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Interaction Between PKR and PACT Mediated by LPS-Inducible NF-κB in Human Gingival Cells

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ABSTRACT

The double-stranded RNA-dependent protein kinase (PKR) is a serine/threonine kinase expressed constitutively in mammalian cells. PKR is activated upon virus infection by double-stranded RNA (dsRNA), and plays a critical role in host antiviral defense mechanisms. PKR is also known to regulate various biological responses, including cell differentiation and apoptosis. However, whether PKR is involved in the progress of periodontitis is not clear. The present study explained the phosphorylation of PKR by LPS in the human gingival cell line, Sa3. Expression of genes encoding LPS receptors was detected in Sa3 cells and treatment of cells with 1 μg/mL LPS for 6 h caused PKR phosphorylation. LPS elevated the expression of the protein activator of PKR (PACT) mRNA and protein, followed by the enhanced association between PACT and PKR within 3 h. In addition, LPS treatment induced the translocation of NF-κB to the nucleus after 30 min, and inhibition of NF-κB decreased the PACT–PKR interaction induced by LPS. The level of pro-inflammatory cytokine mRNA, including interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα), appeared within 45 min and reached at the maximal levels by 90 min after the addition of LPS. This induction of pro-inflammatory cytokines was not affected by RNAi-mediated silencing of PKR and a pharmacological inhibitor of PKR, whereas the inhibition of NF-κB decreased it. These results indicated that LPS induces PKR phosphorylation and the PACT–PKR association in Sa3 cells. Our results also suggest that NF-κB is involved in the PACT–PKR interaction and the production of pro-inflammatory cytokines in periodontitis. J. Cell. Biochem. 113: 165–173, 2012.

KEY WORDS: PKR; LPS; PACT; PERIODONTITIS

The double-stranded RNA-dependent protein kinase (PKR) is a serine/threonine kinase that is expressed constitutively in mammalian cells. During viral infection, PKR is converted from its latent form to an active protein kinase upon binding viral double-stranded RNA (dsRNA), which leads to dimerization and autophosphorylation of PKR [Patel et al., 1995; Romano et al., 1998]. Activated PKR phosphorylates the α subunit of eukaryotic translation initiation factor-2 (eIf2α), which results in the inhibition of protein synthesis in virally infected cells. Thus, PKR plays a critical role in the antiviral defense mechanisms of host cells.

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Because PKR also regulates other aspects of cellular physiology, such as differentiation and apoptosis, that are independent from the binding of the kinase to viral dsRNA, the presence of other PKR activating mechanisms is likely. A protein activator of PKR (PACT), which is an RNA-binding protein, was identified as an activator of PKR in the absence of dsRNA [Patel and Sen, 1998]. The activation of PKR by PACT is mediated by the binding to a PACT-binding motif present in the kinase domain of PKR [Peters et al., 2001; Li et al., 2006]. PACT itself is phosphorylated in response to various chemical stresses and growth factor withdrawal [Peters et al., 2006]. In fact, stress signals induce rapid phosphorylation of PACT, followed by its association with PKR, which leads to the activation of PKR [Patel et al., 2000]. Furthermore, PACT is phosphorylated in response to tunicamycin, an inhibitor of protein glycosylation, inducing the association with PKR and causing its activation [Singh et al., 2009]. The association between RAX, a mouse homologue of PACT, and PKR has been reported to be promoted by ethanol in the central nervous system [Chen et al., 2006]. However, the mechanisms of activation of PACT itself and its interaction with PKR are still obscure.

Many studies suggest that PKR plays pivotal roles in inflammation. Activated PKR has been detected in liver biopsies of patients with chronic hepatitis C [Asselah et al., 2005]. In multiple sclerosis, a chronic inflammatory disease of the central nervous system, PKR activates NF-κB, leading to the production of pro-inflammatory cytokines [Chakrabarty et al., 2004]. Periodontitis, which is also a chronic inflammatory disease, is induced by specific Gram-negative anaerobic bacteria and their products. Lipopolysaccharide (LPS), a major component of the outer membrane of *Poryphyromonas gingivalis*, is a well-known causative agent for periodontitis. LPS binds to cell surface receptors and stimulates the secretion pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα), in a range of cell populations in gingival tissue [Roberts et al., 1997]. LPS-induced pro-inflammatory cytokines cause destruction of the supporting osseous and connective tissue of the teeth [Nair et al., 1996]. Because LPS is one of the factors that can activate PKR, and periodontitis-associated pro-inflammatory cytokines are also known to be regulated by PKR, it is likely that PKR may play a role in periodontitis. However, little is known about the potential involvement of PKR in the progress of periodontitis.

The present study investigated the mechanisms underlying the phosphorylation of PKR induced by LPS in the human gingival cell line Sa3. The results showed that PKR was phosphorylated 6 h after stimulation with 1 μg/mL LPS. LPS induced the expression of PACT and its interaction with PKR in an NF-κB-dependent manner. In addition, the level of pro-inflammatory cytokine mRNA, including IL-6 and TNFα, reached at the maximal levels by 90 min after the addition of 1 μg/mL LPS. This induction of pro-inflammatory cytokines was decreased by inhibition of NF-κB but not PKR. These results indicated that LPS leads to PKR phosphorylation and increases the association between PACT and PKR in human gingival cells. They also suggested that NF-κB plays an important role in the PACT–PKR interaction and the production of pro-inflammatory cytokines in periodontitis.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's medium (DMEM) and OPTI-MEM were purchased from Sigma Chemical Co. (St. Louis, MO) and Invitrogen (Carlsbad, CA), respectively. LPS and 2-aminopurine (2-AP) were obtained from Sigma. Caffeic acid phenethyl ester (CAPE) was purchased from Calbiochem (San Diego, CA). Antibodies against PKR (ab32052) and PACT (ab50916) were obtained from Abcam (Cambridge, MA). Antibody against phosphor-Ser/Thr/Tyr was obtained from AnaSpec (San Jose, CA). Antibodies against Eps15 (C-20), B23(C-16), and Protein A/G PLUS-Agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-V5 antibody was purchased from Nacalai Tesque (Kyoto, Japan).

CELL CULTURE

Sa3 cells, which are gingival epithelial cells derived from human oral cancer, were provided by the RIKEN BRC through the National Bio-Resource of MEXT, Japan. Sa3 cells were cultured in plastic dishes containing DMEM supplemented with 20% FBS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were subcultured every 3 days by treatment of the cells with 0.25% trypsin together with 1 mM EDTA in Ca^{2+}-, Mg^{2+}-free phosphate-buffered-saline (PBS). For experiments, cells were plated in plastic dishes at a density of 10,000 cells/mL and incubated for the desired periods.

RNA PREPARATION AND REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION AND REAL-TIME PCR

Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously [Qiu et al., 2007]. Briefly, total cellular RNA was isolated from Sa3cells using ISOGEN (Nippon Gene, Tokyo, Japan), followed by phenol extraction and ethanol precipitation. The purified RNA was further incubated with DNase I (Sigma) to digest the contaminating DNA. The cDNA was synthesized by using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, Uppsala, Sweden). RT-PCR was performed on the cDNA with the following sense and antisense primers: human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_001001303): forward, 5′-GACCCCTTCATTGACCTCAAC-3′, reverse, 5′-CTTCTCCATGGTGGTGAAGA-3′, human TLR2 (NM_009283): forward, 5′-GGCCAGCAAATTACCTGTGT-3′, reverse, 5′-CCACTTGCCAGGAATGAAGT-3′, human TLR4 (NM_145978): forward, 5′-TACAGAAGCTGGTGGCTGTG-3′, reverse, 5′-CCAGAACCAAACGATGGACT-3′.

Real-time PCR was performed with the 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA) using SYBR Premix Ex Taq™ (Takara Bio, Kyoto, Japan). The sequences of primers were as follows: human GAPDH (NM_007743): forward, 5′-GCACCGTCAAGGCTGAGAAC-3′, reverse, 5′-TGGTGAAGACGCCAGTGGA-3′, human PACT (NM_003690.3): forward, 5′-GCTGCAGAGGCTGCCATAAAC-3′, reverse, 5′-GGAAGTCTCCAGCCATGATGAATAG-3′, human TNFα (NM_000594): forward; 5′-GACAAGCCTGTAGCCCATGTTGTA-3′, reverse; 5′-CAGCCTTGGCCCTTGAAGA-3′, human IL-6 (NM_000600.3): forward; 5′-AAGCCAGAGCTGTGCAGATGAGTA-3′, reverse; 5′-TGTCCTGCAGCCACTGGTTC-3′.

DNA CONSTRUCTS AND RNAi

The human PACT coding region (NM_003690) was amplified by RT-PCR using the forward 5′-CACCATGTCCCAGAGCAGGCACCGC-3′ and reverse 5′-CTTTCTTTCTGCTATTATCTT-3′ primers. The PCR product was cloned into pcDNA™3.1D/V5-His-TOPO® (Invitrogen) to generate V5-fusion PACT. The plasmid DNA was transferred into cells by electroporation with an Amaxa® Nucleofector II using the program T-20 (Amaxa Biosystems, Cologne, Germany).

An RNA duplex targeting the sequence 5′-UUUACUUCACG-CUCCGCCUUCUCGU-3′ (#1); 5′-UAAGUAUCUCAACAGCUAAU-UUGGC-3′ (#2); 5′-AGGACAGGUAGUCAGAUUUCACUGA-3′ (#3); of human PKR (NM_007614.3), and scrambled control oligonucleotide, 5′-AATTCTCCGAACGTGTCACGT-3′, were synthesized. Ten microliters of Lipofectamine 2000 and 40 nM small interfering RNA or 40 nM control oligonucleotide were used for transfection.

IMMUNOPRECIPITATION AND WESTERN BLOT ANALYSIS

For analysis of the interaction of PKR with PACT, Sa3cells were transfected with plasmids expressing V5-tagged PACT. After transfection, cells 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 lysates were incubated overnight at 4°C with primary antibodies and further incubated with Protein A/G PLUS-Agarose. An aliquot of the lysates was subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes for immunoblotting. PKR proteins were precipitated using anti-PKR. V5 tagged-PACT proteins were precipitated with anti-V5 antibody. Normal rabbit IgG and normal mouse IgG were used as negative controls. Immunoprecipitated complexes were detected by western blot analysis using anti-V5 and anti-PKR antibodies according to the protocol described previously [Okamura et al., 2009]. For analysis of PKR phosphorylation, Sa3 cells were scraped into TN lysis buffer. PKR was precipitated by an anti-PKR antibody and detected by anti-phospho-Ser/Thr/Tyr antibodies as described above.

FRACTIONATION AND IMMUNOFLUORESCENCE MICROSCOPY

Sa3 cells were cultured for 5 days and treated with or without LPS. Nuclear and cytoplasmic proteins were isolated using the CelLytic™ NuCLEAR™ EXTRACTION KIT (Sigma Chemical).

Sa3 cells were cultured on sterile 18-mm round coverslips. For immunocytochemistry, the cells were fixed with 3.0% formalin for 30 min and then permeabilized with 0.1% Triton X-100 in PBS for 1 min at 4°C. After blocking of nonspecific binding sites, cells were incubated with anti-NF-κB antibody overnight at 4°C, followed by Alexa 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR), diluted 1:500 in 4% BSA in PBS for 60 min at ambient temperature. Then, cells were exposed to 10 mg/L Hoechst 33342 for 60 min at room temperature and washed with PBS. The samples were mounted and examined under a microscope equipped with epifluorescence illumination. Photomicrographs were recorded on a computer.

STATISTICAL ANALYSIS

Data are expressed as means (SD). The statistical significances of the differences between the controls and the experimental groups were determined by Student's *t*-test using the SPSS 11.0 for Windows statistical software package (SPSS Inc., Chicago, IL).

RESULTS

LPS INDUCES PKR PHOSPHORYLATION

Since LPS signaling is known to be mediated by binding Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) [Darveau et al., 2004], we first investigated whether the receptors for LPS exist in Sa3 cells. RT-PCR analysis detected the expression of TLR2 and TLR4 mRNA in Sa3 cells. LPS treatment for 60 min increased the expression of TLR2 and TLR4 mRNA (Fig. 1A).

To examine the effect of LPS on PKR phosphorylation in Sa3 cells, cells were treated with 1 μg/mL LPS for 3 h and PKR proteins were immunoprecipitated from cell lysates. After equalizing for the amount of PKR proteins, the phosphorylation of PKR was detected

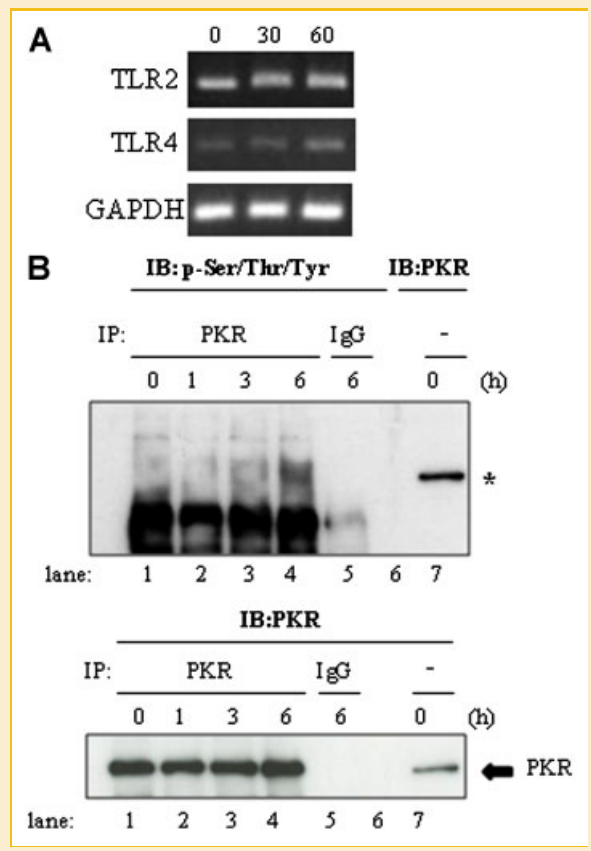

Fig. 1. Effect of LPS on phosphorylation of PKR in Sa3 cells. A: Expression of TLR2 and TLR4 mRNA in Sa3 cells. Sa3 cells were cultured for 5 days and total RNA was isolated. Expression of TLR2 and TLR4 mRNA was assessed by RT-PCR. B: The phosphorylation of PKR was induced by treatment with LPS. Sa3 cells were cultured for 5 days and treated with LPS (1 μg/mL) for the indicated times. PKR protein was precipitated using an anti-PKR antibody (PKR) or normal rabbit IgG (IgG) from whole cell lysates (–). PKR phosphorylation was detected using anti-phospho-Ser/Thr/Tyr antibody. To indicate the position of bands corresponding to PKR, input controls (Ipt) were blotted on the same membrane. The membrane was cut and incubated with an anti-PKR antibody to visualize PKR (68 kDa) (upper panel). Blots were reprobed for PKR expression as a loading control (lower panel).

with an anti-phospho-Ser/Thr/Tyr antibody, which recognizes serine, threonine, and tyrosine phosphorylated proteins. Endogenous PKR proteins were precipitated with an anti-PKR antibody (Fig. 1B, lower panel, lanes 1–4), while incubation with normal rabbit IgG did not show any immunoprecipitated complexes (Fig. 1B, lower panel, lane 5). The same samples were separated by SDS-PAGE and transferred to a PDVF membrane. To visualize the position of the PKR protein (68 kDa), the membrane was cut between lanes 5 and 7 and the pieces were incubated with anti-phospho-Ser/Thr/Tyr (IB: p-Ser/Thr/Tyr) and anti-PKR antibodies (IB: PKR), respectively. Bands corresponding to the phosphorylated PKR proteins were increased by LPS treatment for 6 h in Sa3 cells (Fig. 1B, upper panel, lane 4). These results suggest that LPS induces the phosphorylation of PKR in human gingival cells.

LPS INDUCES PACT EXPRESSION AND ITS ASSOCIATION WITH PKR

The possible involvement of PACT in the phosphorylation of PKR induced by LPS in Sa3 cells was investigated by real-time PCR analysis, which showed that the expression levels of PACT mRNA were significantly increased at 45 min upon stimulation with LPS in Sa3 cells (Fig. 2A). Furthermore, treatment of LPS led to an increase in the amount of PACT protein at 60 min (Fig. 2B).

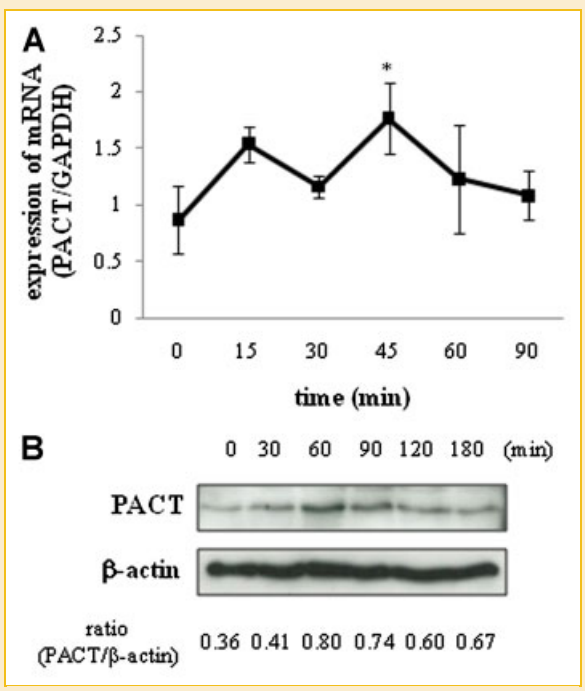

Fig. 2. Effect of LPS on PACT expression in Sa3 cells. A: PACT mRNA was increased by LPS treatment. Sa3 cells were stimulated with 1 μg/mL LPS for the indicated times. Total RNA was isolated and PACT mRNA levels were determined by RT-PCR. Data represent mean (SD) of triplicate measurements. Asterisks indicate statistical significance ($^{*}P < 0.05$). B: LPS increased PACT protein expression in Sa3 cells. Sa3 cells were stimulated with 1 μg/mL LPS for the indicated times. PACT proteins were detected by western blot analysis using an anti-PACT antibody. β-Actin was used as a loading control. The densitometry ratio of PACT which was normalized by that of β-actin using NIH image was shown.

Because PACT binds to PKR, leading to PKR phosphorylation and activation, the effect of LPS on inducing the association between PACT and PKR was examined in Sa3 cells. To detect the interaction between exogenous PACT protein with PKR, an expression vector encoding V5-tagged PACT was constructed and transfected into Sa3cells. An anti-PKR antibody was used to precipitate PKR and associated proteins, and the binding of V5-tagged PACT with PKR was detected by western blot analysis of Sa3 cells treated with or without LPS. The amount of PKR proteins interacting with V5-tagged PACT increased with LPS treatment for 3 h (Fig. 3A). The PKR protein and its binding form with V5-tagged PACT were not detected in the samples immunoprecipitated with normal rabbit IgG. To confirm these results, V5-tagged PACT proteins were also precipitated with anti-V5 antibody, and then PKR protein binding to V5-tagged PACT proteins were detected by anti-PKR antibody.

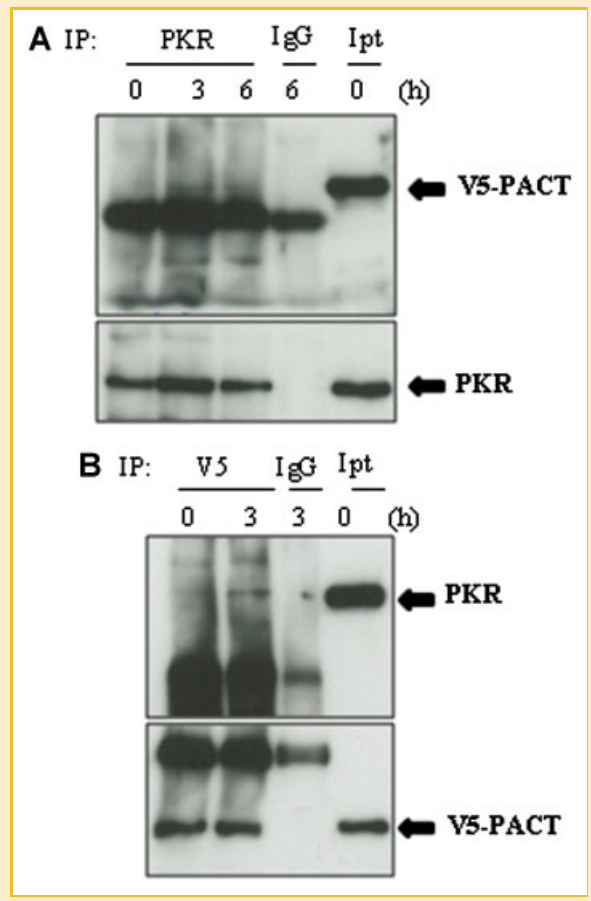

Fig. 3. Effect of LPS on the binding of PACT to PKR in Sa3 cells. Sa3 cells were transfected with a V5-PACT expressing plasmid. Forty-eight hours post-transfection, cells were treated with 1 μg/mL LPS for the indicated times. A: Cell extracts were incubated with an anti-PKR antibody or normal rabbit IgG over-night. The immunoprecipitated samples were separated on PAGE gels and blotted using anti-V5 (upper panel; V5-PACT) or anti-PKR (lower panel; PKR) antibodies. The input control is also shown (Ipt). B: Cell extracts were incubated with an anti-V5 antibody or normal mouse IgG over-night. The immunoprecipitated complexes were subjected to immunoblot analysis with anti-PKR (upper panel; PKR) or anti-V5 (lower panel; V5-PACT) antibodies. Input control is also shown (Ipt).

Consistent with the results shown in Figure 3A, association of exogenous PACT proteins with PKR was increased in LPS-treated cells (Fig. 3B).

THE ASSOCIATION OF PACT WITH PKR INDUCED BY LPS IS DEPENDENT ON THE NF-κB PATHWAY

NF-κB is a well-known transcription factor, which is activated and translocated to the nucleus in response to LPS in many types of cells. We therefore examined whether NF-κB is implicated in the LPS-induced interaction between PACT and PKR in Sa3 cells. Sa3 cells were treated with LPS and fixed and immunostained with an anti-NF-κB antibody. Immunofluorescence analysis showed that NF-κB was enriched in the cytoplasm of Sa3 cells in the absence of LPS stimulation (Fig. 4A, a). In contrast, intense fluorescence for NF-κB was observed in the nucleus in the cells treated with LPS for 30 and 60 min (Fig. 4A, b, c). To confirm these results, cytosolic and nuclear proteins were prepared from the cells treated with or without LPS and analyzed by immunoblotting. Figure 4B shows the presence of the NF-κB protein in the cytoplasmic fraction of untreated cells, while after LPS treatment for 60 min, a fraction of the NF-κB protein was detected in the nuclear fraction (upper). The purity of the nuclear fraction was confirmed using an antibody against B23, which is present in the nuclear fraction but not in the cytosolic fraction (bottom). The purity of cytoplasmic fraction was also confirmed using an anti-Eps15 antibody (middle). These results indicate that LPS induces the translocation of NF-κB to the nucleus in Sa3 cells.

We next investigated whether NF-κB regulates the LPS-induced interaction of PKR with PACT. The activation of NF-κB was inhibited with CAPE, a pharmacological inhibitor of NF-κB [Song et al., 2008; Lee et al., 2010]. Pretreatment with CAPE blocked the nuclear localization of NF-κB induced by LPS (Fig. 5A). These results suggest that CAPE may inhibit NF-κB activity in our experimental models. Sa3 cells expressing V5-tagged PACT were pretreated with 200 μM CAPE for 30 min and then stimulated by LPS for 3 h. PKR proteins were immunoprecipitated by an anti-PKR antibody and the interaction of PACT proteins with PKR was detected using an anti-V5 antybody. In agreement with the results shown in Figure 3,

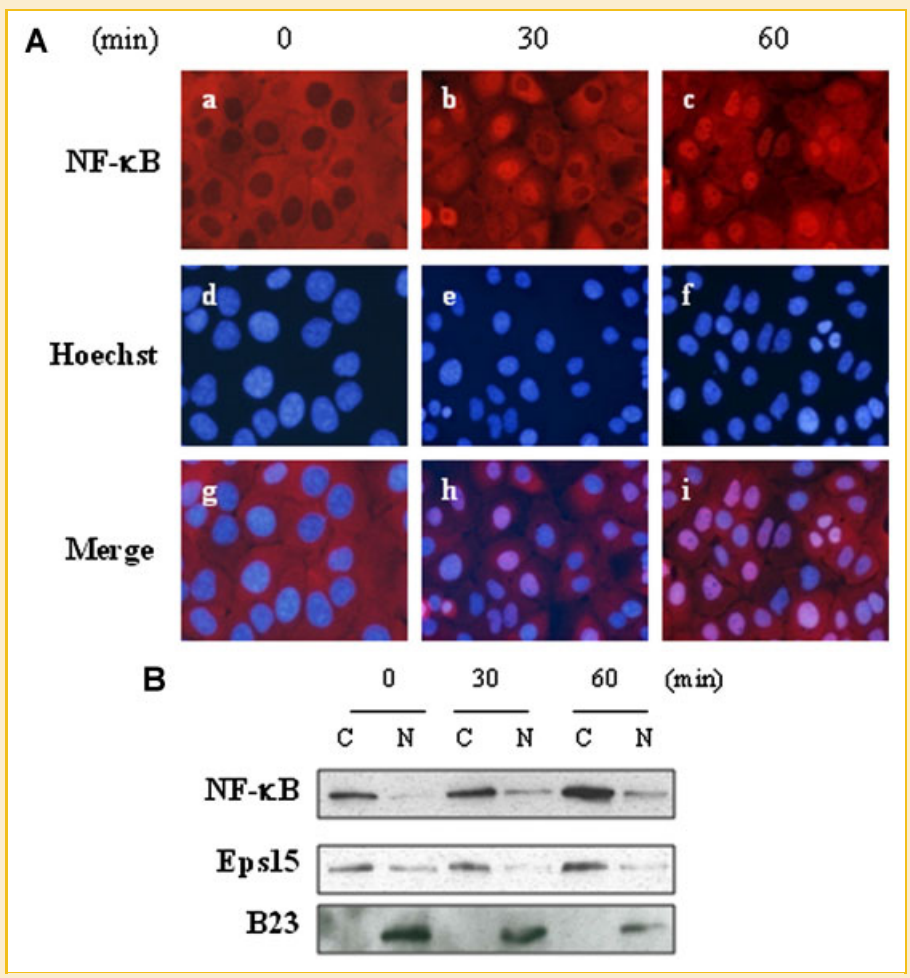

Fig. 4. Changes in NF-κB localization induced by LPS in Sa3 cells. A: Sa3 cells were cultured on cover slips for 3 days and treated with or without 1 μg/mL LPS for 30 or 60 min. Then, cells were subjected to indirect immunofluorescence with an anti-NF-κB antibody (a, b, c). Hoechst 33342 was used to stain nuclei (d, e, f). Microscopic images of the same field were taken and merged (g, h, i). B: Sa3 cells were treated with or without LPS (1 μg/mL) for 30 or 60 min. Cytoplasmic (C) and nucleic proteins (N) were prepared from the cells. Each sample was subjected to SDS-PAGE and immunoblot analysis with anti-NF-κB (top), anti-Eps15 (middle), and anti-B23 antibodies (bottom). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

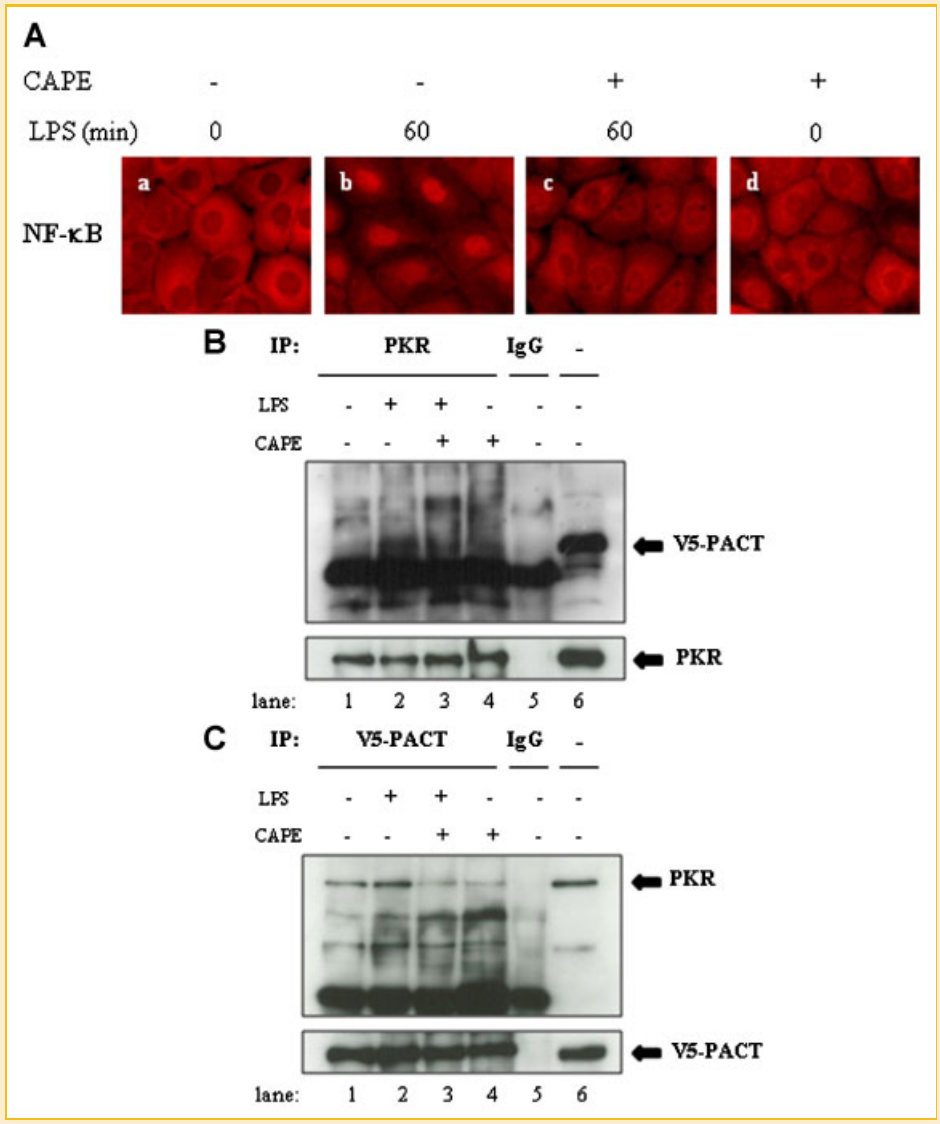

Fig. 5. NF-κB-dependent interaction of PACT with PKR in LPS-stimulated Sa3 cells. A: Sa3 cells were cultured on cover slips for 3 days. Cells were pretreated with (c, d) or without (a, b) 200 μM CAPE for 30 min and then treated with 1 μg/mL LPS for 60 min (b, c). Cells were then subjected to indirect immunofluorescence with an anti-NF-κB antibody. B: Sa3 cells were transfected with a V5-PACT expressing plasmid. After 48 h, Sa3 cells were pretreated with 200 μM CAPE, an inhibitor of NF-κB, for 30 min, and then stimulated with 1 μg/mL LPS for 3 h. PKR protein was precipitated from cell lysates using an anti-PKR antibody. The immunoprecipitated complexes were separated by SDS-PAGE and blotted. Binding of the PACT protein with PKR was detected by anti-V5 antibody (upper panel). The membrane was stripped and then PKR protein was detected with an anti-PKR antibody (lower panel). The input control is indicated by Ipt. C: V5 tagged-PACT proteins were immunoprecipitated with an anti-V5 antibody. PKR proteins binding to the V5 tagged-PACT protein were detected by an anti-PKR antibody (upper panel) as described above. In the same membrane, the V5 tagged-PACT protein in the immunoprecipitated complexes was detected with an anti-V5 antibody (lower panel). The input control is also shown (Ipt). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

treatment of cells with LPS for 3 h increased the binding of PKR to V5-tagged PACT (Fig. 5B, lane 2). In contrast, CAPE reduced this LPS-stimulated interaction between PKR and V5-tagged PACT (Fig. 5B, upper panel, lane 3). To confirm these results, V5-tagged PACT was precipitated with an anti-V5 antibody and PKR proteins included in the immunoprecipitated complexes were detected by western blot analysis using an anti-PKR antibody. LPS treatment for 3 h enhanced the binding of V5-tagged PACT protein to PKR (Fig. 5C, upper panel, lane 2) and this increase was prevented by treatment with CAPE (Fig. 5C, upper panel, lane 3). These results indicate that NF-κB activation can augment the association of PKR and PACT induced by LPS in Sa3 cells.

THE INDUCTION OF CYTOKINES BY LPS IS AFFECTED BY NF-κB BUT NOT PKR

Furthermore, we investigated the expression levels of pro-inflammatory cytokines in LPS-treated Sa3 cells were analyzed by real-time PCR. The treatment of LPS led to an increase in the expression of IL-6 and TNFα mRNA within 45 min. The peak levels of both IL-6 and TNFα mRNA were seen at 90 min after the addition

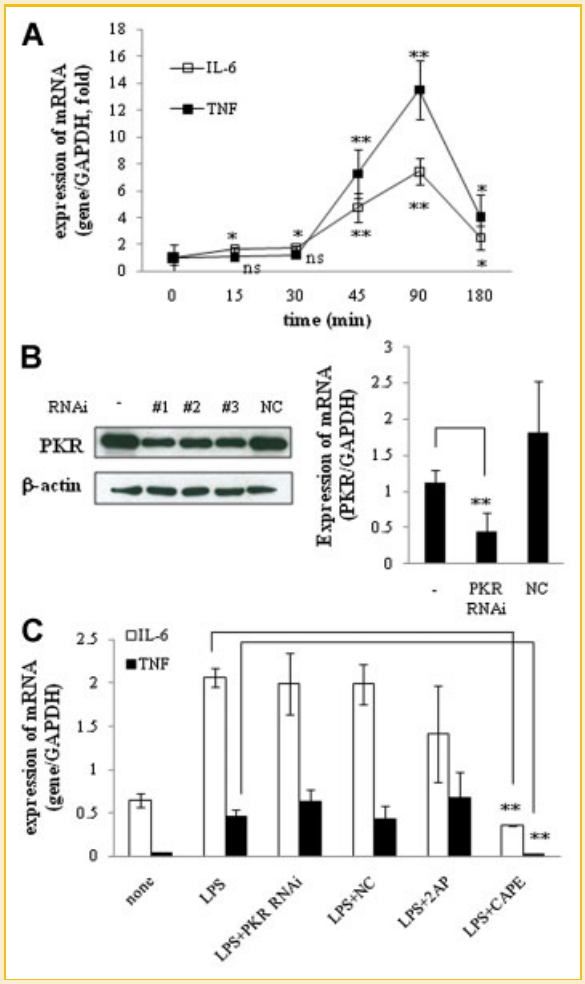

Fig. 6. Changes in cytokine production induced by LPS in Sa3 cells. A: Sa3 cells were cultured for 5 days and treated with 1 μg/mL LPS for indicated periods. Total RNA was isolated and IL-6 and TNFα mRNA levels were determined by RT-PCR. Data represent mean (SD) of triplicate measurements. Asterisks indicate statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$) and ns indicate no significance compared with the control at 0 min. B: Sa3 cells were treated with three individual RNAi duplexes targeting PKR gene (#1, #2, and #3) for 48 h. Control oligo nucleotides with a similar GC content (48%) were used as negative control, which has no effect on PKR (NC). Whole cell lysates were extracted analyzed by Western blotting with anti PKR and β-actin antibodies. Total RNAs were isolated and the expression of PKR mRNA was determined by Real-time PCR. Data represent mean (SD) of triplicate measurements. Asterisks indicate statistical significance ($^{**}P < 0.01$). C: Sa3 cells were treated with RNAi duplexes (PKR RNAi) or scramble nucleotides (NC) for 48 h, and then incubated with LPS for 90 min under pre-treatment with CAPE or 2-AP. Total RNAs from Sa3 cells were analyzed by Real-time PCR for the expression of IL-6 and TNFα. Data represent mean (SD) of triplicate measurements. Asterisks indicate statistical significance compared with LPS-treated Sa3 cells ($^{**}P < 0.01$).

of LPS, followed by a decrease in expression to basal levels up to 180 min (Fig. 6A).

To determine whether PKR was involved in the LPS-induced cytokine production, we inhibited the expression of PKR by RNAi in Sa3 cells. Indeed, in these cells, all of three individual RNAi duplexes targeting PKR gene (#1, #2, and #3) suppressed the level of PKR protein compared with that in control cells. The scramble nucleotides (NC) did not change the amount of PKR protein (Fig. 6B). We observed that the number 1 (#1) of RNAi duplexes also decreased the expression of PKR mRNA (Fig. 6B).

Next, we treated Sa3 cells with RNAi duplexes or scramble nucleotides for 48 h. Then, Sa3 cells were incubated with 1 μg/mL LPS for 90 min after pre-treatment with CAPE or 2-AP, a pharmacological inhibitor of PKR. As shown in Figure 6C, pretreatment with CAPE significantly inhibited the increase of IL-6 and TNFα mRNA after 90-min incubation with LPS, whereas PKR depletion by using RNAi did not affect that. Similarly, the expressions of IL-6 and TNFα mRNA induced by LPS were not changed when PKR activity was suppressed by treatment with 2-AP (Fig. 6C).

DISCUSSION

In this study, we examined the effect of NF-κB on the PACT–PKR interaction induced by LPS because NF-κB is an important factor involved in both cytokine production and the PKR activation pathway. As shown in Figure 4, in response to LPS treatment, the NF-κB protein shifted from the cytoplasmic fraction to the nuclear fraction. Because NF-κB is a transcription factor that translocates to the nucleus, where it regulates gene expression under various stimuli, the present results suggest that LPS may play a role in the activation of NF-κB. This translocation of NF-κB was first detected within 30 min after LPS treatment, and then PACT bound to PKR leading to PKR phosphorylation, suggesting that the activation of NF-κB by LPS is involved in PACT–PKR interaction in Sa3 cells. In accordance with this, inhibition of NF-κB by CAPE was correlated with a reduction of the PACT–PKR interaction (Fig. 5). These results suggest that NF-κB may regulate the interaction between PACT and PKR, followed by PKR phosphorylation induced by LPS in Sa3 cells.

Studies using dominant-negative PKR mutant cells or PKR-deficient mice showed that NF-κB is not activated in response to viral infection in the absence of PKR [Kumar et al., 1997; Chu et al., 1999]. These studies indicated that PKR functions up-stream of NF-κB. Our results differ from those of previous studies showing that PKR is essential for NF-κB activation. Although the reasons for this discrepancy are unclear, the differences could be attributed to variations in the signaling pathway, as suggested by some reports. Two distinct factors lead to activation of PKR in mammalian cells, namely dsRNA, which is produced by viruses, and PACT, which is activated by bacterial products and cellular stress [D'Acquisto and Ghosh, 2001; García et al., 2006; Blalock et al., 2010]. In the dsRNA pathway, PKR is phosphorylated and activated by viruses such as HIV and influenza [Demarchi et al., 1999; Balachandran et al., 2000]. The activation of PKR induces the degradation of IκBα (inhibitor of κBα), which activates NF-κB DNA binding activity. The activation of NF-κB results in apoptosis and inflammation. These findings suggest that the activation of PKR induced by dsRNA is directly involved in the activation of the NF-κB pathway.

In contrast, the molecular mechanisms of the pathway activated by PACT are not known. To the best of our knowledge, the present study is the first report showing that the regulation of PKR

activation by NF-κB occurs in an upstream pathway involving PACT. PACT was considered to interact with PKR and activate it in vitro in the absence of dsRNA [Patel and Sen, 1998]. On the other hand, studies using siRNA silencing of PACT/RAX or cells expressing phosphorylation mutants of PACT/RAX clearly revealed that PKR requires PACT/RAX to be activated by virus infection in vivo [Bennett et al., 2006]. These reports suggest that the activation of PKR by PACT involves a complex mechanism. In the present study, it remains to be determined which residues of PKR are phosphorylated by LPS. Moreover, we have no direct evidence that LPS increases PKR activity. It would be of interest to study how PACT interacts with PKR in response to LPS and whether its association is actually required for the induction of PKR activity.

In the present study, the question of how activated PKR might be engaged in the pathogenesis of periodontitis remains unanswered. The expression of pro-inflammatory cytokines is increased in gingival tissue from patients with chronic periodontitis [Garlet et al., 2006], indicating that LPS-induced cytokines are among the major pathogens of periodontitis. In this regard, the involvement of LPS-activated PKR in cytokine production would help understand how PKR is involved in periodontitis.

In Figure 6, we observed that IL-6 and TNFα mRNA expression appeared within 45 min and reached maximal levels by 90 min after the addition of LPS, followed by a decrease in expression to basal levels up to 180 min. This rapid induction of pro-inflammatory cytokines was suppressed by inhibitor of NF-κB but not PKR. Moreover, RNAi-mediated silencing of PKR did not affect the production of pro-inflammatory cytokines induced by LPS (Fig. 6C).

It is well known that NF-κB regulates the expression of IL-6 and TNFα in periodontal disease [Patil and Kirkwood, 2007]. Moreover, several reports have showed that both IL-6 and TNFα can activate PKR. These reports and the present observations raise the possibility that LPS rapidly activates NF-κB within 30 min, and then increases IL-6 and TNFα production 90 min after LPS stimulation. The increase in the levels of these pro-inflammatory cytokines could stimulate the association between PACT and PKR at 3 h, followed by PKR phosphorylation at 6 h. The possibility that the rapid production of cytokines is required for the interaction of PACT with PKR is currently under investigation.

In this study, the expression of pro-inflammatory cytokines induced by LPS was decreased to basal levels at 180 min (Fig. 6A). It would be of interest to study how PACT–PKR interaction has effect on the production of cytokines at later stage. Furthermore, additional studies are required to clarify whether PKR affect the production of another causative agent for periodontitis, including prostaglandin E_2 and matrix metalloproteinases [Liu et al., 2010].

In conclusion, LPS induced NF-κB activation and sequential PACT–PKR association, and PKR phosphorylation in Sa3 cells. Our present study also demonstrates that NF-κB is an important factor for the PACT–PKR association and the production of pro-inflammatory cytokines in human gingival cells. These findings provide new insight into LPS-induced phosphorylation of PKR in periodontitis.

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