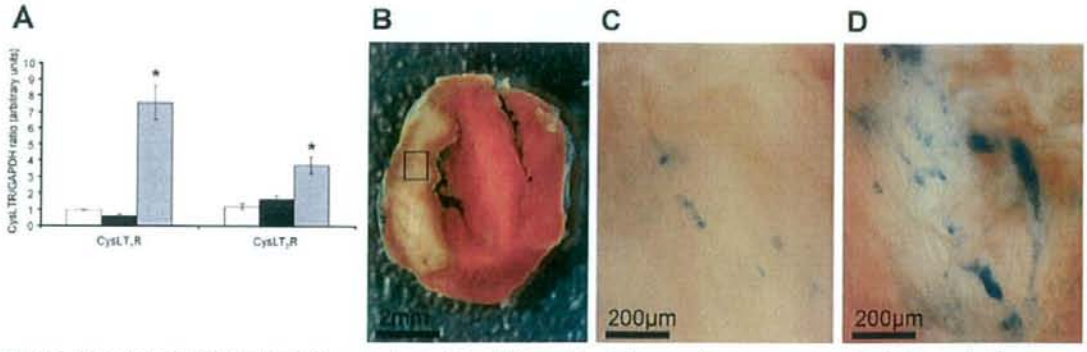

Figure 1. Expression of CysLT$_1$R and CysLT$_2$R in mouse hearts. **A:** Quantitative real-time PCR was used to assess gene expression for the two CysLT receptors relative to GAPDH expression in ntg mouse hearts ($n = 3$) as described in the Materials and Methods section. **Open bars**, sham-operated mice; **black bars**, hearts 3 hours after I/R; **gray bars**, hearts 48 hours after I/R. *$P < 0.05$ compared to sham-operated controls. **B:** TTC-stained heart slice from a CysLT$_2$R-deficient LacZ mouse 3 hours after I/R injury. **C:** Representative CysLT$_2$R expression in **boxed region** of the slice shown in **B** detected via the reporter gene LacZ with blue X-gal staining. **D:** CysLT$_2$R expression (via reporter LacZ/X-gal staining) in a heart slice from a CysLT$_2$R-deficient LacZ mouse having undergone 48 hours of I/R. Similar patterns of expression were observed in two additional mice at 3 and 48 hours after I/R.

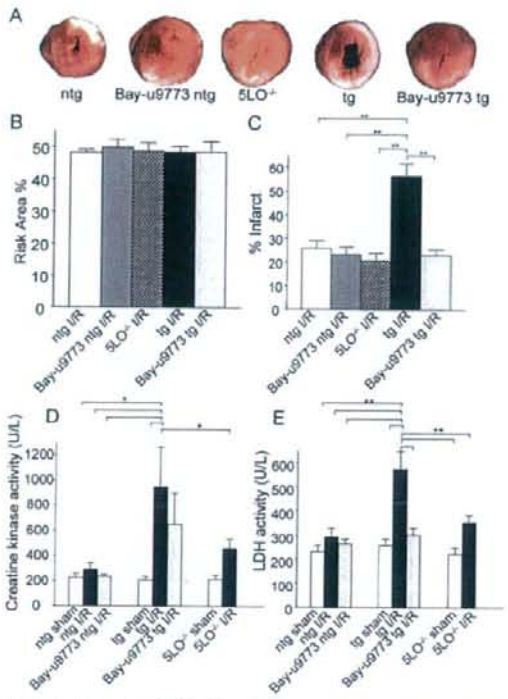

Figure 2. Effect of endothelial $CysLT_2R$ overexpression on LV infarct size after acute I/R injury. **A:** Representative TTC-stained ventricular sections from ntg, tg, and $5LO^{-/-}$ mice at 48 hours after I/R. Representative sections of ntg and tg mice treated with the nonselective dual $CysLT_1R/CysLT_2R$ receptor antagonist Bay-u9773 are also shown. **B:** Morphometric analysis of LV area at risk (**B**) and infarct size (**C**) in the five groups mentioned above. **D** and **E:** Serum levels of CK (**D**) and LDH (**E**) in sham and infarcted ntg, tg, and $5\text{-}LO^{-/-}$ mice at 48 hours after reperfusion. $^*P < 0.05$; $^{**}P < 0.01$; $n = 8$ for groups in **A–C**; $n = 6$ for groups in **D** and **E**.

hybridization in normal mouse heart.[15] After 48 hours of I/R injury, staining intensity increased in the infarct and peri-infarct zones (Figure 1D), which was in harmony with the PCR data.

Endothelial $CysLT_2R$ Overexpression Increases Myocardial Infarct Size after LAD Occlusion and Reperfusion

The effect of endothelial overexpression of $CysLT_2R$ on myocardial I/R injury is shown in Figure 2. Gross histological analysis of TTC-stained sections 48 hours after reperfusion showed a larger necrotic area in tg animals compared to ntg littermates and 5-lipoxygenase-null $5LO^{-/-}$ mice (Figure 2A). Histomorphometric analysis revealed that infarct size in $CysLT_2R$ tg mice was increased by 114% relative to ntg mice (56 ± 15% versus 26 ± 9%, $n = 8$, $P < 0.01$) (Figure 2C), despite comparable risk area in all groups (Figure 2B). Infarct size in the $5LO^{-/-}$ null mice was comparable to ntg mice (21 ± 9% versus 26 ± 9%). Treatment of tg mice with the nonselective dual $CysLT_1R/CysLT_2R$ antagonist Bay-u9773, at a dose tested empirically to evoke $CysLT_2R$ antagonism, markedly reduced infarct size by nearly 60% (56% versus 23%, $n = 8$, $P < 0.05$) to levels comparable to ntg and $5LO^{-/-}$ mice (Figure 2, A and C). The antagonist had no additional effect on infarct size in ntg mice.

We measured serum levels of CK and LDH 48 hours after reperfusion. CK (Figure 2D) and LDH (Figure 2E) activities in infarcted ntg mice were increased by ~26% compared to the baseline levels in sham-operated controls. In contrast, CK and LDH levels were markedly elevated by 357% and 123%, respectively, in tg mice subjected to I/R compared to tg sham controls (Figure 2, D and E). Compared to ntg I/R mice, the levels of CK and LDH were elevated by ~230% and 100%, respectively, in tg mice. In concordance with the histopathological findings, treatment with Bay-u9773 reduced levels of CK and LDH after reperfusion in the tg animals (Figure 2, D and E), while having no significant effect on these markers in ntg mice. I/R increased the levels of CK and LDH in $5LO^{-/-}$ mice but this was significantly smaller than in tg mice (Figure 2, D and E).

Endothelial $CysLT_2R$ Overexpression Increases Permeability in the Infarcted Region of Transgenic Mouse Hearts

Previously, we detected enhanced vascular permeability responses to leukotriene challenge and passive cutaneous anaphylaxis in mouse ear vasculature of tg mice.[16] To examine if similar vascular responses occur in the coronary endothelium after myocardial I/R, we assessed the histopathology of the infarct. In addition, we measured extravasation of ^{125}I-BSA in the ischemic and remote areas of the left ventricle at 48 hours after reperfusion. Microscopic examination of the infarct in hematoxylin and eosin (H&E)-stained sections showed minimal accumulation of erythrocytes in the infarcted region of ntg mice (Figure 3A). In contrast, tg mice presented significant accumulation of red cells in the interstitium, resulting in hemorrhage of the infarcted area (Figure 3B). Basal coronary endothelial permeability to ^{125}I-BSA did not differ significantly between ntg and tg mice (Figure 3C). I/R injury led to significant interstitial accumulation of ^{125}I-BSA in both ntg and tg mice. However, the increase in coronary circulation permeability was more pronounced in tg versus ntg mice (202% versus 93%, Figure 3C). No differences in permeability were seen in the nonischemic region of the myocardium.

Endothelial $CysLT_2R$ Overexpression Increases $CD45^+$ Leukocyte Infiltration after I/R in Transgenic Mouse Hearts

We used immunostaining of the pan leukocyte cell surface marker CD45 to determine whether the enhanced permeability of coronary endothelium leads to increased leukocyte infiltration of the infarcted region after I/R injury. Figure 4A shows representative cross-sections from the peri-infarct region in ntg and tg mice. The tg mice showed greater density of CD45-positive cells than ntg mice. Morphometric analysis showed

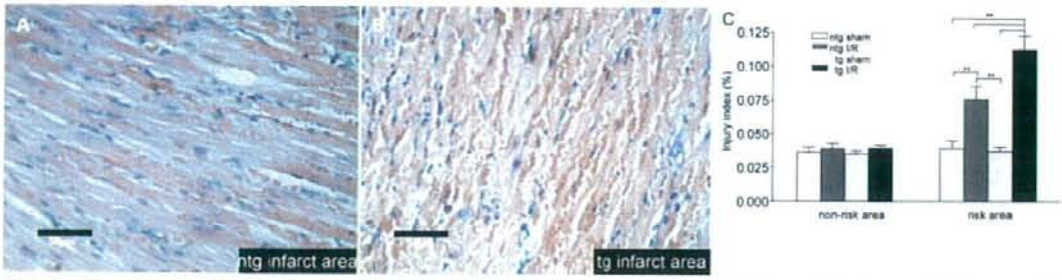

Figure 3. Effect of endothelial CysLT$_2$R overexpression on myocardial histopathology and coronary endothelial permeability after acute I/R injury. **A** and **B:** Microscopic appearance of infarcted left ventricle in H&E-stained paraffin sections from ntg (**A**) and tg (**B**) mice 48 hours after acute I/R injury. **C:** Permeability of coronary endothelium to ^{125}I-BSA in the at-risk and nonrisk regions of the left ventricle of ntg and tg mice. $^{**}P < 0.01$; $n = 6$. Scale bars = 50 μm.

>100% increase in the number of infiltrating leukocytes in tg compared to ntg mice (1289 ± 113/mm^2 versus 528 ± 131/mm^2) (Figure 4).

Endothelial CysLT$_2$R Overexpression Increases Egr-1, ICAM, and VCAM-1 Gene Expression in Transgenic Mouse Hearts

To examine potential molecular correlates for the I/R-induced histopathological and permeability alterations seen in tg mice, we determined myocardial mRNA expression of adhesion molecules ICAM and VCAM-1, as well as Egr-1 transcription factor (Figure 5). These genes have been implicated in the myocardial inflammatory response to I/R injury. No significant genotype-related differences were seen in basal expression of these genes. Myocardial expression of ICAM (Figure 5A), VCAM-1 (Figure 5B), and Egr-1 (Figure 5C) were increased significantly in both ntg and tg mice 3 hours after reperfusion. However, the I/R-induced increase in expression of these genes was greater in the tg mice (Figure 5).

Endothelial CysLT$_2$R Overexpression Increases Cardiomyocyte Apoptosis in Transgenic Mouse Hearts

Because apoptosis plays a central role in myocardial cell loss after I/R, we determined whether endothelial overexpression of CysLT$_2$R influences the number of apoptotic nuclei in cardiomyocytes in the peri-infarct region of tg and ntg mice after I/R. We found increased apoptosis of cells with cardiomyocyte morphology in both groups at 48 hours after reperfusion (Figure 6, A and B). However, the number of apoptotic nuclei in the peri-infarct region of tg animals was significantly greater than in ntg animals (641 ± 222 TUNEL-positive myocytes/10^4 nuclei versus 84 ± 21/10^4 nuclei) (Figure 6B). At 48 hours after reperfusion, cardiomyocyte apoptosis was confined primarily to the peri-infarct region, although at earlier time points (ie, 6 to 24 hours after reperfusion), apoptosis is typically elevated in the infarct core. The number of apoptotic nuclei in the noninfarcted region was markedly lower than in the peri-infarct region and did not differ between ntg and tg mice (Figure 6B). It should be noted that apoptotic nuclei in noncardiomyocytes were observed; however, the precise cell types were not identified nor were they quantified in the present studies.

Endothelial CysLT$_2$R Overexpression Accelerates Left Ventricular Remodeling after I/R

We used two-dimensional echocardiography to examine early (2 week) changes in LV wall and chamber dimensions after I/R. We chose the I/R model of myocardial infarction because it recapitulates some of the features of pathology after infarction seen in humans with reperfused MI, namely slow-developing LV remodeling that is generally complete by 3 to 6 weeks in rodents.[36,37] Repre-

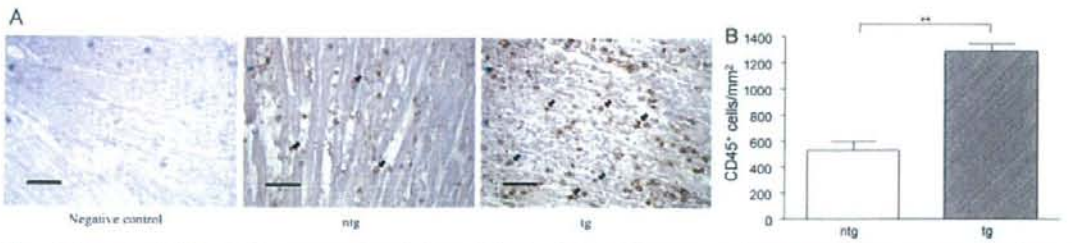

Figure 4. Effect of endothelial CysLT$_2$R overexpression on leukocyte infiltration after I/R. **A:** Representative photomicrographs showing immunohistochemical detection of pan-leukocyte cell surface marker CD45 in the peri-infarct region of the LV in ntg and tg mice at 48 hours after reperfusion. **Arrows** indicate CD45-positive cells. **B:** Quantitative morphometric analysis of leukocyte infiltration. $^{**}P < 0.01$; $n = 4$. Scale bars = 50 μm. Original magnifications, ×400.

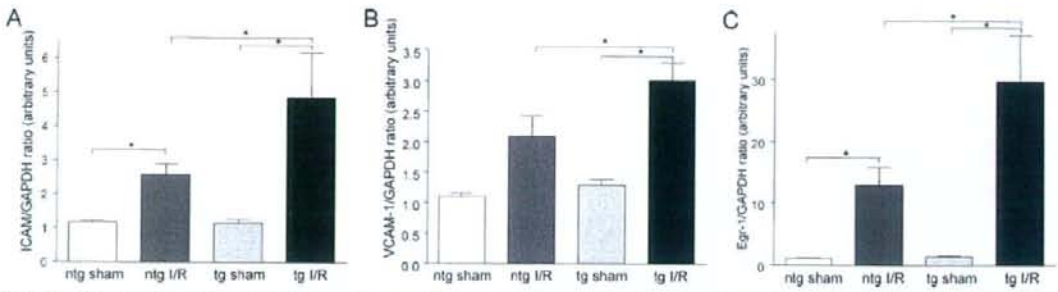

Figure 5. Effect of endothelial $CysLT_2R$ overexpression on proinflammatory gene expression. **A–C:** Quantitative real-time PCR evaluation of ICAM (**A**), VCAM-1 (**B**), and Egr-1 (**C**) gene expression in total RNA extracted from the ischemic area of mouse hearts at 3 hours after reperfusion. $^*P < 0.05$; $n = 3$.

sentative M-mode frames taken before and 2 weeks after acute I/R injury are shown in Figure 7, and echocardiographic data are summarized in Table 1. Pre-I/R wall and chamber dimensions did not differ significantly between ntg and tg mice, with the exception of left ventricular diastolic dimension (LVDd) that was found to be slightly increased in tg mice (Figure 7, A and C; Table 1). Two weeks after reperfusion the tg mice presented significant thinning of the anterior wall/interventricular septum, whereas the anterior wall remained relatively unchanged in ntg mice (Figure 7, B and D; Table 1). Systolic and diastolic LV chamber dimensions after infarction remained relatively unchanged from preinfarction values in ntg mice (Figure 7, C and D; Table 1). However, tg mice showed a trend toward greater LV systolic dimension after infarction than ntg mice (21% versus 12% increase with respect to preinfarction values; Figure 7, A and B, and Table 1).

Endothelial $CysLT_2R$ Overexpression Impairs Left Ventricular Function after I/R

We also assessed the effect of endothelial $CysLT_2R$ overexpression on LV function using a microtip pressure catheter (Table 2). Basal LV function did not differ significantly between ntg and tg mice. Furthermore, both types of mice responded comparably to an acute bolus injection of dobutamine with increases in heart rate, LV pressures, and maximal and minimal values of the first derivative of LV pressure (Table 2). Two weeks after I/R, function remained relatively unchanged in ntg mice. In contrast, tg animals showed a trend toward decreased LV +dP/dt and LV −dP/dt and a significant increase in the time constant of isovolumic relaxation (τ), indicating the presence of both systolic and diastolic dysfunction (Table 2). Interestingly, both genotypes showed refractoriness of heart rate and LV pressures to dobutamine after infarction.

Discussion

The endothelium plays a pivotal role in maintaining vessel homeostasis by elaborating a variety of vasoactive, anti-inflammatory and antithrombotic factors that help maintain coronary vessel tone and protect the vessel wall against inflammatory cell and platelet adhesion.[38] Endothelial dysfunction plays a central role in the pathogenesis of myocardial I/R injury[1,5,16] and is characterized by impaired vessel relaxation, and enhanced expression of

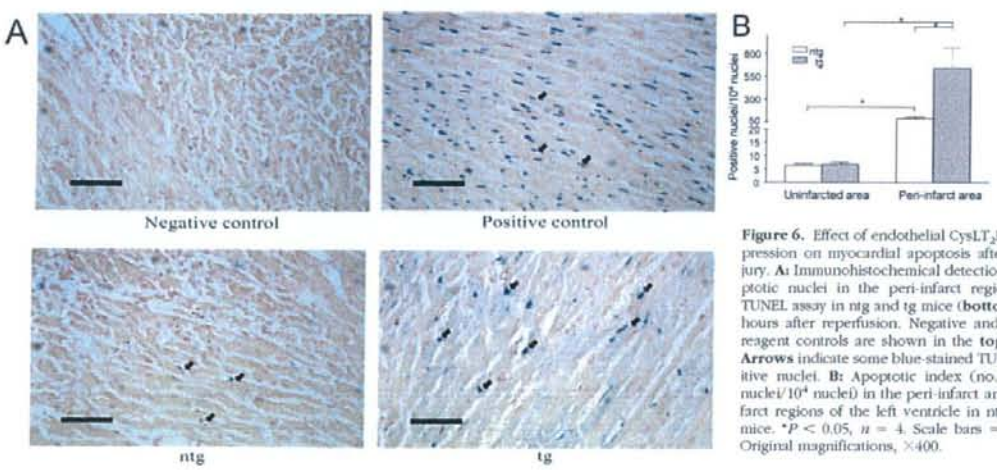

Figure 6. Effect of endothelial $CysLT_2R$ overexpression on myocardial apoptosis after I/R injury. **A:** Immunohistochemical detection of apoptotic nuclei in the peri-infarct region using TUNEL assay in ntg and tg mice (**bottom**) at 48 hours after reperfusion. Negative and positive reagent controls are shown in the **top** panels. **Arrows** indicate some blue-stained TUNEL positive nuclei. **B:** Apoptotic index (no. positive nuclei/10^4 nuclei) in the peri-infarct and noninfarct regions of the left ventricle in ntg and tg mice. $^*P < 0.05$, $n = 4$. Scale bars = 50 μm. Original magnifications, ×400.