Lipopolysaccharide from *Porphyromonas gingivalis* promotes autophagy of human gingival fibroblasts through the PI3K/Akt/mTOR signaling pathway

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ABSTRACT

Aims: Lipopolysaccharide (LPS) is a major component of cell wall in gram-negative bacteria and has been proved to be a predominant pathogenic factor in periodontitis. *Porphyromonas gingivalis* (*P.g*) was abundant in patients with periodontitis and was associated with patient clinic-pathological characteristics. Furthermore, autophagy is a potential mechanism in inflammatory disease. In this study, we hypothesized that LPS from *P.g* may affect the physiological functions of human gingival fibroblasts (HGFs) through activating cellular autophagy. However, it remains unclear what molecular basis related to LPS-induced autophagy in HGFs.
Main methods: Here, we initially addressed the contribution of LPS from *P.g* in inducing autophagy in HGFs. Through a combination of morphology and quantification approaches involving autophagosomes formation observation as well as microtubule-associated protein light chain 3 (LC3)-II conversion. We further evaluated whether the PI3K/Akt/mTOR signaling could mediate LPS-induced autophagy in HGFs.
Key findings: Our results revealed that autophagy was more obvious in LPS-treated cells compared with that in control groups. Finally, our results demonstrated that LPS from *P.g* promoted autophagy in HGFs and was negatively regulated by PI3K/Akt/mTOR.
Significance: Analysis of these data implicates that LPS from *P.g* has a significant impact on the autophagy of HGFs by suppressing PI3K/Akt/mTOR signaling pathway.

1. Introduction

Autophagy is a physiologically self-degradation cellular process in which portions of proteins and damaged organelles are enwrapped inside a double-membrane vehicle called an autophagosome and directed to lysosomes for digestion [1,2]. The formation of these autophagosomes requires two ubiquitin-like conjugation systems: the autophagy-related protein 5 (ATG5) and the microtubule-associated protein light chain 3 (LC3) [3]. In a normal basal condition, all cells undergo physiological levels of autophagy to maintain a balance. The complete autophagic pathway can be divided into five highly regulated phages: induction, elongation, maturation, transport to lysosomes, and degradation [4,5].

There have been enormous studies focusing on the relationship between autophagy and immunological functions [6]. These findings suggest that autophagy functions as a modulator of immunity via regulating microorganism invasion, control of immune signaling and the secretion of inflammatory mediators [7–9]. One line of recent studies showed that autophagy is usually increased in infected cells, resulting

in elimination of pathogens [10]. Furthermore, autophagy participates in negative regulation of inflammasome activation [11,12].

Periodontitis is initiated by multifactorial disorders including oral biofilms and host immune response mechanisms. *Porphyromonas gingivalis* (*P.g*) is believed to be a predominant Gram-negative pathogen that initiate and maintain chronic periodontitis [13]. Lipopolysaccharide (LPS), a component of Gram-negative bacterial cell wall, plays a crucial role in the activation of inflammatory response in chronic periodontitis [14,15]. It is also implicated in the induction of autophagy. Bullon et al. found that LPS obtained from *Porphyromonas gingivalis* leads to reactive oxygen species (ROS)-mediated autophagy [16]. This result is consistent with the finding that *P.g* participates in ROS generation that contributes to enhanced levels of LC3 proteins and conversion of LC3-I to LC3-II [17]. Taken together, these studies suggest that the activity of cellular autophagy in response to infection of periodontal pathogen is associated with subsequent periodontal immune inflammation. The exact molecular mechanisms of LPS from *P.g*-induced autophagy are poorly understood.

There are several signal pathways underlying the regulation of cell

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autophagy including the PI3K/Akt/mTOR, Bcl2/beclin1, MAPK/Erk1/ 2, and AMPK signaling pathways. Accumulating evidence suggests that the PI3K/Akt/mTOR pathway is a critical mediator in the regulation of cell autophagy [18,19]. In the present study, we identified that LPS from *P.g* activated human gingival fibroblasts (HGFs) autophagy and the role of autophagy in the generation of inflammatory cytokines induced by LPS from *P.g*, in which the PI3K/Akt/mTOR pathways were involved. These data provide evidence of the possibility that excessive autophagy is responsible for the regulation of pathogenicity of LPS from *P.g*, as well as a novel mechanism by which autophagy connects to immunity.

In this present study, we found that LPS derived from *P.g* enhanced the autophagy of human gingival fibroblasts by inhibiting the PI3K/ Akt/mTOR pathway. These findings will provide a new insight into the role of autophagy in periodontitis.

2. Materials and methods

2.1. Human gingival fibroblasts (HGFs) isolation and culture

Human gingival tissues were collected from patients (30–45 years old) undergoing crown-lengthening surgery in the Department of Periodontology, Peking University Hospital and School of Stomatology. Informed consent was provided according to protocols approved by the Review Board and Ethics Committee of Peking University Health Science Center (PKUS-SIRB-2013017) and then human gingival fibroblasts were isolated and cultured by previously reported method [20]. Obtained cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin under standard conditions.

2.2. Treatment of cells

Human gingival fibroblasts were grown in 6-well dishes up to 80% confluence. Cells were washed with phosphate-buffered saline (PBS) to remove serum residues. Then cells were incubated in DMEM medium containing 2% FBS with or without 1 μg/ml *P.g* LPS (InvivoGen, San Diego, CA; ATCCs 33277) for 12 h. 5 mM 3-methyladenine (3-MA, Sigma) was pre-added into culture medium for 2 h as an inhibitor of autophagosome formation. 14 μM SC79 (Akt-specific agonist, Selleck, Houston, Texas, USA) was added into medium for 2 h before LPS stimulation. 30 μM 740Y-P (PI3K activator, R&D systems, Minneapolis, MN, USA) was also added into medium for 2 h before LPS treatment.

2.3. Transduction of HGFs with adenovirus GFP-LC3

Cells at 50–60% confluence were transduced with the adenovirus GFP-LC3 (Invitrogen, USA) at indicated infection concentration for 48 h. After treatments as provided in the figure legend, the cells were fixed with 4% paraformaldehyde. The number of GFP-LC3 puncta was calculated by manual counting. Values were evaluated from at least 20 cells/sample.

2.4. Electron microscopy

To analyze the localization of autophagic vesicles in HGFs, cells were fixed in 2.5% glutaraldehyde, washed with PBS three times, then were post-fixed in 1% osmium tetroxide for 2 h, dehydrated in graded alcohol and acetone, and embedded in epoxy resin. The ultrastructures of cells undergoing autophagy were obtained and viewed under the electron microscope (JEOL, Tokyo, Japan).

2.5. Quantitative real-time polymerase chain reaction analysis

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse transcribed with ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative real-time polymerase chain reaction (q-PCR) was carried out with the gene-specific primers and the Toyobo THUNDERBIRD SYBR qPCR Mix in a PikoReal 96 real-time PCR system according to the manufacturer's instructions. Primer sequences are as followed: GAPDH, 5′-GGAGCGAGATCCCTCCAAAAT-3′, 5′-GGC TGTTGTCATACTTCTCATGG-3′; ATG5, 5′-AAGCTGTTTCGTCCTGT GGC-3′, 5′-AGCCACTGCAGAGGTGTTTC-3′; LC3, 5′-TCGCCGACCGCT GTAAGGAG-3′, 5′-CGCCGGATGATCTTGACCAACTC-3′. The expression levels of each mRNA were shown after normalization against the glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.6. Western blot

Western blot was performed as previous description [21]. Cells extracts were lysed in rothlysis containing protease inhibitor cocktail (Roche, Basel, Switzerland) and quantified using BCA Protein Assay Kit (Thermo Fisher Scientific, West Palm Beach, FL). Equal amounts of protein were separated on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS- PAGE) and, following transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were blocked in 5% nonfat dry milk for 1 h and then incubated at 4 °C overnight with primary antibody. Secondary HRP conjugated anti-rabbit antibody was blotted (Cell Signaling Technology) and the signals were detected by enhanced chemiluminescence kit (West Pico; Thermo Fisher Scientific). Subsequently, the protein bands were analyzed by ImageJ 1.43 software. β-Actin was used as a control for whole-cell lysates.

2.7. Cytokines analysis

Human interleukin-1β (IL-1β), tumor necrosis factor (TNF-α) in the supernatants were determined with the enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendation. The experiment was performed in triplicate and repeated 3 times.

2.8. Statistical analysis

Data were reported as means ± SD. All experiments were performed at least 3 times. Differences were assessed using the Student's *t*-test to compare different treatments between and among groups. $p < 0.05$ was considered significant.

3. Results

3.1. Lipopolysaccharide from Porphyromonas gingivalis induced autophagy of human gingival fibroblasts

We first assessed the direct effect of Lipopolysaccharide (LPS) from *Porphyromonas gingivalis* (*P.g*) on autophagy in human gingival fibroblasts (HGFs). We measured autophagy-related 5 (ATG5) and light chain 3 (LC3) mRNA and protein expression in LPS-treated cells and control cells by quantitative PCR and Western blot. As shown in Fig. 1A and B, LPS stimulation significantly increased ATG5 and LC3-II expression compared with control groups. We then confirmed the number of LC3 puncta in these cells by immunofluorescence microscopy using adenovirus GFP-LC3 (Fig. 1E) and finally cells expressed GFP-LC3 punctate signals were observed. In line with our previous results, the number of LC3 puncta was significant higher in the LPS-stimulated cells than control cells (Fig. 1E). Moreover, autophagosomes of cells were more notable in LPS stimulation than control groups as indicated by transmission electron microscope (Fig. 1C). To further investigate the
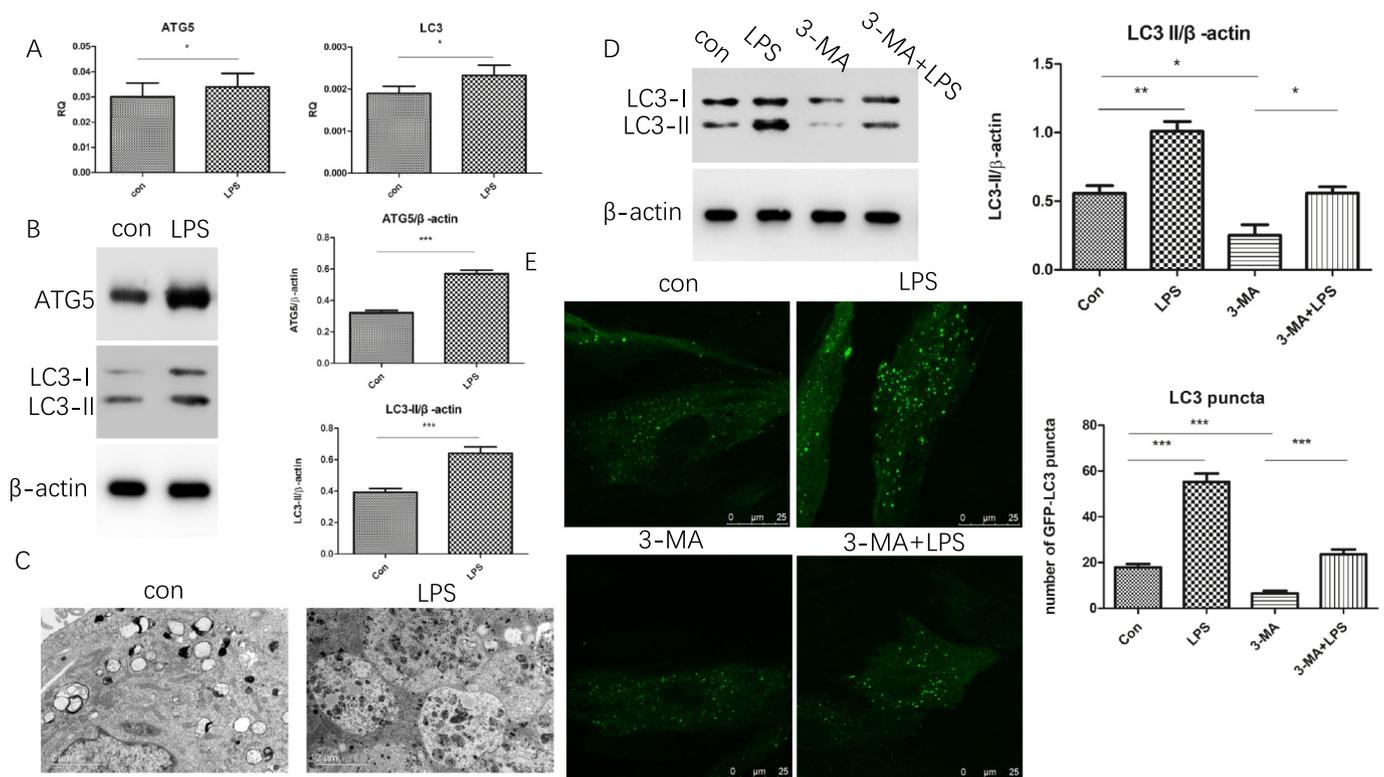
Fig. 1. LPS from *P.g* induces autophagy of HGFs. HGFs were cultured in DMEM medium containing LPS (1 μg/ml) from *P.g* ATG5 and LC3 were measured after 12 h stimulation. (A) We quantified ATG5 and LC3 RNA expression by real-time quantitative PCR and normalized to GAPDH expression levels. RQ: relative quantity. (B) ATG5 and LC3-II protein expression was identified by Western blotting. Results were further quantified by densitometric analysis, normalized by the level of β-actin. (C) Autophagosomes were detected by electron microscope. Scar bars = 2 μm. (D) Inhibition of autophagy by 3-MA was performed and then followed by LPS stimulation. LC3-II protein levels were measured by Western blotting. Results were further quantified by densitometric analysis, normalized by the level of β-actin (E) and adenovirus GFP-LC3 were transduced into HGFs, puncta (green fluorescence) were detected by confocal analysis. The number of puncta was calculated from at least 20 cells each sample. Scar bars = 25 μm. Values of this figure represent the mean ± SD of triplicate assays. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

LPS-induced autophagy on HGFs, 3-MA, a selective autophagy inhibitor was added before LPS stimulation. As shown in Fig. 1D, the addition of LPS to HGFs potently rescued the suppression of autophagy by 3-MA compared with that in the 3-MA-treated alone cells.

3.2. Lipopolysaccharide from Porphyromonas gingivalis repressed activity of PI3K/Akt/mTOR in HGFs

As shown in Figs. 2A–B and 3A–B, LPS down-regulated the protein expression levels of phosphorylated-PI3K (p-PI3K), phosphorylated-Akt (p-Akt) and mTOR after LPS stimulation, with significant differences between LPS-treated and control cells. The results (Figs. 2A–B and 3A–B) thus suggested that LPS possessed inhibition of PI3K/Akt/mTOR signal pathway ability.

740Y-P (PI3K activator) and SC79 (Akt-specific agonist) was further used to analyze whether the decreased expression of PI3K/Akt/mTOR was associated with the LPS infection. We found that the higher protein expression of p-Akt and mTOR observed in 740Y-P compared with 740Y-P combined using LPS groups (Fig. 2C–D). The increased expression of mTOR by SC79 was identified rescued in SC79 and LPS combined groups (Fig. 3C–D). The results showed that PI3K/Akt/mTOR pathway was regulated by LPS and likely to play potential roles in LPS-induced autophagy.

3.3. LPS induced excessive autophagy of HGFs via repressing PI3K/Akt/mTOR signaling pathway

To evaluate whether the PI3K/Akt/mTOR signaling pathway involved in the excessive autophagy of HGFs triggered by LPS. We assessed the LC3-II protein expression and LC3 puncta as well as

autophagosomes. We exposed HGFs to 30 μM 740Y-P or 14 μM SC79. Comparison of the LC3-II protein level revealed a decrease in 740Y-P and SC79 group compared with control group (Fig. 4A). Combining 740Y-P or SC79 with LPS infection showed a lower expression of LC3-II compared with LPS stimulation alone (Fig. 4A). The LC3 puncta number in Fig. 4B indicated that LPS combined with 740Y-P or SC79 significantly down-regulated the number of LC3 puncta, as compared with the LPS-only group. Identification a decrease of autophagosomes in 740Y-P or SC-79 groups has proved the previous results (Fig. 4C). These data suggest that PI3K/Akt/mTOR axis may act as a negative regulator of autophagy in HGFs.

3.4. LPS from P.g-induced autophagy prevented inflammatory cytokines secretion

To assess the role of autophagy in the inflammatory cytokines generation, we first examined the effects of autophagy inhibition on TNF-α and IL-1β secretion. As shown in Fig. 5A, we performed ELISA studies. The secretion of TNF-α and IL-1β displayed a lower level in LPS group without stimulation by 3-MA compared with LPS stimuli with 3-MA stimulation. To further explore the relationship between PI3K/Akt/mTOR signaling and pro-inflammatory cytokines generation, we investigated the levels of TNF-α and IL-1β of supernatants in HGFs stimulated with 740Y-P or SC79. The results showed that TNF-α and IL-1β levels were upregulated by using 3-MA, 740Y-P or SC79 (Fig. 5B).

4. Discussion

A previous study of peritoneal mesothelial cells exposed to LPS from *Escherichia coli* (*E. coli*) enhanced the autophagy [22]. Another previous
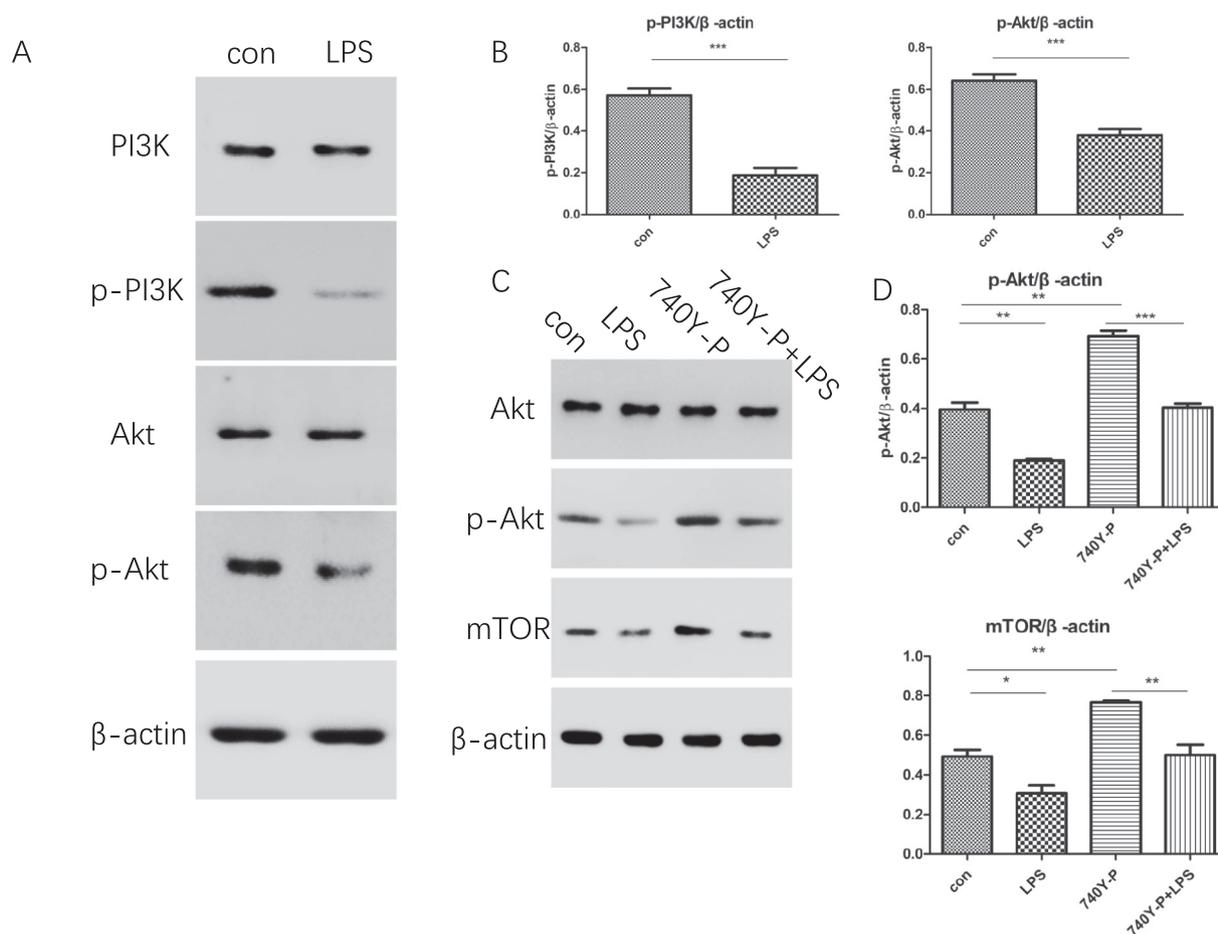
Fig. 2. LPS repressed activity of PI3K/Akt pathways in HGFs. (A) Representative images of Western blotting on unstimulated or LPS-stimulated HGFs, no significant changes were observed on the expression of PI3K or Akt between two groups. Lower expression of p-PI3K, p-Akt was detected on LPS-stimulated HGFs (B), which was clearly quantified by densitometric analysis, normalized by the level of β-actin. (C) Representative images of Western blotting on unstimulated or LPS-stimulated HGFs in the absence or presence of 740Y-P, a PI3K activator. While no changes were observed on Akt expression among groups, p-Akt and mTOR were detected significantly decreased in LPS-stimulated cells. The expression of p-Akt and mTOR was found increased in 740Y-P-stimulated cells, (E) which was quantified by densitometric analysis, normalized by the level of β-actin. Values of this figure represent the mean ± SD of triplicate assays. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

report showed that *P.g* induced autophagy in THP-1-derived macrophages [17]. In this present study, we examined the effects of LPS from *P.g* on the autophagy of HGFs and found that exposure to LPS (1 μg/ml) during the period of 12 h enhanced the autophagy. Interestingly, when we treated the cells with inhibitor of autophagy combining LPS, this condition strongly up-regulated the autophagic process compared with that in autophagy-inhibited group. Therefore, we conclude from our present findings that exposure to LPS from *P.g* induces the autophagy of HGFs.

To date, research to unravel the complex relationship between autophagy-related (ATG) protein and the process of autophagy has been identified [23]. Sixteen ATG proteins have been reported as "key" proteins involved in both non-selective and selective autophagy [24]. ATG5 is one protein that is responsible for the autophagosome formation and maturation [25]. It has been linked to ATG12-ATG5-ATG16L1 complex located at autophagosomal membrane before the autophagome fuses with lysosomes [26]. In our present study, the RNA and protein expression levels of ATG5 in HGFs were increased under LPS stimulation. This result in the presence and absence of inhibitor of autophagy is higher than the inhibitor of autophagy-only and control cells respectively. It may indicate LPS affected the autophagy of HGFs. Another regulator of autophagy is LC3. LC3 (the microtubule-associated protein 1 light chain 3) belongs to the family of mammalian homolog of yeast ATG8 that plays a critical role in the entire autophagic pathway including the growth of autophagic membranes, recognition of

autophagic cargoes, and the fusion of autophagosomes with lysosomes [27–29]. As a result, increased lapidated LC3 (called LC3-II) can be as a molecular marker indicating accumulation of autophagosomes [30] in HGFs. Remarkably, our analysis of LC3-II suggested that LPS induced changes in the protein expression levels of LC3-II related to an increased autophagic response. Meanwhile, analysis of our data showed more obvious GFP-LC3 puncta in LPS-treated group. Such results observed may be related to a more autophagosomes formation and maturation [31]. 3-MA blocked the early stage of autophagy. Therefore, our results are in agreement with studies demonstrating the down-regulated autophagy by-3-MA [32]. Interestingly, LPS positively regulated LC3-II protein expression and GFP-LC3 puncta even in the presence of 3-MA, thus suggesting the regulatory role of LPS in autophagy of HGFs. We do not know why LPS promotes the autophagy of HGFs. Further studies are performed to reveal the mechanism that underlies the enhanced autophagic process of HGFs by LPS observed. We believed that understanding of the characteristics and mechanisms of LPS-induced autophagy in depth might shed light on the relationship between autophagy and periodontitis.

Previous reports have revealed that the PI3K/Akt/mTOR pathway is a critical mediator in signal transduction pathways potentially involved in autophagic process [33]. In addition, inhibition of PI3K/Akt/mTOR was related to autophagic death in cardiomyocytes [18]. Akt, a protein kinase B, is a central regulator in the PI3K/Akt/mTOR signaling pathway and manifests as downstream of PI3K in the signaling way
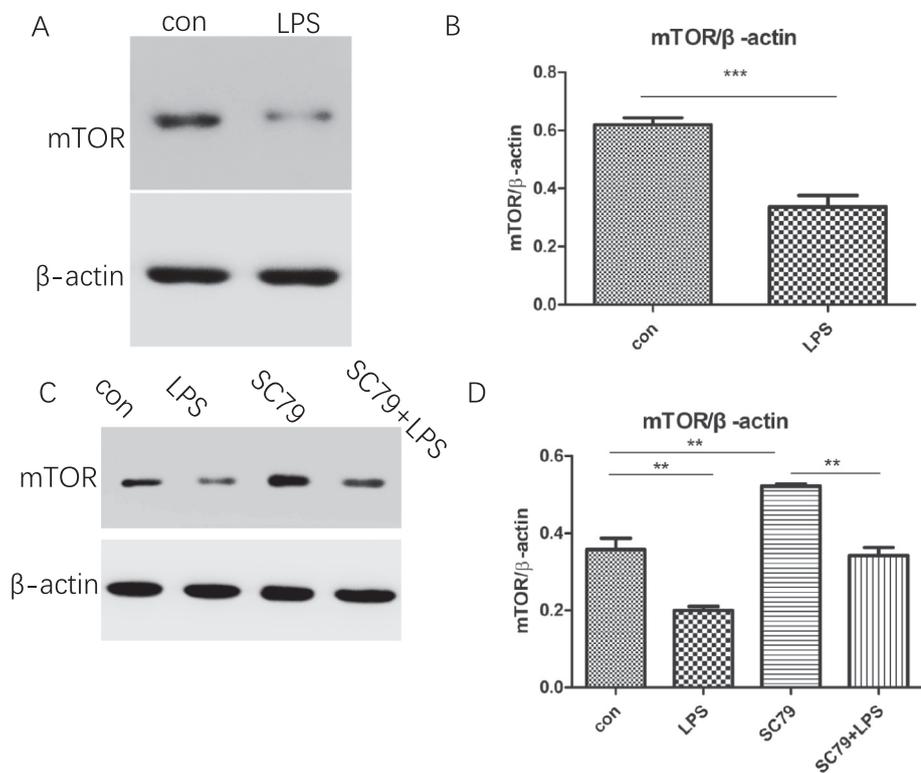
Fig. 3. LPS stimulation decreased activity of mTOR in HGFs. (A) Representative images of Western blotting on control or LPS-treated cells, mTOR was downregulated after LPS stimulation. (B) Results were further quantified by densitometric analysis, normalized by the level of β-actin. (C) Activation of Akt by SC79 increased mTOR expression, LPS combined with SC79-treated cells exhibited decreased mTOR expression compared with SC79 stimulation alone. (D) The densitometric values among four groups were measured, normalized by the level of β-actin. Data of this figure represent the mean ± SD of triplicate assays. $**p < 0.01$, $***p < 0.001$.
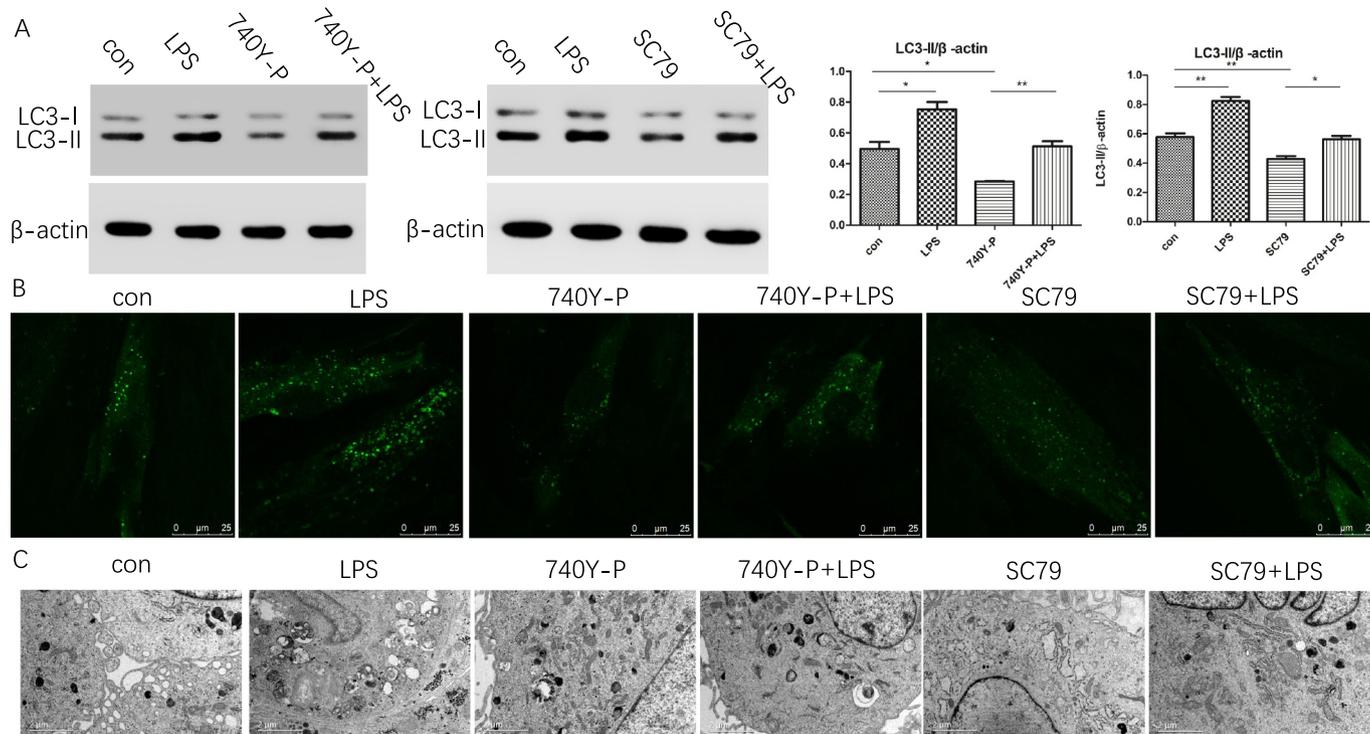
Fig. 4. LPS promoted autophagic process in HGFs through downregulating PI3K/Akt/mTOR pathway. (A) Representative images of Western blotting showed that LPS upregulated LC3-II expression. Both 740Y-P and SC79 upregulated LC3-II expression, LPS combined 740Y-P or SC79 stimulation decreased LC3-II expression. Results were further quantified by analyzing densitometric values and normalized by β-actin. Error bars indicated the SD obtained from triplicate measurements. $*p < 0.5$, $**p < 0.01$. (B) LPS increased the number of GFP-LC3 puncta compared with control cells. 740Y-P and SC79 significantly decreased the number of GFP-LC3 puncta in HGFs. HGFs co-cultured with LPS and 740Y-P or SC79 showed more GFP-LC3 puncta compared with 740Y-P or SC79-only stimulated cells. Scale bar = 25 μm. (C) We observed autophagosomes formation by electron microscope. LPS-treated cells exhibited more autophagosomes. 740Y-P or SC79 stimulation showed that the autophagosomes became less, but LPS rescued their inhibition of autophagosomes. Scale bar = 2 μm.
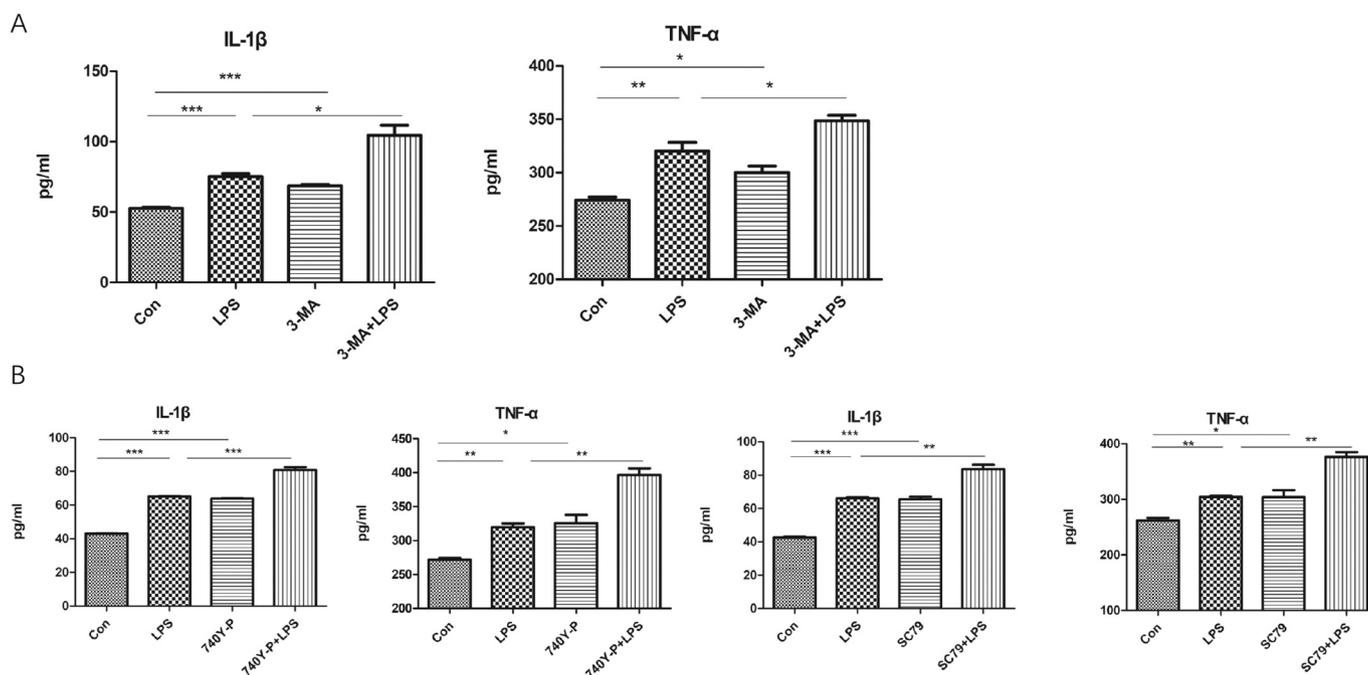
Fig. 5. LPS from *P.g*-induced autophagy prevented inflammatory cytokines secretion. ELISA experiments were performed by using cell supernatants. (A) IL-1β and TNF-α were secreted more by LPS stimulation than control cells. 3-MA, an inhibitor of autophagy, also increased the levels of IL-1β and TNF-α compared with control group. When HGFs were co-incubated with 3-MA and LPS, the levels of IL-1β and TNF-α in HGFs were significantly increased. (B) The levels of IL-1β and TNF-α were upregulated after 740Y-P or SC79 stimulation. Combining 740Y-P or SC79 with LPS showed the enhanced IL-1β and TNF-α generation. Error bars indicated the SD obtained from triplicate assays. $^{*}p < 0.5$. $^{**}p < 0.01$, $^{***}p < 0.001$.

[34]. It is activated by phosphorylation [35]. The kinase mammalian target of rapamycin (mTOR) is a principal mediator of the autophagic process and known to be a downstream target of the PI3K/Akt pathway [36]. Phosphorylated Akt, the activated form of Akt, was capable of inducing another intracellular mTOR [37]. The inhibition of autophagy is through promoting mTOR signaling [19,38]. Recent studies have found that silica nanoparticles induced autophagy in endothelial cells via the PI3K/Akt/mTOR signaling pathway [39]. It is concluded from these studies that deactivation of the PI3K/Akt/mTOR signaling pathway may result in the autophagy. Therefore, we hypothesized that LPS facilitated the autophagy in HGFs through depressing PI3K/Akt/ mTOR signaling pathway. Our data supported this notion because of the deactivated PI3K/Akt/mTOR observed after LPS stimulation, which presumably were related to the enhanced autophagic process. Furthermore, specifically activation of PI3K and Akt by 740Y-P and SC79 respectively down-regulated the autophagic process in HGFs. Given the fact, we speculated that LPS might rescue the decreased autophagic process by 740Y-P or SC79. Our data from LC3-II protein level and GFP-LC3 puncta together with autophagosomes observed in electron microscope all confirmed that LPS indeed promoted autophagy in HGFs even in the presence of 740Y-P or SC79. These results indicated that LPS from *P.g* induced autophagy of HGFs via PI3K/Akt/mTOR signaling pathway. However, further studies are needed to testify whether other signal pathways also participate in the autophagic process.

It has been established that LPS, the major pathogenic factor of all Gram-negative bacteria, is known to induce a strong inflammatory response by firstly interacting with Toll-like receptor (TLR) 2 or 4 [40], notably leading to the downstream classical nuclear factor (NF)-κB pathway activation causing production of pro-inflammatory cytokines and chemokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1β [41]. Recent studies also revealed that suppressing mTOR can negatively regulate the LPS-induced pro-inflammatory cytokines secretion process and NF-κB phosphorylation. This suggests that the PI3K/Akt/mTOR signal pathway can influence the activation of NF-κB [42–44]. *P.g*, the main Gram-negative periodontal pathogenic

bacteria, is involved in the initiation and progression of periodontitis [45]. In this study, using LPS from *P.g* stimulated HGFs to conduct an ELISA assay. Our results are in agreement with studies demonstrating the up-regulation of pro-inflammatory cytokines. Additionally, here we clearly demonstrated that HGFs generated more TNF-α and IL-1β in the supernatant after LPS stimulation combined inhibition of autophagy. Several studies have found a link between autophagy and inflammasome activation [46]. An inflammasome is a multiple protein complex and related to recognize microorganism, resulted in the generation of the mature cytokines such as IL-1β, TNF-α to defense against bacteria [47]. Recent studies have demonstrated NLRP3 inflammasome was strengthened after autophagy was repressed by 3-MA, offering a new perspective that autophagy is important in host defense through eliminating *P.g* within cells [17,48]. This work added to the current knowledge that when autophagy was blocked, HGFs increased their secretion ability of TNF-α, IL-1β. However, further studies are needed to investigate whether inflammasome components are involved in LPS from *P.g*-mediated autophagic process.

Taken together, our findings indicate, for the first time, that LPS from *P.g* stimulation of human gingival fibroblasts leads to up-regulation of autophagy via reducing PI3K/Akt/mTOR signaling pathway, which may, at least in part, be responsible for immune response. This may provide a new insight into the association between autophagic process and inflammation.

5. Conclusions

In summary, we have shown that LPS from *P.g* induced autophagy of HGFs by suppressing PI3K/Akt/mTOR signaling pathway. Moreover, autophagy can participate in limiting pro-inflammatory cytokines secretion. These results enhanced our understanding of autophagy link to inflammation. Further studies should be undertaken to confirm the possible mechanisms between autophagy and inflammation in depth.

Acknowledgments

Conflict of interests

The authors declared no conflict of interests regarding the publication of this manuscript.

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