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# Membrane-Bound Prostaglandin E Synthase-1-Mediated Prostaglandin $E_2$ Production by Osteoblast Plays a Critical Role in Lipopolysaccharide-Induced Bone Loss Associated with Inflammation

**Masaki Inada,* Chiho Matsumoto,* Satoshi Uematsu,† Shizuo Akira,† and Chisato Miyaura[1]***

**$PGE_2$ acts as a potent stimulator of bone resorption in several disorders including osteoarthritis and periodontitis. Three PGE synthases (PGES) were isolated for $PGE_2$ production, but which PGES has the major role in inflammatory bone resorption is still unclear. In this study, we examined the role of $PGE_2$ in LPS-induced bone resorption using membrane-bound PGES (mPGES)-1-deficient mice (*mPges*$1^{-/-}$). In osteoblasts from wild-type mice, $PGE_2$ production was greatly stimulated by LPS following the expression of cyclooxygenase 2 and mPGES-1 mRNA, whereas no $PGE_2$ production was found in osteoblasts from *mPges*$1^{-/-}$. LPS administration reduced the bone volume in wild-type femur that was associated with an increased number of osteoclasts. In *mPges*$1^{-/-}$, however, LPS-induced bone loss was reduced. We next examined whether mPGES-1 deficiency could alter the alveolar bone loss in LPS-induced experimental periodontitis. LPS was injected into the lower gingiva and bone mineral density of alveolar bone was measured. LPS induced the loss of alveolar bone in wild-type, but not in *mPges*$1^{-/-}$ mice, suggesting an mPGES-1 deficiency resistant to LPS-induced periodontal bone resorption. To understand the pathway of LPS-induced $PGE_2$ production in osteoblast, we used C3H/HeJ mice with mutated *tlr*4. Osteoblasts from C3H/HeJ mice did not respond to LPS, and $PGE_2$ production was not altered at all. LPS-induced bone loss in the femur was also impaired in C3H/HeJ mice. Thus, LPS binds to TLR4 on osteoblasts that directly induce mPGES-1 expression for $PGE_2$ synthesis, leading to subsequent bone resorption. Therefore, mPGES-1 may provide a new target for the treatment of inflammatory bone disease.** ***The Journal of Immunology,*** **2006, 177: 1879–1885.**

The lipid mediator $PGE_2$ is produced by various cells and plays an important role in a wide range of physiological and pathological processes. It is known that $PGE_2$ is mainly produced by osteoblasts and acts as a potent stimulator of bone resorption (1–3). Inflammatory stimulants, such as IL-1 and IL-6, induce $PGE_2$ production by osteoblasts. $PGE_2$ stimulates adenylate cyclase through its receptor EP4, and accumulates cellular cAMP in osteoblasts, which induces the expression of a receptor activator of NF-κB (RANK)[2] ligand (RANKL), a critical molecule for osteoclast differentiation (4, 5). It is well accepted that osteoblasts express RANKL on their cell surface and that osteoclast precursors possess RANK, a receptor for RANKL (6–9). Recognition among RANK-RANKL induces the differentiation of osteoclast precursors into osteoclasts. Therefore, $PGE_2$ produced by osteoblasts supports osteoclast formation and may play a key role in the bone resorption associated with inflammation.

$PGE_2$ synthesis is regulated by three metabolic steps: the release of arachidonic acid from the membranous phospholipids by phospholipase $A_2$ ($PLA_2$), the conversion of arachidonic acid to $PGH_2$ by cyclooxygenase (COX), and the synthesis of $PGE_2$ by PGE synthase (PGES) (10–12). We have reported that the purpose of cytosolic $PLA_2$ ($cPLA_2$) expression in osteoblasts is to release arachidonic acid following $PGE_2$ production (13, 14). Two isoforms of the COX enzyme, COX-1 and COX-2, have been identified, and previous studies have shown that COX-1 is a constitutive enzyme and COX-2 is an enzyme induced by various stimuli. In osteoblasts, the expression of COX-2, but not COX-1, is markedly induced by IL-1 and LPS (13). The terminal step for $PGE_2$ synthesis is catalyzed by PGES for the conversion of $PGH_2$ to $PGE_2$. Recent studies have identified three forms of PGES, cytosolic PGES, membrane-bound PGES (mPGES)-1, and mPGES-2 (12, 15–17). The expression of cytosolic PGES is constitutively detected in a wide variety of cells and the enzyme is functionally linked to COX-1 to promote immediate $PGE_2$ production. In contrast, mPGES-1 is markedly induced by inflammatory stimuli, and functionally coupled with COX-2 for the accumulation of $PGE_2$ in the stage of inflammation. The mPGES-2 enzyme is constitutively expressed in a variety of cells and is functionally coupled with both COX-1 and COX-2. Thus, it is unlikely mPGES-2 responds to acute inflammatory stimulation. Recent studies have shown that IL-1 and LPS, an inducer of $PGE_2$, stimulate the mRNA expression of mPGES-1, but not cytosolic PGES, in osteoblasts (14, 18). Therefore, the coordinate induction of COX-2 and mPGES-1 may be essential for $PGE_2$ production by osteoblasts treated with inflammatory stimulants. Nevertheless, the in vivo functional role of mPGES-1 in inflammatory bone resorption is still not known.

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[2] Abbreviations used in this paper: RANK, receptor activator of NF-κB; RANKL, RANK ligand; $PLA_2$, phospholipase $A_2$; $cPLA_2$, cytosolic $PLA_2$; PGES, PGE synthase; mPGES, membrane-bound PGES; COX, cyclooxygenase; TRAP, tartrate-resistant acid phosphatase; BMD, bone mineral density; BV, bone volume; TV, tissue volume; TRAF, TNFR-associated factor.



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To clarify the role of mPGES-1 in PGE production and its biological significance, we generated mPGES-1-deficient mice (*mPges1*$^{-/-}$). The *mPges1*$^{-/-}$ mice are fertile and develop normally from birth to adulthood. A report has shown the critical role of mPGES-1 in LPS-induced $PGE_2$ production in macrophages, although the mice were not impaired in terms of LPS-induced cytokine production and LPS-induced endotoxin-shock (19). However, it is not known in bone whether mPGES-1-mediated $PGE_2$ production has a major role in LPS-induced bone resorption.

LPS is a structural component of the outer membrane in bacteria and is known to stimulate the production of inflammatory cytokines by monocyte/macrophages. LPS is a known pathogen for periodontitis, an infectious disease of mixed Gram-negative bacteria that is associated with bone resorption. Recent studies have shown that LPS signaling in target cells is mediated by TLRs. TLRs play a critical role in innate immune responses in mammals, and 11 members of the TLR family (TLR1–TLR11) have been identified (20). The TLRs are thought to be receptors for recognition of various ligands, including bacterial components and nucleic acid that are involved in the host defense mechanisms against pathogens (20). Previous studies have indicated that TLR2 is involved in LPS signaling (21, 22), but recent studies have shown TLR4 is the most essential LPS receptor for the signal transduction (23). Not only immune systems, but also infectious diseases such as periodontitis, are thought to be involved in LPS-induced inflammation and the ensuing tissue destruction (24).

In the present study, we demonstrated the role of mPGES-1-mediated $PGE_2$ production in LPS-induced bone destruction in femur and mandibular alveolar bone using *mPges1*$^{-/-}$ mice. To further our understanding of the pathway of LPS-induced $PGE_2$ production, C3H/HeJ mice with mutated *tlr4* genes were also examined.

## Materials and Methods

### *Animals and reagents*

Newborn and 6-wk-old mice of *ddy*, C3H/HeN, and C3H/HeJ strains were obtained from Japan SLC. The *mPges1*$^{-/-}$ mice and littermate wild-type mice were established by gene targeting as previously described (19). All procedures were performed in accordance with institutional guidelines for animal research. LPS (*Escherichia coli* O55:B5) was purchased from Difco. $PGE_2$ was obtained from Sigma-Aldrich.

### *Culture of primary mouse osteoblastic cells*

Primary osteoblastic cells were isolated from newborn mouse calvariae after five routine sequential digestions with 0.1% collagenase (Wako Pure Chemical) and 0.2% dispase (Godo Shusei) as previously described (14). Osteoblastic cells collected from fractions 2–4 were combined and cultured in $\alpha$-modified MEM ($\alpha$MEM) supplemented with 10% FCS at 37°C under 5% $CO_2$ in air. Osteoblastic cells were cultured for 24 h in $\alpha$MEM containing 1% FCS, then treated with LPS.

### *Measurement of $PGE_2$ content*

The concentrations of $PGE_2$ in the cultured medium were determined using an enzyme immunoassay (Amersham Biosciences). The Ab had the following cross-reactivity when calculated using the bound to free ratio: $PGE_2$, 100%; $PGE_1$, 7.0%; 6-keto-$PGF_{1\alpha}$, 5.4%; $PGF_{2\alpha}$, 4.3%; and $PGD_2$, 1.0%.

### *Northern blot analysis*

Total RNA was extracted from the cultured mouse osteoblastic cells using the acid guanidium-phenol-chloroform method (14). For Northern blotting, 20 $\mu$g of total RNA was resolved using electrophoresis on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane, which was then hybridized with a $^{32}$P-labeled cDNA probe as reported previously (5). Mouse COX-1 and COX-2 cDNA probes were purchased from Oxford Biomedical Research. A 983-bp fragment of mouse G3PDH cDNA was prepared using RT-PCR and used as the probe (5).

### *RT-PCR analysis*

cDNA was synthesized from 10 $\mu$g of total RNA by reverse transcriptase (Superscript II Preamplification System; Invitrogen Life Technologies) and amplified using PCR. The primers used in PCR for the mouse mPGES-1 gene were sense 5′-ATG CCT TCC CCG GGC CTG-3′ and antisense 5′-TCA CAG ATG GTG GGC CAC-3′. The reaction condition for PCR (mouse mPGES-1) was 25 cycles, denaturation at 94°C for 45 s, annealing at 51°C for 45 s, and extension at 72°C for 2 min. The primers used in PCR for the mouse cytosolic PGES gene were sense 5′-AGG AAG CGA TAA TTT TAA GC-3′ and antisense 5′-ACC CAT GTG ATC CAT CAT CTC-3′. The reaction conditions for PCR (mouse cytosolic PGES) were 25 cycles, denaturation at 94°C for 45 s, annealing at 64°C for 45 s, and extension at 72°C for 2 min. The PCR product was run on a 1% agarose gel and stained with ethidium bromide.

### *Bone-resorbing activity in organ culture of mandibular alveolar bone*

Mandibular alveolar bone was collected from mouse lower gingiva under a microscope and cultured for 24 h in BGJb containing 1 mg/ml BSA. After 24 h, alveolar bone was transferred into new medium, with or without LPS, and cultured for 5 days at 37°C under 5% $CO_2$ in air. The bone-resorbing activity was determined by measuring the concentration of calcium in the conditioned medium using a calcium kit (Calcium C test; Wako Pure Chemical) as reported previously (5). The bone-resorbing activity was expressed as an increase in medium calcium, which is consistent with the osteoclastic bone resorption as shown in the previous studies (5, 25).

### *Treatment with LPS in mice*

Six-week-old mice were i.p. injected with LPS (5 mg/kg body weight) on days 0 and 4. The LPS was dissolved in PBS for injection. The mice in the control group were injected with PBS. On day 8 after the first injection of LPS or PBS, the femurs were collected. As a model for experimental periodontitis, LPS (25 $\mu$g) was injected into the mouse lower gingiva on days 0, 2, and 4. As the control, PBS was injected into the lower gingiva at each time point. The mandibular alveolar bone was collected on 7 days after the first injection.

### *Radiographic analysis of the femur*

The bone mineral density (BMD) of mandibular alveolar bone and femurs was measured by dual x-ray absorptiometry (model DCS-600R; Aloka) as reported previously (14). The bone mineral content of the femurs was closely correlated with the ash weight (14). The BMD was calculated by dividing the bone mineral content of the measured area by the area.

### *Histological analysis of the femoral cancellous bone*

The distal metaphysis of the femur was fixed with 70% ethanol and embedded in glycol methacrylate, and undecalcified 3-$\mu$m sections were prepared and stained for tartrate-resistant acid phosphatase (TRAP) as reported previously (14). The trabecular bone density (bone volume (BV)/tissue volume (TV)), the mean number of osteoclasts in each millimeter of the trabecular bone surface (osteoclast number (Oc no.)/bone surface (BS), per millimeter), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th) were determined in the cancellous bone tissue at the secondary spongiosa of the distal metaphysis.

### *Statistical analysis*

The data are expressed as the mean ± SEM. The significant differences were analyzed using Student's *t* test.

## Results

### *LPS induces COX-2 and mPGES-1 mRNAs in mouse osteoblasts*

We first examined the effects of LPS on the mRNA expression of COX-1, COX-2, cytosolic PGES, and mPGES-1 in cultured mouse osteoblasts collected from *ddy* mice by Northern blotting and RT-PCR analysis (Fig. 1, *A* and *B*). LPS markedly induced the mRNA expression of COX-2 and mPGES-1 in osteoblasts at 3–12 h, and the peak of mRNA expression was 3 h in COX-2 and 12 h in mPGES-1, respectively. The expression of COX-1 and cytosolic PGES mRNAs was detected in osteoblasts and not affected by LPS until 24 h. When the level of $PGE_2$ in the conditioned medium was measured at 24 h, we detected the marked elevation of $PGE_2$ in the conditioned medium (Fig. 1*C*). We also examined the effects of

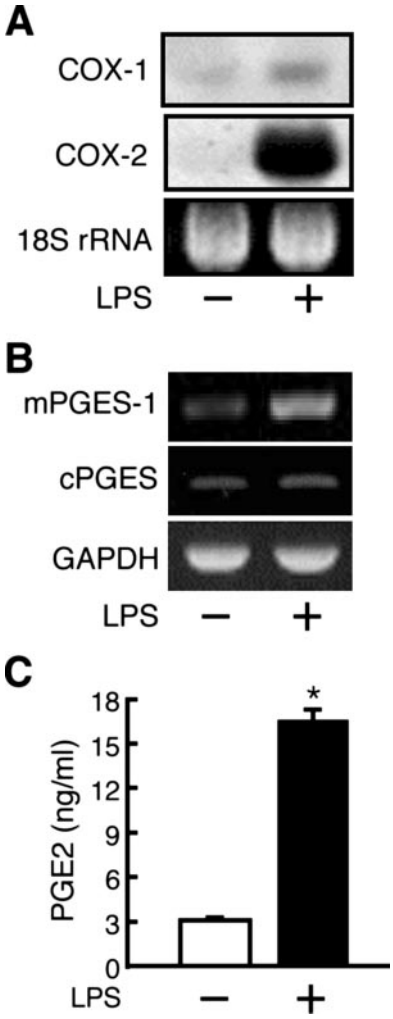


**FIGURE 1.** Effects of LPS on $PGE_2$ production and mRNA expression of COX-1, COX-2, mPGES-1, and cytosolic PGES in osteoblastic cells collected from *ddy* mice. *A*, Osteoblastic cells were cultured for 3 h with (+) or without (−) 100 ng/ml LPS, and total RNA was extracted. Northern blotting was performed using $^{32}P$-labeled cDNA probes for COX-1 and COX-2. *B*, Osteoblastic cells were cultured for 12 h with or without 100 ng/ml LPS, and the total RNA was extracted. The expression of mPGES-1, cytosolic PGES (cPGES), and GAPDH mRNA was analyzed by RT-PCR as described in *Materials and Methods*. *C*, Osteoblastic cells were cultured for 24 h with or without 100 ng/ml LPS. The concentration of $PGE_2$ in the cultured medium was determined using an enzyme immunoassay. Data are expressed as the mean ± SEM of three-wells. Significantly different (*, $p < 0.001$) from control without LPS.

LPS on osteoclast formation and $PGE_2$ production in cocultures of mouse osteoblasts and bone marrow cells. Adding LPS to the cocultures induced the production of $PGE_2$ and formation of osteoclasts, and the osteoclast formation was completely suppressed by adding indomethacin, an inhibitor of PG synthesis (data not shown).

### *Contribution of mPGES-1 to $PGE_2$ synthesis by osteoblastic bone marrow stromal cells*

We first observed that *mPges1*$^{-/-}$ mice were born phenotypically normal, and the growth of the mice was similar to that of wild-type siblings. To examine $PGE_2$ synthesis by osteoblastic cells, the bone marrow cells were collected from *mPges1*$^{-/-}$ and wild-type mice and cultured for 10 days to obtain the osteoblastic stromal cells. When the osteoblastic cells derived from wild-type mice were treated with LPS, the expression of COX-2 and mPGES-1 mRNAs was markedly induced (Fig. 2*A*). In the cells collected from *mPges1*$^{-/-}$ mice, the expression of COX-2 was elevated by adding LPS, but the mRNA of mPGES-1 could not be detected (Fig. 2*A*). The mRNA expression of COX-1, cytosolic PGES, and GAPDH was similarly detected and not affected by LPS in all conditions. In wild-type mice, the addition of LPS to osteoblastic cells markedly induced the production of $PGE_2$. In *mPges1*$^{-/-}$ mice, however, the LPS-induced $PGE_2$ synthesis could not be detected in osteoblastic stromal cells (Fig. 2*B*). These results indicate that the impaired $PGE_2$ production in *mPges1*$^{-/-}$ mice is due to the lack of conversion of $PGH_2$ to $PGE_2$.

### *Role of mPGES-1 in LPS-induced bone loss with increased bone resorption*

We have reported that LPS administration could induce the severe loss of trabecular bone in distal femoral metaphysis in mice (14). To determine the influence of mPGES-1-dependent $PGE_2$ synthesis in bone loss induced by LPS in vivo, *mPges1*$^{-/-}$ and wild-type mice were injected with LPS and the femurs were collected on day 8 for histological analysis. The sections of the distal femoral metaphysis were prepared and stained for TRAP (Fig. 3*A*). In wild-type mice, the bone density (BV/TV) of the trabecular bone and the trabecular bone thickness (Tb.Th) were significantly reduced, whereas the trabecular bone separation (Tb.Sp) increased with the

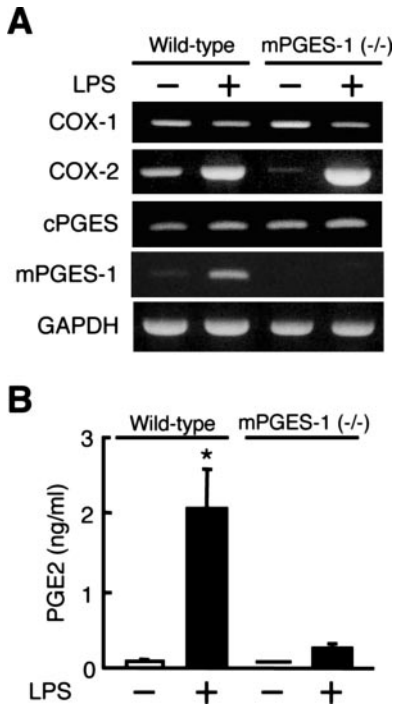


**FIGURE 2.** Effects of LPS on $PGE_2$ production and mRNA expression of COX-1, COX-2, cytosolic PGES, and mPGES-1 in osteoblastic stromal cells collected from *mPges1*$^{-/-}$ and wild-type mice. *A*, Osteoblastic cells were collected from *mPges1*$^{-/-}$ and wild-type mice and cultured for 3 h with (+) or without (−) 10 ng/ml LPS, and the expression of COX-1, COX-2, and GAPDH mRNA was analyzed by RT-PCR. To detect cytosolic PGES (cPGES) and mPGES-1 mRNA by RT-PCR, osteoblastic cells were collected from *mPges1*$^{-/-}$ and wild-type mice and cultured for 12 h with or without 10 ng/ml LPS. *B*, Osteoblastic cells were collected from *mPges1*$^{-/-}$ and wild-type mice, and cultured for 24 h with or without 10 ng/ml LPS. The concentration of $PGE_2$ in the cultured medium was determined using an enzyme immunoassay. Data are expressed as the mean ± SEM of three-wells. Significantly different (*, $p < 0.001$) from control without LPS.

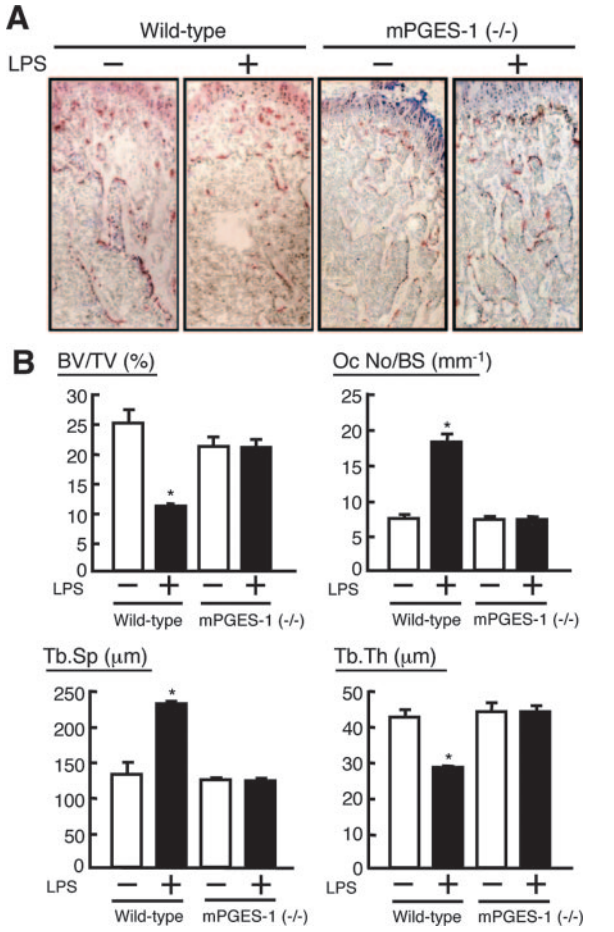


**FIGURE 3.** Histological analyses of the femoral trabecular bone collected from *mPges1*$^{-/-}$ and wild-type mice injected with LPS. Mice were injected with (+) LPS or PBS (−) i.p. on days 0 and 4, and the femurs were collected on day 8 after the first injection to prepare the sections of femoral distal metaphysis. *A*, The sections of trabecular bone were stained for TRAP to detect osteoclasts. *B*, Using the TRAP-stained sections, histomorphometric analyses were performed to calculate bone density (BV/TV), mean number of osteoclasts in each millimeter of the trabecular bone surface (Oc no./BS), trabecular separation (Tb.Sp), and trabecular thickness (Tb.Th) as described in *Materials and Methods*. Significantly different (*, $p < 0.001$) from the control mice injected with PBS in *mPges1*$^{-/-}$ and wild-type mice, respectively. Data are expressed as the mean ± SEM of three to five mice.

LPS administration (Fig. 3*B*). The increase in the trabecular bone separation indicates that the osteoclastic bone resorption was stimulated, resulting in enhanced intertrabecular space. In fact, the number of TRAP-positive multinucleated osteoclasts was significantly increased in the trabecular bone obtained from wild-type mice treated with LPS in vivo (Fig. 3). In *mPges1*$^{-/-}$, however, the LPS administration did not influence the bone density, trabecular bone separation and thickness, and the number of osteoclasts at all (Fig. 3). Therefore, mPGES-1 is essential for LPS-induced osteoclastic bone resorption, which results in bone loss associated with inflammation.

### *Influence of mPGES-1-mediated PGE synthesis in a new model for periodontitis*

Periodontal disease, such as periodontitis is a typical bone disease accompanying inflammation and loss of alveolar bone. To examine the influence of mPGES-1-dependent PGE synthesis in the pathogenesis of periodontitis, we developed a new experimental model for periodontitis in vitro and in vivo. Mandibular alveolar bone was collected from mice (Fig. 4*A*), and cultured with or without LPS to detect bone resorption. We detected the increase in bone-resorbing activity in LPS-treated alveolar bone collected from wild-type mice (Fig. 4*B*). Alveolar bone collected from *mPges1*$^{-/-}$ mice did not respond to LPS, indicating that mPGES-1-dependent $PGE_2$ synthesis is critical for the bone resorption induced by LPS in mandibular alveolar bone (Fig. 4*B*). In in vivo experiments, LPS was injected into the gingiva in the lower mandible, and the alveolar bone was collected from mice on day 7 for the measurement of BMD by dual x-ray absorptiometry. LPS administration induced a significant decrease in BMD of mandibular alveolar bone in wild-type mice (Fig. 4*C*). In *mPges1*$^{-/-}$ mice,

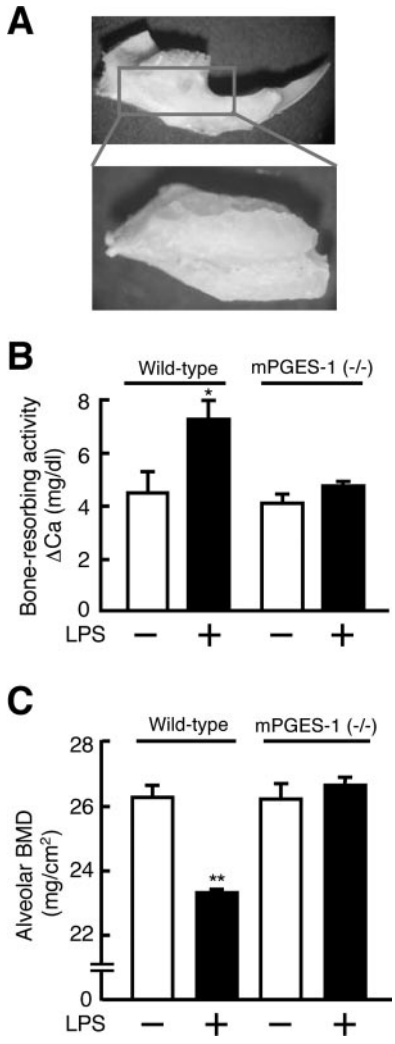


**FIGURE 4.** Lack of LPS-induced bone loss of mandibular alveolar bone in *mPges1*$^{-/-}$ mice. *A*, Alveolar bone was collected from mouse lower mandible. *B*, Alveolar bone was collected from *mPges1*$^{-/-}$ and wild-type mice, and cultured for 5 days with (+) or without (−) 100 ng/ml LPS to detect bone resorption. The bone-resorbing activity was measured by medium calcium, as described in *Materials and Methods*. Significantly different (*, $p < 0.05$) from the control without LPS. Data are expressed as the mean ± SEM of three cultures. *C*, LPS or PBS was injected into the gingiva in the lower mandible of *mPges1*$^{-/-}$ and wild-type mice, as described in *Materials and Methods*, and the alveolar bone was collected from respective mice on day 7 for the measurement of BMD. Significantly different (**, $p < 0.001$) from control mice injected PBS. Data are expressed as the mean ± SEM of six mice. Note that a significant decrease in BMD was observed in the alveolar bone in the LPS-injected wild-type mice, but not in *mPges1*$^{-/-}$ mice.

however, the LPS administration did not influence the BMD of alveolar bone at all (Fig. 4*C*).

*LPS induces $PGE_2$ production by osteoblasts via TLR4*

To examine the role of TLR4 in LPS-induced $PGE_2$ production by osteoblasts, we isolated osteoblasts from C3H/HeJ mice that contained a nonfunctional mutation in the *tlr4* gene. We also isolated osteoblasts from C3H/HeN mice as a control. Adding LPS markedly induced the mRNA expression of COX-2 and mPGES-1, and $PGE_2$ production by osteoblasts was greatly enhanced by LPS in C3H/HeN mice (Fig. 5). In osteoblasts collected from C3H/HeJ mice, however, LPS did not induce the expression of COX-2 or mPGES-1, and $PGE_2$ synthesis was not elevated by LPS at all (Fig. 5). When bone marrow cells were isolated from C3H/HeN and C3H/HeJ mice, and cocultured with osteoblasts derived from the respective mice with or without LPS, LPS-induced osteoclast formation in the cocultures of C3H/HeN mice, but not in that of C3H/HeJ mice (data not shown). These results indicate that LPS acts on osteoblasts to stimulate $PGE_2$ synthesis via TLR4 expressed on their surface.

*TLR4-mediated bone loss by LPS in vivo*

Using histological sections of the distal femoral metaphysis, we examined the role of TLR4 in the bone loss induced by LPS administration in vivo. In C3H/HeN mice, the bone density (BV/TV) of the trabecular bone was significantly reduced, and the number of TRAP-positive osteoclasts significantly increased by the administration of LPS (Fig. 6). In C3H/HeJ mice, however, LPS did not influence the bone density or the number of osteoclasts at all (Fig. 6). When we measured the femoral BMD, the BMD was significantly reduced by the LPS administration in C3H/HeN mice, but not in C3H/HeJ mice (data not shown). These results indicate that TLR4 is a functional receptor for LPS to induce bone resorption in vivo.

## Discussion

In the present study, we showed that *mPges1*$^{-/-}$ is resistant to LPS-induced bone resorption in the femur and mandibular bone. LPS-induced PGE synthesis was reduced in TLR4 mutated C3H/HeJ mice. We previously reported that $PGE_2$ binds to the EP4 receptor in osteoblasts and induces RANKL expression on their surface, which is critical for osteoclast differentiation. Therefore, we now show the pathway, the stream of LPS-TLR4-$PGE_2$ has a crucial role in inflammatory bone destruction (Fig. 7). $PGE_2$ production by osteoblasts is regulated by three metabolic steps; the release of arachidonic acid from the membranous phospholipids by $cPLA_{2\alpha}$, the conversion of arachidonic acid to $PGH_2$ by COX-2

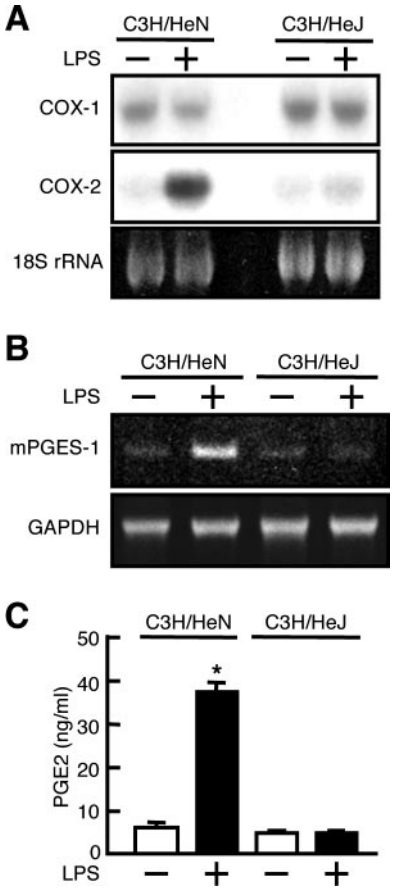


**FIGURE 5.** Effects of LPS on $PGE_2$ production and mRNA expression of COX-1, COX-2, and mPGES-1 in osteoblastic stromal cells collected from C3H/HeN and C3H/HeJ mice. *A*, Osteoblastic cells were collected from C3H/HeN and C3H/HeJ mice, and cultured for 3 h with (+) or without (−) 10 ng/ml LPS. Total RNA was extracted, and Northern blotting was performed using $^{32}$P-labeled cDNA probes for COX-1 and COX-2. *B*, Osteoblastic cells were collected from C3H/HeN and C3H/HeJ mice and cultured for 12 h with or without 10 ng/ml LPS. Total RNA was extracted, and the expression of mPGES-1 and GAPDH mRNA was analyzed by RT-PCR. *C*, Osteoblastic cells were collected from C3H/HeN and C3H/HeJ mice, and cultured for 24 h with or without 10 ng/ml LPS. The concentration of $PGE_2$ in the cultured medium was determined using an enzyme immunoassay. Data are expressed as the mean ± SEM of three wells. Significantly different (*, $p < 0.001$) from the control without LPS.

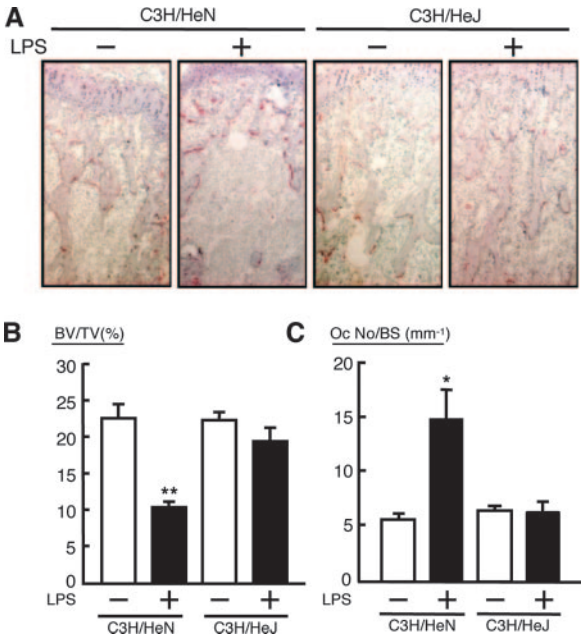


**FIGURE 6.** Histological analyses of the femoral trabecular bone collected from C3H/HeN and C3H/HeJ mice injected with LPS. C3H/HeN and C3H/HeJ mice were injected with LPS or PBS i.p. on days 0 and 4, and the femurs were collected on day 8 after the first injection to prepare the sections of femoral distal metaphysis. The sections of trabecular bone were stained for TRAP to detect osteoclasts (*A*). Using the TRAP-stained sections, histomorphometric analyses were performed to calculate bone density (BV/TV) (*B*) and the mean number of osteoclasts in the trabecular bone surface (Oc No/BS) (*C*) as described in *Materials and Methods*. Significantly different from the control mice injected with PBS in C3H/HeN (*, $p < 0.01$) and C3H/HeJ (**, $p < 0.001$) mice, respectively. Data are expressed as the mean ± SEM of four to five mice.

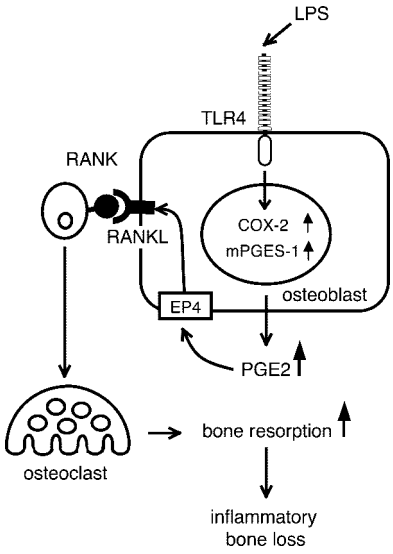


**FIGURE 7.** Proposed cascade of PGE production and bone resorption in inflammatory bone loss.

and the synthesis of $PGE_2$ by PGES. We have reported that $cPLA_{2\alpha}$ expression in osteoblasts is indispensable to the arachidonic acid release for $PGE_2$ synthesis in the bone (13, 14). Thus, in $cPLA_{2\alpha}$-deficient mice, the LPS-induced bone resorption was attenuated due to inhibiting $PGE_2$ production by osteoblasts (14). It is known that COX-2 expression is markedly induced by cytokines, such as IL-1 and IL-6 in osteoblasts (13, 18, 26–28). The expression of mPGES-1 is linked to COX-2 expression that was induced by inflammatory cytokines in various cells (12). Because mPGES-1 is an inducible terminal enzyme for the $PGE_2$ biosynthesis, the gene transcriptional regulation is important to understand for the biological significance in target tissues. The mRNA expression of mPGES-1 is coupled with COX-2, and the induction of COX-2 mRNA proceeded rather than mPGES-1 after adding LPS. Previous studies have shown that the mouse COX-2 gene promoter possesses functional regulatory elements for NF-κB, NF-IL-6, AP-1, and c/EBPα. In contrast, the mouse mPGES-1 gene promoter possesses AP-1 and c/EBP, but not NF-κB (29). Further studies are needed to define the transcriptional regulation of mPGES-1 in osteoblasts.

Recently, three types of PGES, mPGES-1, mPGES-2, and cytosolic PGES, have been identified (12, 15–17). The data in this study suggested that the LPS-induced $PGE_2$ synthesis in osteoblasts was mediated by mPGES-1, rather than cytosolic PGES (Fig. 2*A*). Saegusa et al. (18) have reported the induction of mPGES-1 expression by IL-1α, TNF-α, and fibroblast growth factor-2 in mouse osteoblasts. The cells collected from $mPges1^{-/-}$ mice could not produce $PGE_2$, suggesting that the conversion of $PGH_2$ to $PGE_2$ mediated by mPGES-1 is an inevitable step for $PGE_2$ synthesis in osteoblasts (Fig. 2*B*). Previous studies using $PGE_2$ production related gene deficient mice, such as $cPLA_{2\alpha}$, COX-2, and EP4 receptor show a normal skeletal phenotype in development and growth. $mPges1^{-/-}$ also showed normal skeletal development, suggesting that $PGE_2$ production in bone tissue is enhanced in inflammatory bone loss with a pathological condition, which may be dispensable in a normal physiological condition. To determine whether LPS challenge could alter the bone phenotype, we administered LPS to wild-type and $mPges1^{-/-}$ mice. In bone morphometry, sections from wild-type distal femoral metaphysis clearly showed an increased number of TRAP-positive multinucleated osteoclasts, observed in the trabecular bone. The bone density and the trabecular bone thickness were also significantly reduced, whereas the trabecular bone separation was increased by LPS administration (Fig. 3). In $mPges1^{-/-}$ mice, however, LPS administration did not influence the bone density, trabecular bone separation and thickness, or the number of osteoclasts (Fig. 3). These result indicated that $mPges1^{-/-}$ is resistant to LPS-induced bone resorption following osteoclast formation in the femur.

Periodontitis is a bone destructive disease accompanied with inflammation. In this study, we demonstrated a new model for mouse periodontitis that associated with bone resorption in alveolar bone, LPS administration to ex vivo in organ culture and in vivo injection to alveolar bone. The model was successfully detected bone loss induced by LPS. The data showed that $mPges1^{-/-}$ has no resistance to LPS-induced bone loss either ex vivo or in vivo (Fig. 4). Our previous study indicated that treatment with indomethacin suppressed the LPS-induced loss of alveolar bone in wild-type mice, and that $PGE_2$ induced osteoclast formation in $mPges1^{-/-}$ mice (C. Miyaura, unpublished data). A recent review by Salvi and Lang (30) showed the evidence from animal experiments and clinical trials documents that selective and nonselective nonsteroidal anti-inflammatory drugs (NSAIDs) were responsible for the stabilization of periodontal conditions by reducing the rate of alveolar bone resorption. Therefore, the production of $PGE_2$ is responsible for the LPS-induced loss of alveolar bone, and the application of NSAIDs may help to protect the loss of alveolar bone in periodontal diseases.

Recently, several TLRs have been cloned and identified as receptors for bacterial components (20). LPS has also been reported to recognize TLR2 and TLR4 (21–23). To examine which TLR mediates LPS signals in osteoblasts, we used C3H/HeJ mice that contained a nonfunctional mutation in the *tlr4* gene and C3H/HeN mice as controls. $PGE_2$ synthesis by osteoblasts was not observed in C3H/HeJ mice in vitro (Fig. 5), and LPS-induced bone loss was impaired in C3H/HeJ mice (Fig. 6). Takeuchi et al. (23) reported that TLR4-deficient mice showed LPS unresponsiveness, whereas the response of macrophages in TLR2-deficient mice is comparable to that of wild-type mice. These results indicate that TLR4 is essential for LPS signaling. Besides LPS, some bacterial components might be involved in the pathogenesis of periodontitis. Therefore, it is possible that other TLRs also contribute to inflammatory bone loss in human periodontitis. Recent studies have shown that the cytoplasmic domain of TLR possesses a similarity with that of the IL-1R and is known as the Toll/IL-1R homology domain (20, 31, 32). The Toll/IL-1R family is thought to share a common cell signaling pathway, leading to the activation of NF-κB. After ligand binding, TLR2 and TLR4 activate IL-1R-associated kinase and phosphorylate TNFR-associated factor (TRAF) 6. Lomaga et al. (33) reported the generation of TRAF6-deficient mice that showed osteopetrotic bone and macrophage impaired NO production in response to LPS and IL-1. It is possible that TRAF6 is involved in LPS signaling, but further studies are needed to define the mechanism of LPS action to osteoblasts. We previously reported that $PGE_2$ signaling in osteoblasts was mediated by the EP4 receptor and that it induced RANKL expression to support osteoclastogenesis (4, 5). In EP4-deficient mice, the LPS induced bone loss was impaired in the femurs (C. Miyaura, unpublished data). Therefore, $PGE_2$ induced RANKL expression through EP4 is the major stream in LPS-induced bone resorption (Fig. 7).

Using $mPges1^{-/-}$ mice, previous studies have shown the role of mPGES-1 in pain sensitivity, febrile response, rheumatoid arthritis and the formation of granulation tissue (34–36). In the present study, we showed a definitive role of mPGES-1 on LPS-induced inflammatory bone destruction in the femur and mandible. LPS-TLR4 recognition on osteoblasts induce mPGES-1 expression and

$PGE_2$ synthesis, which is dominant in the bone destruction associated with inflammation. These results suggest that mPGES-1 is a key mediator in the bone destruction associated with inflammation, such as periodontal disease and osteoarthritis. Because mPGES-1 is a terminal enzyme in the biosynthesis of $PGE_2$, the inhibition of mPGES-1 may provide a new therapeutic approach to inflammatory bone disease with less side effects compared with other NSAIDs.

## Disclosures

The authors have no financial conflict of interest.

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