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<td>Journal</td>
<td>Archives of Oral Biology, 56(3): 244-250</td>
</tr>
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<td><a href="http://hdl.handle.net/10130/2325">http://hdl.handle.net/10130/2325</a></td>
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Exposure of P. gingivalis to noradrenaline reduces bacterial growth and elevates ArgX protease activity

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Running title: Noradrenaline enhances RgpB production
Key word: Noradrenaline; Gingipain; Porphyromonas gingivalis; Quorum sensing; Periodontitis

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ABSTRACT

Objective: Periodontitis, an infectious disease caused by periodontopathic bacteria, including Porphyromonas gingivalis and spirochetes, is reported to be accelerated by stress, under which noradrenaline levels are increased in the bloodstream. The purpose of this study was to evaluate the effects of noradrenaline on \textit{P. gingivalis}.

Design: \textit{P. gingivalis} was incubated in the presence of 25 µM, 50 µM, or 100 µM adrenaline or noradrenaline at 37°C for 12, 24 or 36 h and growth was evaluated by OD\textsubscript{660}. Auto-inducer-2 (AI-2) was measured by luminescence of \textit{Vibrio harveyi} BB 170. Expression of \textit{P. gingivalis} genes was evaluated using a microarray and RT-PCR. Rgp activity of arg-gingipainA and B (Rgp) was measured with a synthetic substrate.

Results: Growth of \textit{P. gingivalis} FDC381 was inhibited by noradrenaline at 24 and 36 h. Growth inhibition by noradrenaline increased dose-dependently. Inhibition of growth partially recovered with addition of propranolol. AI-2 production from \textit{P. gingivalis} showed a marked decrease with addition of noradrenaline compared with peak production levels in the control group. Microarray analysis revealed an increase in expression in 18 genes and a decrease in expression in 2 genes. Among these genes, expression of the protease arg-gingipainB (RgpB) gene, a major virulence factor of \textit{P. gingivalis}, was further analyzed. Expression of \textit{rgpB} showed a significant increase with addition of noradrenaline, which was partially reduced by addition of propranolol. Cell-associated Rgp activity also increased with addition of noradrenaline.

Conclusions: These results suggest that stressors influence the expression of the virulence factors of \textit{P. gingivalis} via noradrenaline.
1. Introduction

Periodontal disease is caused by Gram-negative anaerobic rods, including *Porphyromonas gingivalis* (1-3). *P. gingivalis* has several virulence factors, including fimbriae, gingipains and lipopolysaccharide (4). A great deal of evidence suggests that stress-induced neurohormones play a critical role in the outcome of infections (5-8). Epidemiological studies have also demonstrated a strong association between periodontal disease and several risk factors other than periodontopathic bacteria such as age, sex, genetic factors, stress and smoking (9). Among these factors, a strong association has been demonstrated between periodontal disease and psychological stress (9-13), although the mechanism underlying this relationship remains to be fully clarified.

Catecholamines such as adrenaline and noradrenaline were reported to increase under stress. Takada et al. (14) reported that adrenocorticotropic hormone levels and bone loss were higher in rats subjected to restraint stress than those in control rats in a rat periodontitis model. It is possible that adrenocorticotropic hormones affect the development of periodontitis. Noradrenaline and adrenaline were reported to affect gene expression in the locus of enterocyte effacement pathogenicity islands in enterohemorrhagic *Escherichia coli* (15). It has been reported that catecholamines such as noradrenaline inhibited growth of periodontal bacterial species, including *P. gingivalis* (16). It is possible that hormones such as noradrenaline increase in the bloodstream and local tissues in patients in response to stress and that this increase affects the composition or phenotype of subgingival periodontopathogens. In this study we evaluated the effect of noradrenaline on *P. gingivalis* gene expression to investigate the virulence of *P. gingivalis* in a host subjected to stress.
2. Materials and Methods

2.1. Media and Growth Conditions

*P. gingivalis* FDC381 was maintained on Tryptic soy agar containing 5 μg/ml hemin, 0.5 μg/ml menadione and 10% horse blood (Becton Dickinson Microbiology System, Cockeysville, MD) at 37°C under anaerobic conditions (N2: 80%, CO2: 10%, H2: 10%).

2.2. Growth suppression by noradrenaline

*P. gingivalis* was precultured in Tryptic soy broth (Becton Dickinson Microbiology System) at 37°C for 24 h, diluted to 1:40 with the broth and incubated in the presence of 25 μM, 50 μM, or 100 μM adrenaline (Sankyo, Tokyo, Japan) or noradrenaline (Sankyo, Tokyo, Japan) at 37°C for 12, 24 or 36 h. After incubation, growth of *P. gingivalis* was evaluated by OD$_{660}$. After evaluating the effect of dose-dependency on growth of *P. gingivalis*, 50 μM noradrenaline was used for further experiments. To evaluate the effect of propranolol, a noradrenaline antagonist, 100 μM propranolol was then added to the broth together with noradrenaline and growth of *P. gingivalis* evaluated as described above.

2.3. Influence of noradrenaline on gene expression by DNA microarray

To clarify the effect of noradrenaline on *P. gingivalis*, its gene expression profile was investigated. *P. gingivalis* FDC 381 was incubated with or without 50 μM noradrenaline at 37°C under anaerobic conditions for 30 h. Total RNA of *P. gingivalis* was extracted using the TRIzol Max Bacterial RNA Isolation Kit (Invitrogen, Carlsbad, CA) and cleaned up with the RNeasy Mini Kit (Qiagen, Valencia, CA). Expression of RNA was analyzed using the *P. gingivalis* array manufactured by Nimble Express containing 12,000 probe sets based on *P. gingivalis* W83 whole genome (282,000 unique features, Affymetrix, Santa Clara, CA). cDNA synthesized from total RNA of
*P. gingivalis* was biotinylated and hybridized over 16 h with the *P. gingivalis* array. The hybridized array was washed and stained using the Gene Chip Hybridization, Wash and Stain Kit (Affymetrix) according to the manufacturer’s protocol. After staining, the microarray chips were scanned using the Gene chip scanner 3000 (Affymetrix). Obtained images were analyzed with the GCOS (Affymetrix).

2.4. Quantitative analysis of gene expression by real-time PCR

The arg-gingipainB gene (*rgpB*) selected by microarray analysis was further analyzed by RT-PCR. Primers and Taqman probe for RT-PCR for *rgpB* and 16S rRNA are listed in Table 2. The Taqman probe for *rgpB* was designed based on those regions non-homologous with *rgpA*. cDNA was synthesized from total RNA of *P. gingivalis* with Omniscript RNA transcriptase (Qiagen) and specific primers for *rgpB* or 16S rRNA at 37°C for 1 h, and RNA transcriptase was inactivated at 93°C for 5 min. RT-PCR was performed under the following conditions: 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec, with final holding at 40°C for 8 min using SD7700 (Applied Biosystems), and fluorescence was measured. Expression of *rgpB* was normalized using 16S rRNA levels, and was calculated as a fold modulation over the control (17).

2.5. Quantitative analysis of activity of arg-gingipainA and B (Rgp)

*P. gingivalis* FDC381 precultured in TS broth at 37°C for 24 h was diluted 1:40 with TS broth and incubated in the presence of 50 μM noradrenaline at 37°C for 24 or 36 h. After incubation, growth of *P. gingivalis* was evaluated by absorbance at 660 nm and cells washed with PBS. Rgp activity in the *P. gingivalis* cells was determined by measuring the hydrolysis of N-Benzol-L-Arginine-p-Nitroanilide (BAPNA) according to the method of Hasegawa et al. (18) with minor modification. Briefly, number of
cells was adjusted to 0.05 at OD$_{660}$. Cell suspension was used to make a reaction mixture containing 100 mM Tris-HCl and 10 mM cysteine, which was then incubated at 37°C for 30 min. Released cleaved $p$-nitroanilide was then evaluated at OD$_{405}$ using a microplate reader (Biopad, Hercules, CA). Protease activity was determined by absorbance at 660 nm of cell density to normalize all values per OD$_{660}$ unit.

2.6. Effect of AI-2 on rgpB expression

Auto-inducer-2 (AI-2) is known to regulate the gene expression of microorganisms. It is possible that rgpB expression is up-regulated by reduction in AI-2 production. Therefore, AI-2 levels in the culture supernatant of \textit{P. gingivalis} with or without noradrenaline were evaluated using the \textit{Vibrio harveyi} reporter strain BB170 (kindly provided by Dr. Bassler, B.L., Princeton University) by a luminometer (Microtec-Nichion, Funabashi, Japan) as described previously (19). Data reported as fold activation were obtained by dividing the light produced by the reporter after addition of culture fluid by the light output of the reporter when growth medium alone was added.

2.7. Statistical analysis

To evaluate the results of absorbance, multiple comparisons were performed with the Scheffe test. Comparison of expression level after addition of noradrenaline was performed with the Mann-Whitney U-test. Comparison of Rgp activity was performed with the Student $t$-test.

3. Results

3.1. Growth suppression by noradrenaline
Fig. 1 A shows the effect of noradrenaline on growth of *P. gingivalis* FDC381. Noradrenaline reduced the growth rate of *P. gingivalis* from 24 to 36 h (p < 0.05). Attenuation of growth increased dose-dependently. Growth inhibition by noradrenaline was partially reversed with addition of propranolol. No change in growth was observed with addition of propranolol alone (p < 0.05) (Fig.1B).

3.2. Influence of noradrenaline on gene expression by DNA microarray and real-time PCR

*P. gingivalis* FDC381 genes showing a change in expression of more than 0.5-fold under noradrenaline exposure are listed in Table 1. An increase and decrease were detected in 18 and 2 genes, respectively. An increase was observed in a variety of genes, including in those coding for enzymes involved in arginine hydrolysis and proteins involved in response to oxygen stress and metabolism. From these, we chose cysteine protease RgpB, a major virulence factor of *P. gingivalis*, for further analysis. The primers and Taqman probes used to evaluate *rgpB* expression by RT-PCR are listed in Table 2. RT-PCR revealed that *rgpB* expression in the noradrenaline-treated cells was almost 2 times greater than that in the control group (Fig. 2A), and that this increase was inhibited by noradrenaline inhibitor (Fig. 2B). In addition to *rgpB*, 2-oxoglutarate oxidoreductase, an enzyme involved in metabolic pathways, and the F subunit of alkyl hydroperoxide reductase, an enzyme which shows an increase under oxidative conditions, also showed an increase.

3.3 Quantitative analysis of Rgp activity

To evaluate Rgp activity, *P. gingivalis* FDC381 was incubated in the presence of 50 μM noradrenaline for 24 or 36 h as described above. After washing the cells with PBS, Arg-X-specific cysteine proteinase activity in the cells was determined by measuring
hydrolysis of BAPNA. Protease activity was normalized based on concentration of bacterial cells to 0.5 at 660 nm of cell density. Addition of 50 μM noradrenaline enhanced Rgp activity (Fig. 3).

3.4. Al-2 levels in noradrenaline-treated P. gingivalis

The profile of Al-2 levels in P. gingivalis treated with noradrenaline given in Fig. 4 shows that levels were different from those in the control. Peak level of Al-2 in noradrenaline-treated P. gingivalis was lower than that in the control. Levels of Al-2 in the control decreased rapidly, while Al-2 levels in noradrenaline-treated P. gingivalis showed no decrease, with levels higher than those in the control at 36 and 48 hr.

Discussion

In this study, growth of P. gingivalis showed an approximately 40% reduction with addition of adrenaline or noradrenaline (p < 0.05) at 24 and 36 h. This suggests that catecholamines inhibit the growth of periodontopathic bacteria. In addition, growth inhibition increased dose-dependently with concentration of catecholamine. This suggests that noradrenaline exerts a direct effect on P. gingivalis. This reduction in growth agrees with the results of another report on growth of periodontopathic bacteria (16). Ishisaka et al. reported that cortisol levels were associated with severity of periodontitis (12). Oral challenge with P. gingivalis under increased cortisol levels due to superior cervical ganglionectomy accelerated alveolar bone loss and osteoclastic activity (20). In the present study, expression of the virulence factor rgpB showed an increase in the presence of noradrenaline.

Change in expression in Rgp and reduction in growth may be explained by two major events resulting from exposure of oral pathogens to noradrenaline: noradrenaline
acting as a cue to prime expression of virulence factors, and noradrenaline interacting with lactoferrin or transferrin (or both). The partial recovery brought about by supplementation with propranolol in this study suggests that \textit{P. gingivalis} possesses a catecholamine sensor. A similar phenomenon has been observed in \textit{Escherichia coli} O157H7 (15). Recently, Clarke (21) reported that histidine kinase QseC was a sensor of noradrenaline in \textit{E. coli}. The antagonistic effect observed in the present study suggests that \textit{P. gingivalis} cells are able to sense the presence of noradrenaline. Further study is needed to clarify whether this microorganism senses catecholamine by means of a sensor protein such as QseC.

It is possible that noradrenaline affects acquisition of iron by microorganisms and that this is involved in the reduction in growth observed in this study. Noradrenaline interacts with lactoferrin or transferrin, converting it to a useful iron source (22, 23). Catecholamine has also been reported to chelate iron (24). It is possible that change in the concentration of iron affects growth of \textit{P. gingivalis}. In the present study, \textit{P. gingivalis} was grown in nutrient-rich medium, whereas in the previous study, Serum-SAPI medium was used (16, 25). Serum-SAPI medium consists of minimum salts medium supplemented with 30% bovine adult serum, and exposes bacteria to conditions similar to those experienced inside a host, including similar levels of iron, nutrients and host immune components (26). Although the effect of lactoferrin or transferrin can not have been involved in the reduction in growth observed in the present study, as they were absent from the medium used, chelation of iron, on the other hand, can not be ruled out. Further study employing iron-limited conditions is required to clarify the regulation mechanism involved.
Many species of microorganism have a system of cell-to-cell signaling known as quorum sensing (QS) (27). Among the signaling molecules of this system, AI-2 is involved in cell-to-cell sensing, both among and between species (28). It is possible that AI-2 is involved in growth rate and \( \text{rgpB} \) expression. Production of AI-2 from \( E. \ coli \) by noradrenaline stimulation was reported to suppress growth of \( P. \ gingivalis \) by 9.6% in minimal medium (25). According to the present results, AI-2 was reduced in noradrenaline-treated \( P. \ gingivalis \). This suggests that decrease in AI-2 production was not a major cause of the growth reduction observed in the present study. In this experiment, expression of \( \text{rgpB} \) was increased by addition of noradrenaline, whereas expression of AI-2 was reduced. \( \text{rgpB} \) was not included in the quorum-sensing regulated genes reported by Yuan et al (29). James et al. (30) reported that LuxS did not affect expression of \( \text{rgp} \). Gingipain activity was reported to decrease in LuxS-deficient mutant (31). On the other hand, Chung et al (32) reported that RgpA and hemin acquisition protein were up-regulated by inactivation of the LuxS gene. In \( E. \ coli \) O157 H7, the signal induced by noradrenaline was different from that induced by AI-2, and was associated with expression of its virulence gene (15). In this study, AI-2 levels in the culture supernatant of adrenaline-treated \( P. \ gingivalis \) were lower than those of non-treated \( P. \ gingivalis \). It is possible that signaling other than that of AI-2 induced by noradrenaline is associated with increase in \( \text{rgpB} \). Roberts et al (25) found that the effect of AI-2 response was less than that of limiting nutrients within the spent medium. It is possible that nutrient conditions make it difficult to clarify the effect of AI-2. Further study employing different nutrient conditions which mimic the condition of the subgingival crevice is required to clarify the regulation mechanisms.
involved, including those regulating AI-2 production in *P. gingivalis* by exposure to noradrenaline.

We observed a reduction in growth rate and an increase in expression of several genes, including *rgpB*, after exposure to noradrenaline. An enzyme required for adaptation to oxygen stress, the F subunit of alkyl hydroperoxide reductase, showed an increase. Alkyl hydroperoxide reductase was reported to be up-regulated under oxidative conditions due to the presence of *oxyR* (33). It is possible that stimulation by noradrenaline induced up-regulation of genes involved in response to oxidative conditions. Gingipain, a major virulence factor of this microorganism involved in colonization, impairs host defense systems and damages host tissue (34). Rgp have been reported to be induced by environmental conditions such as oxygen stress (35). In addition, an enzyme involved in metabolic pathways, 2-oxoglutarate oxidoreductase, showed an increase. This enzyme is involved in the metabolism of glutamate to butylate (36). Taken together with the present results, this suggests that expression of *rgpB* is induced by response to oxygen stress or regulation of genes encoding proteins involved in the metabolic pathway, although further analysis is required to confirm this. These changes in enzyme expression suggest noradrenaline-induced adaptation by this microorganism. The reduction in growth rate and increase in proteolytic enzymes seen in this study may be explained by adaptation to a temporary reduction in gingival crevicular fluid caused by noradrenaline-induced vasoconstriction. Further analysis, however, is required to clarify the relationship between expression levels of *rgpB* in periodontal lesions and noradrenaline concentrations in serum to confirm this possibility. Taken together, these findings show that noradrenaline induced phenotypical change in
*P. gingivalis*, including growth reduction and increase in *rgpB*, suggesting that the virulence of *P. gingivalis* is affected by host-pathogen communication induced by stress response.

**Conflict of interest statement**

We have no conflict of interest to declare.

**Acknowledgements**

This work was partially supported by a Grant HRC7 from the Oral Health Science Center of Tokyo Dental College, and a “High-Tech Research Center” Project for Private Universities: matching fund subsidy from MEXT, 2006-2010. The authors would like to thank Associate Professor Jeremy Williams, Tokyo Dental College, for his assistance with the English of the manuscript.

**Competing interest: No conflicts of interest.**

**Ethical approval: Not required.**
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324-328.


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Table 1. *P. gingivalis* genes regulated by exposure to noradrenaline

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Genes showing a level change of more than 50% after addition of noradrenaline, except
where signal was less than 50.

*(With noradrenaline) / (Without noradrenaline)*
Table 2. Primers and TaqMan probes for RT-PCR

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<td>TaqMan probe 5’-ATGACTGTAGTGAAAAACCGTCTTTCCCTTC-3’’</td>
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Figure legends

Fig. 1. Effect of noradrenaline on growth of *P. gingivalis* FDC381

(A) *P. gingivalis* FDC381 was incubated in presence of 25, 50 or 100 mM noradrenaline for 12 to 36 hr. Growth of *P. gingivalis* was evaluated by absorbance at 660 nm.

(B) *P. gingivalis* FDC381 was incubated in the presence of 50 mM noradrenaline and 100 mM propranolol for 12 to 36 hr. Growth of *P. gingivalis* was evaluated by absorbance at 660 nm. Results are presented as mean ± SD of triplicate assays.

*p < 0.05 by Scheffe’s test

Fig. 2 Effect of noradrenaline on *rgpB* expression

(A) *P. gingivalis* FDC381 was incubated in the presence of 50 mM noradrenaline for 12 to 36 hr. Control: *P. gingivalis* FDC381 incubated without noradrenaline. Expression of *rgpB* was expressed as relative amount to that of ribosomal RNA. Results are
presented as mean ± SD of triplicate assays.

* $p < 0.01$ by Student t test

(B) *P. gingivalis* FDC381 was incubated in the presence of 50 mM noradrenaline for 24 hr with or without 100 mM propranolol. Control: *P. gingivalis* FDC381 incubated without noradrenaline. Expression of *rgps* was expressed as relative amount to that of ribosomal RNA. Results are presented as mean ± SD of triplicate assays.

* $p < 0.05$ by one-way ANOVA followed by Turkey's test

Fig. 3. Rgp activity in *P. gingivalis* FDC381

*P. gingivalis* FDC381 was incubated in the presence of 50 mM noradrenaline for 12 to 36 hr and Rgp activity was evaluated using BAPNA. Control: *P. gingivalis* FDC381 incubated without noradrenaline. Results are presented as mean ± SD of triplicate assays.

Fig. 4. Growth and level of AI-2 in *P. gingivalis* FDC381 in the presence of noradrenaline. Control: *P. gingivalis* FDC381 incubated without noradrenaline. Results are presented as mean ± SD of triplicate assays.

* $p < 0.05$ by Scheffe’s test
Fig. 2
Fig. 3
Fig. 4