PKR induces the expression of NLRP3 by regulating the NF-κB pathway in *Porphyromonas gingivalis*-infected osteoblasts

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Abstract

The double-stranded RNA-dependent kinase (PKR), which is activated by double stranded RNA, induces inflammation by regulating NF-κB signaling. The NLR family pyrin domain-containing 3 (NLRP3) inflammasome also modulates inflammation in response to infection. *Porphyromonas gingivalis* (*P.gingivalis*) is an oral bacterium which is implicated in the pathogenesis of periodontal diseases. We previously reported that PKR is a key modulator of bone metabolism and inflammation in the periodontal tissue. PKR was also reported to induce inflammation in response to microbes by regulating the NLRP3 inflammasome, suggesting that PKR could affect inflammation along with NLRP3 in periodontal diseases. In this study, we investigated the effects of PKR on NLRP3 expression and NF-κB activity in *P. gingivalis* infected osteoblasts. We first constructed a SNAP26b-tagged *P.gingivalis* (SNAP-*P. g.*) and traced its internalization into the cell. SNAP-*P. g.* increased the activity of PKR and NF-κB and also induced NLRP3 expression in osteoblasts. Inhibition of NF-κB attenuated SNAP-*P. g.*-induced NLRP3 expression. The knockdown of PKR using shRNA decreased both the activity of NF-κB and the expression of NLRP3 induced by SNAP-*P.g.*. We therefore concluded that in osteoblasts, *P. gingivalis* activated PKR, which in turn increased NLRP3 expression by activating NF-κB. Our results suggest that PKR modulates inflammation by regulating the expression of the NLRP3 inflammasome through the NF-κB pathway in periodontal diseases.

Keywords: Double-stranded RNA-dependent kinase; inflammasomes; NF-kappa B; osteoblasts; *Porphyromonas gingivalis*

1. Introduction

The serine-threonine kinase PKR is ubiquitously expressed in mammalian cells and was originally reported to be activated by binding to the double-stranded RNA derived from viruses. In virus-infected cells, activated PKR inhibits protein synthesis and promotes apoptosis through phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α), resulting in an induction of the immune response [1]. PKR is now known to mediate inflammation, cancer [2] and obesity [3, 4] in response to a variety of factors, including cytokines [5], growth factors, lipopolysaccharide (LPS) [6] and oxidative stresses [7].

The diverse functions of PKR are considered to be dependent on its ability to act as a signal transducer. For example, activated PKR affects multiple pathways mediated by several kind of transcriptional factors such as interferon regulatory factor 3 (IRF3), nuclear factor κB (NF-κB), c-Jun, and activating transcription factor 2 (ATF2), leading to regulation of inflammation and immunity [8]. Among these pathways, the mechanisms by which PKR regulate NF-κB signaling has been well studied. PKR is autophosphorylated and activated by the inflammation-induced stimuli, and directly interacts with the IκB kinase (IKK) β subunit of IKK complex to phosphorylate it [9, 10]. The activated IKK then phosphorylates the inhibitory of κB (IκB) which retain NF-κB in the cytoplasm, while the IκB is degraded by the 26S proteasome in an ubiquitin-dependent manner. This degradation allows NF-κB to translocate to the nucleus where it promotes the transcription of genes that regulate inflammation. Thus, PKR is considered to be a crucial factor in modulating inflammation by regulating NF-κB signaling.

The NLR family pyrin domain-containing 3 (NLRP3) inflammasome- a multiprotein complex comprising of NLRP3, the adaptor apoptosis-associated speck-like protein (ASC) and pro-caspase-1, is also reported to modulate inflammation. In viral and bacterial infected cells, NLRP3 inflammasome is activated, which in turn recruits pro-caspase-1 and cleaves it into caspase-1 p20

and p10 subunits. The subunits assemble to form active caspase-1 hetero-dimers that catalytically cleave pro-IL-1β into the biologically active form. IL-1β is one of most potent proinflammatory cytokines and the increase of IL-1β secretion by the inflammasomes is related to the pathogenesis of several diseases [11, 12].

NLRP3 inflammasome is known to be activated by two steps—an initial priming step and an activation step. Recently, NF-κB has been found to be required for the priming step [11]. In response to stimuli, the priming step is mediated through the Toll-like receptors (TLRs), which activates NF-κB and increases the expression of NLRP3, which in turn convert the pro-IL-1β to its functional form. In the activation step, the induced NLRP3 is subsequently able to assemble and form the complex of NLRP3, ASC and pro-caspase-1, resulting in an increase in cleavage of pro-caspase-1 and IL-1β secretion. Indeed, the inhibition of either TLRs or NF-κB resulted in the reduction of NLRP3 expression and caspase-1 cleavage in macrophages [13], suggesting that the priming step is important to activate inflammasomes. However the molecular mechanism regulating this remains poorly understood.

Porphyromonas gingivalis (*P.gingivalis*) is an oral microorganism which is implicated in the pathogenesis of periodontal disease, a chronic inflammatory disorder of the periodontal tissue. Progress in periodontal disease induces inflammation and resorption of alveolar bone, resulting in tooth loss. An understanding of how bacterial infection affects bone metabolism and induces bone resorption is therefore necessary to prevent periodontal disease.

Little is known about the relationship between PKR and periodontal disease. We previously reported that PKR positively regulates the differentiation of osteoblasts by inducing GSK-3β activity using mouse osteoblastic cell line MC3T3-E1 cells, which have the capacity to undergo osteoblastic differentiation and mineralization *in vitro* [14, 15]. We also showed that LPS isolated from *E. coli* phosphorylates PKR to increase the expression of pro-inflammatory cytokines through NF-κB signaling in human gingival cells [6]. Our results suggested that PKR might be a key modulator for

bone metabolism and inflammation in periodontal tissue. However, the effects of the periodontal bacteria on PKR activity and PKR-regulated inflammation in osteoblasts still remain unknown.

Several studies have also indicated that NLRP3 is involved in the pathogenesis of periodontal disease. Bostanci *et al.* reported that NLRP3 is expressed at significantly higher levels in gingival tissue from patients with periodontal disease [16]. They also showed that *P. gingivalis* regulates the NLRP3 inflammasome complex by increasing NLRP3 and down-regulating NLRP2 and ASC expression in human myelomonocytic cell line Mono-Mac-6 [16]. It was found that in human monocytic cell line THP-1, *P. gingivalis* activates the NLRP3 inflammasome via TLR2 and TLR4 [17] pathways. Infection with another periodontal bacteria *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) also promotes apoptosis of human osteoblastic MG63 cells by activating the NLRP3 inflammasome [18]. Moreover, *A. actinomycetemcomitans* enhances NLRP3 and reduces NLRP6 expression leading to the release of IL-1β in human mononuclear leukocytes [19].

Recently, PKR has been reported to induce the immune response and inflammation in response to microbes by regulating multiple inflammasomes [20]. PKR directly interacts with inflammasome components such as NLRP3, NLRP4, and AIM2 to mediate inflammasome activity [21]. Inhibition of PKR by $p58^{IPK}$ suppressed the activity of NLRP3 inflammasomes in macrophages [22].

These observations suggest that PKR, along with NLRP3 affects inflammation in osteoblasts during periodontal diseases. We therefore hypothesized that PKR could be activated by *P. gingivalis* and contribute to inflammation by regulating the NLRP3 in osteoblasts. In this study, we therefore investigated the effects of PKR on NLRP3 expression and NF-κB activity in *P. gingivalis*-infected osteoblasts.

2. Materials and Methods

2.1. Materials

Caffeic acid phenethyl ester (CAPE) was purchased from Calbiochem (San Diego, USA). Antibodies against phosphorylated eIF2a (Ser51), NLRP3 (D4D8T), IκB-α and GAPDH (14C10) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against PKR (M-515), phospho-PKR (Thy 446, sc-101783), NF-κB p65 (F-6), caspase-1 p10 (M-20), IL-1β (H-153) and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the SNAP-tag® rabbit polyclonal antibody was obtained from New England Biolabs.

2.2. Establishment of SNAP-P.g.

SNAP-*P.g.* was constructed by transforming *P. gingivalis* ATCC33277 as described previously [23]. Briefly, *SNAP26b* was amplified from the bacterial expression plasmid pSNAP-tag® (T7). The promoter (*protrxB*) and terminator (*termtrxB*) regions of *trxB* (Gene ID 6330860) were also amplified. The three PCR fragments were ligated into pBlueScript®II Phagemid Vector (pSK), and the resulting recombinant plasmids (pSK- protrxB_SNAP26b_termtrxB) were transformed into *E. coli* Single Step (KRX) competent cells. The protrxB_SNAP26b_termtrxB (*pst*) fragment was cloned into a shuttle vector, pT-COW and the resulting plasmid (pT-COW_pst) was introduced into *P. gingivalis* strain ATCC33277 by electroporation.

2.3. Bacterial cultures

SNAP-*P.g.* was cultured in Brain Heart Infusion (BD Bioscience, Franklin Lakes, NJ) containing 0.5% yeast extract (BD Bioscience), 10 μg/mL hemin (Wako Chemicals, Osaka, Japan), 1 μg/mL 2-methyl-1, 4-naphthoquinone (vitamin K3) (Tokyokasei, Tokyo, Japan) and 5 μg/mL tetracycline in an anaerobic condition at 37°C.

2.4. Cell culture and treatment with SNAP-*P.g. preparations*

The mouse osteoblastic cell line MC3T3-E1 cells were plated at a density of 3×10^3 cells/ mL and cultured in plastic dishes in α-MEM supplemented with 10% FBS for 5 days as previously described [24]. For experiments, the medium was changed to α-MEM supplemented with 10% FBS containing 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate, and the cells were infected with SNAP-*P.g.* at a multiplicity of infection (MOI) of 100 for the desired time period.

2.5. SDS-PAGE and western blot analysis

Cells were washed with PBS and were scraped into TN lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% NP-40) in the presence of protease inhibitors (4 μg/mL aprotinin, 1 μg/mL leupeptin, 0.2 mM PMSF). The protein extracts obtained were subjected to immunoblotting according to the protocol described previously [25].

2.6. Real-time PCR

Total RNA was isolated from MC3T3-E1 cells using ISOGEN (Nippon Gene, Tokyo, Japan), followed by phenol extraction and ethanol precipitation. The cDNA was synthesized using Prime Script™ RT (Takara Bio, Kyoto, Japan) as per manufacturer's protocol. The levels of mRNA were measured by the real-time reverse transcription-PCR method using a 7300 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) and SYBR Premix Ex Taq™ (Takara Bio) for detection. The primer sequences were as follows: mouse GAPDH (NM_001001303): forward, 5'-TGTGTCCGTCGTGGATCTGA-3', reverse, 5'-TTGCTGTTGAAGTCGCAGGAG-3'; mouse NLRP3 (NM_145827.3): forward, 5'-ACTGAAGCACCTGCTCTGCAAC-3', reverse, 5'-AACCAATGCGAGATCCTGACAAC-3'.

2.7. Luciferase assay

The NF-κB promoter reporter assay was performed by transfection of the luciferase plasmid pNF-κB-Luc containing 5 × binding sites (TGGGGACTTTCCGC) (Agilent Technologies, La Jolla, CA). One μg of pNF-κB-Luc and 0.05 μg of pRL-TK *Renilla* luciferase vector (Promega) expressing *Renilla* luciferase (as control of transfection efficiency) were used for electroporation into MC3T3-E1 cell using an Amaxa® Nucleofector II -program T-20 (Lonza). The luciferase activity of NF-κB was measured by Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's directions and normalized to the control *Renilla* luciferase activity.

2.8. Immunocytochemistry

MC3T3-E1 cells were cultured on sterile 18-mm round coverslips and immunocytochemistry was performed as described previously [6]. Briefly, the cells were fixed with 3% formalin for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 2 min on ice. After blocking with 4% BSA in PBS for 1 h, the cells were incubated with anti-NF-κB p65 antibody overnight at 4°C, followed by Alexa Fluor 488-conjugated anti-mouse IgG. The samples were mounted and observed using an inverted fluorescence microscope (ECLIPSE Ti-U, Nikon, Tokyo). Images were acquired using ECLIPSE Ti-U microscope and analyzed with its NIS-Elements software (Nikon, Tokyo, Japan).

2. 9. shRNA

MC3T3-E1 cells were plated at a density of 0.5×10^4 cells in 96-well plates and infected with MISSION® shRNA lentiviral transduction particles specific for mouse PKR (NM_011163, Sigma-Aldrich) according to the manufacturer's directions. The infected cells were selected as stable clones by treatment with 5 μg/mL of puromycin. MISSION® Non-Target shRNA lentiviral transduction particles were used as negative control.

2. 10. Statistical analysis

All data are expressed as means ± SD with a minimum of 3 independent experiments performed for each assay. The Student's *t*-test was used for statistical analysis.

3. Results

3.1. SNAP-P.g. activates PKR and induces NLRP3 expression in osteoblasts

We first established a SNAP26b-tagged *P.gingivalis* (SNAP-*P. g.*) in order to specifically trace the internalization of *P. gingivalis* into osteoblastic cells. MC3T3-E1 cells were infected with SNAP-*P.g.* at a MOI of 100 for 0 to 24 h, and SNAP-*P.g.* was detected by western blotting using antibody against the SNAP26b protein. As shown in Fig. 1A, the intensity of the band corresponding to SNAP26b increased at 1 h and remained increased up to 24 h post infection, indicating that SNAP-*P.g.* internalized into MC3T3-E1 cells. We then examined whether the infection of SNAP-*P. g.* induced PKR activation in these cells. The level of total PKR was increased at 1 h and persisted up to 6 h post infection. The phosphorylation of PKR at threonin 446, however, did not change upon SNAP-*P.g.* infection (Fig. 1A). We therefore examined the effect of SNAP-*P.g.* on PKR phosphorylation at earlier time points. As shown in Fig. 1B, SNAP-*P.g.* induced the phosphorylation of PKR at threonin 446, 15 min after infection. eIF-2α, the substrate phosphorylated by PKR, was also phosphorylated after 15 min of infection (Fig. 1B). β-actin, used as an internal control, remained unchanged for the samples (Fig. 1A, 1B).

Next, the cells were incubated with or without SNAP-*P.g.* (at a concentration of 100 MOI) for 0 to 24 h and the effect of SNAP-*P.g.* on NLRP3 expression was analyzed by real-time PCR. The mRNA levels of NLRP3 increased and reached maximum expression 6 h post infection, after which it reduced. No increase in the mRNA expression was observed at the 12 h time-point and there was only a slight increase in its level 24 h post-infection (Fig. 1C). Furthermore, the expression of NLRP3 protein in SNAP-*P.g.* treated MC3T3-E1 cells was examined by western blotting. The intensity of the band corresponding to SNAP26b increased in a time-dependent manner up to 24 h (Fig. 1D, middle panel). The level of NLRP3 protein was increased by SNAP-*P.g.* infection from 6 to 12 h.

Consistent with the results obtained by real-time PCR, the increase of NLRP3 was very little in SNAP-*P.g.* treated MC3T3-E1 cells 24 h post infection (Fig. 1D, upper panel).

3.2. SNAP-P.g. activates NF-κB by inducing IκB degradation in osteoblasts

It is reported that NLRP3 expression is increased through the NF-κB pathway in the priming step. We therefore investigated whether NF-κB is implicated in the up-regulation of NLRP3 expression induced by SNAP-*P.g.* in MC3T3-E1 cells. We infected cells with SNAP-*P.g.* for 60 min and then detected the phosphorylation of p65, a component of NF-κB, at serine 536 by western blotting. As shown in figure 2A, the level of phosphorylated p65 was increased upon infection (Fig. 2A, upper panel). Because NF-κB phosphorylates in the cytoplasm and translocates to the nucleus only after the degradation of inhibitor IκB (which bind to NF-κB), we examined the effect of SNAP-*P.g.* on the level of IκB protein. As expected, SNAP-*P.g.* infection reduced the IκB levels in MC3T3-E1 cells (Fig. 2A, middle panel). We then analyzed the localization of p65 in cells with or without infection by immunocytochemistry using an antibody for p65. In control cells, p65 was observed in cytoplasm (Fig. 2B, a, c), while in cells infected with SNAP-*P.g.*, the major part of p65 translocated from cytoplasm to nucleus (Fig. 2B, b, d, e). We then assessed the transcriptional activity of p65 using a dual luciferase reporter assay. The luciferase plasmid pNF-κB-Luc and pRL-TK *Renilla* luciferase vector were transfected into MC3T3-E1 cells. After transfection, the cells were treated with SNAP-*P.g* for up to 3 h, and then luciferase activity was assessed by a luminometer. The transcriptional activity of p65 was not altered 1 h post infection, however, it was significantly increased at 3 h ($p < 0.01$) (Fig. 2C).

3.3. NF-κB regulates SNAP-P.g.-induced NLRP3 expression in osteoblasts

To clarify whether the NF-κB activation induced by SNAP-*P.g.* was involved in the increase of NLRP3 expression, we pre-treated cells with BAY11-7085, an inhibitor of IκB phosphorylation, and

then infected the cells with SNAP-*P.g.* for 1 h. As shown in figure 3A, BAY11-7085 inhibited the SNAP-*P.g.*-induced IκB degradation and p65 phosphorylation. These inhibitions also partly decreased the mRNA expression of NLRP3 (Fig. 3B). Consistent with the results obtained by real-time PCR, BAY 11-7085 also inhibited the protein level of NLRP3 induced by SNAP-*P.g.* (Fig. 3C).

3.4. Reduction of PKR alters NF-κB activation and NLRP3 expression induced by SNAP-P. g. in osteoblasts

To clarify whether PKR is implicated in the molecular mechanism by which SNAP-*P.g.* increased NLRP3 expression, PKR knockdown cells were constructed by infecting MC3T3-E1 cells with a lentivirus expressing a shRNA specific for PKR. We established two clones of the PKR knockdown cells (#1, #2), and confirmed the efficiency of shRNA knockdown in them. Figure 4A shows that PKR knockdown cells (#1, #2) expressed lower levels of PKR protein when compared to the untransfected wild type cells (-). The lower level of PKR protein was not observed in the cells infected with non-target shRNA as negative control (NC) (Fig. 4A). Next, we examined the effect of PKR on the IκB degradation and NF-κB phosphorylation in SNAP-*P.g.*-infected cells. The infection with SNAP-*P.g.* for 1 h induced IκB degradation and NF-κB phosphorylation in (-) and NC cells, however the effect was not observed in PKR knockdown cells (#1, #2) (Fig. 4B). The transcriptional activity of NF-κB was also significantly increased by SNAP-*P.g.* infection in wild type (-) and NC cells. However, SNAP-*P.g.* did not affect the transcriptional activity of NF-κB in PKR knockdown cells (#1, #2) (Fig. 4C).

We later examined if the NF-κB inactivation by shRNA specific for PKR affects the expression of NLRP3. In wild type and NC cells, infection with SNAP-*P.g.* for 3 h significantly increased the mRNA expression of NLRP3. This increase was not observed in PKR knockdown cells (#1, #2) (Fig. 4D, left). 24 h post infection, SNAP-*P.g.* slightly increased the mRNA expression of NLRP3 in(-) and NC cells. In PKR knockdown cells (#1, #2), the NLRP3 expression was same as compared to that in

(-) and NC cells (Fig. 4D, right). Figure 4E shows that SNAP-*P.g.* increased the level of NLRP3 protein in (-) and NC cells 6 h post infection, while the expression of the protein was repressed by the knock down of PKR (Fig. 4E, #1, #2).

4. Discussion

In this study, SNAP-*P. g.* increased the activity of PKR and NF-κB, and induced NLRP3 expression in osteoblasts. The inhibition of NF-κB attenuated SNAP-*P. g.*-induced NLRP3 expression, suggesting that SNAP-*P. g.* increases NLRP3 expression through the NF-κB signaling pathway. Finally, the knockdown of PKR by shRNA decreased both the activity of NF-κB and expression of NLRP3 induced by SNAP-*P. g.*. Based on these observations, we concluded that PKR was activated by SNAP-*P. g.* and then increased the expression of NLRP3 by activating NF-κB in osteoblasts.

Bacteria and its individual components such as LPS are known to induce PKR activation and inflammation in different kinds of cells [26]. We also observed that LPS from *E. coli* induces PKR activation, resulting in the production of pro-inflammatory cytokines in human gingival cells [6]. However, it was not known whether the infection of oral microorganisms implicated in periodontal diseases can activate PKR in osteoblasts. In this study, PKR was phosphorylated 15 min after *P. gingivalis* infection followed by the phosphorylation of eIF-2α, (Fig. 1B), suggesting that *P. gingivalis* activates PKR in osteoblasts.

In contrast, the mechanism by which *P. gingivalis* activates PKR remains unknown. Since *P. gingivalis* has been known to internalize into cells and cause chronic inflammation [27], it was initially expected that *P.gingivalis* affect PKR after its invasion of osteoblasts. We therefore used *P. gingivalis* labeled with SNAP26b tag-protein (SNAP-*P.g.*) in order to trace its internalization. In SNAP-*P.g.*-treated osteoblasts, SNAP26b was detected starting from 1 h up to 24 h post infection (Fig. 1A). In contrast, the phosphorylation of PKR and eIF-2α was observed as early as at 15 min (Fig. 1B) but not 1 h after the internalization of *P.gingivalis* (Fig. 1A). These results indicated that *P. gingivalis* activates PKR without invading the osteoblasts, and therefore other mechanisms are implicated in *P. gingivalis* induced-PKR activation.

We hypothesized that TLRs might mediate PKR activation in osteoblasts infected with *P. gingivalis*. TLRs have been reported to recognize the extracellular milieu and the pathogen-associated molecular pattern (PAMP) such as flagellin and LPS. LPS from *P. gingivalis* is known to be an agonist of TLR2, and it enhances the production of receptor activator of NF-κB ligand (RANKL) in a TLR2/NF-κB dependent manner in mouse parietal osteoblasts [28]. Moreover, the stimulation of TLRs promotes the priming signal of NLRP3 and increases the release of IL-1β release in human monocytes [29]. Thus, it is likely that TLR2 could be involved in the activation of PKR induced by *P. gingivalis*, resulting in the increase of NLRP3 expression. Further studies are needed to address this possibility.

Our results showed that *P. gingivalis* activated PKR almost immediately after infection leading to an increase in both NF-κB phosphorylation and activity within 3 h (Fig. 2). Following NF-κB activation, the expression of NLRP3 mRNA increased at 3 h and reached maximum expression at 6 h, and then decreased and exhibited only a modest level of increase after 24 h of *P. gingivalis* infection (Fig. 1C). In agreement with the report that the priming of NLRP3 is regulated by NF-κB [11], the induction of NLRP3 mRNA by *P. gingivalis* at 3 h was partially dependent on NF-κB in these osteoblasts (Fig. 3B, C). PKR is well known to be essential for NF-κB activation in inflammatory processes [30]. In consistent with those reports, we also observed the knockdown of PKR by shRNA markedly reduced the NF-κB activity (Fig. 4D, left), showing that PKR might increase NLRP3 expression by regulating NF-κB activity. Interestingly, the regulation of PKR on NLRP3 mRNA expression was observed only at 3 h (Fig. 4D, left), but not 24 h after *P. gingivalis* infection (Fig. 4D, right). These results suggested that PKR might play a critical role in NLRP3 expression, especially in early stages of infection.

We concluded that PKR is required for the priming step for NLRP3 in *P. gingivalis*-infected osteoblasts. However as to whether the increase of NLRP3 expression by PKR can lead to the

inflammasome activation remains unanswered in the present study. In agreement with the report showing that IL-1β production is tightly controlled [31], the level of IL-1β expression in osteoblasts was very low and infection with *P. gingivalis* did not increase its extracellular release (data not shown). Moreover, we observed that *P. gingivalis* induced cleavage of pro-caspase-1 into p10 subunit up to 6 h post infection and the PKR shRNA treatment did not affect it (data not shown). Therefore, it is likely that PKR is necessary for the *P. gingivalis*-induced NLRP3 expression in the priming step, but not sufficient for the subsequent activation of NLRP3 in osteoblasts. These results agree with the previous report that PKR was dispensable for caspase-1 activation, processing of IL-1β and secretion of IL-1β induced by LPS in macrophages [32]. In contrast, other studies demonstrated that PKR activates inflammasomes in macrophages treated with LPS [21, 22]. Due to these conflicting data, the role of PKR in the activation of NLRP3 inflammasome still needs further clarification.

The danger signals, such as ATP, released from infected cells are considered to be one of the factors that regulate NLRP3 activation. It has been reported that the activation of inflammasomes requires a second signal such as exogenous ATP in macrophages [13, 33]. In gingival epithelial cells, LPS from *P. gingivalis* induced IL-1β gene, but the cytokine was not secreted unless co-stimulated with LPS and ATP [34]. Recently, the reactive oxygen species (ROS) induced by ATP-$P2X_7$ receptor has been reported to positively regulate the inflammasome activation in gingival epithelial cells infected with *P. gingivalis* [35], and the expression of $P2X_7$ receptor, IL-1β and NLRP3 was also modulated in human chronic periodontitis [36]. Moreover, it was reported that periodontal pathogens such as *P. gingivalis*, *Treponema denticola* and *Tannerella forsythia* induces release of ATP in macrophages [37]. These observations suggest that ATP and ROS produced by oral bacterial infections may contribute to the inflammasome activation, thereby promoting periodontal diseases [38, 39]. To validate the roles of PKR in NLRP3 activation in periodontal diseases, further experiments must be conducted using PKR specific shRNA to show the effects of PKR on caspase-1 cleavage and IL-1β release and the difference in inflammasome activation in the presence of ATP.

Moreover, to clarify the role of PKR in the pathogenesis of periodontal diseases, the effect of biofilms on PKR needs to be further investigated; in this study, *P. gingivalis* was specially evaluated. The formation of biofilms, which contain multiple species of oral bacteria, result in periodontal diseases. Contrary to our results here, subgingival biofilms have been reported to downregulate the expression of NLRP3 mRNA and *IL-1β* in primary human gingival fibroblasts [40]. Notably, the exclusion *of P. gingivalis* from the biofilm reportedly partially rescues this downregulated expression of NLRP3 mRNA and *IL-1β* [41]. In contrast, our results show that *P. gingivalis* may accelerate inflammation by increasing NLRP3 inflammasomes. These observations suggest that *P. gingivalis* is implicated in the host immune system by reducing the expression of NLRP3 inflammasomes in response to an oral biofilms challenge, and that these distinct roles of *P. gingivalis* are important in the pathogenesis of periodontal diseases.

5. Conclusion

We demonstrated that PKR rapidly induces NF-κB in response to infection with *P. gingivalis,* leading to the up-regulation of NLRP3 expression in osteoblasts. The present study suggests that PKR is responsible for *P. gingivalis*-induced NLRP3 expression in the priming step, even though its effects on NLRP3 activation remain unclear. Our findings provide further understanding of the molecular mechanisms by which PKR regulates the process of inflammation in periodontal diseases.

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Figure legends

Fig. 1. SNAP-*P.g.* activates PKR and induces NLRP3 expression in osteoblasts.

(A and B) Western blot analysis of MC3T3-E1 cells infected with SNAP-*P.g.* (MOI =100) for 0, 1, 3, 6 and 24 h (A), for 15, 30 and 60 min (B). (C) Real-time PCR analysis of NLRP3 mRNA expression in MC3T3-E1 cells with (closed squares) or without (opened squares) SNAP-*P.g.* infection for the indicated time periods. Values represent the mean ± S.E.M. (n = 4). ** $p < 0.01$ compared to non-infected MC3T3-E1 cells. (D) The changes in NLRP3 protein levels in the cells infected with SNAP-*P.g.* were analyzed by western blotting.

Fig. 2. SNAP-*P.g.* activates NF-κB by degrading IκB in osteoblasts.

MC3T3-E1 cells were treated with SNAP-*P.g.* at MOI of 100 for the indicated time points. (A) The levels of p65 phosphorylation at serine 536 and IκB were analyzed by western blotting. (B) p65 was detected using an anti-p65 antibody and observing under a fluorescence microscope. a, b; Lower magnification (× 1,000) images of cells non-treated (a) or infected cells (b). The scales were set to 50 μm. c, d, e; Higher magnification images of a and b (× 10,000) in SNAP-*P.g.*-infected (d, e) and control (c) cells. The scales were set to 10 μm. (C) MC3T3-E1 cells were infected with SNAP-*P.g.* at MOI of 100 for 0 to 3 h. The transcriptional activity of NF-κB in SNAP-*P.g.* was assessed by a luminometer. Values represent the mean ± S.E.M. (n = 4). ** $p < 0.01$ when compared to non-infected MC3T3-E1 cells.

Fig. 3. NF-κB regulates SNAP-*P. g.*-induced NLRP3 expression in osteoblasts.

MC3T3-E1 cells were pre-treated with BAY11-7085 and then either infected with SNAP-*P.g* or left uninfected for (A) 1 h (B), 3 h (C) and 6 h. (A) The levels of phospho-p65 and IκB were analyzed by western blotting. (B) The mRNA levels of NLRP3 was measured by real-time PCR. Data were presented as the ratio of *P.gingivalis* and/or BAY11-7085–treated to non-treated cells. Values

represent the mean ± S.E.M. (n = 4). ** $p < 0.01$ compared to individual controls. (C) The expression of NLRP3 protein was analyzed by western blotting.

Fig. 4. Reduction of PKR alters NF-κB activation and expression of NLRP3 induced by SNAP-*P. g.* in osteoblasts.

The two clones of PKR knockdown cells (#1, #2) and non-target shRNA-transfected cells (NC) were established by infecting MC3T3-E1 cells with a lentivirus producing either a shRNA or non-target control shRNA, respectively. (A) The expression of PKR was analyzed by western blotting. The wild type MC3T3-E1 cells (-) were used as a control. (B) The cells were either infected with (+) or left uninfected (-)for 1 h, and subjected to western blot analysis using specific antibodies for phospho-p65, IκB and β-actin. (C) Luciferase activity in MC3T3-E1 cells either infected (closed bar) or uninfected (opened bar) with SNAP-*P.g.* for 3 h. Values represent the mean ± S.E.M. (n = 4). ** $p < 0.01$ compared to individual controls. (D) The expression of NLRP3 mRNA in MC3T3-E1 cells either infected (closed bar) or uninfected (opened bar) for 3 h (left panel) or 24 h (right panel). Values represent the mean ± S.E.M. (n = 4). * $p < 0.05$, ** $p < 0.01$ compared to individual controls. (E) The cells were either infected with (+) or left uninfected (-). for 6 h, and the expression of NLRP3, SNAP26b and β-actin was assessed by western blotting.

Figure 1. SNAP-*P. g.* activates PKR and induces NLRP3 expression in osteoblasts.

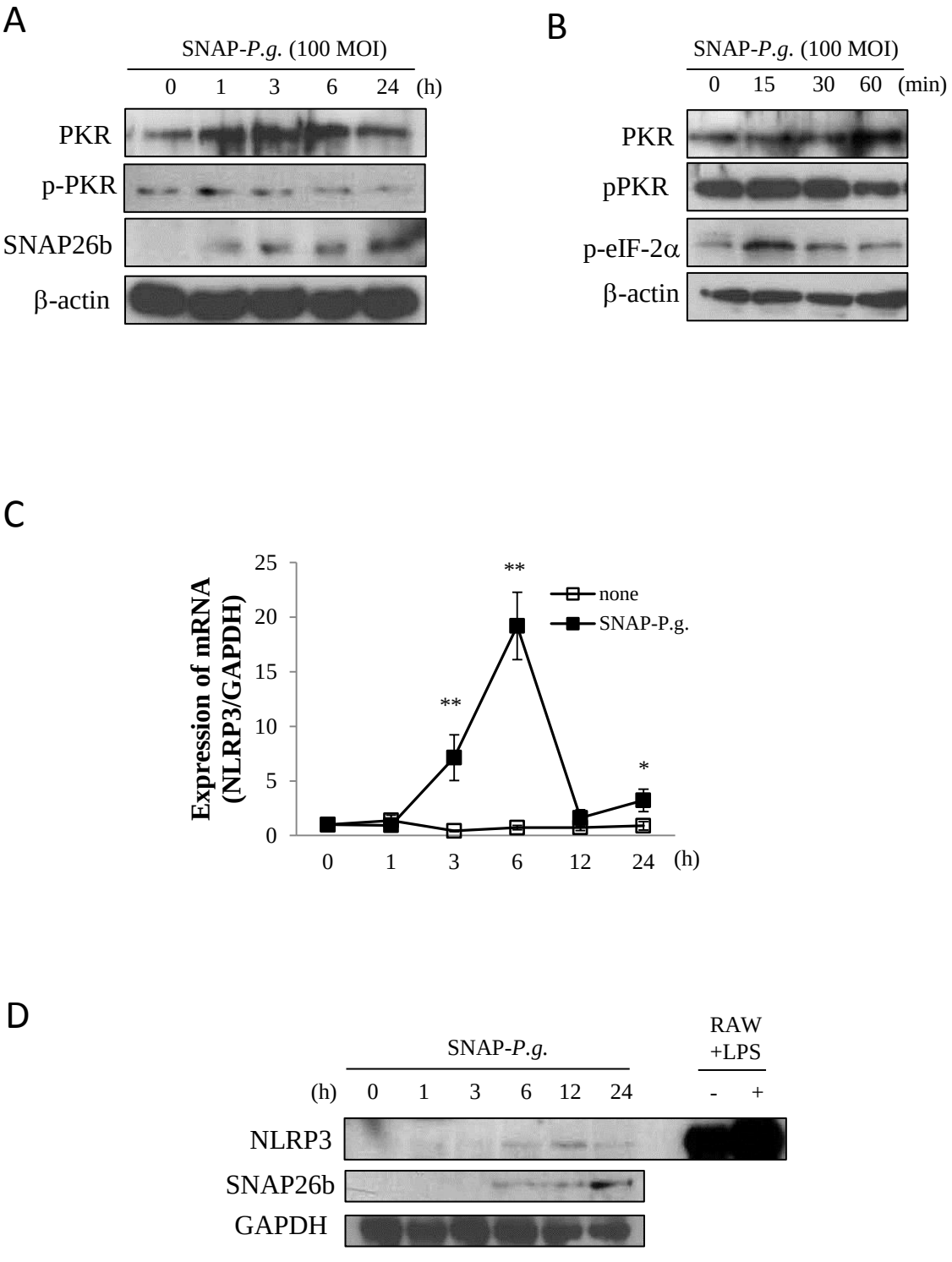
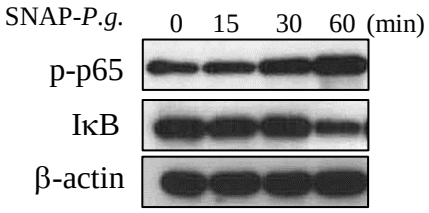


Figure 2. SNAP-*P. g.* activates NF-κB by inducing IκB degradation in osteoblasts.

A



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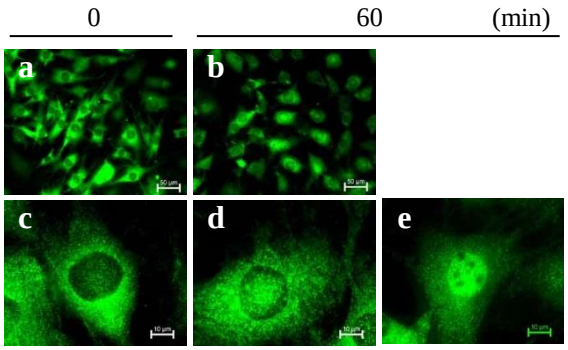

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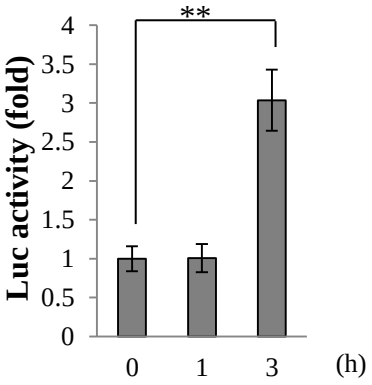
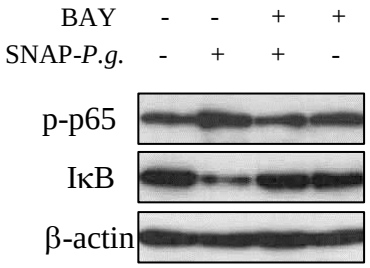


Figure 3. NF-κB regulates SNAP-*P. g.*-induced NLRP3 expression in osteoblasts.

A



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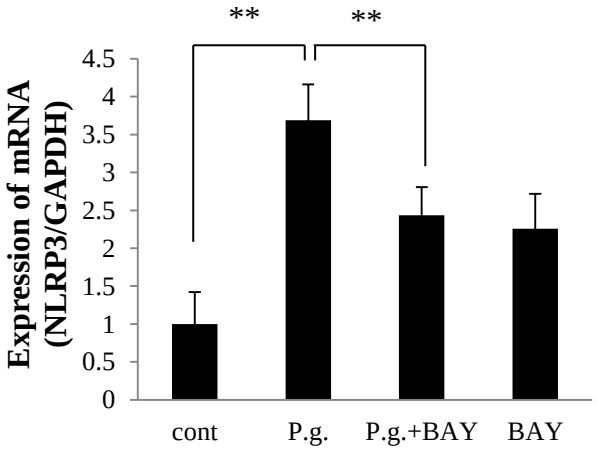

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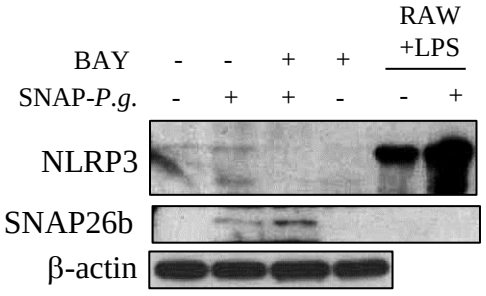
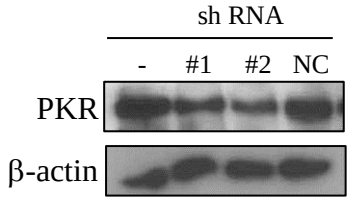


Figure 4. Reduction of PKR alters NF-κB activation and NLRP3 expression induced by SNAP-*P. g.* in osteoblasts.

A



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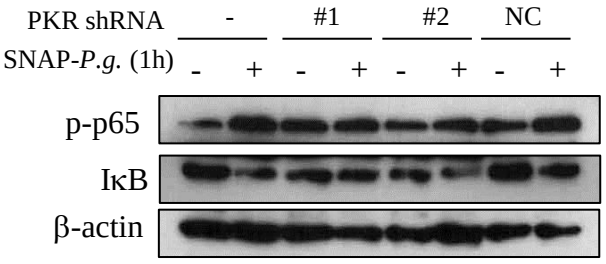

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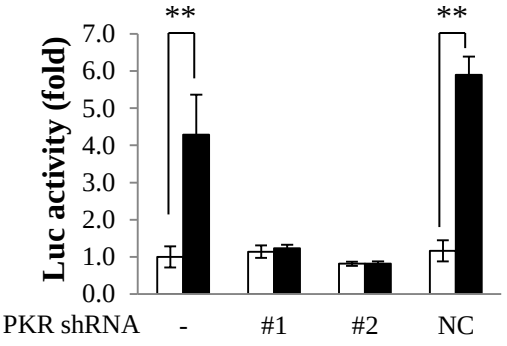
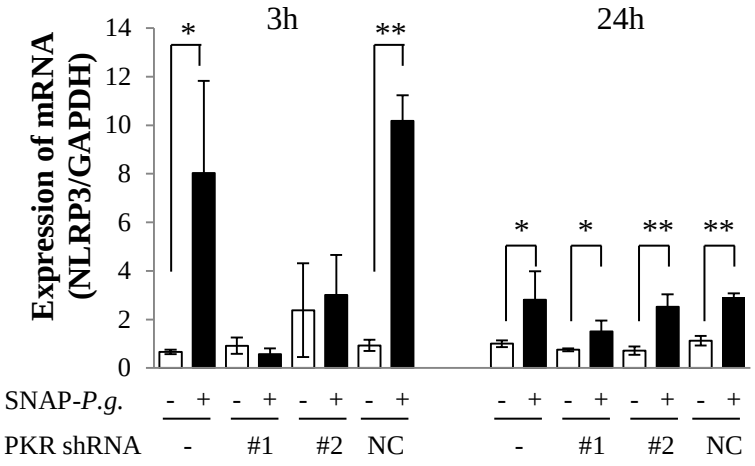


Figure 4. Reduction of PKR alters NF-κB activation and NLRP3 expression induced by SNAP-*P. g.* in osteoblasts.

D



E