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Porphyromonas gingivalis infection modifies oral microcirculation and aortic vascular function in the stroke-prone spontaneously hypertensive rat (SHRSP)

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ABSTRACT

The functional modulation of vascular endothelial cells associated with stroke and periodontal disease has not yet been clarified. The objective of this study is to analyze the vascular endothelial function of periodontitis and stroke animal models. We examined endothelial function and gingival blood flow in oral microcirculation *in vivo* and measured the isometric tension *in vitro* of the aorta in animal models for lifestyle-related diseases, such as periodontitis and stroke. Gingival reactive hyperemia (GRH) was measured using laser Doppler flowmetry. Wistar Kyoto rats (WKY) were used as control animals; *Porphyromonas gingivalis* (*P. gingivalis*) infected WKY (WKY + *Pg*) as the periodontitis model; stroke-prone spontaneously hypertensive rat (SHRSP) as the stroke model; and a final group consisting of *P. gingivalis* infected SHRSP (SHRSP + *Pg*). Furthermore, for each group, the relaxation of descending aortic ring preparations was measured using a force transducer. The GRH was estimated by maximum response (peak), time taken for the maximum response to fall to one half ($T_{1/2}$), and increased total amount of blood flow (mass). The relative change in $T_{1/2}$ and mass increased in SHRSP + *Pg* compared to WKY. However, mass significantly increased in WKY (758.59 ± 88.21 ml/min/100 g s to 1755.55 ± 226.10 ml/min/100 g s) and SHRSP (1214.87 ± 141.61 ml/min/100 g s to 2674.32 ± 675.48 ml/min/100 g s) after treatment with acetylcholine. In addition, $T_{1/2}$ and mass significantly increased in WKY + *Pg* (624.18 ± 96.36 ml/min/100 g s to 2629.90 ± 612.01 ml/min/100 g s) and SHRSP + *Pg* (1116.36 ± 206.24 ml/min/100 g s to 1952.76 ± 217.39 ml/min/100 g s) after treatment with nitroglycerin. Furthermore, the endothelium-dependent relaxation of ring preparations, evoked by acetylcholine, was attenuated in SHRSP compared with WKY, but not in SHRSP + *Pg*. This attenuation effect in SHRSP could be prevented by superoxide dismutase pretreatment. Our results suggest altered endothelial function may occur in gingival tissue in animal models experiencing both periodontitis and stroke. Therefore, these results indicate the disruption of vascular function in oral microcirculation may be caused by the interaction between the oxidative stress induced by periodontitis and nitric oxide in periodontitis, similar to the interactions present in stroke cases.

1. Introduction

The significant association between cardiovascular and periodontal diseases is receiving increasingly more attention. Epidemiological studies show periodontal disease may be a risk factor for systemic diseases, such as hypertension and diabetes

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[1–4]. Periodontal diseases can significantly affect systemic diseases, which can inversely be risk factors for periodontal diseases. The relationship between periodontal and systemic diseases is a basic concept in periodontal medicine [5], increasing the prevalence of this field [6,7]. Periodontal disease is characterized by inflammation and bone resorption; the study of the relationship between inflammation and bone resorption has resulted in a new field of study, providing context for a better understanding of the pathogenesis of periodontal disease [7]. Garrett et al. demonstrated that the formation of osteoclasts was stimulated in bone by the generation of reactive oxygen species (ROS) and that bone resorption occurred both *in vivo* and *in vitro* [8].

Oxidative stress arises when the generation of ROS exceeds the capacity for the cell to detoxify potentially injurious oxidants using endogenous antioxidant defense systems [9]. Conditions associated with oxidative stress induced by ROS include hypertension [10–12] and stroke [13,14].

We previously developed an electron spin resonance (ESR)-based technique to assess oxidative stress, including ROS, in biological systems [15–22]. We demonstrated increased generation of ROS in the brain of the stroke-prone spontaneously hypertensive rat (SHRSP), where ROS ultimately contributed to the mechanisms causing hypertension or stroke [16,19–22]. Additionally, vascular endothelial cell function has been previously estimated by flow-mediated dilation (FMD) and plethysmography, verified by reactive hyperemia of the forearm. We previously reported that measuring reactive hyperemia in oral microcirculation could be used to estimate the vascular endothelial function of general circulation, similar to using FMD or plethysmography of the forearm [23]. Periodontal disease-reduced gingival vascular reactivity could be accelerated by diabetes due to increased oxidative stress in the microcirculation of the oral and maxillofacial regions of the rodent model [15]. A recent study also suggested that periodontitis might be associated with endothelial dysfunction in individuals without cardiovascular risk factors, as well as in hypertensive patients [24].

Porphyromonas gingivalis (*P. gingivalis*) is an anaerobic gram-negative coccobacillus associated with periodontal disease progression, including bone and tissue destruction [25]. Gram-negative bacterial lipopolysaccharides (LPS) are known to induce tissue damage and injury via the generation of ROS [26]. Therefore, we hypothesized that oxidative stress induced by ROS may play a critical role in altering oral vascular function due to periodontal disease caused by *P. gingivalis*. However, the relationship between alteration of oral vascular function due to periodontal disease and a vascular disease model such as SHRSP, which is a model associated with increased oxidative stress, has not yet been examined. We have previously reported using animals orally challenged with *P. gingivalis* as a chronic inflammation model. Our previous results suggested that *P. gingivalis*-induced alveolar bone loss could occur in periodontitis and also “hypertension and stroke” animal models, such as SHRSP [27].

This study investigates the effects of *P. gingivalis* in the SHRSP rodent model, representing hypertension or stroke. By measuring reactive hyperemia in the oral microcirculation, we examined *in vivo* endothelial function and gingival blood flow in the oral microcirculation animal models of lifestyle-related diseases, such as periodontitis or stroke. Furthermore, we measured isometric contraction changes using ring preparations that we extracted from these model animals *in vitro*. We examined *P. gingivalis*-induced alteration of oral vascular function in both SHRSP and WKY, and found that vascular function changed in SHRSP infected with *P. gingivalis*.

2. Materials and methods

2.1. Animals

In this study, male Wistar Kyoto rats (WKY, 4–25 weeks old, weighing 65–450 g) were used as control animals and SHRSP rats (4–25 weeks old, weighing 65–350 g) were used as an animal model for stroke. For both groups, 3-week-old male animals were purchased from a commercial farm (Nihon SLC, Shizuoka, Japan). The procedures used in this study were in accordance with the guidelines of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1985). Our protocols were approved by the Animal Care Committee of Kanagawa Dental University (Yokosuka, Japan).

Animals were housed in groups of five per cage in a room maintained under standardized light (12:12 h light–dark cycle) and temperature (22 ± 3 °C) conditions with free access to food pellets and tap water. Animals were divided into four groups: Group 1, *P. gingivalis*-non-infected WKY; Group 2, WKY infected with *P. gingivalis* (WKY + *Pg*); Group 3, *P. gingivalis*-non-infected SHRSP; and Group 4, *P. gingivalis* infected SHRSP (SHRSP + *Pg*). Each group comprised 5–8 rats.

2.2. Bacteria and culture conditions

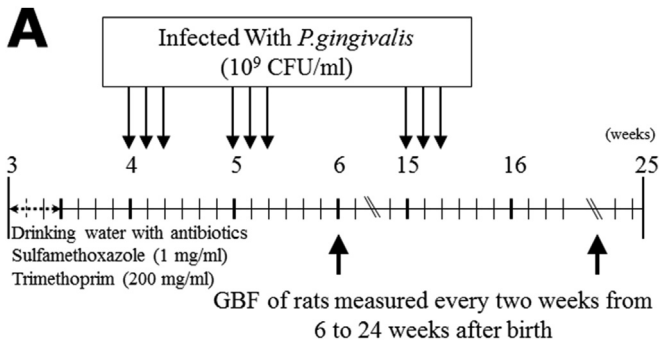
The bacterial strain used was *P. gingivalis* American Type Culture Collection (ATCC) 33277. *P. gingivalis* was grown at 37 °C for 18 h in an anaerobic chamber (Anaerobox, Hirasawa, Tokyo, Japan) with an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 in a brain heart infusion broth (Difco, Detroit, MI, USA) supplemented with 5 mg/ml yeast extract, 5 μg/ml hemin, and 0.2 μg/ml vitamin K_1.

2.3. P. gingivalis infection in rats

As shown in Fig. 1A, rats were given sulfamethoxazole (1 mg/ml) and trimethoprim (200 μg/ml) in their drinking water for three days *ad libitum* to reduce the original oral flora, followed by a 4-day antibiotic-free period before *P. gingivalis* infection. Each rat received 0.5 ml (1.5×10^9 cells per ml) *P. gingivalis* ATCC 33277 suspended in 5% carboxymethylcellulose (Sigma Chemical, St. Louis, MO, USA) by oral gavage three times per week at the ages of 4, 5, and 15 weeks [27,28].

2.4. Gingival blood flow

Animals were anesthetized with sodium pentobarbital (45 mg/kg, IP) and were subsequently given small maintenance doses as necessary. After determining body weight, each rat was laid on a wooden board (20 × 24 cm) in the supine position. All limbs were fixed at an angle of 45° to the body midline with adhesive tape and the upper and lower jaws were anchored in an open position with a thin rope via the incisors. Gingival blood flow (GBF) was measured at the palatal gingiva by a laser Doppler flowmeter (TBF-LN1, Unique Medical Co., Ltd., Tokyo, Japan) with a laser Doppler probe (diameter 2.0 mm). Heart rate was monitored to determine the effects on systemic hemodynamics of the administered agents or gingival reactive hyperemia (GRH). To evaluate vascular endothelial and smooth muscle functions, 100 mg/ml acetylcholine (ACh) or 5 mg/ml nitroglycerin (NTG) were topically absorbed from an area of gingival mucosa approximately 2 mm in diameter for 1 min before gingival compression for 1 min. Reactive hyperemia was elicited at the same place as ACh and NTG application by the release of occlusive gingival compression by the laser Doppler probe for 1 min. As shown in Fig. 1B, the reactive hyperemia in the gingival circulation was estimated by basal blood flow (basal), maximum



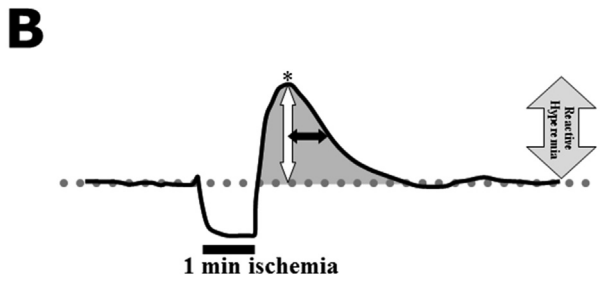


Fig. 1. Experimental design. GBF: gingival blood flow, CFU: colony-forming unit (A). Typical trace of GBF during reactive hyperemia, illustrating the parameters of basal blood flow (gray dotted line), maximum response (* Peak), time taken for the maximum response to fall to one half (dark arrow: $T_{1/2}$), and increased total amount of blood flow (shaded area; mass) (B).

response (peak), time taken for the maximum response to fall by half ($T_{1/2}$), and increased total amount of blood flow (mass) [7,8]. The output signals from the flowmeter were recorded on a computer hard disc through an A/D converter and displayed simultaneously on the monitor. Recorded GBF was analyzed using data analysis software (Chart v. 5.0.1 AD Instruments, Inc., Colorado Springs, CO, USA). These GBF measurements were performed twice a month on the rats 6–24 weeks old. The animals were returned to the same cage after GBF measurements.

2.5. Vessel preparation and isometric tension recording

In accordance with our institutional Animal Care Committee guidelines, 25-week-old animals (weighing 300–450 g) were killed by bleeding under anesthesia (pentobarbital sodium, 45 mg/kg, IP), after which the descending aortas were dissected away and cut into rings. A ring of muscle (1.5–2.0 mm in diameter, 4.5–5.0 mm in length) was prepared in Krebs–Ringer solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, and 11.0 mM glucose) aerated with 95% O_2–5% CO_2 (pH 7.4).

The rings were suspended in a 10 ml water-jacketed tissue bath (37 °C) with one end tied to a fixed point and the other to a force transducer (TB-651T, Nihon Koden, Tokyo, Japan). The change in isometric force was amplified (EF-6021G, Nihon Koden, Tokyo, Japan). The output signals were recorded on a computer hard disc through an A/D converter and displayed simultaneously on the monitor. The recorded tension was analyzed using data analysis software (Chart v. 5.0.1 AD Instruments, Inc., Colorado Springs, CO, USA). Before the start of the experiment, the rings were allowed to equilibrate for 60 min in Krebs–Ringer solution, which was changed at 15 min intervals. During this time, the rings were stretched to a passive tension of 1.0 g. All tracings presented are typical results.

2.6. Drugs

The following drugs and chemicals were used: DL-norepinephrin hydrochloride (NA; Sigma Chemical, St. Louis, MO, USA), acetylcholine chloride (Sigma Chemical, St. Louis, MO, USA), nitroglycerin 50mg/100 ml (Nihon Kayaku, Tokyo, Japan), superoxide dismutase (SOD; from bovine blood, 3300 U/mg protein, Sigma Chemical, St. Louis, MO, USA).

2.7. Statistical analysis

An analysis of variance (ANOVA) and multiple comparison tests

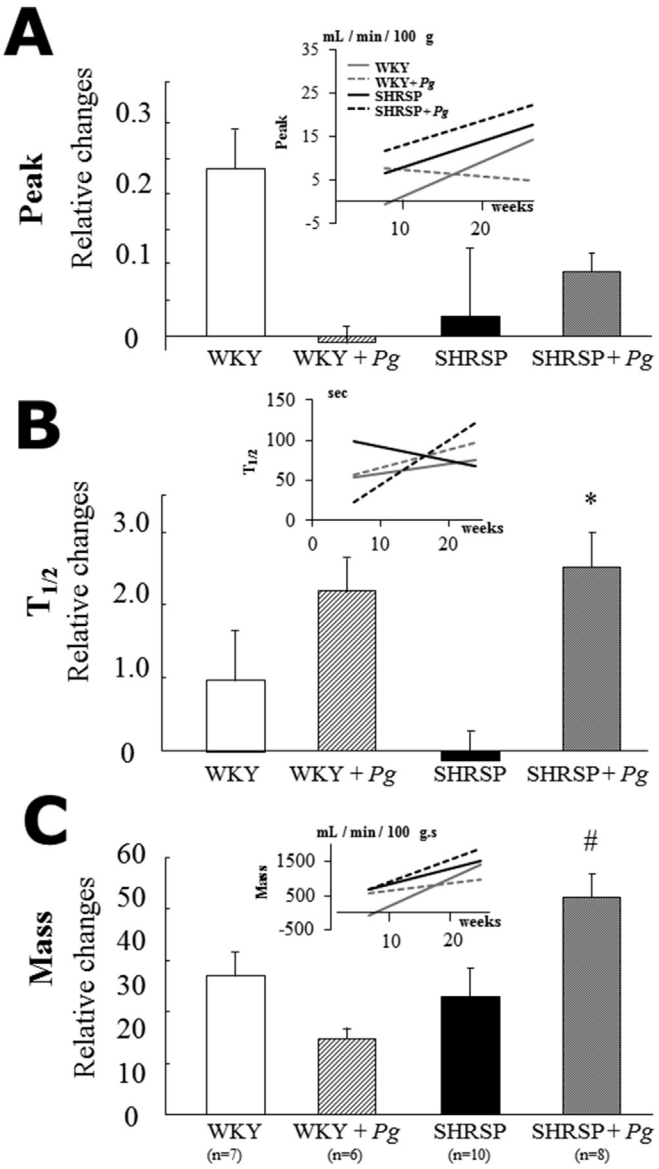


Fig. 2. Alterations in gingival reactive hyperemia in WKY, WKY + *Pg*, SHRSP, and SHRSP + *Pg*. Peak (A), $T_{1/2}$ (B), and mass (C) in gingival reactive hyperemia plotted against time. Inserted figures represent regression lines fitted to a typical plot of laser Doppler flowmetry data obtained from WKY, WKY + *Pg*, SHRSP, and SHRSP + *Pg* groups. Columns show the slope of parameters against time. Data are presented as mean ± SEM (n = 6–10). $^{*}P < 0.05$ vs. SHRSP, $^{\#}P < 0.05$ vs. WKY + *Pg*.

using Tukey's method were applied to determine differences among the four groups. When comparing only one pair, we used Student's paired *t*-test and ANOVA. Data are expressed as the mean ± SEM. A p-value less than 0.05 was considered statistically significant. Data were analyzed using Microsoft Excel 2003.

3. Results

3.1. Evaluation of vascular responses using GRH in the periodontitis and stroke models

In experiments using non-infected WKY, compression of gingival tissue for 1 min resulted in an immediate decrease in GBF. One minute after the release of gingival tissue compression, reactive hyperemia was observed in the gingiva. Peak, $T_{1/2}$, and mass of GBF were increased by gingival compression for 1 min (Fig. 1B). Previously, we reported topical oral mucosal administration of ACh and NTG significantly enhanced the temporary increase of GBF [15]. This previous report also demonstrated that cardiac function was not influenced by GRH or the topical oral mucosal administration of ACh and NTG used in this study [15]. These results suggest that GRH could be measured as a vascular endothelial response that does not affect systemic circulation. In the present study, WKY that were orally challenged with *P. gingivalis* showed a remarkable reduction in GRH throughout the experimental period, which lasted approximately 24 weeks. $T_{1/2}$ in WKY + *Pg* increased, but peak and mass measurements showed decreasing tendencies compared to the WKY controls. Compared to controls, there was a tendency for decreased gingival microcirculation in the SHRSP group (Fig. 2). Futhermore, mass significantly increased in WKY and SHRSP after treatment with ACh (Fig. 3A). In addition, $T_{1/2}$ and mass significantly increased in WKY + *Pg* and SHRSP + *Pg* after treatment with

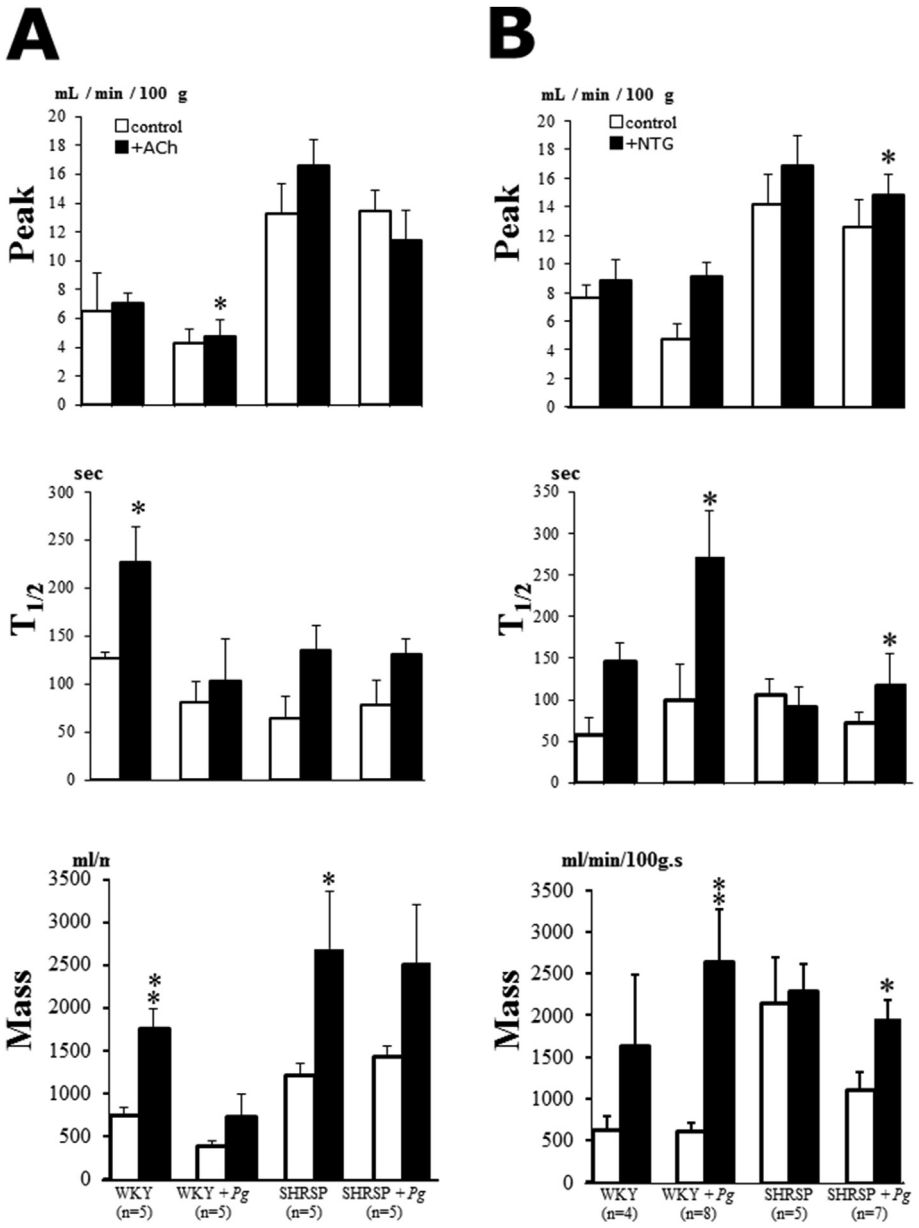


Fig. 3. Evaluation of vascular endothelial and smooth muscle function in WKY, WKY + Pg, SHRSP, and SHRSP + *Pg*. Alterations in peak, $T_{1/2}$, and mass of gingival reactive hyperemia before (open columns) and after (solid columns) treatment with ACh (A; in 24-week-old animals) and NTG (B; in 22-week-old animals). Data are presented as mean ± SEM (n = 4–8). *$P < 0.05$ vs. pretreatment with ACh or NTG.

NTG (Fig. 3B). The alteration of the GRH might depend on the modulation of gingival vascular endothelium function in the *P. gingivalis* infection models.

3.2. Evaluation of vascular responses of the ring preparations isolated from periodontitis and/or stroke model

Rat descending aortic ring preparations were contracted to a stable plateau tension by the addition of 10^{-5} M NA. To observe the endothelium-dependent relaxation response, we measured contraction during the addition of 10^{-5} M NA and relaxation responses to ACh (10^{-8} to 10^{-4} M). In addition, we added NTG (10^{-6} to 10^{-5} M) to confirm the function of vascular smooth muscle (Fig. 4A).

We determined the SHRSP background and *P. gingivalis* altered the endothelium-dependent relaxation induced by ACh. The relaxation responses to the stepwise cumulative addition of ACh and NTG were determined for all groups. In SHRSP, ACh elicited a significantly attenuated response, showing decreased maximum relaxation (Fig. 4B). However, the endothelium-independent relaxation induced by 10^{-6}–10^{-5} M NTG was not significantly affected (data not shown). We next determined the effect of SOD on the attenuation of endothelium-dependent relaxation of ring preparations associated with 10^{-5} M ACh. This concentration of ACh produces approximately maximum relaxation in a single dose, which we determined experimentally (Fig. 4B). We then added 100 U/ml SOD 30 min before inducing precontraction by 10^{-5} M NA. The endothelium-dependent relaxation evoked by adding ACh to the ring preparations was attenuated in SHRSP compared to WKY, but not attenuated in SHRSP + *Pg* (Fig. 4B). This SHRSP attenuation effect was prevented by pretreatment with SOD (Fig. 5A, n = 5, $^{***}P < 0.001$). However, the endothelium-independent relaxation induced by 10^{-6} M NTG was not significantly affected (Fig. 5B, n = 5).

4. Discussion

P. gingivalis is an anaerobic, gram-negative coccobacillus associated with periodontal disease progression, including bone and tissue destruction via the production of lipopolysaccharides (LPS). LPS, as a component of the cell wall of gram-negative bacteria, induces the production of proinflammatory cytokines, such as IL-1,

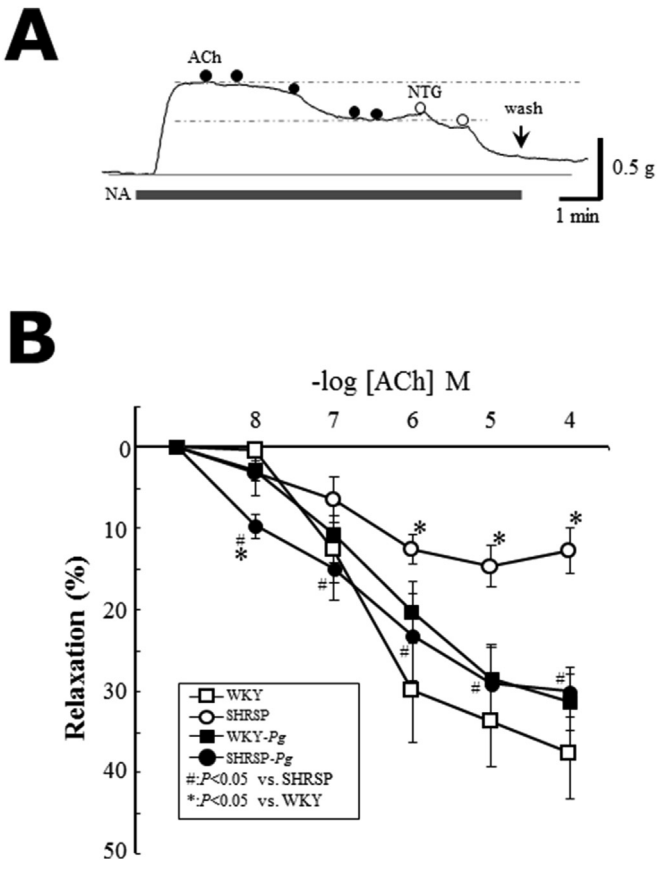


Fig. 4. Relaxation of descending aortic ring preparations in WKY, WKY + Pg, SHRSP, and SHRSP + *Pg*. Relaxation responses to the stepwise cumulative addition of ACh (10^{-8}–10^{-4} M, solid circles) and NTG (10^{-6}–10^{-5} M, open circles) were determined in rings contracted to a stable plateau tension by the addition of 10^{-5} M NA (A). The effects of *P. gingivalis* infection on the concentration–relaxation curve for ACh (B). Data are presented as mean ± SEM (n = 5 in each group). $^{*}P < 0.05$ vs. WKY, $^{\#}P < 0.05$ vs. SHRSP.

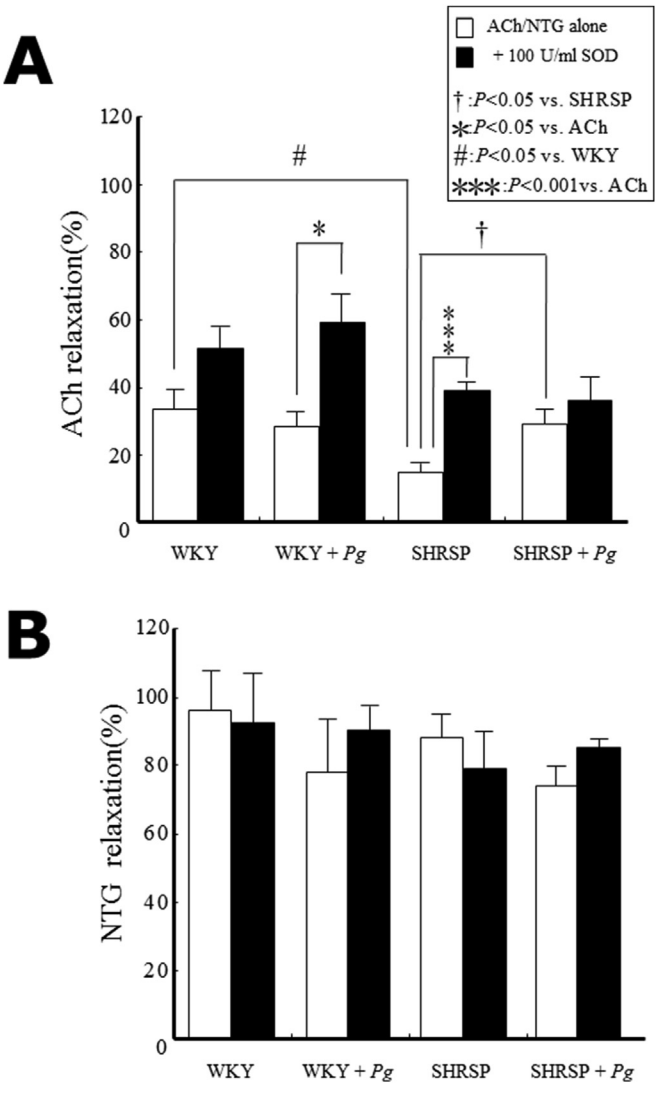


Fig. 5. The effects of *P. gingivalis* infection on the ACh- and NTG-induced relaxation in WKY and SHRSP descending aortic ring preparations. The endothelium-dependent relaxation evoked by ACh (A) in the ring preparations. The endothelium-independent relaxation induced by 10^{-6} M NTG (B). We added 100 U/ml SOD 30 min before inducing precontraction by 10^{-5} M NA (solid columns). Data are presented as mean ± SEM (n = 5 in each group). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. ACh alone, $^{\#}P < 0.05$ vs. WKY, $^{\dagger}P < 0.05$ vs. SHRSP.

TNF-α, and IL-6 in macrophages, lymphocytes, and endothelial cells [29,30]. Inflammation induced by these cytokines is an important event in the development of vascular diseases, such as hypertension, atherosclerosis, and stroke. Furthermore, LPS is known to activate the complement cascade through classical and alternative pathways. This reaction contributes to endotoxin shock [31]. Meanwhile, LPS from *P. gingivalis* is an agonist of both Toll-like receptor (TLR)2 and TLR4 [32]. Several studies have shown the nitric oxide (NO)-ROS pathway is not sufficient to mediate LPS-induced inflammatory responses, but may serve to inflate LPS signaling along the classic TLR4 pathway [33–38]. Namely, TLR4 mediates LPS-induced signal transduction. Therefore, it is possible that *P. gingivalis* infections affect vascular functions in addition to causing alveolar bone loss.

The magnitude of the effect oxidative stress ROS causes on inflammatory pathways in the pathogenesis of periodontitis has previously received attention [8,39,40]. Garrett et al. demonstrated that ROS, and particularly the superoxide anion, are intermediaries in forming and activating osteoclasts [8]. Furthermore, the detection of ROS oxidation products, along with elevated levels of iron and copper ions, may catalyze the production of the most reactive ROS, such as hydroxyl radicals. These induce an imbalance in oxidant/antioxidant activity within periodontal pockets, suggesting a significant role for ROS in periodontal tissue destruction [39]. We have previously reported the use of animals orally challenged with *P. gingivalis* as a chronic inflammation model. We suggested that *P. gingivalis*-induced alveolar bone loss could occur in periodontitis and in "hypertension and stroke" animal models, such as SHRSP [27]. Furthermore, the reactivity of blood vessels in the oral cavity was modulated by *P. gingivalis* infection in this experiment. As described above, *P. gingivalis* is found in the lesions of atherosclerosis and can also penetrate the vascular endothelium [41]. In addition, vascular contraction is affected by ROS [42,43]. Therefore, *P. gingivalis* invasion into blood vessels may induce modulation in vascular function. Interestingly, the effects on vascular functions were noted in specimens removed from the descending aorta, as well as the intraoral vessels, suggesting that *P. gingivalis* infection in the oral cavity may show systematic effects.

Although the relaxation response was suppressed in the excised vessels of SHRSP, the reaction, inhibited by SOD (Fig. 5A), is reversible. This also confirmed that $O_2^{\cdot-}$ was involved. The *P. gingivalis* infection induced vasodilatation in SHRSP. This may be caused by a relative reduction of $O_2^{\cdot-}$ involved in contractions via reactions between NO and $O_2^{\cdot-}$. One cause may be a large amount of non-physiological NO derived from inducible nitric oxide synthase, as iNOS is induced in macrophages stimulated by the LPS of *P. gingivalis*.

As can be inferred from the results, blood vessels exhibit a relaxation reaction when *P. gingivalis* infection is induced in SHRSP, which may present as restored reactivity of the blood vessels. In previous experiments, *P. gingivalis* infection induced in SHRSP showed decreased blood pressure, consistent with our results [23]. However, if this phenomenon is caused by the balance between physiologic or non-physiologic (*P. gingivalis* infection-induced) NO and $O_2^{\cdot-}$, inflammatory vascular dysfunction caused by ROS may be simultaneously progressing, while suggesting an apparent recovery. Thus, if patients predisposed to any systemic lifestyle related diseases are infected with *P. gingivalis*, periodontal tissue and other systemic vascular dysfunctions and failures may also slowly progress. We can infer from the present results that the involvement of ROS may be the pathological mechanism linking periodontal disease and cardiovascular disease. We recommend further studies using antioxidants to characterize the ROS pathway involved in periodontitis and stroke.

5. Conclusion

Our results suggest that altered endothelial function may occur in gingival tissue in both periodontitis and stroke animal models. Oxidative stress induced by ROS may be related to the vascular effects in periodontitis and stroke models. Therefore, it is likely that altered vascular function in oral microcirculation could be caused by the interaction between ROS and NO in periodontitis, similar to what occurs in stroke.

Conflict of interest

The authors declare no potential conflicts of interest with this study.

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