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Periodontal ligament fibroblasts migration injury via ROS/TXNIP/Nlrp3 inflammasome pathway with *Porphyromonas gingivalis* lipopolysaccharide

Dawei Lian[a,1], Linfeng Dai[a,1], Zhaoyu Xie[a], Xing Zhou[a], Xiaohong Liu[a], Yang Zhang[b], Yi Huang[c,*], Yang Chen[a,**]

[a] School of Pharmaceutical, Guangzhou University of Chinese Medicine, No. 232 Waihuan Dong Rd., Guangzhou University Town, Panyu District, Guangzhou, 510000, China
[b] Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, 3455 CULLEN BLVD, Houston, TX, 77204-5037, USA
[c] Department of Stomatology, The First Affiliated Hospital, The school of Dental Medicine, Jinan University, No. 613W. Huangpu Avenue, Guangzhou, 510630, China

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ABSTRACT

Inflammasomes serve as an intracellular machinery to initiate inflammatory response to various danger signals. However, the chronic periodontitis pathological relevance of this inflammasome activation, particularly in periodontal ligament fibroblasts, remains largely unknown. The present study demonstrated that Nlrp3 inflammasome components abundantly expressed in cultured mouse periodontal ligament fibroblasts (mPDLFs). In addition, our data demonstrated that P.g-LPS (*Porphyromonas gingivalis* Lipopolysaccharide), a major injurious factor during chronic periodontitis, could induce the mPDLFs migration dysfunction and the inhibition of Nlrp3 inflammasome by Isoliquiritigenin (ISO) markedly recovered the migration dysfunction in mPDLFs. And Nlrp3 inflammasome components could be aggregated to form an inflammasome complex on stimulation of P.g-LPS, as shown by fluorescence confocal microscopy. Correspondingly, P.g-LPS induced Nlrp3 inflammasome activation, caspase-1 activation, IL-1β and HMGB1 release, which were blocked by Nlrp3 inflammasome inhibitor (ISO). Interestingly, reactive oxygen species, TXNIP protein and TXNIP binding to Nlrp3 were markedly increased in mPDLFs with P.g-LPS. Furthermore, ROS generation inhibitor (Apocynin; APO) significantly reduced Nlrp3 inflammasome formation and IL-1β production in mPDLFs with P.g-LPS. And APO attenuated P.g-LPS-induced TXNIP protein expression and mPDLFs injury. In conclusion, our results demonstrate that ROS/TXNIP/Nlrp3 Inflammasome pathway is a key initiating mechanism necessary for P.g-LPS-induced subsequent mPDLFs inflammatory response leading to chronic periodontitis.

1. Introduction

Chronic periodontitis, whose morbidity and severity increase with age (Graves et al., 2001) and usually without noticeable, is a common disease that can occur at any age. It is a chronic inflammation of periodontal tissues, including gingival inflammation, 'attachment loss' and alveolar bone resorption, eventually cause teeth loss in adults(Graves and Cochran, 2003). Periodontal ligament fibroblasts, the primary cell type of the periodontal ligament tissues, play an important role in the development of periodontitis, including tissue repair and reconstruction (Buckley et al., 2001; Koka and Reinhardt, 1997). This cellular impaired process is closely related to the *Porphyromonas gingivalis* (P.g) invasion (Socransky and Haffajee, 2005). The study found that the subgingival plaque from P.g is important resident which is attached on teeth roots and may lead to destruction of periodontal ligament when the immune response is out of balance and the pathogen is at an advantage (Kuboniwa and Lamont, 2010). At the same time, LPS, which is the major dangerous factor from P.g, contributes to the initiation and development of periodontitis (Golz et al., 2014), and administration of P.g-LPS (*Porphyromonas gingivalis* Lipopolysaccharide) could lead to different kind of disease including cognitive disorders (Zhang et al., 2018), chronic kidney disease (Harada et al., 2018), cardiovascular disease (Bullon et al., 2011) and worsen atherosclerosis (Huck et al., 2012) and so on.

* Corresponding author.
** Corresponding author.
E-mail addresses: 675836257@qq.com (D. Lian), mister.seventeen@qq.com (L. Dai), 508565512@qq.com (Z. Xie), 825815023@qq.com (X. Zhou), 50590752@qq.com (X. Liu), yhuang312@jnu.edu.cn (Y. Huang), ychen8@gzucm.edu.cn (Y. Chen).
[1] These authors have contributed equally to this work.

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As we know, the progress of the periodontal ligament fibroblasts inflammation results in a decline in the connective tissue recovery ability and the stability of teeth, which eventually leads to teeth loss (Latz et al., 2013). Recently, Nlrp3 inflammasome has been reported to plays a critical role in the development of periodontitis (Belibasakis et al., 2013; Shibata, 2018). It has been known that the NLRP3 inflammasome contains NLRP3, ASC and the effector cysteine protease Caspase 1 (dos Santos et al., 2015; Strowig et al., 2012). NLRP3 inflammasomes form a high-molecular-weight inflammasome complex through oligomerization. In the oligomer, Pro-caspase-1 is converted to its active form, caspase-1, which would subsequently cleave its substrates such as pro-interleukin-1β (IL-1β) to bioactive IL-1β (Ma and Damania, 2016). At the same time, it's reported that Nlrp3 inflammasome activation leads to the regulatory or pathogenic actions in cells or tissues beyond the classical inflammatory response (Boini et al., 2014, 2016; Xia et al., 2014; Yang et al., 2014), for instance, through permeability (Chen et al., 2015), pyroptosis (Misawa et al., 2013; Schroder and Tschopp, 2010) or lipid handling (Koka et al., 2017; Li et al., 2014). Increasing evidence prove that these non-inflammatory effects of inflammasome activation may also be important in the regulation of cell function and in the mediation of different cells types (Chen et al., 2016; Zhang et al., 2015). However, the molecular mechanisms activating the Nlrp3 inflammasome in mouse periodontal ligament fibroblasts (mPDLFs) and the relevance of this inflammasome activation is far from clear. Here, we aim to investigate the molecular activation of Nlrp3 inflammasomes in mPDLFs by P.g-LPS and address the implication of mPDLFs inflammasomes in the development of chronic periodontitis.

In the present study, we first determind whether the different domain of Nlrp3 inflammsome has been express in mPDLFs and the Nlrp3 inflammsome can be activated in response to P.g-LPS in mPDLFs. We then explored molecular mechanisms mediating Nlrp3 inflammasome activation by P.g-LPS. The effects of reactive oxygen species (ROS) mediated TXNIP induced-Nlrp3 inflammasome activation in mPDLFs. It is suggested that Nlrp3 inflammasome activation in mPDLFs during P.g-LPS stimulation may trigger and promote the mPDLFs dysfunction during periodontitis and that ROS-TXNIP is critical for mPDLFs function due to its action to inhibit inflammasome activation.

2. Materials and methods

2.1. Cell culture

The Mouse periodontal ligament fibroblasts (mPDLFs) was purchased from Procell (CP-M199). mPDLFs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA), containing 10% of fetal bovine serum (Gibco,USA) and 1% penicillin–streptomycin (Gibco, USA). The cells were cultured in a humidified incubator admixture at 37 °C with 5% CO_2 and 95% air. Cells were passaged by trypsinization (Trypsin/EDTA; Sigma, USA). Simulate the cells by LPS (Sigma), and the cells were treated without or with LPS at varying concentrations (0.5 mM, 1 mM, 2 mM) or Nlrp3 inhibitor Isoliquiritigenin (ISO) (Zeng et al., 2017) or ROS inhibitor Apocynin (APO) (Vejrazka et al., 2005) then incubated for 24 h.

2.2. Real-time PCR

Total RNA was isolated from the cells by using RNAiso Plus (TaKaRa, Japan), and the concentrations of the extracted RNA were measured spectrophotometrically at 260 nm. RNA quality was assessed based on the absorbance ratio at 260 and 280 nm. A260/A280 values ranging from 1.9 to 2.1 were considered acceptable. Total RNA was reverse transcribed into cDNA using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan). Reverse transcription polymerase chain reaction (RT-PCR) was performed by SYBR Green Realtime PCR Master Mix (TaKaRa,Japan) according the conditions provided in the instructions. The primers were synthesized as follows: 5′-ATTRACCRCGCRCCGRAGARAAGRG-3′ (forward primer) and 5′-TCGRCAGRCAARAGARTCCRACARCAG-3′ (reverse primer) for the mouse NLRP3 gene, 5′-GAGCTGATGTTGACCTCAGAG-3′ (forward primer) and 5′-CTGTCAGAAGTCTTGTGCTCTG-3′ (reverse primer) for the mouse Caspase-1 gene,5′-GGCGAGAGAGGTGAACAAGG-3′ (forward primer) and 5′-GCCAAGGTCTCCAGGAACAC-3′ (reverse primer) for the mouse ASC gene. The internal reference control was β-actin, 5′-CCCATCTATGAGGGTTACGC-3′(forward primer), 5′-TTTAATGTCAC GCACGATTTC-3′ (reverse primer). When finished the PCR reaction, the total PCR product (10 μl) of Nlrp3, Caspase-1 and ASC were separated by DNA gel electrophoresis.

2.3. Western blot analysis

Total protein was extracted using RIPA buffer (Thermo, USA). The supernatant was transfered after 10,000 g for 15 min at 4 °C and the concentration was measured with the BCA Protein Assay KIT (Beyotime, China). Cell homogenate was denatured with 5Xprotein loading buffer for adjusting consistency and boiled in metal bath for 5 min at 95 °C. Forty micrograms of total protein per lane were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto 0.2 μm polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA).The membranes were incubated in primary antibodies at 4 °C overnight and then treated with corresponding second, such as anti-rabbit IgG (1:2000; CST, Massachusetts, USA) for 2 h at room temperature. The primary antibodies were anti-NLRP3 (1:1000; CST, Massachusetts, USA), anti-Caspase-1 (8:5000; Santa cruz, USA), anti-TXNIP (1:2000; CST, Massachusetts, USA) and anti-ASC (1:2000; Santa cruz, USA). Anti-β-actin (1:1000; CST, Massachusetts, USA) was used as an internal control. The target bands were detected by ANALYTIK JENA AG (Jena, Germany) and analyzed using Image J software.

2.4. In vitro scratch assay

Cultured cells are plated into 6 wells plates and incubated properly for approximately 6 h at 37 °C, allowing cells to adhere and spread on the substrate completely. A straight scratch was made with removing scratches that can be removed, to simulate a wound. Remove the debris and smooth the edge of the scratch by washing the cells once with 1 ml of the growth medium and then replace with 5 ml of medium specific for the in vitro scratch assay. Acquire the first image of the scratch by a phase-contrast microscope. Then, place the plates in a cell cultured incubator at 37 °C for 24 h. After the incubation, place the plates under a phase-contrast microscope, align the photographed region acquired and acquire a second image. The images acquired for each sample can be further analyzed quantitatively using Image J software (NIH, USA)

2.5. Cell proliferation assay

We used the Cell Counting Kit-8 (CCK-8) to assay cell proliferation (Guo et al., 2009). Fibroblasts were seeded in the 96-well plates at the density of 2000 cells/well. After 24 h stimulation of LPS, add 10 ul CCK-8 every well and then incubate the plate in a humidified incubator about 1 h. Measure the absorbance at 450 nm using a microplate reader.

2.6. Confocal microscopy of inflammasome proteins in Priodontal Ligament Fibroblasts of mice

The cells adherent to round glass coverslips were fixed with 4% buffered paraformaldehyde and permeabilized with 0.1% Triton X-100. Then the cells were incubated with following primary antibodies: goat anti-Nlrp3 (1:200; Abcam, Cambridge, England), mouse anti-caspase-1 (1:200; Santa cruz, USA), rabbit anti-ASC (1:100; Santa cruz, USA), rabbit anti-Txnip (1:200, Abcam, Cambridge, England). After incubation with primary antibodies, the dishes were washed and labeled with