Nucleotide-binding oligomerization domain 1 regulates *Porphyromonas gingivalis*-induced vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 expression in endothelial cells through NF-κB pathway

**Wan M, Liu J, Ouyang X.** Nucleotide-binding oligomerization domain 1 regulates *Porphyromonas gingivalis*-induced vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 expression in endothelial cells through NF-κB pathway. *J Periodont Res* 2015; 50, 189–196. © 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

**Background and Objective:** *Porphyromonas gingivalis* has been shown to actively invade endothelial cells and induce vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) overexpression. Nucleotide-binding oligomerization domain 1 (NOD1) is an intracellular pattern recognition reporter, and its involvement in this process was unknown. This study focused on endothelial cells infected with *P. gingivalis*, the detection of NOD1 expression and the role that NOD1 plays in the upregulation of VCAM-1 and ICAM-1.

**Material and Methods:** The human umbilical vein endothelial cell line (ECV-304) was intruded by *P. gingivalis* W83, and cells without any treatment were the control group. Expression levels of NOD1, VCAM-1, ICAM-1, phosphorylated P65 between cells with and without treatment on both mRNA and protein levels were compared. Then we examined whether mesodiaminopimelic acid (NOD1 agonist) could increase VCAM-1 and ICAM-1 expression, meanwhile, NOD1 gene silence by RNA interference could reduce VCAM-1, ICAM-1 and phosphorylated P65 release. At last, we examined whether inhibition of NF-κB by Bay117082 could reduce VCAM-1 and ICAM-1 expression. The mRNA levels were measured by real-time polymerase chain reaction, and protein levels by western blot or electrophoretic mobility shift assays (for phosphorylated P65).

**Results:** *P. gingivalis* invasion showed significant upregulation of NOD1, VCAM-1 and ICAM-1. NOD1 activation by meso-diaminopimelic acid
increased VCAM-1 and ICAM-1 expression, and NOD1 gene silence reduced VCAM-1 and ICAM-1 release markedly. The NF-κB signaling pathway was activated by \textit{P. gingivalis}, while NOD1 gene silence decreased the activation of NF-κB. Moreover, inhibition of NF-κB reduced VCAM-1 and ICAM-1 expression induced by \textit{P. gingivalis} in endothelial cells.

\textbf{Conclusion:} The results revealed that \textit{P. gingivalis} induced NOD1 overexpression in endothelial cells and that NOD1 played an important role in the process of VCAM-1 and ICAM-1 expression in endothelial cells infected with \textit{P. gingivalis} through the NF-κB signaling pathway.

The detection of microbial infection by the human innate immune system is highly dependent on the recognition of pathogen-associated molecular patterns, structural components conserved among pathogens and pattern recognition receptors (PRRs). Nucleotide-binding oligomerization domain (NOD) proteins are one type of PRRs. Because NOD proteins localize in the cytoplasm, they are known as intracellular PRRs (1). NOD1, a type of NOD protein, specifically detects \(\text{L-Ala-}\gamma\text{-d-glutamyl-meso-diaminopimelic acid (Tri-DAP)}, \) a compound found in nearly all peptidoglycans (2). In the cardiovascular system, endothelial cells are usually the first structures to be exposed to microbial components, which are detected through PRRs. Because NOD proteins localize in the cytoplasm, they are known as intracellular PRRs (1). 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recovered following antibiotic treatment (9,15). ECV-304 monolayers were infected by *P. gingivalis* at an MOI of 1 : 100 (10⁶ CFU/mL bacteria to 10⁶/mL cells). After incubating for 2 h, half of the cell monolayers were washed by PBS to remove the unattached bacteria and lysed with sterile H₂O for 20 min. Then the lysates were diluted, plated on BHI broth agar and incubated under anaerobic conditions for 7 d. The CFUs of *P. gingivalis* that had adhered to and invaded cells were enumerated. For the other half of the cell monolayers, after *P. gingivalis* infection, PBS douche and antibiotic protection were done, the cells were lysed with sterile H₂O for 20 min. Then the lysates were diluted, plated on BHI broth agar plate and incubated under anaerobic conditions for 7 d. The CFUs of invasive bacteria were counted, and the invasion efficiency was expressed as the percentage of the initial inoculum recovered after invasion assay. The invasion assays were performed in triplicate.

**RNA interference in EVC-304**

RNA interference experiments were done as described previously (17): the siRNAs targeting NOD1 (sense: UUCUGUUUGCCUUGGACACACGCC; antisense: GGCUGUGUCUAAACCCCAAACAGGA) were purchased from Invitrogen (San Diego, CA, USA). ECV-304 cells were transfected for 48 h using Lipofectamine 2000 (Invitrogen) and 100 ps siRNA. Cells transfected with Lipofectamine 2000 plus scrambled siRNA were used as a control group (Roche, Indianapolis, IN, USA). Transfection efficiency was assessed by BLOCK-iT Fluorescent Oligo (Invitrogen). Both control and test groups were infected with *P. gingivalis*. The expression levels of NOD1, VCAM-1 and ICAM-1 were determined using real-time polymerase chain reaction (PCR) and western blotting. The viabilities of ECV-304 cells after transfection, as assessed by 0.2% Trypan Blue exclusion test, were shown to exceed 90%.

**Nucleotide-binding oligomerization domain 1 activation by meso-diaminopimelic acid**

Tri-DAP, an agonist of NOD1, was purchased from InvivoGen (San Diego, CA, USA). EVC-304 cells were stimulated for 24 h at a 10 μg/mL concentration of Tri-DAP. The expression levels of NOD1, VCAM-1 and ICAM-1 were then determined through real-time PCR and western blotting.

**Inhibition of NF-κB signaling pathway**

Bay117082 (inhibitor of NF-κB) was purchased from Sigma (St. Louis, MO, USA). The endothelial cells were treated with 5 μm Bay117082 for 1 h before *P. gingivalis* infection. The expression levels of VCAM-1 and ICAM-1 were then determined through real-time PCR and western blotting.

**Real-time quantitative reverse transcription polymerase chain reaction analysis**

Real-time quantitative reverse transcription (qRT)-PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, LifeTechnologies, Warrington, UK) using SYBR green reagent (Roche). Standard real-time qRT-PCR conditions were as follows: 95°C for 15 s and finally 60°C for 1 min. All samples were performed in duplicate. In addition, all experiments were carried out in triplicate. Expression levels of the target transcripts in each sample were calculated by the 2⁻ΔΔCt method after normalization to β-actin, wherein β-actin was used as the endogenous gene control. The primers specific to NOD1, ICAM-1, VCAM-1 and β-actin are shown in Table 1.

**Western blot analysis**

Cells were harvested and lysed in RIPA lysis buffer containing proteinase inhibitors. Western blot was then performed on cell extracts to detect protein expression levels of NOD1, VCAM-1 and ICAM-1. After measuring concentrations, protein samples were separated by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes by wet blotting. Following wet blotting, the membranes were blocked in 5% nonfat dry milk for 1 h, then exposed to antibodies specific to NOD1 (1 : 1000; Cell Signaling Technology, Danvers, MA, USA), VCAM-1 (1 : 1000; Santa Cruz, CA, USA), ICAM-1 (1 : 1000; Santa Cruz, CA, USA), phosphorylated (p)-P65 (1 : 1000, ZSGB-BIO, Beijing, China). Membranes were maintained at 4°C and antibody exposure continued overnight. The following day they were incubated with secondary antibodies (ZSGB-BIO) for 1 h, and then observed for immunoreactive proteins using an ECL reagent (Thermo, Rockford, IL, USA). Data showed in figures represent one of three independent experiments.

**Table 1. PCR primer used in this study**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence 5' → 3'</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>NOD1</td>
<td>ACAGCAGGCCGAGGATAC</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>AAAGGTGCTAAACGAGAAGG</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>GCCCTGGGAAACACCGAGG</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>GGGTGCCAGTCCACGGGTTTC</td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td>GGACCCACATCTACGTCGACAATGAA</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>TCCAGACCGGGCACTCATAATGACTTCT</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>CCTGGACCCACAGCAATG</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>CCGATCCACACGGAGTACCTTG</td>
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ICAM-1, intercellular adhesion molecule 1; NOD1, nucleotide-binding oligomerization domain 1; VCAM-1, vascular cell adhesion molecule 1.
Electrophoretic mobility shift assays
Nuclear extraction and EMSA were performed as described previously (18). After infected by *P. gingivalis*, ECV-304 monolayer was washed three times with ice-cold PBS then nuclear extracts from cells were detected using the nuclear-cytosol extraction kit (Applygen Technology, Shanghai, China) according to the manufacturer’s instruction. Then 5 μg of each nuclear extracts were subjected to EMSA using a P32-radiolabeled NF-κB oligonucleotide probe (5'AGTTGAGGGGACTTTCCCAGGC-3'). Binding reaction was performed using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA). The DNA–protein complex samples were analyzed on a 6% polyacrylamide gel.

Statistics analysis
Data were evaluated using the Student *t*-test. Throughout the figures, *p* < 0.05 was labeled by one asterisk, *p* < 0.01 by two asterisks.

Results

Invasion efficiency of *P. gingivalis*
The ability of *P. gingivalis* to invade endothelial cells was validated when the invasive *P. gingivalis* was recovered after 7 d incubation on BHI broth agar plate. The CFUs of *P. gingivalis* that had adhered to and invaded cells were enumerated, and about $0.083 \pm 0.005\%$ $(8.27 \times 10^4$ CFU/mL) bacteria had a close contact with ECV-304 cells. The CFUs of invasive bacteria were also counted, and the invasion efficiency was about $0.026 \pm 0.008\%$ $(2.63 \times 10^4$ CFU/mL).

*P. gingivalis* induced nucleotide-binding oligomerization domain 1 expression in endothelial cells
Western blotting detected NOD1 protein expression in ECV-304 cells (Fig. 1B,C). NOD1 expression in cells infected with *P. gingivalis* was at a higher level than those observed in cells that were not infected. The mRNA expression of NOD1 in ECV-304 cells with and without *P. gingivalis* infection was also examined by real-time qRT-PCR (Fig. 1A). NOD1 mRNA levels in infected cells were seen to increase by $2.63 \pm 0.12$-fold (*p* < 0.01) of that seen in healthy cells. These results show that expression of NOD1 increased significantly, both in terms of mRNA and protein levels, when ECV-304 cells were infected with *P. gingivalis*.

*P. gingivalis* induced vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 expression in endothelial cells
Cellular expression levels of VCAM-1 and ICAM-1 were also examined. Compared with the control group, VCAM-1 and ICAM-1 mRNA levels increased by $3.07 \pm 0.22$-fold and $2.67 \pm 0.08$-fold (*p* < 0.01), respectively, in cells infected with *P. gingivalis* (Fig. 1A). The protein expression of VCAM-1 and ICAM-1 also increased (Fig. 1B,C). These results show that the expression of VCAM-1 and ICAM-1 both increased significantly in terms of mRNA and protein levels in ECV-304 cells infected with *P. gingivalis*.
Activating nucleotide-binding oligomerization domain 1 by meso-diaminopimelic acid, an agonist of nucleotide-binding oligomerization domain 1, upregulated the expression of vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 in endothelial cells.

This test was used to see if upregulation of NOD1 played a role in VCAM-1 and ICAM-1 expression in ECV-304 cells. After being activated with 10 μg/mL Tri-DAP for 24 h, real-time qRT-PCR and western blot results showed that Tri-DAP successfully upregulated NOD1 in ECV-304 cells by 2.34 ± 0.15-fold. In addition, mRNA levels showed that VCAM-1 and ICAM-1 were upregulated by 2.91 ± 0.26-fold and 2.00 ± 0.21-fold, respectively (Fig. 2A). As shown in Fig. 2B,C, protein levels were also elevated.

Silence of nucleotide-binding oligomerization domain 1 downregulated the expression of vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 in endothelial cells infected by P. gingivalis.

This test was used to see if downregulation of NOD1 played a role in VCAM-1 and ICAM-1 expression in ECV-304 cells infected with P. gingivalis. Both scrambled siRNA and the NOD1 siRNA did not affect the basal expression of VCAM-1 and ICAM-1 in endothelial cells. After siRNA transfection, cells were infected by P. gingivalis. The cells exposed to siRNA targeting NOD1 that specifically aimed to lower NOD1 expression (Fig. 3). This decreased NOD1 expression also significantly blocked the expression of VCAM-1 and ICAM-1 by nearly two times, both in mRNA (Fig. 3A) and protein levels (Fig. 3B,C). In addition, NOD1, VCAM-1 and ICAM-1 expression levels were not affected by scrambled siRNA.

P. gingivalis induced NF-κB activity in ECV-304, and NF-κB mediated vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 expression by P. gingivalis in endothelial cells.

The NF-κB signaling pathway involved in P. gingivalis infection was measured. As shown in EMSA assay, compared with cells untreated, the NF-κB DNA-binding activity remarkably increased in endothelial cells infected by P. gingivalis (Fig. 4A). However, when NOD1 was downregulated by RNA interference, the NF-κB DNA-binding activity decreased (Fig. 4C). Then the cells were treated with Bay117082 (inhibitor of NF-κB) before P. gingivalis infection. The results showed that after adding 5 μM Bay117082 to the cells, the NF-κB DNA-binding activity decreased (Fig. 4A). Western blot result of p-P65 reconfirmed this result, while the total P65 were used to check equal loading (Fig. 4B,D,G). When NF-κB signaling pathway was inhibited by Bay117082, P. gingivalis-induced VCAM-1 and ICAM-1 was reduced to 0.47 ± 0.048-fold and 0.55 ± 0.02-fold in endothelial cells (Fig. 4E,F,H). The results suggested that NOD1-mediating VCAM-1 and ICAM-1 expression was NF-κB dependent in endothelial cells.

Discussion

P. gingivalis is considered to be an important periodontal pathogen. It is reported as being found in atherosclerotic plaques (7,8). In vitro, P. gingivalis has the capability of invading endothelial cells (9) and inducing increased expression of VCAM-1 and ICAM-1 (13–15). How the cells recognize intracellular P. gingivalis and initiate this response is still unknown. This study proposed that NOD1, an intracellular PRR, might play an important role in how the endothelial cell responds to P. gingivalis infection.
P. gingivalis was demonstrated to induce NOD1 expression in endothelial cells. Then two experiments were used to examine the effect of NOD1 in mediating VCAM-1 and ICAM-1 expression in endothelial cells. Exposure of endothelial cells to Tri-DAP led to an increase in NOD1 activity, which in turn caused an increase in the expression of VCAM-1 and ICAM-1. Silencing of NOD1 with siRNA decreased the VCAM-1 and ICAM-1 expression that would normally be induced by P. gingivalis. These results demonstrated that NOD1 plays an important role in regulating VCAM-1 and ICAM-1 expression in endothelial cells, improving the understanding of how P. gingivalis infection may contribute to endothelial cell activation.

P. gingivalis induces NOD1 expression in endothelial cells. Studies have shown that human endothelial cells can express functional NOD1 when cells have been infected by three particular pathogens: Chlamydophila pneumoniae (4), Listeria monocytogenes (5) and Orientia tsutsugamushi (6). All of these pathogens have been shown to invade endothelial cells and induce increased NOD1 expression. NOD1 is believed to play an important role in detecting infections of these microorganisms (4–6).

P. gingivalis has been shown actively to invade endothelial cells (9). This study demonstrated that in addition to the aforementioned microorganisms, P. gingivalis is also capable of inducing NOD1 expression in endothelial cells, which implies that NOD1 may play a role in sensing P. gingivalis endothelial cell infection. NOD1 expression can also be induced in other cell types by various pathogens, such as in epithelial cells by Shigella (19), Campylobacter (20) and enteroinvasive Escherichia coli (21), and in periodontal fibroblasts by P. gingivalis (17). This study, however, is the first documented instance of P. gingivalis-induced NOD1 upregulation in endothelial cells.

Because P. gingivalis is a relatively common organism of the oral cavity, dental activity has the potential to introduce the bacteria into systemic circulation (22,23). P. gingivalis has been shown to actively invade endothelial cells and induce changes that allow it to participate in the inflammatory response (24). Upregulation of adhesion molecules, such as VCAM-1 and ICAM-1, is one of the core changes of endothelial cell activation that can be linked to P. gingivalis infection (24,25).

NF-κB is an important effector in transduction pathways of the inflammatory response. NOD1 have been known as a sensor to trigger activation of NF-κB (26). It is reported that C. pneumoniae were able to induce NF-κB activation in NOD1-overexpressing HEK293 cells, while extracellular challenge failed to activate NF-κB in these HEK293 cells (4). In our present study, P. gingivalis induced NF-κB activation in endothelial cells; meanwhile, the P. gingivalis-induced activation of NF-κB was reduced by siRNA targeting NOD1. Therefore, these results confirmed the role of NOD1 in P. gingivalis-induced NF-κB activation in endothelial cells. The fact that downregulation of VCAM-1 and ICAM-1 by Bay117082 in P. gingivalis-infected endothelial cells confirmed the involvement NF-κB in this process. Thus, NOD1 appears to contribute to P. gingivalis-mediated endothelial cell activation through the NF-κB signaling pathway.

Adhesion molecules initiate leukocyte-endothelial cell contact, leading to leukocyte rolling and transendothelial migration into the vascular wall (27,28). VCAM-1 and ICAM-1 are members of this adhesion molecule family actively expressed on the cell surface (29). Both play a pivotal role in the initiation of atherosclerosis; thus, serum concentrations of VCAM-1 and ICAM-1 are signifi-
cantly higher in patients with coronary artery disease when compared with control groups (30). Increased soluble-ICAM levels also correlate with an increase in the amount of vascular inflammation seen in patients with atherosclerosis (31,32).

Because *P. gingivalis* has been associated with increased expression of VCAM-1 and ICAM-1, infection may also have close ties to endothelial cell inflammation. For this reason, analyzing the mechanism of *P. gingivalis* infection that leads to the upregulation of VCAM-1 and ICAM-1 is important. Results of this study showed that NOD1 is closely involved in this process, highlighting the importance of NOD1 in endothelial cells.

In conclusion, a signaling pathway involving NOD1 has been suggested in endothelial cells infected with *P. gingivalis*. When triggered, this pathway can lead to upregulation and high expression of VCAM-1 and ICAM-1.
tion of the NF-κB signaling pathway in this process was also confirmed. The data presented may improve our understanding of inflammatory mechanisms in endothelial cells.

Acknowledgements

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References
