Journal of
PERIODONTAL RESEARCH

J Periodont Res 2011; 46: 280–284

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2010.01333.x

Short Communication

Defective nuclear factor -κB-inducing kinase in *aly/aly* mice prevents bone resorption induced by local injection of lipopolysaccharide

N. S. Soysa[1,2], N. Alles[1,3], M. Takahashi[1], K. Aoki[1], K. Ohya[1]

[1]Section of Pharmacology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan, [2]Division of Pharmacology, Faculty of Dental Sciences, University of Peradeniya, Peradeniya, Sri Lanka and [3]Department of Farm Animal Production and Health, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya, Sri Lanka

Soysa NS, Alles N, Takahashi M, Aoki K, Ohya K. Defective nuclear factor-κB-inducing kinase in aly/aly *mice prevents bone resorption induced by local injection of lipopolysaccharide. J Periodont Res 2011; 46: 280–284. © 2010 John Wiley & Sons A/S*

Background and Objective: Nuclear factor-κB (NF-κB) is activated at sites of inflammation in many diseases, including periodontitis. Nuclear factor-κB induces the transcription of proinflammatory cytokines, resulting in increased osteoclastogenesis and bone resorption. Recently, it has been shown that the NF-κB alternative pathway is important for maintainance of physiological bone homeostasis. Activation of this pathway is by processing of the inhibitor p100 into the active subunit p52 by nuclear factor-κB-inducing kinase (NIK). Defective NIK in *aly/aly* mice (NIK^{aly}) causes mild osteopetrosis and blunted RANKL-stimulated osteoclastogenesis *in vivo* and *in vitro*, suggesting that NIK is necessary for basal and stimulated osteoclastogenesis. Nevertheless, the role of NIK in pathological bone resorption is not well investigated. The present study was undertaken to investigate the role of NIK in lipopolysaccharide (LPS)-induced inflammatory bone resorption using *aly/aly* mice.

Material and Methods: Mice were injected with LPS over the calvariae and killed 5 d later. Calvariae were subjected to radiological analysis. Histological sections were stained for tartrate-resistant acid phosphatase, and histomorphometric analysis was performed to quantify the number of osteoclasts and the area of bone resorption.

Results: Lipopolysaccharide-induced inflammation was observed in wild-type and *aly/+* mice but not in *aly/aly* mice. Lipopolysaccharide significantly reduced the calvarial bone mineral density in wild-type and *aly/+* mice, whereas bone mineral density was comparable in LPS- and vehicle-injected *aly/aly* mice. In addition, *aly/aly* mice were resistant to LPS-induced bone resorption and osteoclastogenesis.

Conclusion: Taken together, these data show that NIK is important in the bone-destructive components of inflammation and represents a possible therapeutic target.

Niroshani S. Soysa, BDS, PhD, Section of Pharmacology, Department of Hard Tissue Engineering, Graduate school, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
Tel: +81 3 5803 5461
Fax: +81 3 5803 0190
e-mail: niroshani.hpha@tmd.ac.jp

Key words: *aly/aly* mice; lipopolysaccharide; nuclear factor-κB-inducing kinase

Accepted for publication November 2, 2010

Among other bacterial products, lipopolysaccharide (LPS) appears to be the major mediator of bone resorption accompanying chronic infections such as periodontitis (1). Lipopolysaccharide is found in abundance in the foci of periodontal pockets, and it has been suggested that LPS penetrates gingival connective tissue and induces a local inflammatory response that leads to periodontal bone resorption. In addition to its direct action on osteoclasts, the exclusive bone-resorbing cell of haematopoitic origin, LPS stimulates bone resorption by inducing bone marrow macrophages to secrete cytokines, predominantly tumour necrosis factor-α (TNF-α), which is consistent with the rapid induction of circulating TNF-α within 1–2 d of LPS administration. Nuclear factor-κB (NF-κB) is responsible for a broad range of biological activities, including lymphoid organogenesis and regulation of the immune response. Nuclear factor-κB has two main pathways, called classical (canonical) and alternative (noncanonical) (2). The classical pathway is controlled by upstream kinases, such as the inhibitor of κB kinases, IKK-α and IKK-β, while the alternative pathway is regulated by NF-κB-inducing kinase (NIK) and IKK-α (2). The NIK phosphorylates IKK-α homodimers and processes the inhibitor p100 into active subunit p52 to activate the alternative pathway. Osteopenia is observed in mice lacking p100 ($p100^{-/-}$), indicating that in the absence of inhibitor p100 other subunits, such as p52 and RelB, show increased DNA binding activity (3). This shows that p100 is necessary for maintainance of bone homeostasis. A recent report by Yao *et al*. (4) shows that absence of p100 in TNF-Tg-NF-$\kappa B2^{-/-}$ mice have increased inflammatory response and bone erosion, indicating that NF-κB p100 limits not only differentiation of osteoclasts, but also the number of inflammatory cells attracted to the joints of the mice in response to TNF-α. The important role of the alternative pathway of NF-κB in osteoclasts was revealed by mild osteopetrosis in *aly*/*aly* mice, which have defective NIK (NIK^{aly}) and are unable to process the inhibitor p100 (5). The accumulated inhibitor p100 binds to other subunits and retains them in the cytoplasm, thus reducing their DNA-binding activities (5). In addition to reduced basal osteoclastogenesis, the RANKL-stimulated osteoclastogenesis is blunted in *aly*/*aly* mice, indicating that NIK is necessary for basal as well as stimulated osteoclastogenesis. Several lines of evidence suggest that blockade of the classical NF-κB pathway diminishes the inflammatory response and accompanying bone loss (6,7). In contrast, only a few studies have been carried out to asses the role of the alternative NF-κB pathway in inflammatory settings. Therefore, in this study we used *aly*/*aly* mice to examine the role of NIK in pathological bone resorption using an established LPS-induced inflammatory bone resorption model.

Material and methods

The experimental procedures were reviewed and approved by the Animal Care and Use Committee of the Tokyo Medical and Dental University (Tokyo, Japan). Eight-week-old C57BL/6J wild-type mice (WT), mice heterozygous for *aly* (*aly*/+) and mice homozygous for *aly* (*aly*/*aly*) were divided into the LPS-injected group ($n = 6$) and the vehicle-injected group ($n = 6$). 10 mg/kg LPS or phosphate-buffered saline vehicle were injected subcutaneously over mice calvariae under anesthesia with medetomidine hydrochloride (0.5 mg/kg, Domitor, Meijiseika, Tokyo, Japan) and ketamine hydrochloride (50 mg/kg, Ketalar, Sankyo, Tokyo, Japan). In order to recover from anesthesia quickly, mice were injected with atipamazole hydrochloride (0.25 mg/kg, Antisedan, Meijiseika, Tokyo, Japan). Mice were sacrificed by cervical dislocation under anesthesia 5 d after the injections of LPS. The bone mineral density of calvariae was measured by dual-energy X-ray absorptiometry (DCS-600R; Aloka, Tokyo, Japan) as described elsewhere (8). Soft X-ray photographs of calvariae were taken by using a cabinet X-ray apparatus (TYPE SRO-M50; Sofron Co. Ltd, Tokyo, Japan). Three-dimensional reconstruction images of calvariae were obtained by microfocal computed tomography (ScanXmate-E090; Comscan, Kanagawa, Japan) as previously described (9,10). Calvariae were embedded in mixtures of methyl methacrylate and glycidyl methacrylate resins as described elsewhere (9). These sections were then stained with tartrate-resistant acid phosphatase (TRAP) and counterstained with methyl green. Osteoclasts were designated as the TRAP-positive multinucleated cells located on the bone surface. All data are presented as means ± SD. The statistical significance of differences among groups was assessed using one-way ANOVA. When significant F values were detected, Fisher's PLSD *post hoc* test was performed to compare assay groups. Difference was considered significant when $p < 0.05$.

Results

Reddish inflammatory tissues, which represent inflammatory changes, were observed on the calvariae in WT and *aly*/+ mice following LPS injection, whereas they were not observed in *aly*/*aly* mice compared with the vehicle-injected control group (Fig. 1). Many radiolucent spots on calvariae were observed in soft X-ray images of WT and *aly*/+ mice following LPS injection but not in those of *aly*/*aly* mice (Fig. 2A). These changes were further illustrated by microfocal computed tomography (Fig. 2B). The bone mineral density of calvariae was significantly decreased by LPS in WT and *aly*/+ mice. The bone mineral density of LPS-injected *aly*/*aly* mice was comparable to that of the vehicle-injected group (Fig. 3).

Lipopolysaccharide induces bone resorption by increasing the formation and activity of osteoclasts (8). Histological sections of calvariae stained for TRAP revealed a blunted response to LPS-induced osteoclastogenic stimuli in *aly*/*aly* mice. Both WT and *aly*/+ mice showed an increase in TRAP-positive multinucleated cells along calvarial sinuses upon stimulation with LPS, whereas LPS-injected *aly*/*aly* mice showed only a few TRAP-positive multinucleated cells compared with vehicle-injected control animals (Fig. 4A). Quantitative data showed a significant increase in TRAP-positive cells in LPS-

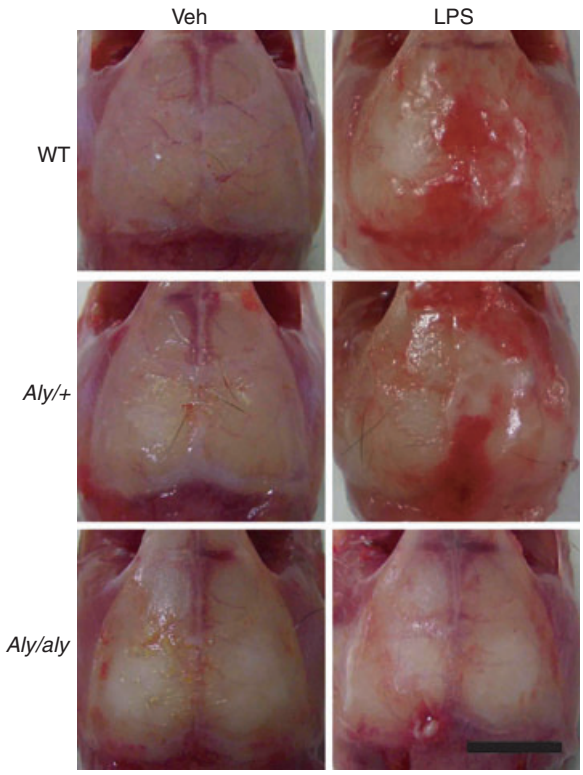

Fig. 1. The degree of inflammation on calvariae is reduced in *aly*/*aly* mice upon stimulation with lipopolysaccharide. Scale bar represents 5 mm. LPS, lipopolysaccharide-injected group; Veh, vehicle-injected group; and WT, wild-type.

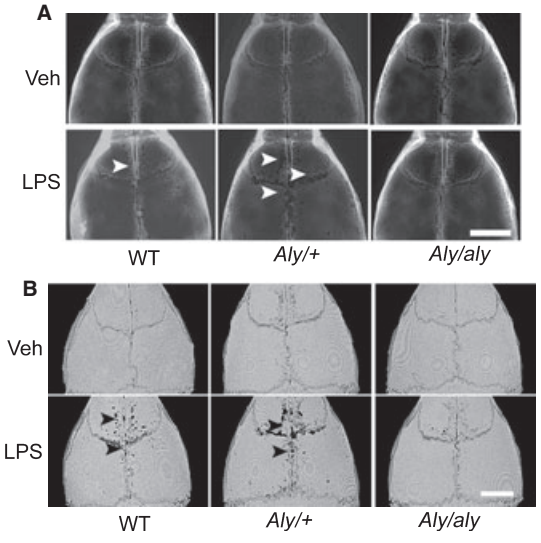

Fig. 2. The degree of bone resorption on calvariae is reduced in *aly*/*aly* mice upon LPS stimulation. The calvariae of WT, *aly*/+ and *aly*/*aly* mice were analysed radiologically. (A) Soft X-ray images of WT, *aly*/+ and *aly*/*aly* calvariae. (B) Microfocal computed tomography reconstruction images of calvariae. Scale bars represent 4 mm. The white and black arrowheads show the resorption lacunae in soft X-ray and microfocal computed tomography images, respectively. LPS, lipopolysaccharide-injected group; Veh, vehicle-injected group; and WT, wild-type.

injected WT and *aly*/+ mice compared with vehicle-injected mice (Fig. 4B). Few TRAP-positive cells were also observed in *aly*/*aly* mice that received LPS compared with vehicle-injected mice (Fig. 4B).

Discussion

Nuclear factor-κB is activated at sites of inflammation in a wide range of diseases, including periodontitis and rheumatoid arthritis, and induces the transcription of proinflammatory cytokines, such as TNF-α and IL-6. Recently, more attention has been focused on osteoporosis and other skeletal disorders, and periodontitis is one of the most prevalent forms of osteopenia. Periodontal bone resorption is due to the products of offending bacteria, such as LPS. Lipopolysaccharide is a cell wall component of gram-negative bacteria and induces bone resorption by its action on osteoclasts (11) and by TNF-α through tumour necrosis factor receptor-1 (8,12). In our previous paper using TNF knockout mice (13) we have shown that LPS induces inflammatory mediators, including TNF and interleukin-6, that induce bone resorption. Administration of LPS to mimic inflammatory bone resorption has been used extensively. Such studies use repeated injections or a single injection of LPS to stimulate an acute inflammatory response. In the present study, we used a single injection of LPS to induce inflammation and bone resorption. As shown in Fig. 1, an LPS-induced inflammatory response was observed in WT and *aly*/+ control mice. This is consistent with a previous report showing that a single injection of 10 mg/kg of LPS is adequate to produce an inflammatory response and bone resorption in calvariae as well as long bones (8). Nuclear factor-κB is responsible for the inflammatory bone resorption in many arthritis models (7,14,15). Inhibition of the components of the classical pathway using NF-κB inhibitors has been tested and shown to reduce bone resorption in these models, whereas the role of the alternative NF-κB pathway in these settings has not been addressed. Using the serum transfer arthritis model, Aya *et al.* (15)

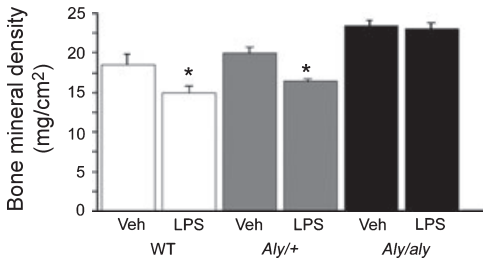

Fig. 3. Bone mineral density of calvariae measured by dual-energy X-ray absorptiometry. Data are presented as means ± SD ($n = 6$). Veh, the vehicle-injected group; LPS, the LPS-injected group. *$p < 0.01$ vs. vehicle-injected group in each genetic background. LPS, lipopolysaccharide-injected group; Veh, vehicle-injected group; and WT, wild-type.

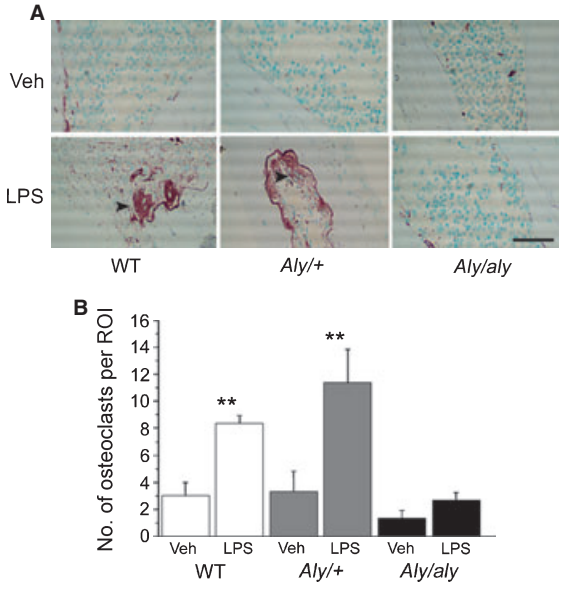

Fig. 4. The *aly*/*aly* mice are resistant to LPS-induced osteoclastogenesis *in vivo*. (A) Tartrate-resistant acid phosphatase (TRAP)-stained coronal sections of calvariae show a dramatic increase in osteoclasts only in WT and *aly*/+ LPS-injected animals. Arrowheads show TRAP-positive multinucleated cells. (B) Quantification of TRAP-positive multinucleated cells. Counting was performed in the region of interest (ROI; 0.29 mm × 0.68 mm) including the sagittal suture. Scale bars represent 50 μm. Data are presented as means ± SD ($n = 6$). **$p < 0.001$ vs. vehicle-injected group in each genetic background. LPS, lipopolysaccharide-injected group; Veh, vehicle-injected group; and WT, wild-type.

showed that $nik^{-/-}$ mice have significantly less periarticular osteoclastogenesis and less bone erosion. In addition, $nik^{-/-}$ mice are completely resistant to antigen-induced arthritis (15), suggesting that NIK is necessary to induce components of the inflammatory response. In the present report, we have shown that *aly*/*aly* mice are protected against the LPS-induced inflammatory response due to NIK^{aly}.

We observed pit formation in the calvariae of WT and *aly*/+ mice injected with LPS, while *aly*/*aly* mice were resistant to LPS-induced bone resorption (Fig. 2). Our previous report shows that *aly*/*aly* mice are resistant to the osteoclastogenic effects of exogenous RANKL (5). In the present study, LPS-induced osteoclastogenesis was blunted in *aly*/*aly* mice (Fig. 4), while WT and *aly*/+ mice showed increased TRAP-positive multinucleated cells. In our previous report, we have shown that RANKL-induced processing of p100 into p52 is absent in *aly*/*aly* osteoclasts compared with those from WT and *aly*/+ mice, thus resulting in impaired RANKL-induced osteoclast formation *in vivo*. Likewise, it has been reported that LPS could not process the inhibitor p100 into active subunit p52 in either $nik^{-/-}$ or *aly*/*aly* bone marrow-derived macrophages (16). Lipopolysaccharide induces accumulation of p100 in bone marrow-derived macrophages of $nik^{-/-}$ and *aly*/*aly* mice in a dose-dependent manner. In addition, NIK is necessary for nuclear translocation of p52 (16) in these cells. Consistent with this observation, TNF-α could not process the p100 in $IKK\alpha^{-/-}$ cells, and accumulation of p100 occurs as a result of TNF-α stimulation in $nik^{-/-}$ pre- osteoclasts (15). Aya *et al.* (15) showed that TNF-α increases osteoclastogenesis in the presence of low levels of RANKL in $nik^{+/+}$ cells, whereas very high doses of RANKL (150 ng/mL), which are greater than the optimal dose for WT osteoclastogenesis, are necessary to synergize with TNF-α to generate some multinucleated cells, suggesting that $nik^{-/-}$ cells are resistant to both RANKL- and TNF-α-induced osteoclastogenesis. Therefore, taken together, these data indicate that the alternative pathway is responsible for LPS-induced bone resorption and osteoclastogenesis by processing the inhibitor p100 into active p52.

The NF-κB pathway is under intense study as a potential drug target to ameliorate inflammatory-mediated diseases, such as rheumatoid arthritis. Our study suggests that periodontitis is another inflammatory-mediated condition that will benefit from NF-κB manipulation. Since NF-κB is involved in normal cellular physiology, the ultimate benefit depends on the balance between suppression of inflammation-induced bone resorption and normal cellular functions.

Acknowledgements

We would like to thank Dr S. M. X. Corea for critical reading of the manuscript. This study was supported by grants from the Japanese Society for Promotion of Science (JSPS) to N.A. and the Ministry of Education, Culture, Sports, Science and Technology of

Japan to M.T. (20791350), K.A. (19390472) and K.O. (19390471).

References

1. Nair SP, Meghji S, Wilson M, Reddi K, White P, Henderson B. Bacterially induced bone destruction: mechanisms and misconceptions. *Infect Immun* 1996;**64:**2371–2380.
2. Soysa NS, Alles N. NF-kappaB functions in osteoclasts. *Biochem Biophys Res Commun* 2009;**378:**1–5.
3. Guo F, Tanzer S, Busslinger M, Weih F. Lack of nuclear factor-kappa B2/p100 causes a RelB-dependent block in early B lymphopoiesis. *Blood* 2008;**112:**551–559.
4. Yao Z, Xing L, Boyce BF. NF-kappaB p100 limits TNF-induced bone resorption in mice by a TRAF3-dependent mechanism. *J Clin Invest* 2009;**119:**3024–3034.
5. Soysa NS, Alles N, Weih D *et al*. The pivotal role of the alternative NF-kappaB pathway in maintenance of basal bone homeostasis and osteoclastogenesis. *J Bone Miner Res* 2010;**25:**809–818.
6. Clohisy JC, Roy BC, Biondo C *et al*. Direct inhibition of NF-κB blocks bone erosion associated with inflammatory arthritis. *J Immunol* 2003;**171:**5547–5553.
7. Jimi E, Aoki K, Saito H *et al*. Selective inhibition of NF-κB blocks osteoclastogenesis and prevents inflammatory bone destruction in vivo. *Nat Med* 2004;**10:**617–624.
8. Hussain Mian A, Saito H, Alles N, Shimokawa H, Aoki K, Ohya K. Lipopolysaccharide-induced bone resorption is increased in TNF type 2 receptor-deficient mice in vivo. *J Bone Miner Metab* 2008;**26:**469–477.
9. Alles N, Soysa NS, Hussain MD *et al*. Polysaccharide nanogel delivery of a TNF-alpha and RANKL antagonist peptide allows systemic prevention of bone loss. *Eur J Pharm Sci* 2009;**37:**83–88.
10. Alles N, Soysa NS, Hayashi J *et al*. Suppression of NF-kappaB increases bone formation and ameliorates osteopenia in ovariectomized mice. *Endocrinology* 2010;**151:**4626–4634.
11. Islam S, Hassan F, Tumurkhuu G *et al*. Bacterial lipopolysaccharide induces osteoclast formation in RAW 264.7 macrophage cells. *Biochem Biophys Res Commun* 2007;**360:**346–351.
12. Abu-Amer Y, Ross FP, Edwards J, Teitelbaum SL. Lipopolysaccharide-stimulated osteoclastogenesis is mediated by tumor necrosis factor via its P55 receptor. *J Clin Invest* 1997;**100:**1557–1565.
13. Tomomatsu N, Aoki K, Alles N *et al*. LPS-induced inhibition of osteogenesis is TNF-alpha dependent in a murine tooth extraction model. *J Bone Miner Res* 2009;**24:**1770–1781.
14. Dai S, Hirayama T, Abbas S, Abu-Amer Y. The IkappaB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks osteoclastogenesis and bone erosion in inflammatory arthritis. *J Biol Chem* 2004;**279:**37219–37222.
15. Aya K, Alhawagri M, Hagen-Stapleton A, Kitaura H, Kanagawa O, Novack DV. NF-(kappa)B-inducing kinase controls lymphocyte and osteoclast activities in inflammatory arthritis. *J Clin Invest* 2005;**115:**1848–1854.
16. Pan Q, Kravchenko V, Katz A *et al*. NF-kappa B-inducing kinase regulates selected gene expression in the Nod2 signaling pathway. *Infect Immun* 2006;**74:**2121–2127.