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Production of nitric oxide-derived reactive nitrogen species in human oral cavity and their scavenging by salivary redox components

UMEO TAKAHAMA[1], SACHIKO HIROTA[2], & TAKAYUKI ONIKI[1]

[1]*Department of Bioscience, Kyushu Dental University, Kitakyushu 803-8580, Japan, and* [2]*Department of Nutritional Science, Kyushu Women's University, Kitakyushu 807-8586, Japan*

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Abstract
Nitrite is reduced to nitric oxide (NO) in the oral cavity. The NO generated can react with molecular oxygen producing reactive nitrogen species. In this study, reduction of nitrite to NO was observed in bacterial fractions of saliva and whole saliva. Formation of reactive nitrogen species from NO was detected by measuring the transformation of 4,5-diaminofluorescein (DAF-2) to triazolfluorescein (DAF-2T). The transformation was fast in bacterial fractions but slow in whole saliva. Salivary components such as ascorbate, glutathione, uric acid and thiocyanate inhibited the transformation of DAF-2 to DAF-2T in bacterial fractions without affecting nitrite-dependent NO production. The inhibition was deduced to be due to scavenging of reactive nitrogen species, which were formed from NO, by the above reagents. The transformation of DAF-2 to DAF-2T was faster in bacterial fractions and whole saliva which were prepared 1–4 h after tooth brushing than those prepared immediately after toothbrushing. Increase in the rate as a function of time after toothbrushing seemed to be due to the increase in population of bacteria which could reduce nitrite to NO. The results obtained in this study suggest that reactive nitrogen species derived from NO are continuously formed in the oral cavity and that the reactive nitrogen species are effectively scavenged by salivary redox components in saliva but the scavenging is not complete.

Keywords: *4,5-diaminofluorescein (DAF-2), nitrite, nitric oxide, reactive nitrogen species, human saliva*

Abbreviations: *DAF-2, 4,5-diaminofluorescein, DAF-2T; Triazolfluorescein, DTCS, N-(dithiocarboxy)sarcosine; ESR, electron spin resonance*

Introduction

Nitrate is a salivary component and the concentration of nitrate in saliva reflects the nitrate intake of the body [1,2]. The nitrate is reduced to nitrite [1] and the nitrite is reduced further to nitric oxide (NO) [3] in the oral cavity by certain bacteria. The NO production in the oral cavity is affected by antimicrobial agents [4], dental plaque deposition and smoking [5]. As a function of NO formed in the oral cavity, antimicrobial activity has been discussed [3,5]. If NO is formed under aerobic conditions, NO can be transformed to NO_2 and N_2O_3 [6], which can contribute to nitration and nitrosation, respectively [7,8]. It has been reported that NO formed under aerobic conditions can be detected using diaminofluoresceins [9]. The detection is based on the transformation of diaminofluoresceins to triazolfluoresceins by reactive nitrogen species which are formed by the reaction between NO and molecular oxygen [9]. It has also been reported that fluorescence quantum efficiency increases more than 180 times after the transformation of diaminofluoresceins to triazolfluoresceins [9]. The results suggest that diaminofluoresceins can be used to detect reactive nitrogen species formed from NO. Measurements of NO formation using diaminofluoresceins has been reported by many authors [10–13]. Then, we designed experiments to estimate the NO-dependent formation of reactive nitrogen

Correspondence: U. Takahama, Department of Bioscience, Kyushu Dental University, Kitakyushu 803-8580, Japan.
E-mail: takahama@kyu-dent.ac.jp

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species in the oral cavity by measuring the transformation of 4,5-diaminofluorescein (DAF-2) to a fluorescent component triazolfluorescein (DAF-2T). The results obtained in this study suggest that reactive nitrogen species derived from NO are effectively scavenged by salivary redox components in whole saliva, but the scavenging is not complete.

Materials and methods

Chemicals

DAF-2 was obtained from Daiichi Pure Chem. Co., Ltd (Tokyo, Japan). Griess-Romijn reagent for nitrite, *N*-(dithiocarboxy)sarcosine (DTCS), (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR 3 or FK409) and 2,3-diaminonaphthalene were from Dojindo (Kumamoto, Japan). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) was from Wako Pure Chem. Ind. (Osaka, Japan).

Preparation saliva

Mixed whole saliva (5 ml) was obtained from four volunteers (35–55 years old) after obtaining their informed consent by chewing parafilm at about 11:00 a.m. Toothbrushing was carried out 20–30 min after lunch and then mixed whole saliva (5 ml) was also collected by chewing parafilm at 0, 1, 2, 3 and 4 h after toothbrushing. No foods but water were taken for 4 h after the brushing. The saliva preparations obtained were passed through two layers of nylon filter nets [380-mesh (32 μm) net, Sansho, Tokyo, Japan] to remove epithelial cells and other particles. The filtrate was used as filtered saliva-I. The filtrate was centrifuged at 20,000*g* for 5 min and the sediment was suspended in 5 ml of 10 mM sodium phosphate (pH 7.0). This fraction mainly contained bacteria when observed using a microscope and was used as bacterial fraction to measure transformation of DAF-2 to a fluorescent component. When NO production was measured using Fe-$(DTCS)_2$, the sediment obtained after the centrifugation was suspended in 1 ml of 10 mM sodium phosphate (pH 7.0).

After toothbrushing, 1 ml of unstimulated mixed whole saliva was also collected and passed through two layers of nylon filter nets as described above. The filtrate was used as filtered saliva-II to measure transformation of DAF-2 to a fluorescent component and to determine the concentration of nitrite in saliva.

Measurements of fluorescence

The transformation of DAF-2 to fluorescent components was measured in the reaction mixture that contained 10 μM DAF-2 in 3 ml of bacterial fraction. After incubating for defined periods under various conditions at 35°C, an aliquot (0.5 ml) of the reaction mixture was withdrawn to measure fluorescence using a spectrofluorometer (RF-550, Shimadzu, Kyoto, Japan). The excitation and emission wavelengths were 485 and 538 nm, respectively, as reported previously [9]. The excitation light was passed through two glass filters ND-13 (11% transmission at 485 nm) and B-440 (18% transmission at 485 nm) from Hoya (Tokyo, Japan). When filtered saliva-II which was prepared from unstimulated mixed whole saliva was used to measure fluorescence, 10 μM DAF-2 was added to 0.5 ml of the filtered saliva and increase in fluorescence were recorded continuously using a recorder at about 30°C. This made possible to measure small changes of fluorescence intensity.

Separation of fluorescence components by HPLC

Fluorescent components, which were separated by HPLC using a Shim-pack CLC-ODS column (i.d. 6 mm × 15 cm; Shimadzu, Kyoto, Japan), could be detected with a spectrofluometric detector (RF-550; Shimadzu) (excitation, 495 nm; emission, 515 nm) and a spectrophotometric detector (SPD-M10A, Shimadzu). The mobile phase was a mixture of 10 mM sodium phosphate (pH 7.2) and acetonitrile (94:6, v/v), and the flow rate was 1 ml min^{-1} [9]. Samples to apply to the HPLC column were prepared by passing through a membrane filter with a pore size of 0.45 μm or by ultrafiltration using Artkiss™ microconcentrator (10,000 molecular weight cutoff) (Advantec, Tokyo, Japan) after incubation of bacterial fractions and filtered saliva-II in the presence and absence of 1 mM sodium nitrite for defined periods.

Mass spectra of fluorescent components, which were separated by the above HPLC column, were measured using an LC/MS spectrometer combined with a spectrophotometric detector with a photodiode array (1100 LC/MSD SL, Agilent Technologies). The mobile phase used for the measurement of mass spectra was a mixture of acetonitrile and 5 mM ammonium acetate (1:10, v/v), pH of which was adjusted to 7.2 by 0.1 M NH_3, and the flow rate was 1 ml min^{-1}.

Measurements of ESR signals

Electron spin resonance (ESR) spectra were measured using a JEOL JES-FE1XG spectrometer at about 25°C with a quartz flat cell (0.05 ml) under the following conditions as reported previously [14,15]: microwave power, 5 mW; line width, 0.2 mT; amplification, 2000-fold; scanning speed, 5 mT min^{-1}. The reaction mixture (0.6 ml) was consisted of 0.3 ml of 10 mM sodium phosphate (pH 7.0) containing 5 mM DTCS and 1.5 mM $FeCl_3$ and 0.3 ml of bacterial fraction prepared for NO detection. When filtered saliva was used, the reaction mixture (0.6 ml) contained 0.3 ml of filtered saliva-I and 0.3 ml of the above solution.

The reaction mixtures were incubated for defined periods at 35°C under various conditions, and then an aliquot (0.05 ml) was withdrawn from the mixture to measure ESR spectra. NO formed was trapped by Fe-$(DTCS)_2$ generating stable NO-Fe-$(DTCS)_2$ complex.

Determination of nitrite concentration

Concentrations of nitrite in filtered saliva-I and -II were measured using a Griess-Romijn nitrite reagent as reported previously [16]. The mixture (1 ml) contained 0.05 ml of sample, 0.1 ml of 1% Griess-Romijn reagent and 0.85 ml of 50 mM KCl–HCl (pH 2.0). The concentration of nitrite was determined from the absorption at 540 nm after 10 min of incubation at 35°C.

Results

Detection of NO

Figure 1 shows nitrite-dependent formation of NO in a bacterial fraction prepared from saliva collected at 11:00 a.m. No ESR signals were detected in the absence of nitrite (trace A), but in the presence of 1 mM sodium nitrite, an ESR signal due to the formation of NO-Fe-$(DTCS)_2$ complex was detected (trace B). The detection of NO by dithiocarbamate derivatives has been reported [17–19]. The signal intensity increased nearly linearly as a function of incubation time for 1 h. The increase in the ESR signal intensity was not affected by redox components contained in saliva such as 0.1 mM ascorbate [20], glutathione [21] and uric acid [21] (trace C). No effects of 1 mM thiocyanate [1] were observed for the increase in ESR signal intensity, either (trace D). Furthermore, supernatant, which was prepared by centrifugation of filtered saliva-I as described in "Materials and Methods" section, did not inhibit the NO production in the presence of 1 mM sodium nitrite. NO was also detected in filtered saliva-I and the NO formation was enhanced by 1 mM sodium nitrite. The concentration of nitrite in the filtered saliva-I used for NO detection was 0.1–0.15 mM.

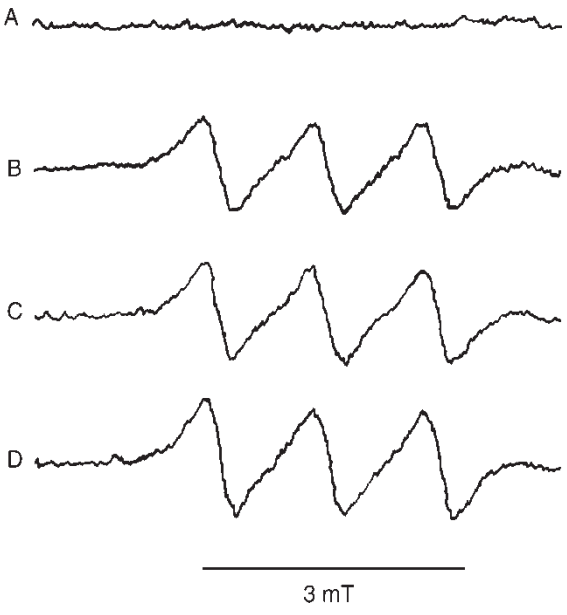

Figure 1. Nitrite-dependent formation of NO. The reaction mixture (0.6 ml) contained 0.3 ml of 10 mM sodium phosphate containing 5 mM DTCS and 1.5 mM of $FeCl_3$ and 0.3 ml of a bacterial fraction prepared for ESR measurement. The reaction mixture was incubated for 30 min and 0.05 ml was withdrawn to measure ESR spectra. A, no addition; B, 1 mM $NaNO_2$; C, B + 0.1 mM uric acid; D, B + 1 mM NaSCN.

Formation of fluorescent component

Figure 2A shows typical time courses of fluorescence increase in a bacterial fraction prepared from saliva collected at 11:00 a.m. In the absence of nitrite and nitrate, a small increase in fluorescence was observed. When 0.3 mM sodium nitrite was added, rapid increase in green fluorescence was observed. The excitation and emission peaks (uncorrected) were observed at 495 and 518 nm, respectively. The values were nearly the same as those of DAF-2T [9]. In the presence of 0.5 mM potassium nitrate, no significant increase in fluorescence was observed during the initial period of incubation, but the fluorescence started increasing after prolonged incubation. These results suggest the reduction of nitrate to NO via nitrite. The nitrite-dependent rapid increase in fluorescence was observed in all preparations of bacterial fraction from four different persons although there were large deviations (Figure 2B).

Initial rates of the increase in fluorescence were independent of the concentration of DAF-2 in its concentration range from 2 to 20 μM, but linearly dependent on dilution of bacterial fraction in the presence of 0.3 mM sodium nitrite. Km value of nitrite for the increase in fluorescence was determined by double-reciplocal plot, and the value was about 1 mM (Figure 3A). Maximal increase in fluorescence was observed around pH 7 (Figure 3B).

Although a significant increase in fluorescence was observed in bacterial fractions, increase in the fluorescence was small in filtered saliva-I which was prepared from saliva collected at 11:00 a.m. (Figure 2B). The fluorescence increase in bacterial fractions was inhibited by the supernatant obtained after centrifugation of the filtrate. The increase in fluorescence in the mixture of the supernatant and a bacterial fraction (1:1, v/v) was 1–2% of that in bacterial fractions. These results suggest that components, which could inhibit the transformation of DAF-2 to a fluorescent component, were included in saliva. Since saliva contained redox components as described above, effects of these components on the increase in fluorescence was studied using bacterial fractions (Figure 4). Ascorbate (0.1 mM), glutathione (0.1 mM), uric acid (0.1 mM) and thiocyanate

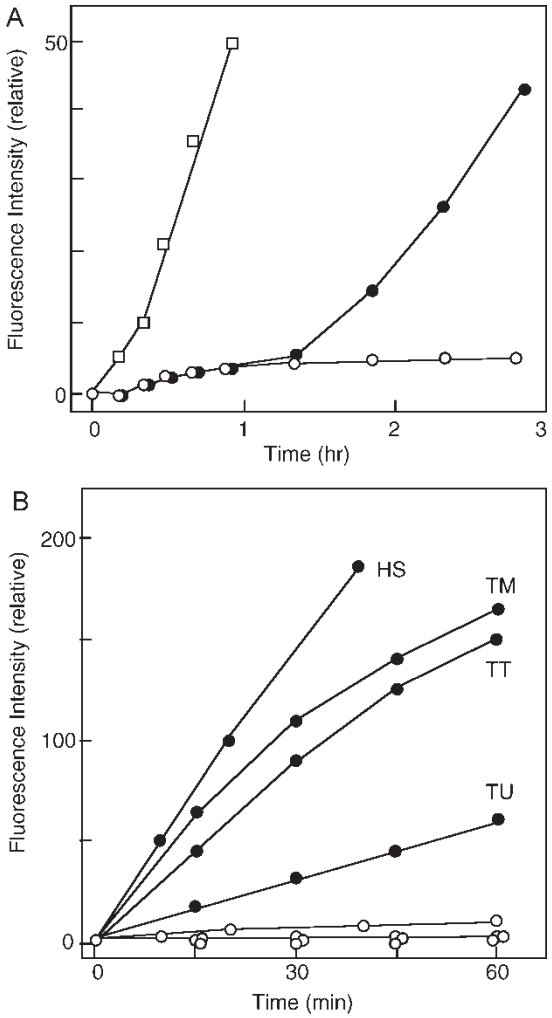

Figure 2. Time courses of fluorescence increase. Panel A: Effects of nitrite and nitrate. The reaction mixture contained 10 μM DAF-2 in 3 ml of bacterial fraction. After incubation for defined periods, 0.5 ml of the sample was withdrawn and fluorescence intensity was measured. Open circles, no addition; closed circles, 0.5 mM potassium nitrate; squares, 0.3 mM $NaNO_2$. Panel B: fluorescence increase in bacterial fraction and filtered saliva-I. The reaction mixture contained 10 μM DAF-2 and 0.3 mM $NaNO_2$ in 3 ml of bacterial fraction or filtered saliva-I. After incubation for defined periods, 0.5 ml of the sample was withdrawn and fluorescence intensity was measured. Closed circles, bacterial fraction; open circles, filtered saliva-I. Saliva preparations from four individuals were used.

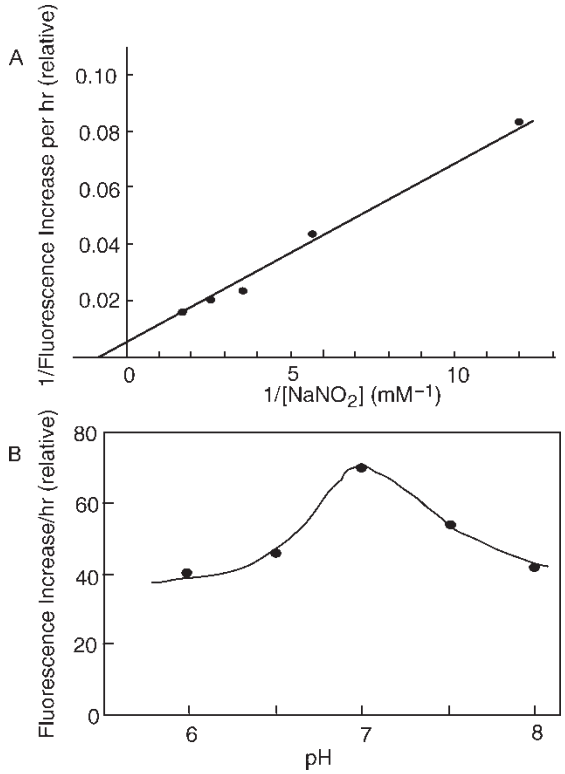

Figure 3. Effects of nitrite concentration and pH on fluorescence increase. Panel A: Double reciprocal plots of rate of fluorescence increase as a function of nitrite concentration. The reaction mixture contained 10 μM DAF-2 in 1.0 ml of bacterial fraction. Panel B: Effects of pH. The reaction mixture contained 10 μM DAF-2 and 0.3 mM $NaNO_2$ in 1.0 ml of bacterial fraction. Rate of fluorescence increase was calculated from fluorescence increase for 30 min.

(1 mM) significantly inhibited the increase in fluorescence. Fifty percent inhibition by these components were observed at about 3, 5, 6 and 100 μM, respectively. The relation between the nitrite-dependent formation of NO-Fe-$(DTCS)_2$ and nitrite-dependent increase in fluorescence was studied using bacterial fractions prepared from saliva collected at 11:00 a.m. There was a nearly linear relation (data not shown) suggesting that NO was transformed to reactive nitrogen species which could react with DAF-2 as reported previously [9].

The formation of reactive nitrogen species was also studied measuring the transformation of

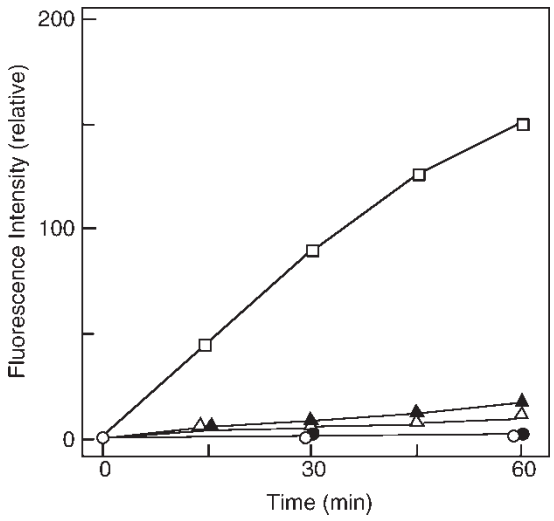

Figure 4. Inhibition of fluorescence increase by salivary redox components. The reaction mixture contained 10 μM DAF-2 and 0.3 mM $NaNO_2$ in 3 ml of bacterial fraction. After defined periods of incubation, 0.5 ml of the sample was withdrawn and fluorescence intensity was measured. Squares, no addition; closed triangles, 1 mM NaSCN; open triangles, 0.1 mM uric acid; open circles, 0.1 mM glutathione; closed circles, 0.1 mM ascorbic acid.

2,3-diaminonaphthalene to 2,3-naphthotirazole (excitation, 365 nm; emission, 460 nm) [22]. Formation of 2,3-naphthotoriazol could be detected using bacterial fractions, but rate of the increase in fluorescence intensity was less than one tenth of that of the increase in fluorescence intensity when DAF-2 was used. The formation of reactive nitrogen species could not be detected using 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). In the following, formation of reactive nitrogen species was measured using DAF-2.

DAF-2 transformation after toothbrushing

Rates of fluorescence increase were measured using bacterial fractions which were prepared from saliva collected at defined periods after toothbrushing. Figure 5A shows data from one person and Figure 5B from three individuals. Rates of fluorescence increase were always larger in bacterial fractions prepared 1–4 h after toothbrushing than in bacterial fractions prepared immediately after toothbrushing independent of the source of saliva. There was a tendency that rate of fluorescence increase increased as a function of time after toothbrushing. The increase in the activity may be due to the increase in population of nitrite reducing bacteria in the oral cavity.

Fluorescence increase in filtered saliva-II

The increase in fluorescence in filtered saliva-I was slow as described above (Figure 2). Then, the increase in fluorescence was recorded continuously using filtered saliva-II prepared from unstimulated saliva. The reason that filtered saliva-II instead of filtered saliva-I was used was that filtered saliva-II might reflect better the aqueous environment in the oral cavity than filtered saliva-I. Figure 6A shows typical time courses of fluorescence increase under various conditions. When 10 μM DAF-2 was dissolved in 50 mM sodium phosphate (pH 6.8 and 7.2), fluorescence due to DAF-2 itself was detected, but no changes of fluorescence intensity were observed after the addition of DAF-2 (traces 1 and 2). This indicates no transformation of DAF-2 to a fluorescent component in the buffer solutions. On the addition of 0.2 mM sodium nitrite to the buffer solutions, slow increase in fluorescence was observed (traces 1 and 2), suggesting slow chemical reaction between DAF-2 and nitrite producing a fluorescent component. When 10 μM DAF-2 was added to filtered saliva-II which was prepared from saliva collected 2 h after tooth brushing, fluorescence due to DAF-2 itself was also detected and then the fluorescence intensity increased slowly (trace 3). The slow fluorescence increase might be due to the transformation of DAF-2 to a fluorescent component. The fluorescence increase was enhanced by 0.2 mM sodium nitrite and the nitrite-dependent

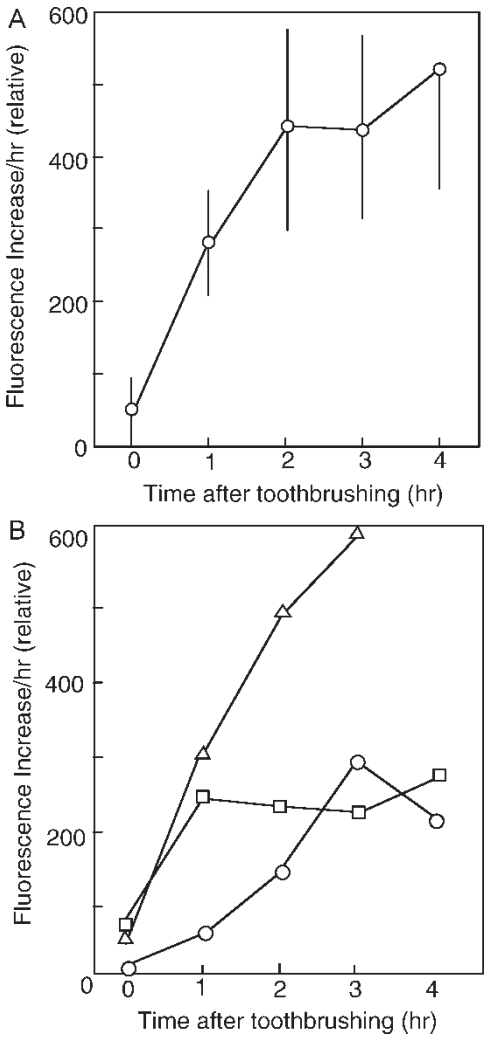

Figure 5. Rate of fluorescence increase as a function of time after toothbrushing. Stimulated saliva were collected at 0, 1, 2, 3 and 4 h after toothbrushing, and bacterial fractions were prepared as described in "Materials and Methods" section. Panel A: Data from one person. Each series of experiment was repeated on different days. Each data point represents mean ± S.D. ($n = 6$). Panel B: Data from three individuals represented by different symbols. Each data point represents mean of two or three experiments.

enhancement in filtered saliva-II (trace 3) was larger than that in buffer solutions (traces 1 and 2).

Figure 6B shows changes in rate of fluorescence increase in filtered saliva-II which was prepared from saliva collected at defined periods after toothbrushing. There were large variations in rate of fluorescence increase depending on saliva preparations, but rates of fluorescence increase increased as a function of time after toothbrushing independent of saliva preparations. The inset shows nitrite-dependent enhancement of fluorescence increase, namely, difference in rates of fluorescence increase after and before the addition of 0.2 mM sodium nitrite. It is clear from the inset that the enhancing effect of nitrite increased as a function of time after toothbrushing. Concentration of nitrite in filtered saliva-II used in the above

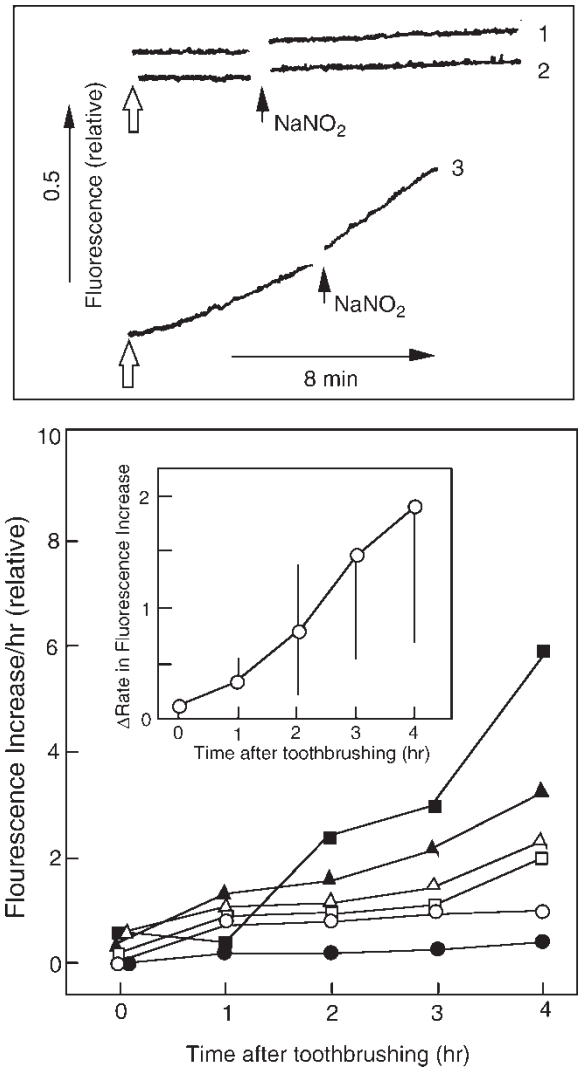

Figure 6. Formation of reactive nitrogen species in filtered saliva-II. Upper panel: Time courses of fluorescence increase. Trace 1, 50 mM sodium phosphate (pH 6.8); trace 2, 50 mM sodium phosphate (pH 7.2); trace 3, filtered saliva-II (pH 7.3). The above buffer solutions and saliva preparations contained 10 μM DAF-2. Immediately after the addition of DAF-2 (white arrows), measurements of fluorescence increase were started. Sodium nitrite (0.2 mM) was added where indicated by black arrows. Lower panel: Rate of fluorescence increase as a function of time after toothbrushing. Filtered saliva-II was prepared from unstimulated saliva collected at 0, 1, 2, 3 and 4 h after toothbrushing from three individuals. Each series of experiment was repeated twice for each person on different days. Inset: Nitrite-induced fluorescence increase. Sodium nitrite (0.2 mM) was added to filtered saliva-II as shown in the upper panel, and then difference in fluorescence increase after and before the addition of nitrite was calculated. Each data point represents mean ± S.D. ($n = 6$).

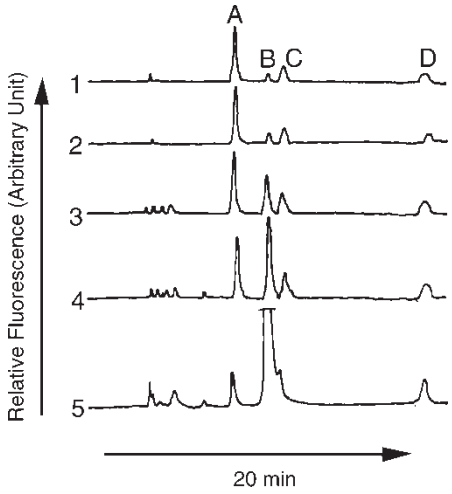

Figure 7. HPLC profiles of DAF-2 and its reaction products. Trace 1, 10 μM DAF-2 in 50 mM sodium phosphate (pH 7.2) (immediately after preparation of the mixture); trace 2, 10 μM DAF-2 and 1 mM $NaNO_2$ in 50 mM sodium phosphate (pH 7.2) (incubated for 30 min); trace 3, 10 μM DAF-2 in filtered saliva-II (pH 7.4) (incubated for 30 min); trace 4, 10 μM DAF-2 and 1 mM $NaNO_2$ in filtered saliva-II (pH 7.4) (incubated for 30 min); trace 5, 10 μM DAF-2 and 1 mM $NaNO_2$ in a bacterial fraction (pH 7.0) (incubated for 20 min).

experiments ranged from 0.06 to 0.3 mM and pH was between 6.7 and 7.5.

Characterization of fluorescent components

Fluorescent component formed during fluorescence increase was analyzed by HPLC (Figure 7). In reagent DAF-2 itself, one major fluorescent component and three minor components were detected (trace 1). Peak A (retention time, 9.2 min) was presumed to be DAF-2, because concentration of this component decreased accompanying the increase in fluorescence (trace 5). This is supported by the results that (i) the component with a retention time of 9.2 min was a only component which could be detected spectrophotometrically when DAF-2 itself was analyzed by HPLC and that (ii) the absorption spectrum of the component (peak, 490 nm) was the same as that of DAF-2 which was dissolved in the mobile phase. During incubation of DAF-2 in the presence of 1 mM nitrite in a buffer solution, only small increase in peak B (retention time, 11.2 min) was observed (trace 2). When incubated in filtered saliva-II, fluorescence intensity of peak B was increased (trace 3) and the increase was enhanced by 1 mM nitrite (trace 4). Fluorescence intensity of peak B was much stronger in bacterial fraction (trace 5) than filtered saliva. The component of peak B was also formed when 10 μM DAF-2 was incubated with 20 μM (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (NOR 3 or FK409), an NO generating reagent [23–25], in 50 mM sodium phosphate (pH 7.2). These results suggest that the component of peak B was DAF-2T. The fluorescent component of peak B was characterized further using an LC/MS system. Its absorption spectrum showed a peak at 495 nm in the mobile phase used for LC/MS and its mass numbers were 372 $[M - H]^-$ and 374 $[M + H]^+$ when ionized by electrospray method, whereas DAF-2 ($C_{20}H_{14}N_2O_5$; molecular weight, 362) had an absorption peak at

490 nm and its mass numbers were 361 $[M-H]^-$ and 363 $[M+H]^+$. This result supports that the fluorescent component of peak B was DAF-2T ($C_{20}H_{11}N_3O_5$; molecular weight, 373).

Discussion

The formation NO in the oral cavity has been reported [3–5], but the transformation of NO to other reactive nitrogen species after reacting with molecular oxygen has not yet been reported in the oral cavity. In this study, nitrite-dependent formation of NO and fluorescence increase due to the formation of DAF-2T from DAF-2 were observed in bacterial fractions prepared from mixed whole saliva (Figures 1, 2 and 7). This result suggests that formation of reactive nitrogen species from NO is possible in the bacterial fractions as reactive nitrogen species derived from NO can transform DAF-2 to DAF-2T under aerobic conditions [9]. Km value of nitrite for the increase in fluorescence was about 1 mM (Figure 3). This value was similar to the Km value of nitrite (about 1 mM) for nitrite-dependent NO production by *Streptococcus salivarius* which had been isolated from human saliva [3]. The formation of DAF-2T was observed in a pH range from 6 to 8 (Figure 3). It has been reported that fluorescence quantum efficiency of DAF-2T decreases as pH is decreased from 7 to 5 and that the efficiency at pH 6 is about a half of that at pH 7 [26,27]. According to this report, it can be deduced that rate of DAF-2T formation at pH 6 was comparable to or larger than that of DAF-2T formation at pH 7. These results suggest that nitrite-dependent formation of reactive nitrogen species from NO can proceed at sites in the oral cavity where the local pHs were below 7.

Soluble salivary components suppressed the increase in fluorescence due to the formation of DAF-2T (Figure 2). Salivary redox components such as ascorbate, glutathione, uric acid and thiocyanate also suppressed the increase in fluorescence without affecting NO production (Figure 4). This result suggests that salivary redox components can scavenge reactive nitrogen species formed from NO since DAF-2T is formed by reactions between DAF-2 and reactive nitrogen species as described above. As a possible mechanism for the scavenging, reduction of reactive nitrogen species like NO_2 and N_2O_3 by the redox components is possible (Figure 8). It has been reported that reduction potential of NO_2/NO_2^- is 0.99 v [28]. If NO_2 is reduced to nitrite by salivary redox components, formation of N_2O_3, which may participate in the transformation of DAF-2 to DAF-2T [9], can be inhibited because N_2O_3 is formed from NO and NO_2 [6,9,28]. The redox components used in this study are present in human saliva in the following concentrations: ascorbate, $< 20\,\mu M$; glutathione, $1-5\,\mu M$; uric acid, $80-280\,\mu M$; SCN^-, $0.3-2$ mM [1,11,20,21]. According to the concentrations of these

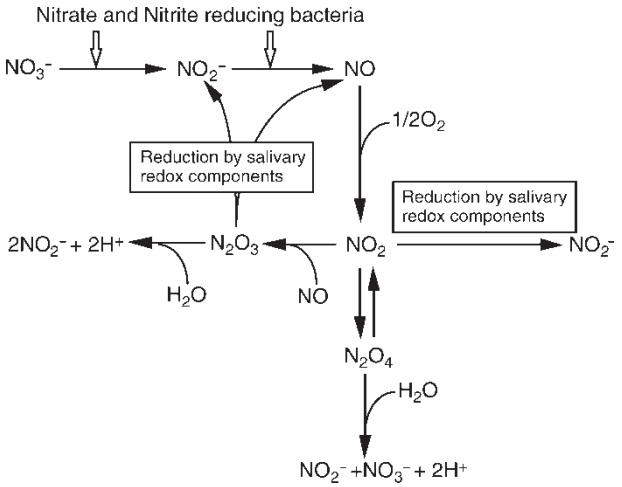

Figure 8. Possible mechanisms of production of reactive nitrogen species and their scavenging in the oral cavity. Nitrate and nitrite are reduced to NO by certain bacteria in the oral cavity. Reactive nitrogen species generated can be scavenged by redox components in the oral cavity such as ascorbate, glutathione, thiocyanate and uric acid.

reagents required for 50% inhibition of DAF-2T formation, the concentrations of uric acid and thiocyanate in saliva are high enough to effectively scavenge reactive nitrogen species. Fluorescence of DAF-2T did not decrease when the above redox components were added to a reaction mixture after a sufficient amount of DAF-2T had been formed. This result excludes the possibility that the suppression of fluorescence increase was due to chemical reactions between DAF-2T and the redox components.

Fluorescence increase was observed in filtered saliva-II without addition of nitrite although the rate in increase was slow, and the fluorescence increase was enhanced by the addition of nitrite (Figure 6). This result suggests that not only nitrite contained in filtered saliva-II itself but also nitrite added could be reduced to NO by nitrite-reducing bacteria in the saliva. The fluorescent component formed in filtered saliva-II was the same as that formed in bacterial fractions (Figure 7). These results suggest that nitrite-dependent formation of reactive nitrogen species can continuously proceed in the oral cavity in spite of the presence of scavengers of reactive nitrogen species such as ascorbate, glutathione, thiocyanate and uric acid. Activity of fluorescence increase also increased gradually in filtered saliva-II after toothbrushing independent of the presence and absence of added nitrite (Figure 6). This may be due to the increase in population of NO producing bacteria, but another possibility for the increase in rate is considered. That is the gradual increase in concentration of nitrite in saliva after toothbrushing, but this possibility is excluded from the result that there were no relations between rate of DAF-2T formation and concentration of nitrite in filtered saliva-II; concentration of nitrite in

saliva increased for the initial 1 h and then decreased gradually as a function of time after toothbrushing (data not shown). The changes in concentration may reflect the changes in the activities of nitrate and nitrite reduction in the oral cavity.

It is known that thiocyanate can be oxidized to $OSCN^-$ by salivary peroxidase in the presence of hydrogen peroxide. The function of $OSCN^-$ in the oral cavity has been discussed in relation to the antimicrobial activity [29]. From the present study, another function of SCN^- in the oral cavity as a scavenger of reactive nitrogen species emerged. Thiocyanate has been suggested to inhibit nitration by scavenging reactive nitrogen species like NO_2 which is formed by peroxidase-dependent reactions [30,31] and acidified nitrite [32]. If the significance of NO formed in the oral cavity can be attributed to its antimicrobial function [3,5], the scavenging of reactive nitrogen species formed from NO in the saliva is a premise for the the antimicrobial function of NO because reactive nitrogen species formed from NO can give damages to oral tissues through nitration, nitrosation, etc. [7,8]. It is concluded from the present study that salivary redox components can scavenge reactive nitrogen species formed from NO but the scavenging is incomplete. According to the above conclusion, it can be deduced that tissues of oral cavity may be damaged when (i) NO production is enhanced by increased population of nitrite-reducing bacteria, (ii) salivary concentration of nitrite is increased, (iii) concentrations of salivary reductants are decreased, or (iv) above two or three events occur at the same. Figure 8 summarizes possible reactions in the oral cavity which may proceed after reduction of nitrate and nitrite to NO.

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