Original Paper

Nicotine Induces the Production of IL-1 β and IL-8 via the α 7 nAChR/NF- κ B Pathway in Human Periodontal Ligament Cells: an *in Vitro* Study

Lizheng Wu Yongchuan Zhou Zhifei Zhou Yingfeng Liu Yudi Bai Xianghui Xing
Xiaojing Wang

State Key Laboratory of Military Stomatology, Department of Pediatric Dentistry, School of Stomatology, The Fourth Military Medical University, Xi'an, Shaanxi, China

Key Words

Nicotine • α 7 nicotinic acetylcholine receptor • NF-kappa B • Periodontal ligament cells • Periodontitis

Abstract

Background/Aims: Tobacco smoking is a major risk factor for the occurrence and progression of periodontitis. We previously demonstrated that nicotine could induce the expression of α 7 nicotinic acetylcholine receptors (α 7 nAChR) in human and rat periodontal tissues. To further examine the signal pathways mediated by α 7 nAChR in periodontal ligament (PDL) cells, we investigated whether nicotine affects interleukin-1 β (IL-1 β) and interleukin-8 (IL-8) via the α 7 nAChR/NF- κ B pathway in human PDL cells. **Methods:** Human PDL cells were pre-incubated with alpha-bungarotoxin (α -BTX) or pyrrolidine dithiocarbamate (PDTC), then cultured with nicotine. Then, we used western blotting, a dual-luciferase reporter, real-time quantitative PCR and an enzyme-linked immunoassay to assess expression of the NF- κ B p65 subunit, NF- κ B activity and production of IL-1 β and IL-8 in human PDL cells. **Results:** Compared with the control group, nicotine could significantly induce production of IL-1 β and IL-8 in human PDL cells and cause the similar effects on the expression of the NF- κ B p65 subunit and NF- κ B activity. **Conclusion:** This study demonstrates that nicotine could induce production of IL-1 β and IL-8 via the α 7 nAChR/NF- κ B pathway in human PDL cells, providing data for a better understanding of the relationships among smoking, nicotine, and periodontitis.

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L. Wu and Y. Zhou contributed equally to the paper.

Prof. Xiaojing Wang, D.D.S., Ph.D.
and Prof. Xianghui Xing, D.D.S., Ph.D.

State Key Laboratory of Military Stomatology, Department of Pediatric Dentistry, School of Stomatology, The Fourth Military Medical University, Xi'an, Shaanxi (China)
E-Mail wxjingpd@hotmail.com and E-Mail xingxh@fmmu.edu.cn

Introduction

Tobacco smoke, which contains numerous chemicals, can contribute to the progression of periodontal diseases [1, 2]. Nicotine, the main component of tobacco, is a natural alkaloid. The average nicotine amount per cigarette ranges from 1 to 1.8 mg, and saliva concentrations could reach 115 ng/ml, as measured by gas-liquid chromatography [3, 4]. Nicotine-induced pathological changes in human periodontal tissue alter the morphology and structure of cells, decrease bone volume and cause loss of teeth [1, 5-8]. $\alpha 7$ nAChR is a predominant subunit of nAChRs, as well as a potent target of the nicotine binding receptor. In previous studies, we demonstrated the functional expression of $\alpha 7$ nAChR in human and rat periodontal tissue. Up-regulation of $\alpha 7$ nAChR upon the administration of nicotine could be inhibited by pre-treatment with α -BTX, the $\alpha 7$ nAChR antagonist [9], suggesting that nicotine could affect smoking-associated periodontitis via $\alpha 7$ nAChR.

Studies have demonstrated that NF- κ B is functionally expressed in oral tissues. NF- κ B is highly activated in subjects with chronic periodontitis compared with healthy controls [10]. Arabaci T et al. found a significantly higher expression of p65 in fibroblasts in a phenytoin overgrowth (PHT-GO) group with respect to gingivitis and in control groups, indicating that NF- κ B is activated in PHT-related gingival overgrowth [11]. Moreover, $\alpha 7$ nAChR activation could mediate the effects of tobacco smoke and pure nicotine on NF- κ B gene expression and transcriptional activity in oral keratinocytes through the Ras/Raf-1/MEK1/ERK steps [12]. To further examine the signal pathways mediated by $\alpha 7$ nAChR in periodontal cells exposed to nicotine, we focused on NF- κ B and investigated its role as a signaling transmitter.

Periodontitis is often caused by over-expression of pro-inflammatory cytokines and inflammatory mediators in the local microenvironment. The loss of bone volume in smoking-associated periodontitis involves various osteolytic mediators such as IL-1 β , IL-8, RANKL, MMP-2, MMP-9 and tPA [13]. IL-1 β , IL-8 and MMP-8 expressions were decreased significantly in gingival crevicular fluid after periodontal treatment [14]. In addition, levels of IL-1 β and IL-8 could be regulated by NF- κ B pathway. Palmitic acid could induce human HaCaT keratinocytes to produce proinflammatory cytokines IL-6, IL-1 β , and TNF- α in a dose-dependent manner via activation of NF- κ B [15]. P. gingivalis induced IL-6, IL-8, and VCAM-1 expression in human gingival fibroblasts and human PDL cells through the NOD1/2-mediated NF- κ B and ERK1/2 signaling pathways [16]. These data suggest that IL-1 β and IL-8 play critical roles in the pathological changes in periodontitis and $\alpha 7$ nAChR/NF- κ B pathway might regulate these cytokines.

We hypothesized that activation of $\alpha 7$ nAChR could up-regulate expression of the NF- κ B p65 subunit and NF- κ B activity, resulting in the up-regulated production of IL-1 β and IL-8 in human PDL cells. This study was designed to investigate the key role of $\alpha 7$ nAChR/NF- κ B pathway in production of IL-1 β and IL-8 in response to nicotine.

Materials and Methods

Cell culture and isolation of human PDL cells

Human PDL cells were isolated from human first premolars (n=5 patients, n=5 teeth, mean age=12 years) that were extracted for orthodontic treatment. Informed consent was obtained from all the patients and their parents. The extracted teeth were stored in Dulbecco's Modified Eagle medium (DMEM) containing 15% fetal calf serum (FCS) and antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin). The periodontal ligament tissues were dissociated from the mid-third of the root using a scalpel to avoid mixing the gingival and dental pulp tissues. Subsequently, the tissues were cultured in DMEM at 37°C in a humidified atmosphere of 5% CO₂. When the cells surrounding the explants reached confluence, the cell layers were collected and sub-cultured. Passage 5 cells were used for all the experiments.

This study was approved without restrictions by the Research Ethics Committee of School of Stomatology, The Fourth Military Medical University.

Cell grouping

A dose-dependent study was conducted to determine the effects of nicotine on the NF- κ B p65 subunit expression and NF- κ B activity in human PDL cells. The cells were incubated with concentrations of nicotine including 0 (control), 10^{-3} M, 10^{-5} M, 10^{-7} M, 10^{-9} M and 10^{-12} M for 2 hr.

In addition, the PDL cells were divided into five groups to determine the effects of PDTC on nicotine-induced NF- κ B p65 subunit expression and NF- κ B activity, with each group receiving one of the following treatments: (1) control, (2) nicotine (10^{-5} M), (3) PDTC (10^{-5} M) for 30 min

followed by nicotine (10^{-5} M), (4) PDTC (3×10^{-5} M) for 30 min followed by nicotine (10^{-5} M), or (5) PDTC (5×10^{-5} M) for 30 min followed by nicotine (10^{-5} M) and then incubated for 2 hr.

Moreover, the PDL cells were incubated with following stimulation for 24 hr: (1) control, (2) nicotine (10^{-5} M), (3) α -BTX (10^{-8} M) for 30 min followed by nicotine (10^{-5} M), (4) PDTC (5×10^{-5} M) for 30 min followed by nicotine (10^{-5} M), (5) α -BTX (10^{-8} M), (6) PDTC (5×10^{-5} M) to determine the effects of nicotine and/or α -BTX/PDTC on expression of the NF- κ B p65 subunit, NF- κ B activity and production of IL-1 β and IL-8. We selected 10^{-5} M nicotine and 5×10^{-5} M PDTC according to the dose-dependent study.

Real-time Quantitative PCR assays

The total mRNA of the human PDL cells was isolated with an RNeasy Kit (Omega, USA), the mRNA concentration was measured by a Nanodrop spectrophotometer (Thermo-Fischer Scientific, USA), and reverse-transcribed to cDNA by an iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, USA). Real-time PCR was performed with iQ SYBR Green Supermix (Bio-Rad, Hercules, USA) and primers for p65, IL-1 β and IL-8 (Takara, Ohtsu, Japan) and analyzed by the 2 $^{-\Delta\Delta C_T}$ method. β -actin served as the housekeeping gene. Real-time PCR was performed on an ABI PRISM 7500 (Applied Biosystems, USA). The primer sequences are listed in Table 1, and all the PCR efficiencies were comparable.

Dual-luciferase Reporter Assay

We used the pNF- κ B luciferase reporter assay system to determine the effects of nicotine and/or α -BTX/PDTC on the NF- κ B activity in human PDL cells. A dual luciferase reporter gene assay kit was purchased from Promega (Madison, WI, USA). pNF- κ B-luc plasmid and pRL-TK Renilla luciferase reporter plasmid were transfected into human PDL cells using Lipofectamine²⁰⁰⁰ (Invitrogen, CA). After transfection for 6 hr, the cells were incubated in DMEM containing 15% FCS for 48 hr, then incubated with nicotine and/or α -BTX/PDTC for 2 hr.

The activity of NF- κ B was assayed in accordance with the manufacturer's instructions in the luciferase reporter gene assay kit. Results were expressed as detected luciferase Fieflly relative light unit (RLU)/Renilla luciferase RLU.

Western blot analyses

The nuclear fraction of human PDL cells was isolated by the Nuclear Extract Kit (Sangon Biotech, China) and measured by a BCA protein assay kit (Pierce, IL). Samples containing equal amounts of protein were separated by 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked for 1 hr at room temperature in 5% nonfat milk in TBST (Tris buffer salt solution containing 0.1% Tween 20), washed 3 times with TBST (5 min each), and incubated overnight at 4°C with a primary antibody specific to the p65 subunit of NF- κ B (1:800 dilution). The membrane was washed and subsequently incubated for 1 hr at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000 dilution). After washing with TBST, an infrared fluorescence image was obtained using the Odyssey infrared imaging system. Image J version 1.41 o software was used to quantitatively analyze protein expression levels.

ELISA analysis

The cell culture supernatants were collected from human PDL cells with different treatments. The concentrations of IL-1 β and IL-8 were quantified using highly sensitive enzyme-linked immunoassay kit

Table 1. Primers Used for Real-time Quantitative PCR

Primers Used for Real-time Quantitative PCR	
β -actin: forward	5'-TGGCACAGCACAATGAA-3'
reverse	5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'
p65: forward	5'-GAGACATCCTTCCGCAAAC-3'
reverse	5'-TCCTTCCTGCCATAATCA-3'
IL-1 β : forward	5'-CACGATGCACCTGTACGATCA-3'
reverse	5'-GTTGCTCCATATCCTGTCCCT-3'
IL-8: forward	5'-TTGCCAAGGAGTGCTAAAGAA-3'
reverse	5'-GCCCTCTTCAAAAACCTTCTCC-3

Fig. 1. Dose-dependent of nicotine on the NF- κ B p65 subunit expression and NF- κ B activity. (A) The mRNA expression of the NF- κ B p65 subunit in PDL cells. (B) The protein expression of the NF- κ B p65 subunit in PDL cells. (C) The NF- κ B activity in PDL cells. * $P < 0.05$, or ** $P < 0.01$, compared with the control.

(Joyee Biotechnics, China) according to the manufacturer's instructions and normalized to the number of cells. The culture supernatants were thawed only once and assayed in the identical run.

Statistical analyses

All data were derived from experiments performed in triplicate and analyzed by one-way analysis of variance (ANOVA), they are depicted as the mean $\pm$ the standard error (SE). The significance of the differences between the two groups was analyzed by the SNK-q test using SPSS 16.0 software. $P < 0.05$ was considered statistically significant.

Results

Dose-dependent study of nicotine on the NF- κ B p65 subunit expression and NF- κ B activity

We used real-time PCR to amplify the mRNA of the NF- κ B p65 subunit in human PDL cells treated with nicotine in a dose-dependent manner. We did not find that nicotine significantly altered the mRNA expression of the NF- κ B p65 subunit in a dose-dependent manner (Fig. 1A, $P > 0.05$) compared with the control group. We used western blot analysis to determine the protein expression of the NF- κ B p65 subunit in the human PDL cells treated with nicotine in a dose-dependent manner and found that nicotine could induce the protein expression of the NF- κ B p65 subunit (10^{-3} M and 10^{-5} M, $P < 0.01$; 10^{-7} M, $P < 0.05$; 10^{-9} M and 10^{-12} M, $P > 0.05$; Fig. 1B) compared with the control group. In addition, nicotine could up-regulate NF- κ B activity (10^{-3} M and 10^{-5} M, $P < 0.01$; 10^{-7} M and 10^{-9} M, $P < 0.05$; 10^{-12} M, $P > 0.05$; Fig. 1C) measured by the luciferase reporter assay system compared with the control group.

Dose-dependent inhibition of PDTC on the nicotine-induced NF- κ B p65 subunit expression and NF- κ B activity

A significant increase in the protein expression of the NF- κ B p65 subunit was observed after incubation for 2 hr with nicotine ($P < 0.01$; Fig. 2A). Pre-incubation with PDTC for 30 min (10^{-5} M and 3×10^{-5} M, $P > 0.05$; 5×10^{-5} M, $P < 0.01$; Fig. 2A) down-regulated the protein expression of the NF- κ B p65 subunit compared with nicotine stimulation.

In addition, the RLU levels were higher in the group with nicotine stimulation compared with the control group ($P < 0.01$; Fig. 2B). Pre-incubation with PDTC for 30 min (10^{-5} M and

Fig. 2. Dose-dependent inhibition of PDTC on the nicotine-induced NF- κ B p65 subunit expression and NF- κ B activity. (A) The protein expression of the NF- κ B p65 subunit in PDL cells. (B) The NF- κ B activity in PDL cells. ** P < 0.01, compared with the control.

Fig. 3. Effects of nicotine and/or α -BTX/PDTC on expression of the NF- κ B p65 subunit and NF- κ B activity. (A) The protein expression of the NF- κ B p65 subunit in PDL cells. (B) The NF- κ B activity in PDL cells. ** P < 0.01, compared with the control.

3x10⁻⁵ M, P > 0.05; 5x10⁻⁵ M, P < 0.01; Fig. 2B) down-regulated the NF- κ B activity compared with nicotine stimulation.

Effects of nicotine and/or α -BTX/PDTC on expression of the NF- κ B p65 subunit and NF- κ B activity

There was a significant increase in the protein expression of the NF- κ B p65 subunit after nicotine stimulation for 2 hr compared with that in the control group (P < 0.01; Fig. 3A). In addition, the RLU levels were higher in the group with nicotine stimulation compared with those in the control group (P < 0.01; Fig. 3B). Pre-incubation with PDTC or α -BTX for 30 min down-regulated expression of the NF- κ B p65 subunit and the NF- κ B activity compared with nicotine stimulation (P < 0.01; Fig. 3A and B).

Effects of nicotine and/or α -BTX/PDTC on the production of IL-1 β and IL-8

We used real-time PCR to amplify the mRNAs of IL-1 β and IL-8 and ELISA to determine the secretions of IL-1 β and IL-8 in the human PDL cells. Nicotine induced the mRNA expressions

Fig. 4. Effects of nicotine and/or α -BTX/PDTC on the production of IL-1 β . (A) The mRNA expression of IL-1 β in PDL cells. (B) The secretion of IL-1 β in culture supernatants of PDL cells. ** P <0.01, compared with the control.

Fig. 5. Effects of nicotine and/or α -BTX/PDTC on the production of IL-8. (A) The mRNA expression of IL-8 in PDL cells. (B) The secretion of IL-8 in culture supernatants of PDL cells. * P <0.05, or ** P <0.01, compared with the control.

of IL-1 β (P <0.01; Fig. 4A) and IL-8 (P <0.01; Fig. 5A) compared with the control. In addition, nicotine also induced the secretions of IL-1 β (P <0.01; Fig. 4B) and IL-8 (P <0.01; Fig. 5B) compared with the control. IL-1 β production was blocked when the cells were pre-incubated for 30 min with α -BTX or PDTC (P <0.01; Fig. 4A and B), and pre-incubated with α -BTX or PDTC caused the similar effect on IL-8 production (P <0.05; Fig. 5A and B).

These results demonstrate a key role of the $\alpha 7$ nAChR/NF- κ B pathway in the nicotine-induced production of IL-1 β and IL-8 in human PDL cells.

Discussion

In previous studies, we demonstrated the functional expression of $\alpha 7$ nAChR in human PDL cells and rat periodontal tissues [9]. In this study, we provided evidence that the expression of the NF- κ B p65 subunit and NF- κ B activity were enhanced after treatment with nicotine in PDL cells. Additionally, there were significant increases in the production of IL-1 β and IL-8 in the PDL cells after nicotine stimulation. These effects could be blocked by pre-

treatment with α -BTX or PDTC, indicating that nicotine might affect PDL cells function via $\alpha 7$ nAChR/NF- κ B pathway.

NF- κ B is a critical regulator of multiple biological functions, including innate and adaptive immunity as well as cell survival [17]. Constitutive NF- κ B activity is observed in many inflammatory diseases. Andresen et al. [18] reported that colonic mucosal NF- κ B is activated in collagenous and ulcerative colitis. NF- κ B is mediated by $\alpha 7$ nAChR in many tissues. For example, nicotine induces neuroprotection of PC12 cells via the $\alpha 7$ nAChR-JAK2-NF- κ B-STAT3-Bcl-2 pro-survival pathway [19]. Recent studies have revealed that NF- κ B is expressed in oral tissues. Compared with healthy controls, NF- κ B is highly activated in subjects with chronic periodontitis [10]. NF- κ B was activated within 1 hr of exposure to periodontopathogens, which are widely regarded as key etiological agents, in oral epithelial cells [20]. Activation of NF- κ B is essential for up-regulation of COX-2 caused by IL-1 β in human gingival fibroblasts [21]. Suppression of NF- κ B p65, ERK and MEK phosphorylations could down-regulate IL-1 β -induced CCL20 and IL-6 production in human PDL cells [22]. In this study, we found a significant increase in expression of the NF- κ B p65 subunit and NF- κ B activity in PDL cells after nicotine stimulation. Enhanced NF- κ B activity is closely related to changes in activation factors such as lipid peroxidation, rather than to increased mRNA [23]. As a result, the difference in the p65 gene expression was not statistically significant and eliminated the detection of p65 in the nucleus. In our study, we demonstrated that pretreatment with α -BTX could suppress this effect, suggesting that the transcription of NF- κ B is mediated by $\alpha 7$ nAChR in human PDL cells.

Another important finding of this study was that nicotine up-regulated the production of IL-1 β and IL-8 in human PDL cells, which could be inhibited by pretreatment with PDTC or α -BTX, suggesting that $\alpha 7$ nAChR/NF- κ B pathway might play a key role in the up-regulation of these cytokines in smoking-associated periodontitis. Inflammatory cytokines, such as IL-1 β and IL-8, in gingival keratinocyte are responsible for periodontitis progression and periodontal tissue destruction, particularly in periodontitis in tobacco users [24]. IL-1 β is a multifunctional cytokine that regulates various cellular and tissue functions. Studies also have confirmed that periodontal bone resorption is predominantly caused by IL-1 β secreted by macrophages [25, 26], and up-regulated expression of IL-1 β could induce RANKL production caused by nicotine in human PDL cells co-cultured with CD4⁺ T cells [27]. Engebretson SP et al. [28] found that there was an increase in the IL-1 β levels in the gingival crevicular fluid of periodontitis patients, and IL-1 β level was decreased significantly in gingival crevicular fluid after periodontal treatment [14]. Additionally, Dongari-Bagtzoglou et al. [29] reported that IL-8 expression is significantly higher in PDL cells from periodontitis patients than in PDL cells from healthy controls, and the level of IL-8 is negatively related to the periodontal treatment [14]. It has also been reported that tobacco smoke could up-regulate pro-inflammatory cytokines such as IL-8 in macrophages [30] as well as bronchial [31] and alveolar [32] epithelial cells. In nicotine-stimulated neutrophils, IL-8 expression was shown to be entirely dependent on NF- κ B activation, as indicated by abrogation of IL-8 production in the presence of the NF- κ B inhibitor, dexamethasone [33, 34]. These data support the results of our study.

In HBE16 cells, the data are different. Nicotine has been shown to restrain lipopolysaccharide-induced TNF- α production, predominantly through an $\alpha 7$ nAChR/MyD88/NF- κ B pathway [35]. These diverse effects in different cell types might be caused by molecular modulation. One of the mechanisms responsible for this effect by nicotine might be that the expression of NF- κ B in different cell lineages is regulated by different signaling pathways.

Although similar results have been reported in human neutrophils and human gingival epithelial cells [36, 37], this report is the first to examine the key role of $\alpha 7$ nAChR/NF- κ B pathway in human PDL cells in response to nicotine, suggesting that $\alpha 7$ nAChR/NF- κ B pathway might take part in the progression of smoking-associated periodontitis. Although we gained some interesting results, these results were based on the responses of cells. Further investigation using an animal model is required to determine whether these findings reflect the processes that occur *in vivo*.

Disclosure Statement

The authors declare that they have no conflict of interest.

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