Substance P aggravates periodontitis by upregulating HIF-1α and RANKL / OPG ratio

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Abstract

Background Both substance P (SP) and hypoxia-inducible factor 1 alpha (HIF-1α) get involved in inflammation and angiogenesis. But the interrelation between SP and HIF-1α in rat periodontitis is still little known.

Methods Ligation-induced rat periodontitis was established to observe the expression of SP and HIF-1α by immunohistochemistry. Gingival fibroblasts and bone marrow macrophages (BMMs) were respectively cultured and stimulated with Porphyromonas gingivalis lipopolysaccharide (LPS). 10 nM SP with or without 1µg/ml LPS was added to elaborate the relationship between SP and HIF-1α in gingival fibroblasts. The effect of SP on osteoclastogenesis was tested by TRAP staining. Western blotting was applied to investigate the expressions of HIF-1α, osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL).

Results The expression levels of HIF-1α and SP were higher in periodontitis than normal tissues. SP could upregulate the level of HIF-1α and RANKL/OPG ratio in LPS-stimulated gingival fibroblasts. SP with or without LPS also facilitated RANKL induced osteoclastogenesis.

Conclusion Substance P aggravates periodontitis by increasing HIF-1α and RANKL / OPG ratio.

Background

Periodontitis is a chronic inflammatory disease with periodontal bone destruction and gingival inflammation[1]{Gölz, 2015 #12; Hou, 2010 #33}. Hypoxia and inflammation are closely interrelated and interact on each other[2]. When periodontal microcirculation was locally damaged because of inflammation, the periodontal tissues may suffer from hypoxia obviously. HIF-1α, a highly conserved transcription factor, plays a key role in mediating
cellular responses to hypoxia[3]. Growing evidences show that SP is involved in the inflammatory and healing responses, and exert a few functions of regulation such as increasing vascular permeability, vasodilatory effect[4, 5], and inducing angiogenesis[6]. It has already been shown that SP may have a role in the pathogenesis of periodontal disease[7].

However, the interrelation between SP and HIF-1α in rat periodontitis is still little known. In this study, recombinant substance P protein was applied to observed the expression of HIF-1α, osteoprotegerin (OPG) and receptor activator of NF-kB ligand (RANKL) to elaborate the relationship between SP and HIF-1α in LPS-stimulated gingival fibroblasts.

Methods

Animals

Twelve male Wistar rats (220–260 g, Laboratory Animal Center, Shandong University) were maintained on a routine diet to acclimate for 1 week before the experiment. The rats were assigned to ligation (L) group and normal (N) group at random. Protocols of the study met approval from Ethics in the Care and Use of Laboratory Animals Committee of the School of stomatology, Shandong University.

Rat experimental periodontitis model

Rats in the L group were under general anesthesia for the experimental periodontitis model. A 4-0 silk suture and an orthodontic ligature wire was placed around the cervical region of the right first lower molars and then ligated firmly. After 6 weeks, all rats in the two groups were euthanized with a lethal dose (150 mg/kg) of sodium thiopental. The gingiva and alveolar bone tissues were collected and fixed in 4% paraformaldehyde for 48 hours. Then specimens were dehydrated, cleared and finally embedded in paraffin. Serial
sections (5-µm thick) were obtained for hematoxylin-eosin staining (HE) staining and SP immunohistochemical staining.

Cell culture and treatment

Fresh healthy gingiva of Wistar rats (80–100 g) were separated after cervical dislocation and washed thrice with phosphate buffer (PBS) with 200 IU/ml penicillin and 200 mg/ml streptomycin (Solarbio, Beijing, China). The gingival tissues were minced by ophthalmic scissors, then digested for one hour at 37 °C constant temperature shaker in a solution of collagenase type I (3 mg/mL; Solarbio) and dispase (4 mg/mL; Sigma Aldrich, St Louis, USA). After enzyme digestion, the filtered single-cell suspension was maintained in α-minimal essential medium (α-MEM; Hyclone, Utah, USA), containing 20% fetal bovine serum (FBS; Biological Industries, Kibbutz, Israel), 100 IU/ml penicillin and 100 mg/ml streptomycin at 37°C in an incubator with 95% O₂-5% CO₂ atmosphere. After confluence, the cells were detached with 0.25% Trypsin-EDTA solution (Solarbio) and subcultured in α-MEM with 10% FBS. The medium was changed every 48 hours. Cells between fourth and sixth passages were used for subsequent experiments.

Primary mouse bone marrow-derived macrophages (BMMs) were isolated from the femurs and tibias of 3-week-old C57/BL6 male mice after cervical dislocation and cultured in complete α-MEM containing 10% FBS and 30 ng/ml Macrophage Colony Stimulating Factor (M-CSF) at 37°C in an incubator with 95% O₂-5% CO₂ atmosphere. 50 ng/ml RANKL was used for 4 days to induce BMMs to differentiate into osteoclasts. To observe the effect of SP on osteoclastogenesis, we added 10 nM SP (Sigma Aldrich) with or without 1µg/ml LPS (Invivo Gen, San Diego, USA) into culture medium (RANKL+10 nM SP group and RANKL+50 nM SP group, only RANKL group as control) and then stained for TRAP.
TRAP staining

The cells were fixed with 4% paraformaldehyde, and then stained for TRAP using a commercially available kit (Joy Tech Bio. Co., Zhejiang, China). Osteoclasts were identified as TRAP-positive multi-nucleated cells containing three or more nuclei.

Immunohistochemical staining

Rabbit polyclonal antibody substance P (diluted 1:200, Abcam, Cambridge, UK), HIF-1α (diluted 1:200, Abcam) were applied as previously described. PBS was obtained as control.

Western blotting analysis

RIPA lysis buffer (Solarbio) was used to extract the total protein. The concentrations of proteins were measured by bicinchoninic acid (BCA) assay kit (Solarbio) according to the manufacturer’s instructions. Equal loading quantity of proteins were separated in a 10% SDS-PAGE and electro-blotted to polyvinylidene difluoride (PVDF) membranes (Millipore, California, USA). Membranes were blocked with 5% non-fat milk dissolved in TBST buffer at room temperature for 1 h, and then incubated overnight at 4°C with primary antibodies, including HIF-1α (diluted 1:500, Abcam), TNF-α (diluted 1:500, Abcam), OPG (diluted 1:500; Bioss, Beijing, China) and RANKL (diluted 1:500; Bioss). After washing with TBST, the membranes were incubated with secondary horseradish peroxidase (HRP)-linked goat-anti rabbit IgG antibody (diluted 1:10000, CWBiotech, Beijing, China) at room temperature for 1 h. Blots were visualized using ECL kit (Milipore).

Statistical analysis

All data values were expressed as mean ± standard error of mean. T tests were conducted with GraphPad Prism 5 software. A value of P < 0.05 was considered statistically significant.
Results

1. Rat experimental periodontitis model

6 weeks after ligation, obvious gingival recession of the first molars was observed.

Gingival inflammatory infiltration and obvious alveolar bone loss between the first and second molars were observed by hematoxylin-eosin staining (Figure 1).

2. Expressions of HIF-1α and SP in rat ligation-induced experimental periodontitis.

After the immunohistochemistry staining was performed in the gingiva of ligation-induced experimental periodontitis, positive expression of HIF-1α was observed. The positive staining region was localized in inflammatory infiltrating cells (Figure 2A, B and C). Comparing with normal gingiva, gingiva of periodontitis expressed higher HIF-1α according to western blotting (*P<0.05) (Figure 2D). In addition, we observed the positive staining of SP was the same with HIF-1α (Figure 3).

3. SP upregulated the level of HIF-1α in gingival fibroblasts and BMMs.

After 24h, both 1µg/ml LPS and 10 nM SP could obviously induce TNF-α expression (*P<0.05) (Figure 4A). 10 nM SP with or without 1µg/ml LPS both could upregulate the level of HIF-1α in gingival fibroblasts (*P<0.05) (Figure 4B).

4. SP promoted RANKL-induced osteoclast differentiation in BMMs and upregulated

In the RANKL+10 nM SP (Figure 5B2) and RANKL+50 nM SP groups (Figure 5B3), more TRAP positive osteoclasts were detected than the RANKL group (Figure 5B1). Both 10 nM and 50 nM SP were able to upregulate osteoclast differentiation induced by RANKL (Figure 5C).

SP upregulated RANKL/OPG ratio in gingival fibroblasts tested by western blotting. The RANKL/OPG ratio was markedly increased in the LPS+SP group, compared to the LPS and
Discussion

SP, an undecapeptide belonging to the tachykinin neuropeptide family and consists of 11 amino acids, could be induced by cytokines and LPS. It can release from peripheral sensory nerve endings and immunological cells, and widely distributed in both the central and peripheral nervous systems[4]. SP is characterized as a pro-inflammatory neurotransmitter and play a key role in inflammation. Periodontitis is a chronic inflammatory disease which has an important neurogenic component[8]. SP, which has been detected in gingival tissues and GCF, could stimulate osteoclasts and promote bone resorption. The amount of local SP is considered to be correlated with periodontal inflammation[9]. Inflammatory infiltration of gingiva is critical for periodontal inflammation destruction. Besides, periodontal tissues have also been shown to be relatively hypoxic and ischemic in periodontitis. HIF, an essential transcription regulator of oxygen homeostasis, allows cells to be adaptive for reduced-oxygen environment[10]. HIF–1 is a member of the basic helix-loop-helix superfamily of transcription factors. Only active as a heterodimer, HIF–1 is composed of 2 subunits: HIF–1α and HIF–1β[11]. In normal oxygen conditions, HIF–1α is hardly undetectable. When cells are in hypoxic condition, level of the HIF–1α protein increases obviously, while the HIF–1β protein expresses in any condition[12]. HIF activation is linked to immunity altering and inflammation. There are several cell type-specific mechanisms which has been acknowledged. For example, in LPS-stimulated macrophages, HIF–1α could enhance IL–1 production and make further efforts to promote inflammatory responses[13]. In neutrophils, it has been demonstrated that HIF–1α was able to increase the activity of NF-κB and inhibit neutrophil apoptosis through this pathway [14, 15]. In this study, increased expressions of HIF–1α and SP were observed in rat ligation-induced
Our previous study has showed upregulating the expression of HIF-1α changes the metabolic pathway of human periodontal ligament cells. Both SP and HIF-1α get involved in inflammation and angiogenesis. But the interrelation between SP and HIF-1α in rat periodontitis is still little known. According to relevant research, 1µg/ml LPS and 10 nM SP[15, 16] were applied in this study. We observed both 1µg/ml LPS and 10 nM SP could obviously induce TNF-α expression. 10 nM SP with or without 1µg/ml LPS both could upregulate the level of HIF-1α in gingival fibroblasts.

Substance P signaling regulates the functions of both osteoblast and osteoclast[17]. It has been clarified that SP inhibits osteoblastic cells differentiation and may be related to bone metabolism in periodontal diseases under conditions of stress[9, 16]. Osteoclasts can express SP receptor, which will bind to SP and then induce osteoclastogenesis. If a certain quantity of SP was produced constantly, it will result in bone resorption[18, 19]. Our results showed RANKL/OPG ratio was markedly increased in the LPS group, SP group, and LPS+SP group (P<0.05), compared with normal group. SP might upregulate RANKL/OPG ratio to enhance bone resorption, which was consistent with the result of Lee et al[20]. There are some other mechanisms through which SP plays a role in periodontitis, such as inducing interleukin-6 (IL-6) synthesis in monocytes, stimulating histamine release from mast cells, regulating the expression of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) and so on[21]. Our study may provide a new mechanism for SP modulating periodontitis.

Conclusions

SP could enhance the level of HIF-1α and increase RANKL/OPG ratio in LPS-stimulated gingival fibroblasts, which may provide a new mechanism for SP modulating periodontitis.
Declarations

Ethics approval and consent to participate

Protocols of the animal study met approval from Ethics in the Care and Use of Laboratory Animals Committee of the School of stomatology, Shandong University, and complied with the guidelines for the use of animals in research.

Consent for publication

Yes

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Competing interests

The authors declare no conflict of interest.

Availability of data and materials

The data used during the current study are available from the corresponding author on reasonable request.

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Contributions

YKX made the outline of this study as part of a Master’s Dissertation and wrote the research protocol in collaboration with LQ, YXJ and LiS. YKX and LiuS contributed to the cell culture, western blotting, and TRAP staining. LQ, TKL and DY contributed to the animal research. All authors took part in the statistical analysis, drafting and revising manuscript, and in the finalization and approval of the submitted version of the manuscript.

YKX and LQ are equal contributors.

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Abbreviations

SP : Substance P

HIF–1α: Hypoxia-inducible factor–1 alpha

BMMs: Bone marrow macrophages

LPS: Lipopolysaccharide

TRAP: Tartrate-resistant acid phosphatase

OPG: Osteoprotegerin

RANKL: NF-kB ligand
L: Ligation

N: Normal

HE: Hematoxylin-eosin staining

PBS: Phosphate buffer

α-MEM: α-Minimal essential medium

FBS: Fetal bovine serum

BMMs: Bone marrow-derived macrophages

M-CSF: Macrophage Colony Stimulating Factor

BCA: Bicinchoninic acid

PVDF: Polyvinylidene difluoride

HRP: Horseradish peroxidase

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Figures
Rat experimental periodontitis model. A: Normal periodontal tissues before ligation; B: A 4-0 silk suture and an orthodontic ligature wire was ligated firmly to the dental cervix of the right first lower molars for 6 weeks. C: 6 weeks after ligation, obvious gingival recession was observed (black arrow). D: Inflammatory infiltration and obvious alveolar bone loss were observed at 6 weeks after ligation.
Expression of HIF-1α in rat ligation-induced experimental periodontitis. The positive staining was localized in inflammatory infiltrating cells (A, B and C) by immunohistochemical staining (red arrow). Higher expression of HIF-1α was observed in periodontitis compared with normal gingiva according to western blotting (*P<0.05).
Expression of SP in rat ligation-induced experimental periodontitis. The positive staining of SP was localized in inflammatory infiltrating cells in the gingiva of rat experimental periodontitis.
SP upregulated the level of HIF-1α in gingival fibroblasts and BMMs. After 24h, both 1µg/ml LPS and 10 nM SP could obviously induce TNF-α expression by western blotting (A). 10 nM SP with or without 1µg/ml LPS both could upregulate the level of HIF-1α in gingival fibroblasts by western blotting (*P<0.05).
Effect of SP on RANKL-induced osteoclastogenesis. Many positive expressions of HIF-1α were observed in the nucleus of BMMs in the SP+LPS group (A). More TRAP positive osteoclasts were observed in the RANKL+SP groups, compared with the RANKL group (B). SP upregulated RANKL-induced osteoclast differentiation (C) (*P<0.05).
Figure 6

SP upregulated RANKL/OPG ratio in gingival fibroblasts by western blotting. The RANKL/OPG ratio was markedly increased in the LPS+SP group, compared to the LPS and SP groups (*P<0.05).
Supplementary Files

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