

N-acetyl cysteine inhibits lipopolysaccharide-mediated synthesis of interleukin-1 β and tumor necrosis factor- α in human periodontal ligament fibroblast cells through nuclear factor-kappa B signaling

Rui Zheng, MD, Yujie Tan, MD, Mengqin Gu, MD, Ting Kang, MD, Hui Zhang, MD, Ling Guo, PhD*

Abstract

Background: The aim of this study was to investigate the role of n-acetyl cysteine (NAC) in the lipopolysaccharide (LPS)-mediated induction of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) synthesis by human periodontal ligament fibroblast cells (hPDLFs). In addition, we aimed to determine the involvement of the nuclear factor-kappa B (NF- κ B) pathway in any changes in IL-1 β and TNF- α expression observed in response to LPS and NAC.

Methods: HPDLFs were obtained by primary culture. The culture medium used in this experiment was Dulbecco's Modified Eagle Medium (DMEM low-glucose). Cells were stimulated with various concentrations of NAC or LPS. Cell proliferation was measured at various time-points with the cell Counting Kit 8 (CCK-8) assay. mRNA levels of IL-1 β and TNF- α were determined by real-time quantitative polymerase chain reaction (RT-qPCR) analysis. Protein levels of IL-1 β and TNF- α were measured by enzyme-linked immunosorbent assay (ELISA). Protein and mRNA expression levels of NF- κ B were measured by western blot and RT-qPCR.

Results: The results showed that LPS treatment in hPDLFs induced mRNA and protein expression of IL-1 β , TNF- α , and NF- κ B. However, these effects were eliminated by pretreatment with NAC. Pretreatment with both NAC (1 mmol/L) and BAY11-7082 (10 μ mol/L) significantly inhibited the NF- κ B activity induced by LPS.

Conclusion: NAC inhibits the LPS-mediated synthesis of tumor TNF- α and IL-1 β in hPDLFs, through the NF- κ B pathway.

Abbreviations: CCK-8 = cell Counting Kit 8, DMEM = Dulbecco's Modified Eagle Medium, ELISA = enzyme-linked immunosorbent assay, hPDLFs = human periodontal ligament fibroblast cells, IL-1 β = interleukin-1 β , LPS = lipopolysaccharide, NAC = n-acetyl cysteine, NF- κ B = nuclear factor-kappa B, RT-qPCR = real-time quantitative polymerase chain reaction, TNF- α = tumor necrosis factor- α .

Keywords: human periodontal ligament fibroblast cells, interleukin-1 β , lipopolysaccharide, n-acetyl cysteine, NF- κ B pathway, tumor necrosis factor- α

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The authors have no conflicts of interest to declare.

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Department of Prosthodontics, Hospital of Stomatology Affiliated to Southwest Medical University, Luzhou, China.

* Correspondence: Ling Guo, Department of Prosthodontics, Hospital of Stomatology Southwest Medical University, No. 2 jiangyang west road, Luzhou 646000, Sichuan, China (e-mail: 372083745@qq.com).

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1. Introduction

Periodontal tissue inflammation (periodontitis) is an infectious disease caused by specific microorganisms. The condition causes gradual destruction of soft tissue and alveolar bone. As a virulence factor of Gram-negative bacteria, lipopolysaccharide (LPS) induces the release of inflammatory cytokines by periodontal ligament cells and stimulates macrophages, monocytes, and fibroblasts to secrete inflammatory mediators. Periodontal inflammation is induced by shifting oral microbiome from symbiotic to dysbiotic state. A number of clinical studies have shown that human periodontal ligament fibroblast cells (hPDLFs) are thought to secrete a variety of inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α).^[1-7] Accumulating evidence suggests that IL-1, including interleukin-1 β (IL-1 β) and interleukin-1 α (IL-1 α), plays a role in bone absorption. Previous studies have found that IL-1 and TNF- α may counteract cytokines and autocrine mediators. At the same time, the 2 have the effect of overlapping each other to exacerbate the inflammation of the tissue. To fully antagonize the osteoclast formation induced by inflammatory factors, it is necessary to antagonize both IL-1 and TNF- α . Drugs designed to inhibit the expression of IL-1 and TNF- α could reduce periodontal inflammation, as well as the absorption and destruction of alveolar bone.^[8]

Table 1**The source of materials.**

Name	Source
lipopolysaccharide (from <i>Escherichia coli</i> serotype 0111:B4)	Sigma-Aldrich Co. LLC. (St. Louis, MO, USA)
n-acetyl cysteine	Sigma-Aldrich Co. LLC. (St. Louis, MO, USA)
Vimentin and cytokeratin	Proteintech Group, Inc (Chicago, USA)
Collagenase-I	Sigma (St. Louis, MO, USA)
Fetal Bovine Serum (FBS)	HyClone Laboratories (HyClone, Logan, Utah, USA)
Dulbecco's Modified Eagle Medium (DMEM low-glucose)	HyClone Laboratories (HyClone, Logan, Utah, USA)
Anti-phospho-NF- κ B (p-p65 and p65)	Cell Signaling Technology (Beverly, Massachusetts, MA, USA)
Bay11-7082	Biomol (Hamburg, Germany)
Cell Counting Kit 8 (CCK-8)	Dojindo (Dojindo Molecular Technologies, Rockville, MD 20850, USA)
Human IL-1 β and TNF- α ELISA kits	Research and Diagnostic Systems (Minneapolis, MN, USA)

Nuclear factor-kappa B (NF- κ B) is expressed in many cells, including hPDLFs, as an early nuclear transcription factor. Few studies to date have addressed the effects of n-acetyl cysteine (NAC) on the inhibition of TNF- α and activation of IL-1 β in hPDLFs. Whether NAC inhibits the LPS-mediated increase in synthesis of IL-1 β and TNF- α in hPDLFs through the NF- κ B pathway remains unknown.

NAC has a potential effect on anti-oxidation as a glutathione precursor and oxidant,^[9] and it also can reverse the effect of NO physiological as a nitric oxide carrier.^[10] It is reported that NAC may inhibit the activation of NF- κ B and the release of TNF- α significantly.^[11] The effect may be explained by the fact that NAC can promote the synthesis of glutathione (GSH), prevent or adjust the imbalance of redox reaction in vivo, inhibit the activation of I- κ B activating enzyme by reactive oxygen species and its derivatives produced by cell stress, thereby inhibiting IL-1 β , TNF- α and other inflammatory cytokines regulated by NF- κ B signaling pathway.^[12-17] However, there is no report related to NAC in hPDLFs.

Porphyromonas gingivalis is the main pathogen of chronic periodontitis, and LPS is one of its most important virulence factors.^[18] Oxidative stress (OS) is caused by increased oxygen free radical production or decreased clearance in the body tissues or cells, resulting in excessive reactive oxygen species (ROS) free radicals, imbalance of oxidation and antioxidant systems, and damage to cells.^[19] LPS can stimulate hPDLFs to produce ROS, which in turn triggers a series of cellular damage caused by oxidative stress.^[20,21] Therefore, NAC as an antioxidant may be able to regulate the inflammatory response in hPDLFs caused by LPS, and an understanding of the underlying molecular mechanisms by which TNF- α and IL-1 β secretion is inhibited by the involvement of the NF- κ B pathway may be useful.

In view of the above reasons, we finally chose to use hPDLFs as the research object to study the effects of NAC as an antioxidant on the inflammatory virulence factor LPS-mediated TNF- α and IL-1 β synthesis. Moreover, we explored the molecular mechanisms of the potential anti-inflammatory effect of NAC by the

involvement of NF- κ B signaling pathway. It is worth mentioning that the above aims have achieved the expected results

2. Materials and methods

2.1. Materials

The source of the materials used in the experiment has been shown in Table 1. The periodontal membranes used for primary culture were obtained from the Stomatological Hospital attached to Southwest Medical University. The research was supported by the Human Subject Ethics Committee of the Affiliated Stomatological Hospital of Southwest Medical University (Table 2).

2.2. Cell culture and immunohistochemistry

The Cell culture and Immunohistochemistry were described in the supplemental material, <http://links.lww.com/MD/D255>.

2.3. Cell counting kit 8 (CCK-8) assay

The cell viability was measured by the CCK-8 assay. And the experiments were divided into blank group, control group and experimental group (NAC and LPS group). Firstly, different concentrations of LPS (0.1, 0.5, 1, 2.5, 5 μ g/ml) were applied to the LPS groups. After choosing the most suitable concentration of LPS, different concentrations of NAC (0.25, 0.5, 1, 2.5, 5 mmol/L) were added to the LPS + NAC group with the optimal concentration of LPS. HPDLFs (density; 1.0×10^5 /well) were seeded in a 96-well plate after culture in 5% CO₂ at 37°C for variable time intervals (24, 48, or 72 hours). The cell viability was measured with the CCK-8 assay. For all measurements of cell viability, hPDLFs were cultured in 5% CO₂ at 37°C for 4 hours. An MCC 340 multi-scan microplate reader (Thermo Fisher Scientific Inc, Pittsburgh, PA) was used to measure the absorbance at a wavelength of 450nm. All the experiments were performed in triplicate.

Table 2**Experimental groups.**

Groups	Control	NAC	LPS	NAC+LPS	BAY11-7082+LPS	BAY11-7082
NAC	-	+	-	+	-	-
LPS	-	-	+	+	+	-
BAY11-7082	-	-	-	-	+	+

2.4. Real-time quantitative polymerase chain reaction (RT-qPCR)

mRNA expression of IL-1 β , TNF- α , and NF- κ B was measured with RT-qPCR. The total RNA was extracted by simple TRIzol reagent (TIANGEN, Beijing, China). Total RNA was finally reverse-transcribed for 1 hour at 42.8°C in the presence of an oligo-primer using a PrimeScriptTM RT kit (Takara, Osaka, Japan) to finally generate cDNA. According to the instructions, RT-qPCR experiments were started through SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan). The experiments were quantified with the 2- $\Delta\Delta$ CT method and performed in triplicate. Primer sequences are shown in the Table 3. β -actin was used as internal reference control.

2.5. Protein expression by enzyme-linked immunosorbent assay (ELISA)

ELISA experiments were performed with fibroblast culture supernatant, TNF- α , and IL-1 β ELISA kits, according to the manufacturer's instructions. The cell culture supernatant (100 μ L) was pipetted into a 96-well plate to incubated for 2 hours, then washed 3 times with washing buffer, then the well plate was dried before 200 μ L of substrate (tetramethylbenzopyridine) was added to every in the well. Let it react for 20 minutes. Plates were read by using an MCC 340 multi-scan plate reader (Thermo Fisher Scientific Inc, Pittsburgh, PA) at a wavelength of 450 nm. The expression of TNF- α and IL-1 β in the samples were determined by comparison with a standard curve generated using a manufacturer-provided standard. The standard curve was completed by plotting average O.D. (450 nm) acquired for each concentration on the "Y" axis and average O.D. (450 nm) for the corresponding concentration on the horizontal ("X") axis.

2.6. Protein isolation and western blot

Total cellular protein was extracted with radioactive immunoprecipitation assay (RIPA) buffer. Samples were then submerged in boiling water; 5 protein loading buffer was added and allowed to react for 5 minutes, before cooling on ice for 5 minutes. Protein were dealt with Sodium dodecyl sulfate (SDS)- Polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. Washing with Tris Buffer Solution Tween (TBST) (0.05% Tween-20) and incubating the PVDF with non-fat dry milk (5%) blocked non-specific protein binding. And incubation with p-65 and p-p65 antibodies for 1 hour at room temperature, and secondary antibody conjugated with HRP incubate the membrane. The internal control was anti- β -actin (1:3000). Image J software was used to measure and analyze target bands.

2.7. Statistical analysis

The data were analyzed using one-way ANOVA (SPSS 17.0) for diversiform comparisons between pairs. $P < .05$ was considered as statistically significant. The resulting data was expressed as the mean and standard deviation of the triplicate determinations.

3. Results

3.1. Primary cell culture and immunohistochemistry

Primary cultured and Immunohistochemistry results were showed in Figure S1, <http://links.lww.com/MD/D255>

3.2. Optimal concentrations of NAC and LPS acting on hPDLF cells

The results in Figure 1A showed the changes of cell viability of hPDLFs after NAC and LPS treatment at different time intervals (24, 48 and 72 hours) and concentrations. NAC-regulated proliferation rate increased from 0 to 1 mM with NAC concentration reaching 1 mM peaked and then attenuated. Considering the maximum cell viability of 1 mmol/L NAC-conditioned hPDLFs, and 1 mmol/L was considered the optimal NAC concentration for hPDLF cells. 1 mM NAC was used to pre-stimulate the cells for 1 hour, and then the hPDLFs were treated with pre-designed concentrations of LPS and NAC mixed solution. A similar trend has been observed in the cell viability of hPDLFs. The proliferation rate increased in direct association with LPS concentration, then started to gradually decrease. At 24, 48, and 72 hours, weak proliferation and weak inhibition were observed for a concentration of 1 μ g/ml. At a concentration of 1 μ g/mL, cell viability decreased, then approached the corresponding peaks at 24, 48, and 72 hours. Based on these results, 1 μ g/ml LPS concentration was considered to be optimal for treatment of hPDLFs.

3.3. mRNA expression of NF- κ B, IL-1 β , and TNF- α

mRNA expression of IL-1 β , TNF- α , and NF- κ B was determined with RT-qPCR. In untreated cells, IL-1 β , TNF- α , and NF- κ B expression levels were low. The expression amount of IL-1 β , TNF- α and, NF- κ B mRNA were significantly increased when treated with LPS (1 μ g/ml) ($P < .05$). BAY11-7082 (10 μ mol/L) or NAC (1 mmol/L) pretreatment significantly inhibited the increases in levels of IL-1 β , TNF- α , and NF- κ B induced by exposure to LPS ($P < .05$). However, there was no comparability between the 2 drugs NAC and BAY11-7082 ($P > .05$). The results of the RT-qPCR are shown in Figure 2.

Table 3
Primer Sequences used for Quantitative real-time PCR.

Gene name	Gene symbol	Primer sequence (5'-3')	Amplicon length
Interleukin-1 β	IL-1 β	5'GCGGCATCCAGCTACGAATCTC-3' 5'GCGGCATCCAGCTACGAATCTC-3'	101
Nuclear factor Kappa B	NF- κ B	5'ACCAGGCTGGCAGCTCTTCTC-3' 5'AGGCACAACCTCTTCATCCTCTCC-3'	120
Tumor necrosis factor- α	TNF- α	5'AGCTGGTGGTGCCATCAGAGG-3' 5'TGGTAGGAGACGGCGATGCG-3'	124
β -actin	β -actin	5'-CCTGGCACCAGCACAAT-3' 5'-GCCGATCCACACGGAGTA-3'	70

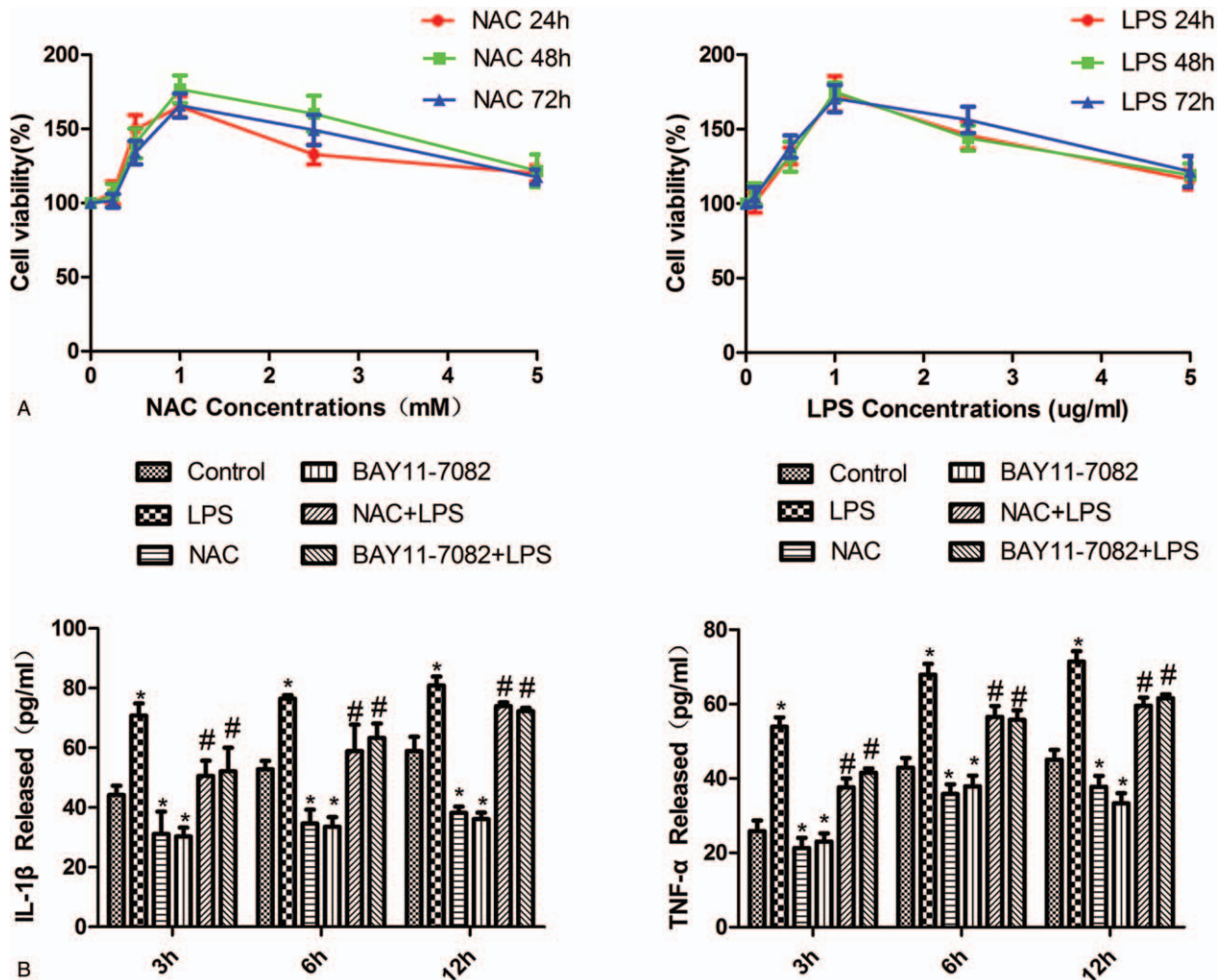


Figure 1. (A) Cell proliferation at three time points (24, 48, and 72 hours) was measured by using CCK8 assay after stimulation of hPDLFs with LPS and NAC. (B) Effects of NAC on TNF- α and IL-1 β expression in LPS-mediated hPDLFs. Firstly, cells were treated with 10 μ mol/L BAY11-7082 or 1 mmol/L NAC for 1 hour, then treated with 1 μ g/ml LPS for 3, 6, 12 hours. The levels of IL-1 β and TNF- α protein were measured by ELISA at 3, 6, and 12 hours. The experiment was repeated 3 times and results were expressed as means \pm SD. In addition, # as $P < .05$ vs LPS group; * as $P < .05$ vs control group.

3.4. Protein expression of IL-1 β , TNF- α , and NF- κ B

We measured protein levels of TNF- α and IL-1 β with ELISA. LPS treatment significantly increased expression of IL-1 β and TNF- α in hPDLFs for all time intervals (3, 6, and 12 hours). Pretreatment of hPDLFs with NAC inhibited the increase in levels of IL-1 β and TNF- α (Fig. 1B).

The effect of NF- κ B on LPS-induced IL-1 β and TNF- α expression was assessed and it was tested whether the effect of NAC on IL-1 β and TNF- α expression is due to inhibition of NF- κ B. The effects of NF- κ B inhibitor BAY11-7082 and NAC on LPS-induced NF- κ B expression were observed. LPS significantly up-regulated phosphorylation of the NF- κ B pathway in hPDLFs. Pretreatment with NAC (1 mmol/L) and BAY11-7082 (10 μ mol/L) significantly inhibited LPS-induced phosphorylation of the NF- κ B pathway, the results of the western blot are shown in Figure 3.

4. Discussion

A classical formula known as NAC has been used clinically as an effective drug for treatment of lung diseases, such as idiopathic

pulmonary fibrosis (IPF).^[22] As a glutathione precursor and antioxidant, NAC might thus exert an anti-inflammatory effect. Our study reports for the first time that NAC inhibits LPS-induced upregulation of NF- κ B signaling in hPDLFs. NAC also inhibits the synthesis of IL-1 β and TNF- α , the genes for which lie downstream of pro-inflammatory cytokines, such as those involved in the NF- κ B pathway. Our current study investigated the effects of NAC on LPS-mediated synthesis of IL-1 β and TNF- α in hPDLFs. We also investigated the effects of the NF- κ B pathway on expression of IL-1 β and TNF- α in response to LPS, NAC, or both, in hPDLFs. Previous research has shown that LPS induces the synthesis of IL-1 β and TNF- α ,^[23-25] which involves phosphorylation of NF- κ B. The data presented above provide additional evidence for the assertion that LPS significantly increases synthesis of IL-1 β and TNF- α in hPDLFs. However, pre-treatment with NAC eliminates this effect.^[26,27] NF- κ B inhibitor BAY11-7082 significantly inhibited synthesis of IL-1 β and TNF- α in LPS-induced hPDLFs.

Previous studies have shown that the secretion of IL1- β and TNF- α is regulated by NF- κ B signaling.^[28-31] Expression of

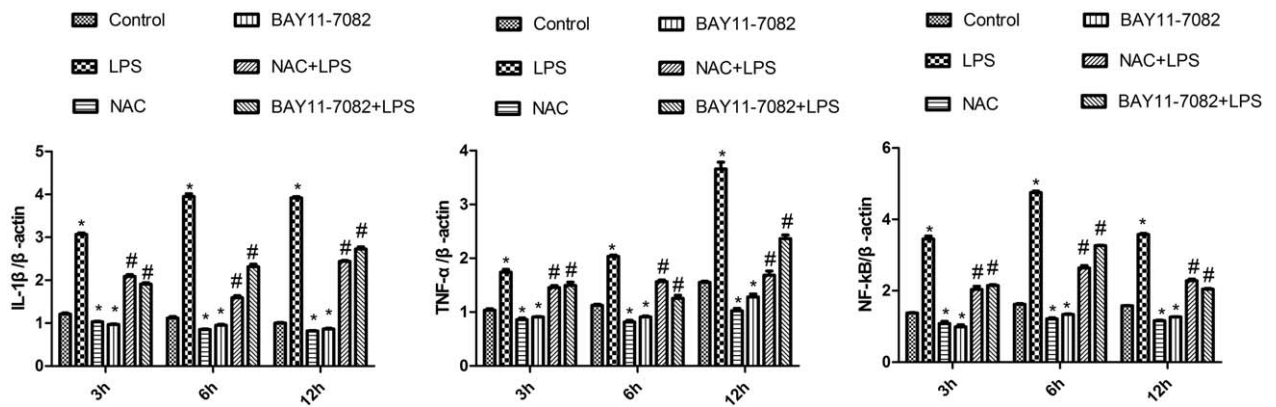


Figure 2. Effects of NAC on expression of IL-1 β , TNF- α , and NF- κ B in hPDLFs stimulated with LPS. Cells were pretreated with 1 mmol/L NAC and 10 μ mol/L BAY11-7082 for 1 hour, then treated with 1 μ g/ml LPS for 3, 6, or 12 hours. Levels of IL-1 β , TNF- α , and NF- κ B mRNA were determined by RT-qPCR. Results are means of 3 independent experiments \pm SD, * P < .05 vs untreated, # P < .05 vs LPS.

TNF- α and IL-1 β was downregulated by NAC in LPS-treated hPDLFs. Exposure to LPS increases the synthesis and activity of NF- κ B, but these effects were completely blocked by treatment with NAC and BAY11-7082. The consequence is consistent with our previous results.^[32] And NAC up-regulated phosphorylation of NF- κ B in the western blot (6 hours) group, compared to the control group, but the results were not statistically significant, perhaps because of inter-operator differences in adherence to the experimental protocol.

Periodontal inflammation is a common oral disease that commonly manifests as the loss of periodontal supporting tissue and the loosening or even shedding of teeth.^[33,34] As a chronic infectious disease, the initial factors of periodontitis are bacteria and their products. The main pathogens associated with periodontitis are gram-negative bacteria such as *P. gingivalis* and *Fusobacterium nucleatum*.^[35] LPS induced inflammatory factors, such as IL-1 β and TNF- α play an important role in the development and progression of periodontitis.^[36] We chose to measure expression of TNF- α and IL-1 β in vitro because TNF- α acts synergistically with IL-1 β in the process of bone resorption.

The results presented above show that LPS significantly increased synthesis of IL-1 β and TNF- α in hPDLFs, while pretreatment with NAC eliminated this effect.

This study showed that low concentrations of LPS promoted the proliferation of hPDLFs, while high concentrations of LPS inhibited proliferation of hPDLFs.^[37,38] Previous studies have established the role of NF- κ B in LPS-induced cytokine expression.^[39,40] However, the contribution of NF- κ B to the effects of NAC on LPS-induced IL-1 β and TNF- α expression in hPDLFs had previously not been investigated. Our study demonstrated that phosphorylation of NF- κ B is involved in the controlling expression of IL-1 β and TNF- α following exposure to LPS. NAC may inhibit phosphorylation of NF- κ B, with effects on expression of IL-1 β and TNF- α after exposure to LPS.

In the in vitro study, NAC inhibited expression of IL-1 β and TNF- α stimulated by LPS in hPDLFs. NAC pretreatment also reduced phosphorylation of NF- κ B in hPDLFs stimulated with LPS. NF- κ B signal transduction may be activated by numerous factors. Currently, 2 main pathways are recognized: the classical pathway and the bypass pathway.^[41,42] In the resting state,

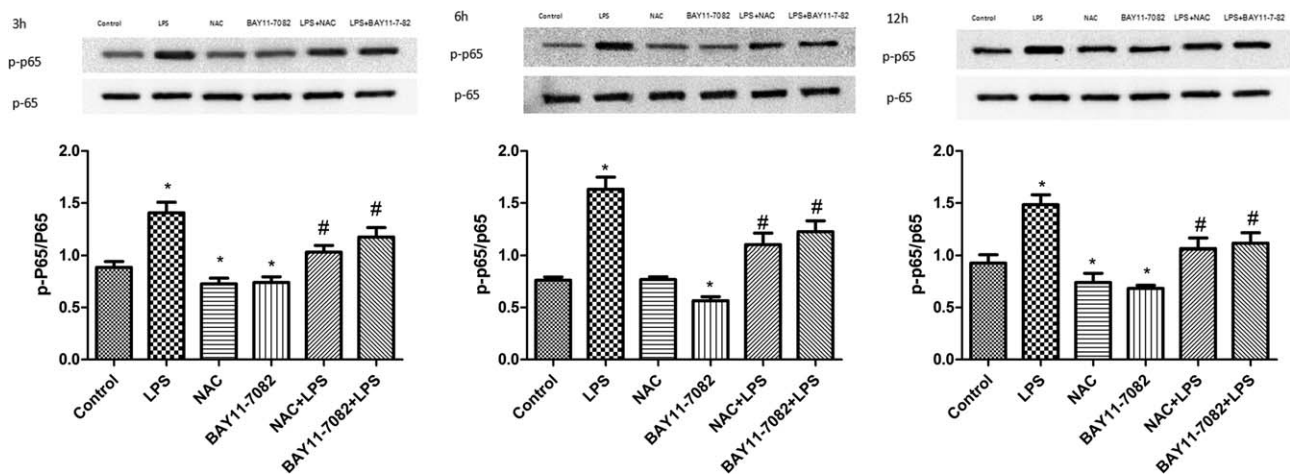


Figure 3. Effects of NAC on NF- κ B expression in hPDLFs stimulated with LPS. Cells were pretreated with 1 mmol/L NAC or 10 μ mol/L BAY11-7082 for 1 hour, then treated with 1 μ g/ml LPS for 3, 6, or 12 hours. Levels of NF- κ B p-65 and p-p65 protein were determined by western blot at 3, 6, and 12 hours. Results are means of 3 independent experiments \pm SD, * P < .05 vs untreated; # P < .05 vs LPS.

cytoplasmic p50/p65 combines with $\text{I}\kappa\text{B}$ to form a trimer, resulting in p50/p65 non-nuclear translocation. In the classical pathway, when cells are affected by cytokines, mitogens, endotoxins, or viral proteins, the inhibitor of nuclear factor kappa-B kinase β (IKK β) subunit of inhibitor of nuclear factor kappa-B kinase (IKK) is activated by phosphorylation, peroxidase, protein kinase C, calcium ionophores, protein synthesis inhibitors, X-rays, or other extracellular signals. Phosphorylation of the IKK β subunit of IKK causes phosphorylation of the Ser36 and Ser32 sites of $\text{I}\kappa\text{B}\alpha$. Phosphorylated $\text{I}\kappa\text{B}\alpha$ is further ubiquitinated and degraded by the 26S proteolytic enzyme complex. The p50/p65 released undergoes nuclear translocation and specifically binds to the κB site of the gene, thus regulating cell function.^[43] NAC may regulate NF- κB signaling through the classical pathway, by inhibiting phosphorylation of NF- κB . Although current studies indicate that phosphorylation of NF- κB is involved in the reduction of IL-1 β and synthesis of TNF- α in LPS-stimulated hPDLFs, the molecular basis by which NAC inhibits LPS-induced expression of IL-1 β and TNF- α in hPDLFs remains uncertain. Further research is required.

5. Conclusion

This study highlights the effect of NAC on expression of TNF- α and IL-1 β in hPDLFs stimulated by LPS. The study had certain limitations. For example, the effects of NAC and BAY11-7082 were not directly compared. Nonetheless, the results described above elucidate the role of NF- κB phosphorylation in LPS-induced expression of TNF- α and IL-1 β . NAC may attenuate LPS-induced expression of TNF- α and IL-1 β by inhibiting phosphorylation of NF- κB . This study provides new evidence for prevention and treatment of periodontal tissue inflammation, by inhibiting expression of TNF- α and IL-1 β . Further research studies and clinical trials are needed to validate the findings presented above.

Author contributions

Conceptualization: Rui Zheng.

Data curation: Rui Zheng, Ling Guo.

Formal analysis: Rui Zheng, Ling Guo.

Funding acquisition: Ling Guo.

Investigation: Rui Zheng, YuJie Tan, Mengqin Gu, Ting Kang, Hui Zhang, Ling Guo.

Methodology: Rui Zheng, YuJie Tan, Mengqin Gu, Ting Kang, Hui Zhang, Ling Guo.

Writing – original draft: Rui Zheng.

Writing – review & editing: Ling Guo.

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