Porphyromonas gingivalis attenuates the insulin-induced phosphorylation and translocation of forkhead box protein O1 in human hepatocytes

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Abstract

Objective: *Porphyromonas gingivalis* (*P. gingivalis*) is a pathogen involved in periodontal disease. Recently, periodontal disease has been demonstrated to increase the risk of developing diabetes mellitus, although the molecular mechanism is not fully understood. Forkhead box protein O1 (FoxO1) is a transcriptional factor that regulates gluconeogenesis in the liver. Gluconeogenesis is a key process in the induction of diabetes mellitus; however, little is known regarding the relationship between periodontal disease and gluconeogenesis. In this study, to investigate whether periodontal disease influences hepatic gluconeogenesis, we examined the effects of *P. gingivalis* on the phosphorylation and translocation of FoxO1 in insulin-induced human hepatocytes.

Design: The human hepatocyte HepG2 was treated with insulin and Akt and FoxO1 phosphorylation was detected by western blot analysis. The localization of phosphorylated FoxO1 was detected by immunocytochemistry and western blot analysis. HepG2 cells were treated with SNAP26b-tagged *P. gingivalis* (SNAP- *P. g.*) before insulin stimulation, and then the changes in Akt and FoxO1 were determined by western blot analysis and immunocytochemistry.

Results: Insulin (100 nM) induced FoxO1 phosphorylation 60 min after treatment in HepG2 cells. Phosphorylated FoxO1 translocated to the cytoplasm. SNAP- *P. g.* internalized into HepG2 cells and decreased Akt and FoxO1 phosphorylation induced by insulin. The effect of insulin on FoxO1 translocation was also attenuated by SNAP- *P. g.*

Conclusions: Our study shows that *P. gingivalis* decreases the phosphorylation and translocation of FoxO induced by insulin in HepG2 cells. Our results suggest that periodontal disease may increase hepatic gluconeogenesis by reducing the effects of insulin on FoxO1.
Highlights:

1. We examined the effects of *P. gingivalis* on FoxO1-regulated hepatic gluconeogenesis.

2. Insulin increased FoxO1 phosphorylation in HepG2 cells.

3. The phosphorylated FoxO1 induced by insulin translocated to the cytoplasm.

4. *P. gingivalis* attenuated the effects of insulin on FoxO1.

5. *P. gingivalis* may increase gluconeogenesis by reducing insulin’s effects on FoxO1.

Keywords:

diabetes mellitus, forkhead box protein O1 (FoxO1), periodontal disease, *Porphyromonas gingivalis*
Introduction

*Porphyromonas gingivalis* (*P. gingivalis*) is one of the primary pathogens involved in periodontal disease, which is chronic inflammation of the periodontal tissues that surround the tooth. Periodontal disease widely affects the adult population and the progress of periodontal disease results in tooth loss by the destruction of periodontal tissues. Thus, the prevention and treatment of periodontal disease have impacts on the patient's quality of life (Needleman, McGrath, Floyd, & Biddle, 2004).

It was recently shown that periodontal disease may not only cause tooth loss but also contribute to the development of systemic inflammation and diseases (Hajishengallis, 2015). Among these, the bidirectional relationship between diabetes mellitus and periodontal disease is well known (Taylor, Preshaw, & Lalla, 2013). For example, poor periodontal tissue status and tooth loss increased the level of glycated hemoglobin A1c (HbA1c) in patients without diabetes mellitus (Demmer et al, 2010). Treatment with local minocycline in the periodontal pocket reduced HbA1c values in patients with type 2 diabetes (Iwamoto et al, 2001). After non-surgical periodontal therapy, *P. gingivalis* in subgingival plaque was detected more frequently in patients with increased HbA1c values compared to those with decreased values (Makiura et al, 2008). The results of these clinical studies suggest that periodontal disease has negative effects on glycemic control (Simpson, Needleman, Wild, Moles, & Mills, 2010). In contrast, many other studies showed that non-surgical periodontal therapy did not improve glycemic control in patients with type 2 diabetes (Christgau, Palitzsch, Schmalz, Kreiner, & Frenzel, 1998; Engebreston et al, 2013), and non-quantitative analysis was considered to cause the discrepant results (Janket, Wightman, Baird, Dyke, & Jones, 2005). The molecular mechanisms by which periodontal disease influences glucose metabolism in diabetes mellitus are not fully understood; thus, the question of whether periodontal disease is a risk factor for diabetes mellitus has not been resolved.

To answer this question, we previously investigated whether oral *P. gingivalis* affects hepatic glycogen synthesis by using *P. gingivalis* tagged with the SNAP26b protein (SNAP-*P. g.*) to allow its
detection in mouse organs and cultured cells. We showed that SNAP-P. g., which is present in the oral cavity, was also detected in the liver, and the accumulation of hepatic glycogen tended to be attenuated in mice. SNAP-P. g. internalized in human hepatic HepG2 cells and decreased insulin-induced glycogen synthesis by inhibiting the Akt pathway (Ishikawa et al, 2013). Our results suggested that periodontal disease may increase the blood glucose levels in diabetes mellitus by inhibiting insulin-induced glycogen synthesis in the liver.

In addition to glycogen synthesis, gluconeogenesis also participates in the maintenance of glucose homeostasis. Gluconeogenesis is a process that induces glucose synthesis in the liver from amino acids and glycerol under fasting conditions (Moore, Connolly, & Cherrington, 1998). In addition, the rate of hepatic gluconeogenesis is considered to increase in patients with type 2 diabetes mellitus. Gluconeogenesis is regulated by transcription factors such as forkhead box protein O1 (FoxO1). For example, after insulin binds to the insulin receptor (IR), insulin receptor substrate 1 (IRS1) is activated by phosphorylation (White, 2003). Activated IRS1 phosphorylates Akt and increases its kinase activity (Guo et al, 2009). Akt activation leads to the phosphorylation of FoxO1 in the nucleus. Phosphorylated FoxO1 then translocates to the cytoplasm and decreases its transcriptional activity, leading to the inhibition of gluconeogenesis by reducing the expression of gluconeogenic genes, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) (Matsumoto, Pocai, Rossetti, Depinho, & Accili, 2007; Ayala et al, 1999). Finally, the inhibition of FoxO1 by insulin results in the attenuation of hepatic gluconeogenesis, and blood glucose levels are kept low in healthy subjects (Dong et al, 2008; Lu et al, 2012).

These data indicate that gluconeogenesis is a critical mechanism to elevate the hepatic glucose output and to induce high levels of blood glucose in diabetes mellitus. However, little is known about the relationship between periodontal disease and hepatic gluconeogenesis. In the present study, we examined the effects of P. gingivalis on the phosphorylation and translocation of FoxO1 in HepG2 cells.
Materials and Methods

Materials

Dulbecco's modified Eagle’s medium (DMEM), human recombinant insulin, and hemin were purchased from Wako Chemicals (Osaka, Japan). Brain heart infusion and yeast extract were obtained from BD Bioscience (Franklin Lakes, NJ, USA). 2-methyl-1,4-naphthoquinone (vitamin K3) was purchased from Tokyo Kasei (Tokyo, Japan). Antibodies against phospho-Akt (Ser473), FoxO1 (C29H4), and phospho-FoxO1 (Thr 24)/FoxO3a (Thr 32) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β-actin and histone H3 were obtained from Sigma Aldrich (St Louis, MO, USA), and antibodies against B23 (C-19) and Eps15 (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Human hepatocyte carcinoma HepG2 cells were plated in plastic dishes at a density of $10^4$ cells/mL and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C under a humidified atmosphere of 5% CO₂. Cells were cultured for 3 days and used for experiments after reaching confluence.

Bacterial culture and treatment with SNAP-P. g.

We used the SNAP26b-tagged *P. gingivalis* (SNAP-P. g.), which was previously constructed. Briefly, a shuttle vector containing the SNAP26b gene cloned under the control of the *P. gingivalis* endogenous *trxB* prompter was transformed into *P. gingivalis* ATCC33277. SNAP-P.g. was cultured in brain heart infusion containing 0.5% yeast extract, 10 μg/mL hemin, and 1 μg/mL 2-methyl-1, 4-naphthoquinone (vitamin K3) in an anaerobic jar at 37°C.

The cultured bacteria were centrifuged and washed twice with phosphate buffered saline (PBS). SNAP-P.g. was then suspended in PBS at 100 multiplicity of infection (MOI) and added to the cultured medium of HepG2 cells. The growth medium was replaced with 2% FBS DMEM and the
cells were incubated overnight before treatment with SNAP- \textit{P. g}. Insulin (100 nM) was added for the indicated periods.

**Cellular fractionation**

HepG2 cells were collected and fractionation was performed using a CelLytic\textsuperscript{TM} NuCLEAR\textsuperscript{TM} Extraction Kit (Sigma Aldrich) according to the manufacturer’s protocol with some modifications. Briefly, cells were scraped into lysis buffer (100 mM HEPES, pH 7.9, 15 mM MgCl\textsubscript{2}, and 100 mM KCL) and incubated on ice for 30 min. Then, 10\% Igepal CA-630 solution was added to the swollen cells in lysis buffer to a final concentration of 0.6\%. Cells were mixed, centrifuged at 10,000\ g for 30\ s, and the supernatants were collected as the cytoplasmic fraction. The crude nucleic pellets were suspended in extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl\textsubscript{2}, 0.42 M NaCl, 0.2 mM EDTA, and 25\% (v/ v) glycerol) containing dithiothreitol (DTT) and protease inhibitor cocktail, mixed for 30 min, and centrifuged for 5 min at 10,000\ g. The supernatants were collected as the nucleic fraction.

**SDS-PAGE and western blot analysis**

Cells were washed twice with PBS and then scraped into radioimmunoprecipitation assay (RIPA) buffer (1 mM DTT, 0.2 mM PMSF, 1 μg/mL leupeptin, 4 μg/mL aprotinin, and 50 mM NaF). Twelve micrograms of each sample were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Medford, MA, USA). The membranes were incubated for 1 h at ambient temperature in 5\% non-fat skim milk in PBS containing 0.05\% Tween-20 (PBS-Tween), and then incubated overnight at 4°C in blocking solution containing specific antibodies (diluted at 1:1000). After washes in PBS-Tween, the membranes were incubated for 1 h at room temperature in blocking solution containing horseradish peroxidase-conjugated secondary antibodies (diluted at 1:5000). The membranes were then washed and the proteins recognized by the antibodies were visualized with an
Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore Corp, MA, USA) according to the manufacturer's instructions.

**Immunocytochemistry**

HepG2 cells were cultured on sterile 18-mm round coverslips, and immunocytochemistry was performed as described previously (Yoshida et al, 2012). Briefly, the cells were fixed with 3% formalin for 30 min and then permeabilized with 0.1% Triton X-100 in PBS for 2 min on ice. After blocking with 4% BSA in PBS for 1 h, the cells were incubated with anti-phospho-FoxO1 antibody or normal rabbit IgG overnight at 4°C, followed by Alexa Fluor 488-conjugated anti-rabbit IgG. The cells were then treated with Hoechst 33342 for nuclear staining. The samples were mounted and observed using an inverted fluorescence microscope (ECLIPSE Ti-U, Nikon, Tokyo). Images were acquired using an ECLIPSE Ti-U microscope with NIS-Elements software (Nikon, Tokyo, Japan).
Results

Insulin increased the phosphorylation and translocation of FoxO1 in HepG2 cells

To examine the effect of insulin on FoxO1 in HepG2 cells, we first treated the cells with 100 nM insulin for 0 to 60 min and determined the expression of phosphorylated-FoxO1 and FoxO1 proteins by western blot. The phosphorylation of FoxO1 on threonine 24 was induced after 60 min of exposure to insulin (Fig. 1A).

FoxO1 is known to translocate to the cytoplasm from the nucleus after its phosphorylation, and its activity as a transcription factor decreases. We therefore determined whether FoxO1 localization changed after insulin treatment in HepG2 cells. The immunocytochemistry results showed that phosphorylated FoxO1 was detected in the nucleus of untreated cells (Fig. 1B, a). In contrast, 30 min and 60 min after treatment with 100 nM insulin, phosphorylated FoxO1 translocated to the cytoplasm (Fig. 1B, b and c). Hoechst 33342 staining of nuclei (Fig. 1B, d, e, and f) and the merged images (Fig. 1B, g, h, and i) are shown.

To confirm the results presented in Figure 1B, cytosolic and nuclear proteins were prepared from the cells treated with or without insulin and analyzed by western blot. As shown in Figure 1C, the presence of the phosphorylated FoxO1 protein in the cytosolic fraction increased 60 min after treatment with 100 nM insulin compared to that in the untreated cells (Fig. 1C, upper panel). The purity of the nuclear (Fig. 1C, middle panel) and cytosolic fractions (Fig. 1C, bottom panel) was confirmed using antibodies against B23 and Eps15, respectively.

SNAP-P. g. internalized into HepG2 cells and attenuated the insulin-induced Akt/FoxO1 pathway

Because P. gingivalis is known to internalize in host cells and express its virulence, we first confirmed whether P. gingivalis also invaded human HepG2 cells. To detect P. gingivalis within the cells, we used the previously constructed SNAP26b-tagged P. gingivalis (SNAP-P.g.) (8). The cells were incubated with SNAP-P.g. at a concentration of 100 MOI for 0 to 6 h, and SNAP-P.g. was
detected by western blot using an anti-SNAP26b antibody. As shown in Figure 2A, the intensity of the band corresponding to SNAP26b increased in a time-dependent manner up to 6 h, indicating that SNAP-\textit{P.g.} internalized into HepG2 cells.

Next, we examined the effects of SNAP-\textit{P.g.} on the insulin-induced Akt/FoxO1 pathway in HepG2 cells. HepG2 cells were treated with insulin for 30 min after incubation with or without 100 MOI of SNAP-\textit{P.g.} for 6 h. Akt phosphorylation on serine 473 was then determined by western blot. The level of phosphorylated Akt increased after insulin treatment (Fig. 2B, lane 2). Pre-incubation with SNAP-\textit{P.g.} decreased the rate of Akt phosphorylation induced by insulin (Fig. 2B, lane 3). Moreover, in HepG2 cells treated with 100 nM insulin for 60 min, FoxO1 phosphorylation on threonine 24 increased compared with that of untreated cells (Fig. 2C, lane 1 and 2). This effect of insulin on FoxO1 phosphorylation was suppressed by pre-incubation with SNAP-\textit{P.g.} for 6 h (Fig. 2C, lane 3). SNAP-\textit{P.g.} itself did not affect FoxO1 phosphorylation (Fig. 2C, lane 4).

**SNAP-\textit{P.g.} inhibited the insulin-induced translocation of FoxO1 in HepG2 cells**

We further verified whether SNAP-\textit{P.g.} affected the translocation of FoxO1 regulated by insulin. HepG2 cells were incubated with or without 100 nM of insulin for 60 min after treatment with 100 MOI of SNAP-\textit{P.g.} for 6 h. FoxO1 localization was then detected by immunocytochemistry and western blot.

In insulin-treated cells, the accumulation of phosphorylated FoxO1 in the cytoplasm was increased when compared with that of untreated cells (Fig. 3A, a and b). SNAP-\textit{P.g.} inhibited the insulin-induced translocation of phosphorylated FoxO1 to the cytoplasm (Fig. 3A, c). The localization of phosphorylated-FoxO1 was not altered in cells treated with SNAP-\textit{P.g.} only (Fig. 3A, d). The nuclei were stained with Hoechst 33342 (Fig. 3A, e to h) and images were merged (Fig. 3A, i to l). Images of cells stained with normal rabbit IgG instead of anti-phospho-FoxO1 antibody (negative control) are shown in Figure 3B.
We next prepared cytosolic and nuclear proteins from the cells and detected the localization of phosphorylated FoxO1 by western blot. Consistent with the results presented in Figure 3A, insulin treatment induced the translocation of phosphorylated FoxO1 to the cytoplasmic fraction (Fig. 3C, lane 1 and 4), and this effect of insulin on phosphorylated FoxO1 translocation was inhibited by SNAP-\textit{P.g.} (Fig. 3C, lane 3).
Discussion

*P. gingivalis* is an oral bacterium that is generally found within gingival tissues and dental plaque. *P. gingivalis* is also known to invade the cardiac endothelium (Deshpande, Khan, & Genco, 1998) and spread into the bloodstream in patients with periodontitis (Castillo et al, 2011). Additionally, *P. gingivalis* is considered to evade circulating phagocytes by adhering to erythrocytes (Belstrom et al, 2011). Indeed, we previously found that SNAP-*P. g.* was detected in the liver of mice orally infected with SNAP-*P.g.* Additionally, it was detected at a high frequency in mice with high blood glucose levels (Ishikawa et al, 2013). These observations suggest that oral *P. gingivalis* probably translocates to the liver through systemic circulation and is implicated in hepatic gluconeogenesis. We therefore used hepatic HepG2 cells to investigate the effects of *P. gingivalis* on gluconeogenesis in the present study. To investigate association between oral bacteria and glucose metabolisms in the liver is important for understanding the mechanisms by which periodontal disease influences diabetes mellitus. However, there are few reports showing the relationship between oral bacteria and hepatic gluconeogenesis. This is the first report indicating that *P. gingivalis* is associated with gluconeogenesis by regulating FoxO1 in insulin-treated HepG2 cells.

FoxO1 increases hepatic glucose production in response to insulin (Matsumoto, Pocai, Rossetti, Depinho, & Accili, 2007). There are four FoxO proteins in mammals: FoxO1, FoxO3, FoxO4, and FoxO6. Among these, FoxO1 is known to participate in glucose production in the liver (Haeusler, Kaestner, & Accili, 2010; Zhang et al, 2012). Thus, in the present study, we focused on FoxO1 in HepG2 cells treated with insulin and/or SNAP-*P.g.*

Akt is phosphorylated in response to insulin and plays critical roles in regulating both glycogen synthesis and gluconeogenesis in the liver. Because FoxO1 contains potential Akt phosphorylation sites (Nakae, Park, & Accili, 1999), we verified the phosphorylation status of both Akt and FoxO1 in insulin-treated HepG2 cells. Our results showed that Akt was phosphorylated 30 min after treatment with insulin (Fig. 2B). In contrast, the phosphorylation and translocation of FoxO1 was
induced 60 min after treatment with insulin following Akt phosphorylation (Fig. 1A). When HepG2 cells were infected with SNAP-P.g. for 6 h, the effect of insulin on Akt phosphorylation was inhibited (Fig. 2B). SNAP-P. g. internalization also decreased the effects of insulin on the phosphorylation and translocation of FoxO1 (Fig. 3A and C). These results suggest that SNAP-P. g. is internalized into HepG2 cells and decreases FoxO1 phosphorylation, probably by inhibiting insulin-induced Akt phosphorylation.

Although the Akt-dependent inactivation of FoxO1 is considered to be essential for the insulin-induced suppression of hepatic glucose output after feeding, Lu et al. (2012) reported that the genetic manipulation of Akt and FoxO1 does not affect insulin signaling and metabolic responses, suggesting an alternative mechanism through which liver responds to insulin signaling under some conditions in vivo. In the present study, we examined the effect of P. gingivalis on insulin signaling using HepG2 cells in vitro but not in vivo. It also remains unknown whether P. gingivalis affects the transcription activity of FoxO1 and the expression of its regulatory genes that are involved in gluconeogenesis, such as PEPCK and G6Pase. Further studies are needed to investigate whether our results shown in this study can be observed in mouse livers infected with P. gingivalis in vivo and whether the inhibitory effects of P. gingivalis on hepatic insulin signaling result in abnormal gluconeogenesis.

Furthermore, the mechanism by which SNAP-P.g. decreases Akt phosphorylation was not elucidated in this study. P. gingivalis presents many virulence factors that promote periodontal disease such as fimbriae, lipopolysaccharide (LPS), and cysteine proteinases. Moreover, it is considered that the ability of P. gingivalis to adhere to and invade host cells is critical to express its virulence (Lamont, & Yilmaz, 2002; Tribble, Mao, James, & Lamont, 2006). Recently, it was reported that gingipains, P. gingivalis cysteine proteinases, inactivated Akt phosphorylation on threonine 308 and serine 473 in human gingival epithelial cells (Nakayama, Inoue, Naito, Nakayama, & Ohara, 2015). LPS from P. gingivalis also suppressed Akt kinase activity in rat sublingual acinar cells (Slomiany, B. L., & Slomiany, A, 2011). Moreover, in our preliminary experiments,
heat-inactivated SNAP-\textit{P.g.}, which was incubated for 10 min at 100°C, did not internalize into HepG2 cells, and insulin-induced Akt phosphorylation was not altered (data not shown). Based on these observations, it is likely that some gingipains and the internalization of SNAP-\textit{P.g.} play important roles in the inhibition of FoxO1 phosphorylation and translocation. To understand the role of \textit{P. gingivalis} in hepatic gluconeogenesis, further studies investigating which \textit{P. gingivalis} factors regulate the Akt/FoxO1 pathway and whether the internalization of \textit{P. gingivalis} into host cells is critical for its effects are needed.

In conclusion, we demonstrated that \textit{P. gingivalis} internalizes into human hepatocytes, HepG2 cells, and attenuates the insulin-induced activation of the Akt/FoxO1 pathway. Our results suggest that periodontal disease may increase hepatic gluconeogenesis by reducing the effects of insulin on FoxO1.
Acknowledgments

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References


Figures

Figure 1: Insulin induces the phosphorylation and translocation of FoxO1 in HepG2 cells
HepG2 cells were treated with 100 nM of insulin for 0 to 60 min. (A) Cells were subjected to western blot analysis with anti-phospho-FoxO1 (pFoxO1), FoxO, and β-actin. A representative data of three separate experiments is shown. (B). Localization of phosphorylated FoxO1 detected by immunocytochemistry using an anti-phospho-FoxO1 antibody (green, a, b, and c). Hoechst 33342 was used to stain the nuclei (blue, d, e, and f). Microscopic images of the same field were taken and merged (g, h, and i). (C). Nuclear (Nucleus) and cytosolic (Cytoplasm) fractions were prepared from the cells. Each sample was subjected to western blot analysis with anti-phospho-FoxO1 (pFoxO1), B23, and Eps15 antibodies. The immunoblots shown are representative of three separate experiments.

Figure 2: SNAP-P.g. internalizes into HepG2 cells and decreases insulin-induced Akt/FoxO1 phosphorylation
(A) HepG2 cells were treated with 100 MOI of SNAP-P.g. for up to 6 h. The presence of SNAP26b within the cells was analyzed by western blot using an anti-SNAP26b and β-actin antibody. The arrow indicates SNAP26b-specific bands; ns, non-specific bands. A representative data of three separate experiments is shown. (B), (C) HepG2 cells were pretreated with 100 MOI of SNAP-P.g for 6 h and then treated with or without 100 nM insulin for 30 min (B) or 60 min (C). Cell lysates were analyzed by western blot using an anti-phospho-Akt (pAkt), Akt, phospho-FoxO1 (pFoxO1), FoxO1, and β-actin antibody. The immunoblots shown are representative of three separate experiments.

Figure 3: SNAP-P.g. inhibits insulin-induced FoxO1 translocation in HepG2 cells
HepG2 cells were pretreated with 100 MOI of SNAP-P.g. for 6 h and then treated with or without 100 nM insulin for 60 min. (A) Phosphorylated FoxO1 (pFoxO1, green, a to d) was detected by immunocytochemistry using an anti-phospho-FoxO1 antibody and a fluorescence microscope. Hoechst 33342 was used to stain the nuclei (blue, e to h). Microscopic images of the same field were
taken and merged (i to l). (B) As a negative control, cells stained with normal rabbit IgG instead of anti-phospho-FoxO1 are shown. (C) Nuclear (Nucleus) and cytosolic (Cytoplasm) fractions were prepared from the cells. Each sample was subjected to western blot analysis with an anti-phospho-FoxO1 (pFoxO1), histone H3, and Eps15 antibody. A representative data of three separate experiments is shown.
Figure 1

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