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Increased Expression of 11β-Hydroxysteroid Dehydrogenase Type 1 in Experimental Periodontitis Induced by Lipopolysaccharide from *Porphyromonas gingivalis*

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Abstract

It has been proposed that 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which activates glucocorticoids, plays a role in chronic inflammatory diseases including metabolic diseases, rheumatoid arthritis, and ulcerative colitis. We have recently reported that the expression of 11β-HSD1 is increased in the gingiva of patients with chronic periodontitis and in that of rats with ligature-induced periodontitis. In this study, to further demonstrate the involvement of 11β-HSD1 in chronic periodontitis, the expression of 11β-HSD1 was investigated in another rat model of experimental periodontitis induced by intragingival injection of lipopolysaccharide from *Porphyromonas gingivalis* (LPS-PG). Alveolar bone loss was observed two weeks after intragingival injection of LPS-PG. The level of 11β-HSD1 mRNA assessed by real-time reverse transcriptase-polymerase chain reaction was significantly elevated in LPS-PG-induced periodontitis compared with controls. The expression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which inactivates glucocorticoids, was not significantly different between control and LPS-PG-induced periodontitis. The expression of 11β-HSD1 was significantly correlated with that of TNF in LPS-PG-induced periodontitis. The increased expression of 11β-HSD1 protein in LPS-PG-induced periodontitis was confirmed by immunohistochemistry using anti-11β-HSD1 antibody. These results further suggest a role for 11β-HSD1 in the pathogenesis of chronic periodontitis.

Keywords

Chronic Periodontitis, 11β-Hydroxysteroid Dehydrogenase Type 1, Lipopolysaccharide, *Porphyromonas gingivalis*

1. Introduction

It is well known that glucocorticoids play various roles in the metabolism of nutrients and immune responses [1]. Among many enzymes involved in the biosynthesis and catabolism of glucocorticoids, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) mainly catalyzes the conversion of inactive glucocorticoids (cortisone in humans and 11-dehydrocorticosterone in rodents) into active glucocorticoids (cortisol in humans and corticosterone in rodents), whereas 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) mainly catalyzes the conversion of active glucocorticoids into inactive glucocorticoids [2] [3]. It has been proposed that 11β-HSDs play a pivotal role in the intracellular regulation of glucocorticoids.

The involvement of 11β-HSD1 in metabolic diseases including obesity and diabetes mellitus has been reported [4] [5] [6]. The expression of 11β-HSD1 was elevated in adipocytes in the visceral fat of patients with obesity. Animal studies revealed that 11β-HSD1-deficient mice were protected against overnutrition [7]. Furthermore, transgenic mice overexpressing 11β-HSD1 selectively in adipose tissue developed metabolic phenotypes including visceral fat obesity, dyslipidemia, insulin resistance, and hypertension [8] [9]. Since several inhibitors of 11β-HSD1 ameliorate metabolic diseases in animal models [10] [11], selective and potent 11β-HSD1 inhibitors have been developed for the treatment of metabolic diseases [12] [13].

Since obesity is regarded as a chronic inflammatory disease of adipose tissue [14] [15], the involvement of 11β-HSD1 in other chronic inflammatory diseases was examined. As expected, the expression of 11β-HSD1 was increased in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel diseases [16] [17] [18] [19]. We recently reported that the expression of 11β-HSD1 was increased in gingival tissues of patients with another chronic inflammatory disease, chronic periodontitis [20]. Moreover, we reported that the expression of 11β-HSD1 was also increased in ligature-induced experimental periodontitis in rats [21]. To further demonstrate the involvement of 11β-HSD1 in chronic periodontitis, another rat model of experimental periodontitis induced by intragingival injection of lipopolysaccharide (LPS) from *Porphyromonas gingivalis* was used in this study.

2. Materials and Methods

2.1. Experimental Animals

Fifty-two six-week-old male Wistar rats were purchased from Shimizu Labora-

tory Supplies (Kyoto, Japan). Rats were maintained under specific pathogen-free conditions in the Osaka Dental University Animal Care facility. The use of animals and experimental protocol were approved by the Committee for Animal Experiments of Osaka Dental University (#17-02018). The experimental procedures were executed in accordance with the Guidelines for Animal Experiments of Osaka Dental University.

2.2. Induction of Periodontitis by Intragingival Injection of LPS from *Porphyromonas gingivalis*

Forty-six rats at seven weeks of age were anesthetized by inhalation of isoflurane (Wako Pure Chemical industries, Osaka, Japan) and intraperitoneal injection of pentobarbital (0.3 mg/kg) (Kyoritsu Seiyaku Corporation, Tokyo, Japan). Periodontitis was induced by intragingival injection of LPS from *Porphyromonas gingivalis* (LPS-PG) (InvivoGen, San Diego, CA, USA). LPS-PG was dissolved in endotoxin-free water at concentrations of 0.01, 0.1, and 1 mg/ml, and 5 μl of LPS-PG (for controls 5 μl of endotoxin-free water) was injected into the palatal gingiva around the second maxillary molars using a 31-gauge needle equipped with a gastight syringe (Hamilton Company, Reno, NV, USA) on day 1, day 3, and day 5.

2.3. Micro-CT Analysis

For micro-CT analysis, 5 μl of 1 mg/ml LPS-PG was injected into the palatal gingiva around the second maxillary molars in three rats on day 1, day 3, and day 5, and rats were euthanized with an overdose of isoflurane and pentobarbital on day 14. Maxillary bones were excised and analyzed using a micro-CT scanner, SMX-130CT (Shimadzu Corporation, Kyoto, Japan) as described previously [21]. Three dimensional images were created using VGStudio MAX 1.2.1 software (Volume Graphics, GmbH, Germany).

2.4. RNA Extraction

For RNA extraction, rats were euthanized with an overdose of isoflurane and pentobarbital on day 9. Palatal gingiva around the second maxillary molars were excised, and were homogenized in 1 ml Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). After incubation for 5 min, 0.2 ml chloroform was added and the mixture was incubated for 2 min. The mixture was centrifuged at 12,000 g for 15 min at 4˚C, and the upper phase was transferred to another tube. After addition of 0.5 ml isopropyl alcohol, the mixture was incubated for 10 min. Then the mixture was centrifuged at 12,000 g for 10 min at 4˚C. The supernatant was removed, and 1 ml of 75% ethanol was added to the RNA pellet. After centrifugation at 7500 g for 5 min at 4˚C, the supernatant was removed and the RNA pellet was air-dried. Then the RNA pellet was dissolved in nuclease-free water (Thermo Fisher Scientific, Waltham, MA, USA). The RNA concentration was determined by measuring the absorbance at 260 nm using a spectrophotometer, Smart Spec 3000 (Bio-Rad, Hercules, CA, USA).

2.5. Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

TaqMan Gene Expression Assays for rat 11β-HSD1 (#Rn00567167_m1), rat 11β-HSD2 (#Rn00492539_m1), rat tumor necrosis factor (TNF) (#Rn999990-17_m1), and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#Rn01-7757-63_g1) were purchased from Applied Biosystems (Foster City, CA, USA). RT-PCR was performed using the Taq Man RNA-to-CT 1-Step Kit and Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). As a template, 20 ng RNA was used for each sample. RNA was mixed with 0.5 μl RT Enzyme Mix, 10 μl RT-PCR Mix, 1 μl Gene Expression Assay, and nuclease-free water (total volume 20 μl). The mixture was incubated for 15 min at 48˚C for reverse transcription, and for 10 min at 95˚C for activation of DNA polymerase. Then the mixture was incubated for 15 sec at 95˚C for denaturing and for 1 min at 60˚C for annealing/extension for 49 cycles. All reactions were carried out in triplicate. For normalization, the ratios of 11β-HSD1, 11β-HSD2, and TNF mRNA versus GAPDH mRNA were calculated in each sample.

2.6. Immunohistochemistry

For immunohistochemistry, 5 μl of 1 mg/ml LPS-PG was injected into the palatal gingiva around the second maxillary molars in three rats on day 1, day 3, and day 5, and rats were euthanized with an overdose of isoflurane and pentobarbital on day 9. After euthanasia, the rats were perfused with 10% formaldehyde neutral buffer solution (Sigma-Aldrich, St. Louis, MO, USA). The maxillary bones were excised and fixed in 10% formaldehyde neutral buffer solution at 4˚C for 7 days. Then the bones were decalcified in a rapid decalcification solution, K-CX (Falma, Tokyo, Japan) at 4˚C for 24 h, followed by conventional dehydration and paraffin embedding. After cutting into 5 μm-thick sections, the specimens were deparaffinized and stained with hematoxylin-eosin (HE) or immunostained with rabbit anti-11β-HSD1 monoclonal antibody (abcam, Cambridge, UK) and Envision + kit/HRP (DAB) (Dako, Glostrup, Denmark). Images were captured using an all-in one microscope, BZ-9000 (Keyence, Osaka, Japan).

2.7. Statistical Analysis

One-way analysis of variance followed by a post-hoc test (Tukey) and significance testing of Pearson's correlation coefficient were performed using SPSS software version 21.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Micro-CT Analysis

In this study, experimental periodontitis was induced by intragingival injection of LPS from *Porphyromonas gingivalis* (LPS-PG) in rats. LPS-PG was injected into the palatal gingiva around the second maxillary molars on day 1, day 3, and day 5. Micro-CT analysis revealed alveolar bone loss around the second maxil-

lary molar on day 14 (Figure 1(b)), demonstrating that experimental periodontitis was induced by LPS-PG injection.

3.2. RT-PCR Analysis of 11β-HSD1, 11β-HSD2, and TNF mRNA

The expression levels of 11β-HSD1, 11β-HSD2, and TNF mRNA in rat gingiva were analyzed using real-time RT-PCR methods as described above. As shown in Figure 2(a), the ratio of 11β-HSD1 mRNA versus GAPDH mRNA was significantly higher in gingiva injected with 1 mg/ml LPS-PG than in controls injected with endotoxin-free water. The ratio of 11β-HSD2 mRNA versus GAPDH mRNA was not significantly increased in LPS-PG-injected gingiva compared with controls (Figure 2(b)). The expression of TNF, a common inflammatory cytokine, was significantly higher in 1 mg/ml LPS-PG-injected gingiva than in controls (Figure 2(c)). As shown in Figure 3, the expression of 11β-HSD1 was positively and significantly correlated with that of TNF, in accordance with our previous report [21].

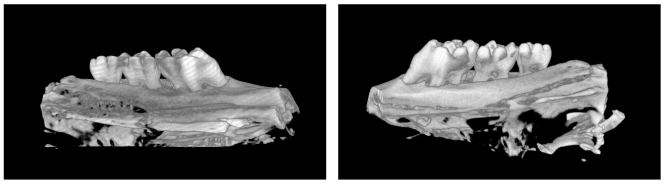
3.3. Immunohistochemistry for 11β-HSD1 Protein

The expression of 11β-HSD1 at the protein level was examined by immunostaining using anti-11β-HSD1 antibody. HE-staining showed that inflammatory cells such as neutrophils were infiltrated in LPS-PG-injected gingiva (Figure 4(b)). Immunostaining demonstrated that many round cells (presumably inflammatory cells such as neutrophils and monocytes) were positively stained with anti-11β-HSD1 antibody in LPS-PG-injected gingiva (Figure 4(d)).

4. Discussion

We recently reported that the expression of 11β-HSD1 as well as the ratio of 11β-HSD1 versus 11β-HSD2 is elevated in gingiva of patients with chronic periodontitis and in ligature-induced experimental periodontitis in rats [20] [21]. In this paper, we have demonstrated that the expression of 11β-HSD1 is elevated in gingiva of rats with LPS-PG-induced experimental periodontitis. These results strongly suggest that 11β-HSD1 plays a role in the pathogenesis of chronic periodontitis.

The expression of 11β-HSD1 at the protein level was confirmed by immunostaining using anti-11β-HSD1 antibody. In our previous report, a polyclonal anti-11β-HSD1 antibody was used for immunostaining [21]. In contrast, a monoc-



(a) (b)

Figure 1. Micro-CT analysis of maxillary bones on the palatal side. (a) Control; (b) LPS-PG-induced periodontitis (on day 14).

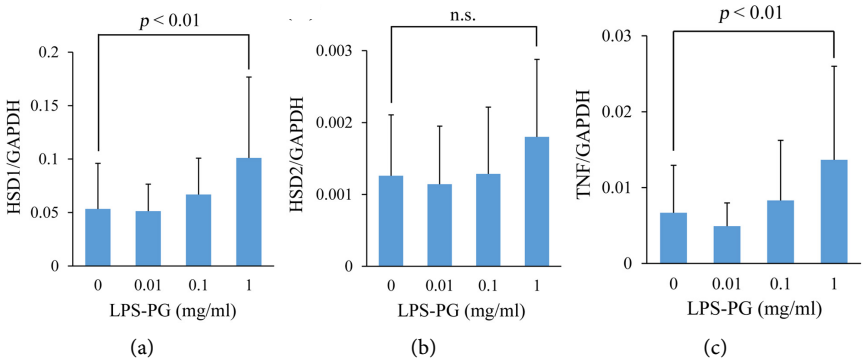


Figure 2. The expression of 11β-HSD1, 11β-HSD2 and TNF mRNA in LPS-PG-induced experimental periodontitis in rats. (a) The ratio of 11β-HSD1 mRNA versus GAPDH mRNA; (b) The ratio of 11β-HSD2 mRNA versus GAPDH mRNA; (c) The ratio of TNF mRNA versus GAPDH mRNA. n = 30 for 0 mg/ml (control); n = 18 for 0.01 and 0.1 mg/ml; n = 20 for 1 mg/ml. Bars indicate standard deviation. n.s., not significant.

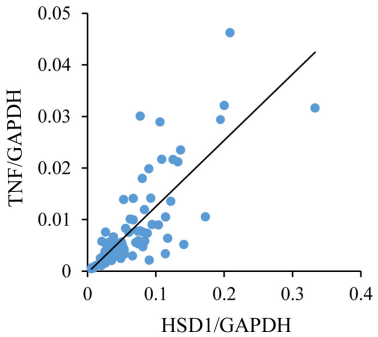


Figure 3. Correlation between 11β-HSD1 mRNA and TNF mRNA (r = 0.779; p < 0.01).

lonal anti-11β-HSD1 antibody was used in this study, since the specificity of monoclonal antibodies is generally higher than that of polyclonal antibodies. Immunostaining revealed that round cells (presumably inflammatory cells) rather than spindle-shaped epithelial cells were stained with anti-11β-HSD1 antibody, suggesting that inflammatory cells express more 11β-HSD1 protein. In accordance with this result, it has been reported that monocytes/macrophages and neutrophils highly express 11β-HSD1 [3] [22].

The involvement of 11β-HSD1 in chronic inflammatory diseases including metabolic diseases, rheumatic arthritis, and inflammatory bowel diseases has been proposed [4] [5] [6] [16] [17] [18] [19]. Since chronic periodontitis is thought to be a chronic inflammatory disease, we investigated the gingival expression of 11β-HSD1. As we expected, the gingival expression of 11β-HSD1 was increased in patients with chronic periodontitis and two rat models of experimental periodontitis. These results further suggest the involvement of 11β-HSD1 in chronic inflammation. However, the precise role of 11β-HSD1 in chronic inflammation remains to be determined. Since 11β-HSD1 mainly converts inactive glucocorticoids into active glucocorticoids, the increased expression of 11β-HSD1 would result in

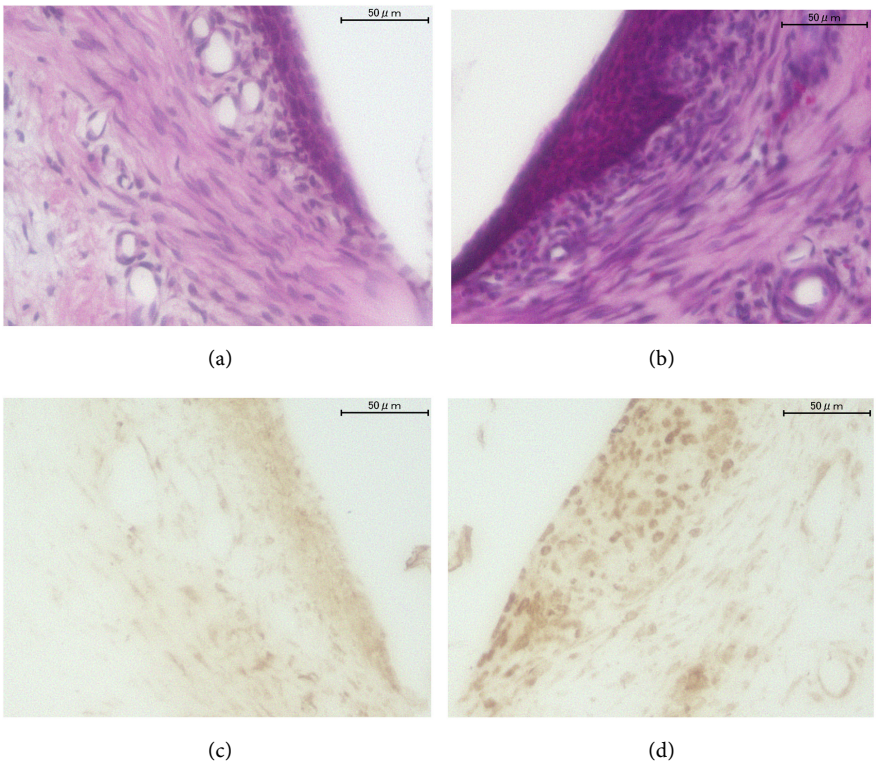


Figure 4. Histological examination of 11β-HSD1 expression in LPS-PG-induced experimental periodontitis. (a) Control (HE staining); (b) LPS-PG (HE staining); (c) Control (immunostaining using anti-11β-HSD1 antibody); (d) LPS-PG (immunostaining using anti-11β-HSD1 antibody).

increased levels of intracellular active glucocorticoids. Although glucocorticoids usually exert anti-inflammatory effects [1], it has been reported that glucocorticoids may have pro-inflammatory effects under chronic inflammatory conditions [17] [23]. The pro-inflammatory effects of 11β-HSD1 have also been reported [24] [25]. Furthermore, the increased expression of 11β-HSD1 might play a role in alveolar bone loss resulting from chronic periodontitis, since glucocorticoids are known to stimulate bone resorption and suppress bone formation [26]. A recent report has suggested that glucocorticoids can potentiate alveolar bone loss in experimental periodontitis in rats [27]. Thus, further studies are required to elucidate the role of 11β-HSD1 in chronic periodontitis and alveolar bone loss.

5. Conclusion

In conclusion, we demonstrated that the expression of 11β-HSD1 is increased in experimental periodontitis induced by intragingival injection of LPS-PG in rats. Since it was previously reported that the 11β-HSD1 expression was elevated in chronic periodontitis in humans and in ligature-induced experimental periodontitis in rats, these results further suggest a role for 11β-HSD1 in the pathogenesis of chronic periodontitis.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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