Porphyromonas gingivalis exacerbates the progression of fatty liver disease via CD36-PPARγ pathway

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Periodontal diseases have been reported to have a multidirectional association with metabolic disorders. We sought to investigate the correlation between periodontitis and diabetes or fatty liver disease using HFD-fed obese mice inoculated with *P. gingivalis*. Body weight, alveolar bone loss, serological biochemistry, and glucose level were determined to evaluate the pathophysiology of periodontitis and diabetes. For the evaluation of fatty liver disease, hepatic nonalcoholic steatohepatitis (NASH) was assessed by scoring steatosis, inflammation, hepatocyte ballooning and the crucial signaling pathways involved in liver metabolism were analyzed. The C-reactive protein (CRP) level and NASH score in *P. gingivalis*-infected obese mice were significantly elevated. Particularly, the extensive lobular inflammation was observed in the liver of obese mice infected with *P. gingivalis*. Moreover, the expression of metabolic regulatory factors, including peroxisome proliferator-activated receptor γ (Ppary) and the fatty acid transporter Cd36, was up-regulated in the liver of *P. gingivalis*-infected obese mice. However, inoculation of *P. gingivalis* had no significant influence on glucose homeostasis, insulin resistance, and hepatic mTOR/AMPK signaling. In conclusion, our results indicate that *P. gingivalis* can induce the progression of fatty liver disease in HFD-fed mice through the upregulation of CD36-PPARγ axis. [BMB Reports 2021; 54(6): 323-328]

INTRODUCTION

Periodontitis is a chronic dental disease with a high risk of recurrence, in which the periodontal tissue is destroyed by bacteria accumulating in the teeth (1). Several risk factors such as poor oral health, hormonal changes, bacterial infection have been reported to be associated with the initiation and progression of periodontal disease (2). Periodontitis is mostly caused by gram-negative bacteria such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia,* and *Bacteroides forsythus* (3). Recent reports have suggested the association of periodontitis with systemic diseases including cardiovascular disease, rheumatoid arthritis, type 2 diabetes mellitus (T2D), hypertension and inflammatory bowel disease (4-6).

Diabetes is accompanied by complications such as hypertension, cardiovascular disease, skin lesions, and obesity (7, 8). A multidirectional relationship between diabetes and periodontitis has been reported, with diabetes increasing the risk of periodontitis and periodontal inflammation negatively affecting control of blood glucose levels (9-11). Non-alcoholic fatty liver disease (NAFLD) is frequently observed in patients with T2D and is known to be associated with increased insulin resistance triggered by fatty acid accumulation, inflammation and reactive oxygen species production (12-14). Moreover, it has been reported in many studies that periodontitis contributes to the progression of NAFLD (15, 16).

It has been shown that hepatic CD36, which acts as a transcriptional regulator of PPARγ, is significantly upregulated in obese rats with periodontitis (17). Depletion of CD36 from hepatocytes attenuates fatty liver disease and ameliorates insulin sensitivity in obese mice (18). As another important fatty acid regulator, the fatty acid-binding protein 4 (FABP4) can induce systemic disease progression (19). FABP4 expression is increased in macrophages upon infection with *P. gingivalis* or *Fusobacterium nucleatum,* affecting both T2D and periodontitis progression (20). In addition, adipocytes from patients with T2D have been reported to contain reduced levels of FOXO1, an important regulator of glucose and lipid homeostasis (21).

The link between periodontitis and T2D or fatty liver disease is associated with the production and expression of inflammatory factors including pro-inflammatory cytokines, chemokines, and metabolic regulators (22). The higher levels of tumor necrosis factor α (TNFα), interleukin (IL)-1β, and CRP induce insulin resistance by impairing insulin action (23, 24). A marked elevation of components of the NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome at both the gene and protein levels have been reported in patients with periodontitis and T2D (25). Moreover, several inflammatory responses mediated by pro-inflammatory cytokines or inflammasome activation have been suggested to play an important role in the induction of hepatic inflammation in NASH (26, 27).

Since obesity is a major cause for chronic diseases including T2D and NAFLD (28), we used HFD-induced obese mice model. To investigate the correlation of periodontitis and T2D or fatty liver disease, HFD-fed mice were inoculated with *P. gingivalis* to develop the periodontitis, and then checked for crucial pathophysiological factors related to the link between the diseases.

RESULTS

Obese mice with *P. gingivalis* infection exhibit alveolar bone loss and increased CRP level

To investigate the correlation between periodontitis and liver malfunction, periodontitis was induced by *P. gingivalis* infection on the mandibular molars of 8-week old mice or by direct administration into the stomach using a feeding catheter. HFD was supplied to the mice to generate a mouse model of obesity (Fig. 1A). Body weight was measured once every 2 weeks during the experiment. *P. gingivalis* treatment increased the body weight of mice. Although the body weight of HFD-fed groups significantly increased compared to the NC group, there was no difference among HFD-fed (HFD), HFD-fed *P. gingivalis*-infected (HFD-p.g.), and HFD-p.g. (P.O.) groups (Fig. 1B). To confirm that the experimental periodontitis was appropriately induced by *P. gingivalis* infection, the extent of alveolar bone loss was measured. The mice infected with *P. gingivalis* in the oral cavity displayed a significant increase in alveolar bone loss compared to the NC group, as indicated by a decrease in the relative amounts of bone around the mandibular molars of the infected mice (Fig. 1C). We next performed serum biochemical analysis by measuring glucose, metabolic disease-related factors (cholesterol, HDL, LDL), liver enzymes (AST, ALT), and the levels of the systemic inflammatory marker (CRP) in blood. The levels of glucose, cholesterol, HDL, LDL, AST and ALT were significantly increased in HFD-fed mice. However, *P. gingivalis* treatment did not further elevate the levels of glucose, cholesterol and lipoproteins, as well as liver enzymes. CRP levels were significantly increased in *P. gingivalis*-treated groups compared to those in the NC group (Fig. 1D). These results indicate that HFD feeding can induce systemic metabolic disease with liver damage and that the *P. gingivalis* infection does not alter the overall levels of serum biochemical factors except the CRP level.

P. gingivalis infection did not affect the glucose metabolism in HFD-fed mice

We next measured blood glucose using intraperitoneal glucose tolerance test (IPGTT) to investigate the bidirectional relationship between periodontitis and glucose homeostasis. After 6 hours of fasting, the blood glucose levels were measured. Fast-
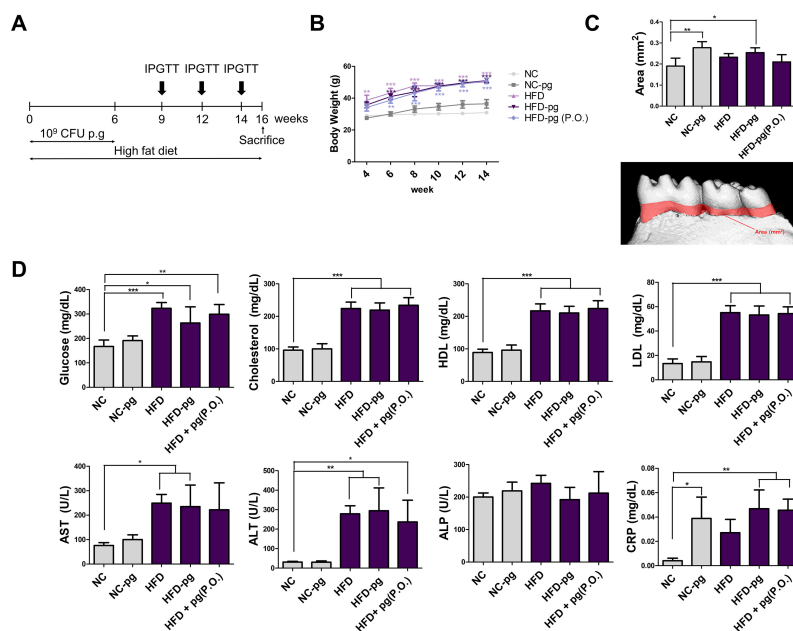
Fig. 1. Induction of periodontitis and serological biochemical analysis in blood samples. (A) Mice were divided into 5 groups: NC, NC-p.g., HFD, HFD-p.g., and HFD-p.g.(P.O.). Mice were maintained on HFD for 16 weeks, and periodontitis was induced by *P. gingivalis* infection of the mandibular first molars or by direct administration into the stomach using a feeding needle catheter 3 times a week. (B) Measurement of body weight every 2 weeks. (C) Hemi-mandible, as reconstructed by micro-computed tomography (micro-CT) analysis, and alveolar bone loss (red area) were measured for each group. (D) Levels of glucose, cholesterol, HDL, LDL, AST, ALT, ALP, and CRP in the blood from mice of each group at 14 weeks. The results are shown as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.
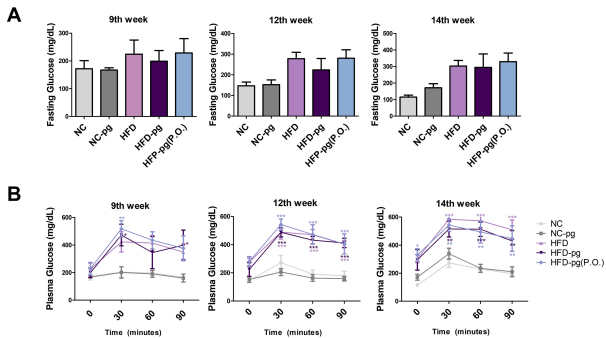
Fig. 2. Detection of blood glucose level in NC and HFD-fed mice. (A) Fasting glucose and (B) plasma glucose at 30, 60, and 90 min after glucose administration. The results are shown as the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

ing blood glucose was higher in all HFD-fed groups than in the NC group but was not altered by *P. gingivalis* administration (Fig. 2A). The IPGTTs also did not show any significant difference in glucose levels in mice infected with *P. gingivalis* via different routes. In the HFD-p.g. (P.O.) group, the glucose level in IPGTT at 9 and 12 weeks was the highest, but these changes were statistically insignificant compared to that in the HFD-fed groups (Fig. 2B). These results suggest that *P. gingivalis* treatment did not significantly aggravate HFD-induced glucose intolerance in our experimental settings.

## *P. gingivalis* exacerbates NAFLD, particularly by up-regulating lobular inflammation

Given that fatty liver disease can be exacerbated in association with periodontitis, we then analyzed lipid accumulation, inflammation and hepatocyte degeneration in liver tissues. Upon histological examination by hematoxylin and eosin (H&E) staining (Fig. 3A), the evaluation of steatosis, inflammation, and hepatocyte ballooning were conducted to calculate NASH score (Fig. 3B-E). Our results showed that livers of NC-p.g. group mice had minimal steatosis, and no signs of lobular inflammation and hepatocyte ballooning (NASH score 0-1). The total score of HFD-fed mice (NASH score 4-7) was significantly elevated compared to that in the NC group (Fig. 3B). The HFD-p.g. group (NASH score 5-9) exhibited significantly increased total score compared with HFD control group (NASH score 4-7). Overall, increased steatosis, lobular inflammation, and hepatocyte ballooning were displayed in the HFD groups (Fig. 3C-E). In the HFD groups infected with *P. gingivalis* (p.g. and p.g.[P.O.]), we observed extensive lobular inflammation foci (Fig. 3A). Consistently, the inflammation score was significantly increased in both *P. gingivalis* inoculated groups (Fig. 3D). This finding indicates that HFD-fed mice have steatohepatitis, which is diagnosed as a total score of 5 or higher, and *P. gingivalis* infection impairs fatty liver disease to a greater extent through the induction of increased inflammatory responses.
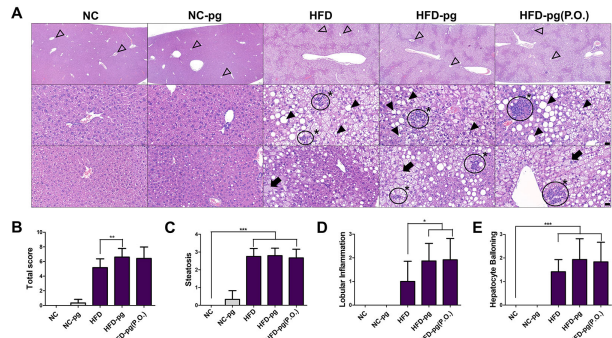
Fig. 3. Histological examination and analysis. (A) Representative H&E-stained liver tissue sections. Bar, 20 µm. The open arrowheads indicate the central vein and filled arrowheads indicate steatosis. The asterisks indicate lobular inflammation foci. The arrows indicate hepatocyte ballooning. (B) Total histological score, (C) steatosis, (D) lobular inflammation, and (E) hepatocyte ballooning were exhibited. The results are shown as the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

P. gingivalis infection can exacerbate fatty liver disease through CD36 and PPARγ upregulation

We next assessed the expression level of several risk factors that play a crucial role in the progression of fatty liver disease. The mRNA expression levels of genes related to inflammation and lipid or glucose metabolism in liver were determined (Fig. 4A). We analyzed the expression of several metabolic regulators including Foxo1, PparγC1α, Cd36, and Fabp4 in the liver. Decreased Foxo1 expression was observed in the HFD and HFD-p.g. groups. Importantly, the expression level of PparγC1α, a key regulator of adipocyte differentiation and lipid metabolism, was significantly upregulated upon *P. gingivalis* infection in HFD-fed mice. Moreover, HFD-fed mice showed increased Cd36 mRNA expression, which increased further following *P. gingivalis* infection. The Fabp4 mRNA expression was high in HFD-fed mice, but did not show additional elevation upon *P. gingivalis* exposure. The expressions of Tnfα and Il-1β were upregulated in HFD-p.g., but this change was statistically insignificant. The expression levels of Il-4 and Il-17 were not altered. The mRNA levels of inflammasome components including Nlrp3 and Casp1 were not changed upon HFD feeding or *P. gingivalis* infection. We next investigated the phosphorylation of Akt, AMPK and mTOR, which represent insulin responsiveness and metabolic regulation, respectively. Akt phosphorylation was slightly decreased in the HFD-fed mice group and, both intraoral and peroral administration of *P. gingivalis* did not alter the level of p-Akt (Fig. 4B). Moreover, the expression levels of p-mTOR and p-AMPK did not show any consistent pattern of change (Fig. 4C). Taken together, our findings demonstrate that *P. gingivalis* infection can exacerbate fatty liver disease by disturbing hepatic lipid metabolism through the CD36-PPARγ pathway.
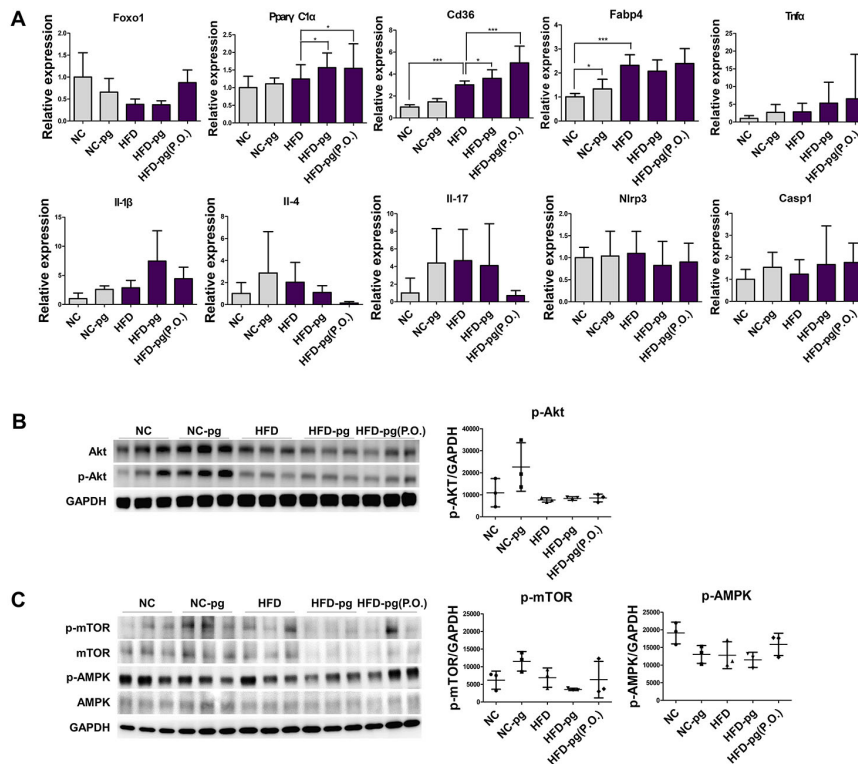
Fig. 4. The mRNA and protein expression in liver tissues. (A) The mRNA expression levels of Foxo1, PparγC1α, Cd36, Fabp4, Tnfα, Il-1β, Il-4, Il-17, Nlrp3, and Casp1 in liver tissue. Western blot analysis of (B) Akt, p-Akt, (C) mTOR, p-mTOR, AMPK, and p-AMPK protein levels in liver tissue (3-5 mice/group). The results are shown as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

DISCUSSION

Periodontitis is an inflammatory disease that leads to alveolar bone loss and further exerts various adverse impacts on systemic health. It is widely accepted that immune responses to peri-odontogenic pathogen play key roles in the progression of metabolic diseases (29). In the present study, we reported that *P. gingivalis*, a major candidate correlating periodontitis and metabolic diseases (30), can aggravate fatty liver signatures in mouse models of obesity and periodontitis.

A previous study has experimentally demonstrated that *P. gingivalis* or Lipopolysaccharide (LPS) from the bacteria can impair HFD-induced insulin resistance and glucose tolerance (31). Although we used slightly modified protocols from this study, however, in our study, neither intraoral nor peroral administration of *P. gingivalis* did not significantly increase the level of fasting glucose or glucose intolerance. This discrepancy might be due to the differences in the environment of animal breeding or virulent potency in *P. gingivalis* strains. Other studies have shown consistent results with our findings. Li *et al.* explored the onset and severity of diabetes using mouse model of both T1D and T2D with *P. gingivalis* infection. Despite the development of alveolar bone loss, periodontitis did not alter the glucose metabolism, determined by fasting glucose, body weight change and glucose tolerance (32). In addition, another study by Wang *et al.* proposed the similar findings. In the

study, *P. gingivalis* inoculation did not significantly regulate the levels of fasting glucose in db/db mice, Tallyho/JngJ mice and streptozotocin-treated mice (33). Kuraji *et al.* suggested that ligature-induced periodontitis could be more effective than the infusion of peri-odontogenic bacteria without ligation (34). Further cumulative studies might be required to establish more reproducible multiple animal models for periodontitis with other systemic diseases, using a ligature-induced model with the inoculation of peri-odontogenic bacteria.

The upregulation of components of various inflammasomes in patients with periodontitis was reported by Bostanci *et al.* and Garcia-Hernández *et al.* (35). More importantly, the latter study demonstrated that NLRP3 inflammasome components were overexpressed in the gingival tissue of patients with periodontitis and uncontrolled T2D (25). Therefore, in the present study, we observed the expression of components of the inflammasome complex, including NLRP3, NLRC4, AIM2, CASP1 and IL-1β, in both periodontal tissues and liver tissues. However, in periodontal tissues, none of the inflammasome components was significantly regulated by HFD feeding or *P. gingivalis* infection (data not shown). One can envision that *P. gingivalis*-mediated inflammatory responses in periodontal tissues might be resolved at the time point when the samples were harvested. In liver tissues, the level of IL-1β expression was elevated in the HFD-fed group with intraoral *P. gingivalis* infection, which may indicate inflammasome activation could

be involved in the regulation of live metabolism.

Despite the slight symptoms in alveolar bone loss and related inflammation, we here observed that the administration of *P. gingivalis* accelerated the progression of fatty liver disease. In particular, inflammation in liver tissue was increased in HFD-fed mice with both intraoral and peroral administration of *P. gingivalis*. Previous studies revealed that an increased level of hepatic CD36 could be a pivotal regulator in the impairment of liver metabolism (36). In addition, a recent study by Ipsen *et al.* reported that CD36 is abnormally increased in patients with NASH and hepatic steatosis (37). Consistent with these findings, our data showed that the level of CD36 in liver tissues was increased by HFD feeding and further increased by *P. gingivalis* administration to a greater extent. Moreover, the expression of PPARγ, a transcription factor in the downstream of CD36, was significantly upregulated in HFD-fed mice with *P. gingivalis* infection, compared to HFD-fed mice. Moreover, a large body of other previous studies have proven that PPARγ/CD36 pathway critically contributes to HFD-induced NAFLD through the increase of free fatty acid uptake and triglyceride synthesis in the liver. These studies collectively demonstrated that the increased expression of PPARγ results in elevated CD36 expression, which aggravates HFD-induced fatty liver and insulin resistance (18, 38, 39).

The limitation of our study is that the animal model we used here is not a perfect model for the study on the correlation between periodontitis and liver metabolism and that symptoms for both diseases are too mild. Therefore, further investigations using severe periodontitis model are required since damages in periodontal tissue and subsequent exacerbating effects of periodontitis on systemic disease are all cumulative. To this end, increased dosage of bacterial infection, shorter interval of bacterial inoculation, or combined treatment with molar ligation might be adopted for future study. In conclusion, our data revealed that the administration of *P. gingivalis* in HFD-fed mice aggravated NASH in mice through the upregulation of CD36 and PPARγ, pivotal regulators in lipid metabolism. These findings might propose the novel therapeutic targets in the treatment of periodontal metabolic liver diseases.

MATERIALS AND METHODS

The detailed methods are described in the "Supplementary Materials and Methods".

Induction of diabetes and periodontitis mice

8-week-old C57Bl/6 wild-type (WT) mice were housed in specific pathogen-free controlled conditions on 12 h day light/dark cycle. Mice were randomly divided into five groups: negative control group (NC), negative control with *P. gingivalis* treated group (NC-p.g.), high-fat diet group (HFD), high-fat diet with *P. gingivalis* treated group (HFD-p.g.) and high-fat diet with peroral administration *P. gingivalis* group (HFD-p.g.[P.O.]). For the *P. gingivalis* group, 10^9/ml colony-forming unit (CFU)

of *P. gingivalis* suspended in 100 μl PBS (Gibco, Waltham, Massachusetts, USA) with 2% carboxy-methylcellulose (Sigma, Saint Louis, Missouri, USA) was treated at the mandibular first molars, three times a week for 6 weeks. Each group was divided into two subgroups and fed with either normal chow or high-fat diet for 14 weeks. The induction of obesity and periodontitis model was confirmed by measuring body weight every 2 weeks and measuring fasting glucose level at 9, 12 and 14 weeks. This study protocol was confirmed by Ethics Committee of Pusan National University (ethics code: PNU-2019-2130). Mice were monitored for reaching criteria of humane endpoint. Mice exhibiting signs of distress, such as panting, breathlessness, abnormal bodily posture and shaggy fur, were sacrificed using CO_2 asphyxiation. At the time points of tissue harvesting, mice were sacrificed by CO_2 asphyxiation. Every effort was made to minimize the suffering of mice.

Histological analyses

To assess hepatic morphology and scoring, liver samples are fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin. Histological scoring was performed by three-stage ranges system as follows: Degree of steatosis (0-3), lobular inflammation (0-3), hepatocyte ballooning (0-2) (40).

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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