Nitration of the salivary component 4-hydroxyphenylacetic acid in the human oral cavity: enhancement of nitration under acidic conditions


4-Hydroxyphenylacetic acid (HPA) and nitrite are present in human mixed whole saliva, and HPA can be nitrated by peroxidase/hydrogen peroxide (H$_2$O$_2$)/nitrite systems in the oral cavity. Thus, the objectives of the present study were to estimate the concentrations of HPA, nitrated HPA [4-hydroxy-3-nitrophénylacetic acid (NO$_2$-HPA)], nitrite, and thiocyanate (SCN$^-$) in saliva from 73 patients with periodontal diseases and to elucidate the conditions necessary to induce nitration of HPA. High concentrations of HPA, nitrite, and SCN$^-$ were found in the saliva of patients older than 50 yr of age. NO$_2$-HPA was detected in seven patients who were older than 60 yr of age. Nitrite-dependent formation of NO$_2$-HPA by a bacterial fraction prepared from mixed whole saliva was faster at pH 5.3 than at pH 7, and increased as the rate of H$_2$O$_2$ formation increased. The formation of NO$_2$-HPA was inhibited by SCN$^-$ and by salivary antioxidants such as uric acid, ascorbic acid, and glutathione. These results suggest that nitration can proceed at an acidic site in the oral cavity where H$_2$O$_2$ is produced under conditions of decreased concentrations of SCN$^-$ and of antioxidants.

Human mixed whole saliva contains 4-hydroxyphenylacetic acid (HPA). The concentration of HPA has been shown to range from 2 to 10 μM in saliva collected from healthy individuals 2–3 h after breakfast (1). It has been reported that HPA is formed by Porphyromonas gingivalis as a metabolic end-product (2). It has also been reported that saliva contains nitrate, the concentration of which reflects the amount of nitrate ingested as a component of food (3). In the oral cavity, nitrate in saliva is reduced to nitrite by nitrate-reducing bacteria and to nitric oxide (NO) by nitrite-reducing bacteria (4–7). Furthermore, certain bacteria and leukocytes in the oral cavity can produce O$_3^-$, which is then converted into hydrogen peroxide (H$_2$O$_2$) (8–10). It has been reported that HPA can be nitrated, producing 4-hydroxy-3-nitrophénylacetic acid (NO$_2$-HPA), by salivary peroxidase/H$_2$O$_2$/nitrite systems (11–13). Nitric oxide formed by the reduction of nitrite-reducing bacteria may also contribute to HPA nitration, as NO is transformed to NO$_2$ and ONOO$^-$ (pKa = 6.8), both of which are nitrating reagents, by reacting with O$_2$ and O$_3^-$, respectively (14). The NO$_2$ formed can be transformed further into N$_2$O$_3$ by reacting with NO and, accordingly, the formation of N$_2$O$_3$ has been suggested to occur in human whole saliva (15).

In this study, we determined the concentrations of HPA, NO$_2$-HPA, nitrite, and thiocyanate (SCN$^-$) in mixed whole saliva from patients with periodontal diseases. In addition, conditions that could induce the formation of NO$_2$-HPA in the oral cavity were also investigated.

**Material and methods**

**Reagents**

4-Hydroxyphenylacetic acid, glucose oxidase from Aspergillus niger, and the Griess–Romijn reagent for nitrite were obtained from Wako (Osaka, Japan). NO$_2$-HPA was obtained from Aldrich (Milwaukee, WI, USA).

**Preparation of saliva**

Mixed whole saliva (approximately 1.5 ml) was collected from 73 patients (age-range: 21–84 yr; 26 men and 47 women) who visited a dental clinic in Kitakyushu City from January to July in 2003. After informed consent was obtained, saliva was collected from patients by having them chew solid paraffin for a few min before any treatment. The saliva collected was stored at ~20°C until used to

**Key words:** human saliva; 4-hydroxyphenylacetic acid; nitration; nitrite; periodontal diseases

Accepted for publication June 2009
determine the concentrations of HPA, NO₂HPA, nitrite, and SCN⁻. Component determination was completed within 3 months of saliva collection. Before collecting saliva, clinical examination of the oral cavity was performed. Oral inflammation was scored by the area of inflammation relative to the entire gingival area. When no inflammation was detected, the inflammation score was expressed as zero, and when inflammation was detected in more than 90% of the entire gingival area, the score was expressed as 10. The severity of inflammation was also taken into consideration for the scoring. Bleeding and oozing of pus from the gingiva were scored as inflammation.

Mixed whole saliva (about 5 ml) was also obtained from four healthy volunteers (age range: 35–55 yr; one man and three women) after informed consent had been obtained. Volunteers were asked to brush their teeth 20–30 min after lunch, and saliva was collected by having them chew parafilm (for 5–10 min) 0, 1, 2, 3, and 4 h after brushing. During the 4 h after initial brushing, only water (no food) was permitted. The saliva preparations were passed through two layers of nylon filter net [380-mesh (32 µm); Sansho, Tokyo, Japan] to remove epithelial cells and other particles, and the filtrates were used to measure the concentration of HPA.

To measure the formation of NO₂HPA in vitro, mixed whole saliva (about 10 ml) was also collected from the volunteers after chewing parafilm (for about 10 min) at 09.00–10.00 h. The collected saliva was passed through two layers of nylon filter nets as described above. The filtrate was centrifuged at 20,000 g for 5 min, and the sediment was suspended in 50 mM sodium phosphate buffer (at either pH 5.2 or pH 7.0) for use as the bacterial fraction. The volume of phosphate buffer used for the suspension was the same as the volume of saliva filtrate. When the sediment was suspended in 50 mM sodium phosphate buffer (pH 5.2), the pH of the bacterial fraction was about 5.3.

Quantification of HPA and NO₂HPA in saliva

4-Hydroxyphenylacetic acid and NO₂HPA were extracted from saliva preparations as follows. Saliva (0.5 ml) was acidified by adding 1.5 ml of 0.67 M HCl. The acidified saliva was extracted twice with 2 ml of ethyl acetate. The ethyl acetate extracts were combined and dried with anhydrous sodium sulphate. After ethyl acetate had been evaporated using a rotary evaporator, the residue was dissolved in 1 ml of a mixture of methanol and 25 mM KH₂PO₄ (1:2, v/v), the pH of which was adjusted to 3.0 by the addition of 4-Hydroxyphenylacetic acid and NO₂HPA were separated from the other components using high-performance liquid chromatography (HPLC), which was performed using a Shim-pack CLC-ODS column (6 mm i.d. × 16 cm; Shimadzu, Kyoto, Japan) combined with an injector (Rheodyne 7125 with a 0.1 ml sample loop; IDEX Health and Science, Oak Harbor, WA, USA) and a spectrophotometric detector with a photodiode array (SPD-M10Avp; Shimadzu). 4-Hydroxyphenylacetic acid and NO₂HPA were separated from the other components using a mixture of methanol and 25 mM KH₂PO₄ [1:2 (v/v), pH 3.0], the flow rate of which was 1 ml min⁻¹. The separated HPA and NO₂HPA components were detected at 280 and 360 nm, respectively, as reported previously (1). The concentrations of these components were determined from peak areas on chromatograms using standard HPA and NO₂HPA.

Quantification of nitrite and SCN⁻ in saliva

Nitrite was quantified using the Griess–Röhmij reagent (1). The reaction mixture (1.0 ml) contained 0.05 ml of saliva, 0.1 ml of 1% Griess–Röhmij reagent, and 0.85 ml of 50 mM KCl–HCl buffer (pH 2.0). After incubation for 20 min at 25°C, the absorbance at 540 nm was determined, and this reading was used to estimate the concentration of nitrite from a standard curve. Thiocyanate was quantified in a reaction mixture (1.0 ml) containing 0.1 ml of saliva, 0.1 ml of 0.1 M FeCl₃, and 0.8 ml of 0.1 M HCl (16). The concentration of SCN⁻ was calculated by measuring the absorbance at 450 nm, and this reading was used to estimate the concentration of SCN⁻ from a standard curve.

NO₂HPA formation in the bacterial fraction

NO₂HPA formation was studied in the presence and absence of glucose/glucose oxidase systems. In the absence of glucose/glucose oxidase systems, the reaction mixture (1 ml) contained 0.1 mM HPA and 1 mM sodium nitrite in the bacterial fraction (at pH 5.3 and pH 7.0). After incubation for 30 min, 1 ml of methanol was added to stop the reaction and then 5 ml of ethyl acetate was added. The mixture was centrifuged at 2,000 g for 2 min, and the upper organic layer was transferred to a test tube. After dehydration with anhydrous sodium sulphate, the organic solvents were evaporated using a rotary evaporator. The residue was dissolved in 0.2 ml of a mobile phase used for HPLC [methanol and 25 mM KH₂PO₄ = 1:2 (v/v); pH 4.5] and 0.1 ml of the solution was applied to the HPLC column described above.

When the formation of NO₂HPA was studied in the presence of glucose/glucose oxidase systems, the reaction mixture (0.5 ml) contained 0.1 mM HPA, 2 mM glucose, and 0.1 µg of glucose oxidase in 0.5 ml of the bacterial fraction (at pH 5.3 and pH 7.0). The mixture absorbed O₂ at a rate of about 2 µM min⁻¹ in 50 mM sodium phosphate buffer (at pH 5.3 and pH 7.0) when measured using a Clark-type oxygen electrode. This result indicated that H₂O₂ was formed at a rate of 2 µM min⁻¹ according to the reaction mechanism of glucose oxidase. After incubation for defined periods of time in the presence of various concentrations of nitrite, the mixture was combined with 0.5 ml of methanol to stop the reaction. The methanolic solution was filtered through a cellulose-acetate membrane filter (0.45 µm; Advantec, Tokyo, Japan), and an aliquot (50 µl) of the filtrate was applied to the HPLC column described above. The mobile phase used was a mixture of methanol and 25 mM KH₂PO₄ [1:2 (v/v), pH 4.5], and the flow rate was 1 ml min⁻¹. Furthermore, the formation of NO₂HPA was also studied by changing the concentration of glucose oxidase in the above reaction mixture in the presence of 0.3 mM sodium nitrite. NO₂HPA separated under the conditions described above (retention time, 7.8 min) was detected at 360 nm using a spectrophotometric detector, as also described above. NO₂HPA was quantified from the peak area on chromatograms.

The decrease in the concentration of HPA was studied in the reaction mixture that contained 0.1 mM HPA, 2 mM glucose, 0.3 mM NaNO₂, and 0.2 µg of glucose oxidase in 1 ml of the bacterial fraction (at pH 5.3 and pH 7.0). After incubation for 30 min, 0.5 ml of methanol was added to 0.5 ml of the reaction mixture and the methanolic solution was filtered as described above. An aliquot (10 µl) of the filtrate was applied to the HPLC column described above. The mobile phases used for the bacterial fractions of
pH 5.3 and pH 7.0 were mixtures of methanol and 25 mM KH$_2$PO$_4$ [1:2 (v/v), pH 4.5 and 1:1 (v/v), pH 3.0, respectively]. The mobile phase of pH 3.0 was prepared as described above. Separated HPA was detected at 280 nm using a spectrophotometric detector and quantified from the peak area on chromatograms. The retention times were 4.3 and 4.1 min in the mobile phases of pH 4.5 and pH 3.0, respectively.

Statistics
When required, data were shown as means ± standard deviations (SDs) and were evaluated statistically using the Student’s t-test. A P-value of < 0.05 was regarded as statistically significant. When statistical analysis was not required, typical data or the averages of two experiments were shown.

Results

Concentrations of HPA, nitrite, and SCN$^-$$^-$
Figure 1A shows the concentration of HPA in saliva obtained from individuals with periodontal disease as a function of age. 4-Hydroxyphenylacetic acid was detected in all patients, and the concentrations ranged from 0.5 to 110 µM [mean ± SD = 18.4 ± 23.6 µM (n = 73)]. Twenty patients had a high concentration of HPA (> 20 µM) and the number of patients with a high concentration of HPA was greater in older patients than in younger patients. It has been reported that the concentration of HPA in saliva from healthy individuals is below 10 µM (1).

Figure 1B shows the concentration of nitrite in saliva of patients with periodontal disease. The concentrations ranged from 0.03 to 0.97 mM (mean ± SD = 0.27 ± 0.21 mM). Nine patients had a concentration of nitrite higher than 0.5 mM, and the number of patients with high concentrations of nitrite increased as a function of age. The concentration of nitrite in saliva obtained from healthy individuals has been reported to range from 0.1 to 0.3 mM (17, 18) and increases to 1–2 mM after the ingestion of nitrate-rich foods (3).

The concentration of SCN$^-$ estimated in this study ranged from 0.12 to 1.86 mM (mean ± SD = 0.57 ± 0.40 mM) (Fig. 1C). The number of individuals with high concentrations of SCN$^-$ was greater in older patients than in younger patients. It has been reported that the SCN$^-$ concentration in saliva from healthy individuals ranges from 0.5 to 2.3 mM (17–19).

In the present study, no clear relationships were found among the concentrations of HPA, nitrite, and SCN$^-$ in patients. In addition, no statistical differences in the concentrations of HPA, nitrite, and SCN$^-$ were found between men and women. Moreover, no statistical relationship could be found between the concentration of each substance and the score of each diagnostic parameter.

We also studied the effects of smoking on the concentrations of HPA, nitrite, and SCN$^-$ in patients. Forty of the patients in our study were non-smokers, 12 were smokers, and the status of the rest was unknown. Statistical analysis showed that smoking caused an increase in the concentration of SCN$^-$ (mean ± SD: 0.44 ± 0.30 for non-smokers and 1.11 ± 0.51 for smokers, P < 0.001), as reported previously (20, 21), but did not show any relationship between smoking and the concentration of nitrite or HPA.

NO$_2$HPA was detected in seven saliva preparations obtained from individuals, older than 60 yr of age, with periodontal diseases. The component was identified by comparison of the retention time (18.8 min) and absorption spectrum (peaks at 215, 275, and 357 nm) with those of standard NO$_2$HPA, as reported previously (1). The concentration of NO$_2$HPA in the seven saliva preparations ranged from 0.03 to 1.7 µM [mean ± SD = 0.40 ± 0.65 µM (n = 7)]. Two of the seven patients were smokers, and no relationships were found between smoking and the concentration of NO$_2$HPA, indicating that the formation of NO$_2$HPA was not related to smoking. The relationships among the concentrations of NO$_2$HPA, HPA, nitrite, and SCN$^-$ were studied, but no significant association could be found. This result suggests that the NO$_2$HPA found in the saliva preparations was not caused by NO$_2$HPA formed during extraction of the compound from saliva. It has been

Fig. 1. Concentrations of 4-hydroxyphenylacetic acid (HPA), nitrite, and thiocyanate (SCN$^-$) in saliva from individuals with periodontal diseases as a function of age. Panel A, HPA; panel B, nitrite; panel C, SCN$^-$.
reported that nitrite can convert HPA to NO$_2$HPA under acidic conditions (1). As no NO$_2$HPA was detected in saliva obtained from healthy individuals, it is unlikely that NO$_2$HPA had been formed by chemical reactions during the extraction of HPA under acidic conditions (1).

Figure 2 shows the change in the concentration of HPA in mixed whole saliva obtained from healthy individuals at different time-points after toothbrushing. The concentration of HPA showed an initial increase, which was followed by a gradual decrease, indicating that the formation of HPA was controlled by unknown mechanisms.

**Formation of NO$_2$HPA in vitro**

The conversion of HPA to NO$_2$HPA was studied using the bacterial fraction and 50 mM sodium phosphate buffer in the presence of 0.1 mM HPA and 1 mM nitrite in order to clarify the conditions of its formation in the oral cavity. When the bacterial fraction (pH 5.3) was incubated for 30 min, a small amount of NO$_2$HPA was formed, and the rate of its formation was calculated to be 4.7 ± 3.7 nM min$^{-1}$ (mean ± SD, n = 4). The rate of NO$_2$HPA formation in 50 mM sodium phosphate buffer (pH 5.2) was calculated to be 0.9 ± 0.3 nM min$^{-1}$ (mean ± SD, n = 3). No detectable formation of NO$_2$HPA was observed in the bacterial fraction when it was incubated in the buffer solution at pH 7.0. However, the formation of NO$_2$HPA was increased by the addition of 2 mM glucose and 0.2 μg of glucose oxidase per ml to the bacterial fraction. The rates of NO$_2$HPA formation at pH values of 5.3 and 7.0 were calculated to be 0.51 ± 0.19 and 0.18 ± 0.05 μM min$^{-1}$ (mean ± SD, n = 4), respectively, and the ratio in the rate of NO$_2$HPA formation at pH 5.3 to that at pH 7.0 was 2.78 ± 0.38 (mean ± SD, n = 4). A small amount of NO$_2$HPA was formed when HPA was incubated in the nitrite/glucose/glucose oxidase system in 50 mM sodium phosphate buffer (pH 5.2) and the rate was about 2% of that in the bacterial fraction at pH 5.3. NO$_2$HPA could not be detected in the buffer solution at pH 7.0 in the nitrite/glucose/glucose oxidase system. This result indicates that both the bacterial fraction and H$_2$O$_2$ were required for the formation of NO$_2$HPA. Because the rate of NO$_2$HPA formation was faster at pH 5.3 than at pH 7.0, further studies were performed at pH 5.3.

Figure 3 (left panel) shows typical time courses of NO$_2$HPA formation in the bacterial fraction. NO$_2$HPA was formed nearly linearly as a function of incubation time after a lag period that was independent of the concentration of nitrite, and NO$_2$HPA formation was faster in the presence of 0.3 mM nitrite than in the presence of 1 mM nitrite. The effects of nitrite concentration on the formation of NO$_2$HPA showed that when the concentration of nitrite was lower than 0.3 mM, the rate of NO$_2$HPA formation increased as the concentration of nitrite was increased, and that when the nitrite concentration was higher than 0.3 mM, NO$_2$HPA formation decreased as the concentration of nitrite increased (Fig. 3, right panel). The inhibition of NO$_2$HPA formation by a high concentration of nitrite was caused by the nitrite-dependent inhibition of glucose oxidase. In fact, 1 mM sodium nitrite inhibited oxygen uptake by about 35% in the presence of 2 mM glucose and 0.2 μg of glucose oxidase per ml in 50 mM sodium phosphate buffer (pH 5.3); however, no significant effects on oxygen uptake were observed for 0.3 mM nitrite. At pH 7.0, 1 mM nitrite did not affect the O$_2$ uptake induced by a glucose/glucose oxidase system.

Figure 4 shows the effects of the concentrations of glucose oxidase and SCN$^-$ on NO$_2$HPA formation. The rate of NO$_2$HPA formation increased as a function of glucose oxidase concentration (left panel), indicating that the rate of NO$_2$HPA formation was dependent on the rate of H$_2$O$_2$ formation, but the increase in the rate seemed to be sigmoidal in nature. Thio cyanate inhibited...
the formation of NO₂HPA induced by a nitrite/glucose/ 
glucose oxidase system in the bacterial fraction (right 
panel). The concentration of SCN⁻ found in saliva 
collected from patients (0.1–1.8 mM) (Fig. 1C) inhibited 
the formation of NO₂HPA by more than 70%. 
Enhancement of the formation of NO₂HPA by H₂O₂ 
and inhibition by SCN⁻ indicate that peroxidase 
contributes to the formation of NO₂HPA. Salivary 
peroxidase bound to bacteria may be responsible for this 
as it has been reported that oral bacteria bind salivary 
peroxidase (8). In addition to salivary peroxidase, 
myeloperoxidase is also a probable candidate because the 
bacterial fraction may have been contaminated with 
salivary leukocytes (12). The contribution of peroxidases 
to the formation of NO₂HPA was supported by the 
result that 1 mM KCN and 1 mM NaN₃ almost 
completely inhibited NO₂HPA induced during incubation 
of the bacterial fraction (pH 5.3) for 30 min in the 
presence of 0.1 mM HPA, 2 mM glucose, 0.3 mM NaN₂O₂, 
and 0.1 μg of glucose oxidase, whereas the formation of NO₂HPA was 3.6 ± 0.1 μM 
(mean ± SD, n = 3) and the decrease in the concentration of HPA was 9.7 ± 0.6 μM (mean ± SD, n = 3) 
when the bacterial fraction was incubated under the 
above conditions at pH 7.0. The ratio of NO₂HPA 
formed at pH 5.3 to that at pH 7.0 was 7. This ratio was 
larger than the ratio obtained in the presence of 
1 mM nitrite (see above). The difference could be 
attributed to the inhibition of glucose oxidase by 
1 mM nitrite at pH 5.3.

### Discussion

*P. gingivalis*, which is found in the human oral cavity as an indigenous bacterium (22), can produce HPA 
during the metabolism of phenylalanine and tyrosine, 
but other indigenous species of *Porphyromonas*, such as 
*Porphyromonas asaccharolytica* and *Porphyromonas 
endodontalis*, cannot produce HPA (2). The concentration 
of HPA in mixed whole saliva obtained from 
healthy individuals was found to be below 10 μM (1), 
whereas an HPA concentration higher than 20 μM was 
found in saliva from individuals with periodontal disease 
(Fig. 1). This increase in the concentration of HPA may 
be attributed to the increased population of *P. gingivalis* 
in patients with periodontal disease (23–26). Therefore, if 
HPA was produced mainly by *P. gingivalis* in the oral 
cavity, the salivary concentration of HPA might be a 
suitable index with which to estimate the population of 
*P. gingivalis* in the oral cavity. In the present study, the 
concentration of HPA in saliva increased after tooth-
brushing, although the increase was small (Fig. 2). Small 
changes in the concentration of HPA in saliva after a 
meal have also been reported in healthy individuals 
whose salivary concentration of HPA is around 1 μM 
(27). These results suggest that ingestion of a meal did 
not significantly affect the population of *P. gingivalis* and 
that toothbrushing might establish growth conditions for 
*P. gingivalis*.

Nitrite in saliva is mainly derived from nitrate that is 
reduced by nitrate-reducing bacteria in the oral cavity 
(4–7). There are reports that the concentration of nitrite 
in dental plaque is higher than the concentration of 
nitrite in saliva (28, 29), and that periodontitis decreases 
(30), or increases (31, 32), the concentration of nitrite 
in saliva. The increased concentration of nitrite has been 
postulated to be caused by the enhanced production of NO by NO synthase (31, 32). Taking the result of 
the present study into account, we postulated that 
periodontal diseases could result in an increase in nitrite 
concentration in saliva under certain conditions, and 
that this increased concentration might be caused by 
an increase in the population of nitrate-reducing bacteria 
(4–7). The latter is deduced from the result that 
the salivary concentration of nitrite is significantly 
affected by the intake of nitrate as a component of food 
(3, 5).
NO$_2$HPA was detected in seven patients, indicating that HPA could be nitrated in the oral cavity. Thus, the formation of NO$_2$HPA might be caused by the faster formation of reactive nitrogen oxide species that could nitrate HPA, as well as by the lower concentrations of antioxidants that could scavenge the reactive nitrogen oxide species. It has been reported that reactive nitrogen oxide species are continuously formed in mixed whole saliva (33, 34). However, in a large number of patients with periodontal disease, NO$_2$HPA was not detected. Furthermore, no NO$_2$HPA has been detected in saliva from healthy individuals, as described in the Results section of this work and in research performed by Pananala et al. (27). Failure to detect NO$_2$HPA suggests that the formation of reactive nitrogen oxide species was slow, and/or that the reactive species formed might be effectively scavenged by salivary antioxidants. It has been reported that the reactive nitrogen oxide species formed are effectively scavenged by antioxidants in saliva (33, 34). When saliva is mixed with gastric juice, NO$_2$HPA is formed from salivary nitrite and HPA (1, 27). Because NO$_2$HPA formed in the stomach is excreted as a component of urine (27), we can deduce that NO$_2$HPA formed in the oral cavity in patients with periodontal disease may also be excreted as a urinary component.

The formation of NO$_2$HPA in the stomach increases with decreasing pH and an increasing concentration of nitrite, suggesting that the formation reaction is nitrous acid-dependent (1, 27). By contrast, in the bacterial fraction, NO$_2$HPA seemed to be produced by peroxidase-catalyzed reactions (11). Various peroxidases, including salivary peroxidase and myeloperoxidase, can nitrate HPA in the presence of nitrite and H$_2$O$_2$ (11, 13, 35). In patients with periodontal disease, the contribution of myeloperoxidase to the formation of NO$_2$HPA could be increased if the diseases resulted in the enhanced exudation of leukocytes into the oral cavity. The peroxidase-dependent nitration of HPA may be initiated by the peroxidase-catalyzed oxidation of nitrite to NO$_2$ and of HPA to its radical, which can react with each other to produce NO$_2$HPA (11, 35). In addition to the NO$_2$ formed by peroxidase/H$_2$O$_2$/nitrite systems, the formation of NO$_2$ by the following reactions is enhanced when the pH in the oral cavity decreases to about 5:

$$\text{2HNO}_2 \leftrightarrow \text{N}_2\text{O}_3 + \text{H}_2\text{O} \leftrightarrow \text{NO}_2 + \text{NO} + \text{H}_2\text{O}$$ \hspace{1cm} (1)$$

$$\text{2NO + O}_2 \rightarrow 2\text{NO}_2$$ \hspace{1cm} (2)

Reaction 1 is possible because about 1% of the nitrite ion is present as nitrous acid (pKa = 3.3) at pH 5.3, and reaction 2 is possible because NO formation by nitrite-reducing bacteria becomes faster with a decrease in pH (36, 37). The NO$_2$ formed by the above two reactions may also contribute to the formation of NO$_2$HPA, because NO$_2$ can oxidize HPA to its radical, which can react with another molecule of NO$_2$ (11, 35).

It has been reported that (i) the decrease in concentration of nitrite, which is produced by the salivary peroxidase-catalyzed reaction, is approximately 30% faster at pH 5.5 than at pH 7.2, that (ii) salivary peroxidase-catalyzed oxidation of HPA at pH 7.2 is about twofold higher than that at pH 5.5, and that (iii) there are two peaks in the rate of formation of NO$_2$HPA at pH values of 5.5 and 7.2 when the reactions occur in the presence of 0.1 mM HPA, 1 mM nitrite, and 0.5 mM H$_2$O$_2$ (13). In this study, the formation of NO$_2$HPA was much faster at pH 5.3 than at pH 7.0. The faster formation of NO$_2$HPA could be explained by the faster oxidation of HPA at pH 5.3 than at pH 7, and the faster formation of NO$_2$ by peroxidase-catalyzed oxidation of nitrite, self-decomposition of nitrous acid, and auto-oxidation of NO, as discussed above. The different effects of pH on the rate of HPA oxidation at high and low concentrations of H$_2$O$_2$ may be attributed to the different effect of H$_2$O$_2$ concentration on the $K_m$ of salivary peroxidase for HPA at pH values of 5.3 and 7.

The decrease in concentration of HPA (29.9 $\pm$ 2.4 $\mu$M 30 min$^{-1}$) was similar to the formation of NO$_2$HPA (25.2 $\pm$ 2.9 $\mu$M 30 min$^{-1}$) at pH 5.3, whereas the decrease in concentration of HPA (9.7 $\pm$ 0.6 $\mu$M 30 min$^{-1}$) was larger than the formation of NO$_2$HPA (3.6 $\pm$ 0.1 $\mu$M 30 min$^{-1}$) at pH 7.0. This result suggests that the HPA radical formed at pH 5.3 could be effectively transformed to NO$_2$HPA, whereas the radical formed at pH 7.0 could not be effectively transformed to NO$_2$HPA, as suggested previously (13). This difference might be explained from H$^+$ dissociation of the carboxyl group of HPA (pKa = 4.43) (38). In addition to NO$_2$ generated by peroxidase/H$_2$O$_2$/nitrite systems and the reactions 1 and 2 shown above, HPA may be nitrated by ONOOH/ONOO$^-$ because bacteria can produce NO as well as O$_2^-$ (8, 39). However, the contribution of ONOOH/ONOO$^-$ to the nitrination of HPA seems to be negligible when the formation of H$_2$O$_2$ is fast. This idea is deduced from the result that the rate of NO$_2$HPA formation was quite slow when glucose oxidase was omitted from the reaction mixture.

According to the earlier discussion, we can assume that salivary peroxidase- and myeloperoxidase-catalyzed nitrination of HPA can proceed faster when the pH of the oral cavity is decreased than when the pH is maintained at approximately 7. In addition to HPA, nitration of tyrosine residues of proteins may also proceed at low pH values because salivary peroxidase can nitrate the tyrosine residues of salivary proteins (11). In the oral cavity, the pH of dental plaque rapidly decreases to about 5.5, on average, after the ingestion of 5% sucrose, and the decreased pH is maintained for about 30 min when the secretion of saliva is slow (40). Under such conditions, demineralization of teeth, as well as damage to tissues by oxidative nitrination, can occur. The decrease in concentrations of salivary antioxidants, which is observed in patients of periodontal disease (41), may enhance oxidative nitrination. Nitration has been reported to be linked to many human diseases such as inflammation and cancer (42, 43).

It can be deduced from the present study that nitrination may be enhanced by increases in nitrite concentration (28, 29) and formation of H$_2$O$_2$ (44) in the dental plaque.
This enhancement may also be observed with a decreased concentration of SCN⁻ (Fig. 4). Because SCN⁻ is an inhibitor and a substrate of salivary peroxidase (45) and a substrate of myeloperoxidase (8), it can inhibit the formation of NO₂HPA by suppressing peroxidase-catalyzed oxidation of nitrite and HPA. Although the increase in the SCN⁻ concentration in saliva because of smoking (20, 21 and this study) would be expected to result in the slowing down of peroxidase-catalyzed nitration of HPA, smoking results in an increase in periodontal disease (46-48). It is known that SCN⁻ reacts with nitrite under acidic conditions, producing ONSCN (37). Although nitrite has been discussed as induced by peroxidase/H₂O₂/nitrite systems (11) and ONSCN can be increased (36, 37, 50, 51). Antioxidants formation, suggesting an effective scavenging of reactive species by antioxidants in the bacterial fraction, NO species by antioxidants in the bacterial fraction in the presence of 0.2 mM nitrite, but in the absence of glucose oxidase was slower when the formation of the reactive NO species was slow. The effective scavenging was supported by the results that there was a lag period for the glucose/glucose oxidase-induced production of NO₂HPA (Fig. 3) and that the increase in the rate of NO₂HPA production as a function of concentration of glucose oxidase showed a sigmoidal curve (Fig. 4).

In conclusion, nitration induced by peroxidase/nitrite/H₂O₂ systems increased with decreasing pH in the oral cavity if the formation of H₂O₂ and the concentration of nitrite were kept constant. By contrast, when the nitration is suppressed by SCN⁻ under acidic conditions, oxidation and nitrosation induced by ONSCN can be increased (36, 37, 50, 51). Antioxidants in saliva prevent oxidation, nitration, and nitrosation induced by peroxidase/H₂O₂/nitrite systems (11) and ONSCN (37). Although nitrite has been discussed as being a ‘prodrug’ with which to increase the concentration of NO in the human body (52–55), the results of this study suggest that oxidation, nitration, and nitrosation stemmed from an increased concentration of peroxidase, nitrite, H₂O₂, and SCN⁻ in the oral cavity as the pH decreased. Moreover, to protect oral tissues from damage induced by oxidation, nitration, and nitrosation, it is essential to keep the pH in the oral cavity at around 7 while maintaining high concentrations of antioxidants.

References


