Butyric acid modulates periodontal nociception in Porphyromonas gingivalis-induced periodontitis

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Abstract

Purpose: Periodontitis progresses with chronic inflammation, without periodontal pain. However, the underlying mechanisms are not well known. Here, the involvement of butyric acid (BA) in periodontal pain sensitivity in Porphyromonas gingivalis (P. gingivalis)-induced periodontitis was examined.

Methods: P. gingivalis was inoculated into the ligature which was tied around the molar (P. gingivalis-L) and the gingival mechanical head withdrawal threshold (MHWT) was measured. Following P. gingivalis-L, the expressions of orphan G protein-coupled receptor 41 (GPR41) in trigeminal ganglion (TG) neurons were examined. The amount of gingival BA was analyzed following the P. gingivalis-L and the changes in the MHWT in complete Freund’s adjuvant (CFA)-injected gingival tissue by gingival BA were examined. The changes in the MHWT following P. gingivalis-L by gingival GPR41 antagonist (HA) were examined.

Results: No change in the MHWT was observed, GPR41-immunoreactive TG neurons were increased following P. gingivalis-L and the gingival BA suppressed the decrease in MHWT following CFA. HA decreased MHWT following P. gingivalis-L.

Conclusion: Gingival BA modulates periodontal mechanical nociception via GPR41 signaling in P. gingivalis-induced periodontitis.

Keywords: Butyric acid, periodontal pain, Porphyromonas gingivalis, primary afferent

Introduction

Periodontal disease which is well known to be a chronic inflammatory disease which is attributable to various bacterial infections originating from dental plaque causes progressive destruction of periodontal tissues such as alveolar bone [1,2]. It is well known that many inflammatory diseases generally progress with pain symptoms, although interestingly, periodontal disease often progresses without evident pain awareness [3]. Hence, many patients with periodontitis are oblivious to the progression of periodontitis, leading to severe periodontal tissue destruction, starting with delayed diagnosis and treatment of periodontitis [4]. However, it remains unclear why periodontitis progresses without pain symptoms in the periodontal tissue.

The leading pathogenic bacteria that causes periodontitis in humans is Porphyromonas gingivalis (P. gingivalis), and the releasing factors from P. gingivalis in dental plaque trigger chronic inflammation of the periodontal tissue [5]. Short chain fatty acids (SCFAs) including butyric acid (BA) which are metabolites of bacteria are known to be released from P. gingivalis in subgingival plaques and produced in large quantities during periodontal disease progression [6]. BA produced in the periodontal tissue of periodontal disease promotes the progression of periodontal disease by promoting overproduction of matrix metalloproteinases or consistent damage to periodontal immunomodulatory cells [7-9]. It has been reported that orphan G protein-coupled receptor 41 (GPR41), which is the receptor of SCFAs, is expressed in primary sensory neurons [10]. This report suggests that BA signaling via GPR41 in the sensory nerve endings of the periodontal tissue may change the somatosensory characteristics of the periodontal tissue in a chronic inflammation state. Nevertheless, it is completely unknown whether BA signaling in sensory nerve endings is involved in the painless progression of periodontal tissue in periodontitis.

This study aimed to elucidate the mechanism by which periodontal pain is not induced in the periodontal tissue in a chronic inflammation state by examining the involvement of BA signaling in primary afferent nerve endings in periodontal mechanical nociception associated with periodontitis using a P. gingivalis-induced periodontitis mouse model.

Materials and Methods

Animals

Male C57BL/6 mice (n = 99, 20-30 g; Japan SLC, Hamamatsu, Japan) were used for all experiments in this study. Mice were reared in a well-controlled laboratory with free intake to water and food. (Light-dark cycle: 12 h/12 h, humidity: 55 ± 5%, ambient temperature: 23 ± 2°C). The Animal Experimentation Committee at Nihon University approved this study (AP18DEN016-1), and it was performed in compliance with the International Association for the Study of Pain guidelines [11]. Every effort has been made to minimize distress in mice and the number of mice used.

Establishment of periodontitis

The mouth of the mouse lying on a temperature-controlled mat (37°C) was opened by mouth opener under anesthesia with intraperitoneal (i.p.) administration of midazolam (4.0 mg/kg; Sandoz, Tokyo, Japan), medetomidine (0.75 mg/kg; Zenoa, Koriyama, Japan), and butorphanol (5.0 mg/kg, Meiji Seika Pharma, Tokyo, Japan). The neck of the right maxillary second molar was coiled with a 5-0 silk ligature while avoiding damage to the surrounding tissues. On days 0 to 2 after ligation, the mice were inoculated P. gingivalis (FDC381, 10^6 colony-forming units/mL) into the silk ligature under anesthesia (1.5% isoflurane, Canonsburg, PA, USA) [12]. Mice inoculated with P. gingivalis on the silk ligature around the neck of the maxillary second molar (P. gingivalis-L) were defined as the P. gingivalis-L group. Mice were separately injected with Complete Freund’s adjuvant (CFA; Sigma-Aldrich, St. Louis, MO, USA) into the gingival tissue with the mouth opening under deep anesthesia, as described above (CFA group) [12]. Mice that underwent the same treatment with the P. gingivalis-L group except for the P. gingivalis inoculation to the ligature were defined as the control group.

Measurement of mechanical nociceptive sensitivity

Under light anesthesia (2% isoflurane, Mylan), the mouth of the mice was kept open by the mouth opener. After interruption of isoflurane inhalation,
it was confirmed that the appropriate level of anesthesia was maintained by inducing the same withdrawal reflex with the same noxious stimuli applied to the hind paw. Immediately after confirming the appropriate level of anesthesia, the gingival tissue was applied graded mechanical stimulation by an electronic von Frey anesthesiometer (Bioshe, Vitrolles, France) and the lowest mechanical intensity required the induction of a head withdrawal reflex was determined as the mechanical head withdrawal threshold (MHWT) in a manner similar to previous study [12]. The mice were free to escape the mechanical stimulation. Each graded mechanical stimulus was performed at 1-minute intervals. The MHWT measurement was performed three times, and the average MHWT was determined as the MHWT for each. All MHWT measurements were completed under blinded conditions.

High performance liquid chromatography

Under the above-described deep anesthesia, mice were transcardially perfused with saline on day 4 following the P. gingivalis-L and control treatment, and the gingival tissue in each was removed. Following the homogenization of gingival tissue, the supernatant was collected. Then, the BA amount was quantified by high-performance liquid chromatography (HPLC) as follows.

Briefly, the HPLC instrument comprised an LC-20AT pump (Shimadzu, Kyoto, Japan), a CTO-10A column oven (Shimadzu), a UV-2075 UV detector (Jasco, Tokyo, Japan), and an LC-Net II/ADC recorder (Jasco). ChromNAV Lite (Jasco) was used for data acquisition and handling. A V-630 spectrophotometer (Jasco) was used to measure the absorbance of all sample solutions. First-grade sulfuric acid (Fujifilm Wako Pure Chemical, Osaka, Japan) was used as the mobile phase. To prepare the standard solution, reagent grade sodium acetate, first-grade sodium propionate, and sodium butyrate were purchased from Fujifilm Wako Pure Chemical. Reagent grade sodium isobutyrate and sodium isovalerate were purchased from Kanto Chemical. Standard solutions (1,000 mg/L) were prepared by diluting the certified standards for several short-chain fatty acids. Ethanol and absolute ethanol (reagent or HPLC grade, Fujifilm Wako Pure Chemical) were used as extraction reagents.

To remove proteins from the real sample solution, 300 μL of a real sample solution was centrifuged at 3,000 rpm for 60 min. After standing, a constant volume was obtained by filling the volumetric flask with ultrapure water to the marked line. The absorbance of each sample solution was measured to confirm the effect of deproteinization, and only solutions with an absorbance of <0.2 were analyzed using HPLC.

Drug administration

Four microliters of 3-hydroxybutyric acid (HA, 50 μg/mL dissolved in 0.01 M phosphate-buffered saline (PBS); #085-03571, Fujifilm Wako Pure Chemical), which is a GPR41 antagonist or 4 μL of BA (50 μg/mL) was administered once daily for 15 days to the gingival tissue after P. gingivalis-L or CFA treatment under the above-described light anesthesia. On the day of MHWT measurement, drug administration into the gingival tissue was performed immediately after MHWT measurement.

Immunohistochemistry

On day 2 before P. gingivalis-L or CFA, 4% hydroxyethylamidated (Fluoro-Gold [FG], Fluorochrome, Denver, CO, USA) dissolved in saline was injected to the gingival tissue to identify the trigeminal ganglion (TG) neurons innervating the gingival tissue under the deep anesthesia described above. On day 8 following the P. gingivalis-L, CFA, or control treatment, the expression of GPR41, a SCFA receptor in TG neurons innervating the gingival tissue with deep anesthesia described above. On day 8 following the P. gingivalis-L, CFA, or control treatment, the expression of GPR41, a SCFA receptor in TG neurons innervating the gingival tissue, was immunohistochemically examined. Briefly, mice were perfused with 4% paraformaldehyde (PFA) under deep anesthesia. After removing the TG and the gingival tissue, these samples were immersed in 4% PFA for 4h. For cryoprotection, these samples were placed in 20% sucrose and embedded at −20°C. These samples were sliced to a thickness of 15 μm, and the sections were pasted onto microscope slides. The sections of the TG were rinsed and reacted with anti-GPR41 polyclonal rabbit antibody (1:100, #G250, AssayBiotech, Fremont, CA, USA) containing 4% normal goat serum (Sigma-Aldrich) with 0.3% TritonX-100 at 4°C overnight. After rinsing, the sections were reacted in Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:200, Fab175471, Thermo Fisher Scientific, Waltham, MA, USA) for 2 h. After washing, the sections were cover-slipped. FG-labeled GPR41 immunoreactive neurons in the TG were detected using a BZ-9000 system (Keyence, Osaka, Japan). The average background intensity was considered immunoreactive. Without the primary antibodies, no specific immunoreactivity was detected. The percentage of FG-labeled neurons expressing GPR41 in five sections of TG was defined as FG-labeled GPR41 immunoreactive neurons in each mouse, respectively. The mean percentage of FG-labeled GPR41 immunoreactive neurons was calculated in each group.

Statistical Analysis

It was confirmed that all data were normally distributed and satisfy the homoscedasticity by the Shapiro-Wilk normality test and the Brown-Forsythe test, respectively. Therefore, all data were presented as mean ± standard error (SEM). For immunohistochemistry and HPLC, dot plots were added to show individual sample sizes. Two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni’s multiple comparison test was conducted to examine the differences in MHWT. One-way ANOVA followed by Tukey’s multiple comparison test was conducted to examine the difference in the number of FG-labeled GPR41 immunoreactive neurons. For the statistical analysis of the amount of BA in the gingival tissue, Student’s t-test was used. A P-value <0.05 was considered significant.

Results

Changes in gingival mechanical sensitivity

In the P. gingivalis-L group, the MHWT did not change and there was no significant difference compared to MHWT in the control group during the experimental period (Fig. 1). On day 2 after the gingival CFA injection, the MHWT in the CFA group was considerably decreased compared with the P. gingivalis-L or control group, and the decrease in the MHWT continued until day 14. During the experimental period, there were no motor deficits or sedation (data not shown).

Amount of BA in the gingival tissue following P. gingivalis-L treatment

On day 4 following P. gingivalis-L or control treatment, chromatograms were obtained from the gingiva under optimal HPLC conditions (Fig. 2a). Prior to measuring the sample solutions of the P. gingivalis-L group and the control group, a standard solution containing only BA was analyzed. It was confirmed in advance that BA was detected at 16 min. Therefore, the peak indicated by the arrow is confirmed to be the peak derived from BA. Amount of BA in the P. gingivalis-L group (69.4 ± 15.7 mg/L) was larger than that of the control group (17.9 ± 6.9 mg/L) on day 4 after these treatments.

Effect of BA on the inflamed gingival mechanical sensitivity

In the P. gingivalis-L group, BA injection into the gingival tissue did not alter the MHWT during the experimental period (Fig. 2b). Although
vehicle administration into the gingival tissue considerably decreased the MHWT after CFA injection, BA administration in the gingival tissue in the CFA group recovered the decrease in the MHWT from day 6 onward. Motor deficits or sedation were not observed during the experimental period (data not shown).

**Expression of GPR41 in TG neurons after the *P. gingivalis*-L treatment**

FG-labeled GPR41 immunoreactive neurons were detected in the TG on day 2 following *P. gingivalis*-L or control treatment. Data represent mean ± SEM. *(P < 0.05, **P < 0.01, §§ P < 0.001, †††† P < 0.0001, vs. pre in *P. gingivalis*-L + vehicle group; †, P < 0.05, ††, P < 0.01) (one-way ANOVA followed by Tukey’s multiple comparison test). Arrows indicate GPR41 immunoreactive TG neurons innervating into the gingival tissue. Scale bar: 50 μm. Data represent mean ± SEM. *(P < 0.05, **P < 0.01) (two-way repeated-measures ANOVA followed by Bonferroni’s multiple comparison test).

**Effect of GPR41 antagonism on the inflamed gingival mechanical sensitivity**

HA administration into the gingival tissue in *P. gingivalis*-L group decreased the gingival MHWT from the 8th day after *P. gingivalis*-L, while vehicle administration did not change the gingival MHWT (Fig. 3b). In contrast, CFA administration increased the gingival MHWT from the 8th day after *P. gingivalis*-L or CFA treatment. Data represent mean ± SEM. *(P < 0.05 vs. pre in *P. gingivalis*-L or CFA + HA group; ††† P < 0.001, vs. pre in *P. gingivalis*-L + vehicle group; †, P < 0.05, ††††, P < 0.0001, vs. pre in CFA + HA group (two-way ANOVA followed by Bonferroni’s multiple comparison test).

**Discussion**

Various chemical mediators released from peripheral tissues associated with local inflammation act on nociceptive nerve endings, which induce an enhancement of peripheral nociceptive nerve excitability. Thus, nerve afferent firings were increased, leading to the development of pain hypersensitivity [13]. In previous studies, local inflammation increased glutamate or calcitonin gene-related peptides at the site of inflammation, and enhancement of their signals induced increased expression of nociceptive receptors, resulting in orofacial pain hypersensitivity dependent on primary afferent neuronal sensitization [14,15]. However, patients with periodontitis, which are chronic inflammations of the periodontal tissue, complain of little periodontal pain [3]. Here, there was little or no change in *P. gingivalis*-L-inflamed gingival mechanical sensitivity, while CFA-induced gingival inflammation caused mechanical pain hypersensitivity. These results indicate that periodontitis progresses without periodontal mechanical pain hypersensitivity in the *P. gingivalis*-L model. Periodontitis in humans progresses without overt periodontal mechanical pain hypersensitivity; thus, this model can be used for assessment of the modulation of periodontal nociception in periodontitis patients because it resembles human periodontitis characterized by progression without overt periodontal pain.

SCFAs, such as BA, are found in sites of bacterial infections like...
periodontal diseases and play important roles in their progress [16]. Small amounts of BA produced by *P. gingivalis* were detected in the gingiva in chronic periodontitis patients and disrupted the tight attachment between epithelial cells, resulting in bacterial invasion into the periodontal tissue and its destruction [17,18]. In previous study, significant inflammatory cellular infiltration in the periodontal tissue was generated by *P. gingivalis*-L treatment with resorption of alveolar bone [12]. It is also reported that BA produced by *P. gingivalis* suppresses cell proliferation and cell cycling progression in gingival fibroblasts by inducing apoptosis in monocytes and macrophages in humans [19]. Therefore, BA is likely to be involved in the pathogenesis of periodontal disease [20,7]. Inflammation induced by CFA causes pain hypersensitivity by increasing the nociceptive sensitivity and causing plastic changes in sensory and spinal cord neurons that innervate inflamed tissues [21-23]. These plastic changes are initiated by a complex pattern of chemical signals that interact with nociceptive neurons at the site of inflammation. Here, the amount of BA in the periodontal tissue in the *P. gingivalis*-L group was larger than that in the control group on day 4 after these treatments. Interestingly, BA administration into the gingival tissue suppressed periodontal inflammatory pain hypersensitivity induced by periodontal CFA, although periodontal CFA significantly enhanced the periodontal mechanical pain sensitivity. BA gingival administration did not alter the periodontal mechanical pain sensitivity in the *P. gingivalis*-L group during the experiment period. Therefore, the suppression of inflammatory pain hypersensitivity by the signal of BA released from *P. gingivalis* may be the reason for the absence of periodontal pain despite tissue inflammation in periodontal disease.

SCFAs, which are essential nutrients, are involved in various cell function processes as signal transduction molecules [24]. It should be noted that SCFAs can activate GPR41 and GPR43 [25,26]. GPR41 is a G-protein-coupled receptor in the Gs/i/o family, and activation of GPR41 by SCFAs suppresses extracellular signal-regulated kinase (ERK) cascade signaling activity and the production of intracellular cyclic adenosine monophosphate (cAMP) [27,28]. GPR41 is expressed in fatty tissues and its signaling induces leptin secretion [29]. At this point, GPR41 is reportedly expressed in primary sensory neurons, although little is known about GPR41 neurophysiological functions [10]. Here, it was defined as TG neurons which innervate inflamed gingival tissues in the *P. gingivalis*-L group expressing GPR41. Successful GPR41 antagonism in the inflamed gingival tissue induced periodontal mechanical pain hypersensitivity in the *P. gingivalis*-L group. In contrast, periodontal CFA administration induced periodontal mechanical pain hypersensitivity, which was not suppressed by successive GPR41 antagonism in the inflamed gingival tissue. Altogether, these findings suggest that the BA signal via GPR41 in inflamed gingival tissue suppresses the periodontal inflammatory pain caused by *P. gingivalis*-L. Contrary to expectations, *P. gingivalis*-L treatment increased the number of GPR41 immunoreactive neurons which innervate the inflamed gingival tissue, although no changes were observed in the CFA and control group. The kind of signal in the *P. gingivalis*-L-inflamed gingival tissue that regulates the expression of GPR41 in TG neurons remains unclear and requires further studies.

In conclusion, *P. gingivalis*-L-induced periodontitis, which progresses unaccompanied by periodontal inflammatory pain hypersensitivity, resembles the clinical condition of periodontitis in humans. This study indicates that *P. gingivalis*-L-induced BA-GPR41 signaling plays an important role in the periodontitis progression without periodontal inflammatory pain. Therefore, *P. gingivalis*-L-induced BA-GPR41 signaling may be a key cascade in the regulation of periodontal nociception in periodontal disease.

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Conflict of interest

The authors declare no potential conflicts of interest.

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