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Chlorogenic Acid in Coffee Can Prevent the Formation of Dinitrogen Trioxide by Scavenging Nitrogen Dioxide Generated in the Human Oral Cavity

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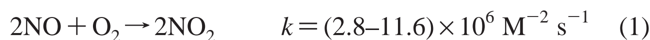
Coffee contains antioxidants like chlorogenic acid and its isomers. In this report, effects of coffee on the nitrite-induced N_2O_3 formation were studied using whole saliva and bacterial fraction prepared from the saliva. The formation of N_2O_3 was measured by fluorescence increase due to the transformation of 4,5-diaminofluorescein to triazolfluorescein. Coffee inhibited the nitrite-induced fluorescence increase, and 50% inhibition was observed at several μg of coffee/mL in bacterial fraction of saliva as well as whole saliva. During the inhibition of the fluorescence increase, concentration of chlorogenic acid and its isomers decreased. It is discussed that the reduction of NO_2 by chlorogenic acid and its isomers contributed to the coffee-dependent inhibition of the fluorescence increase as N_2O_3 is formed from NO and NO_2. When coffee was added to whole saliva, chlorogenic acid and its isomers bound to cells in the saliva. The rate of the fluorescence increase in bacterial fraction, which was prepared at defined periods after the ingestion of coffee, was increased to the rate before the ingestion of coffee with a half-time of about 1 h. This result suggests that chlorogenic acid and its isomers remained in the oral cavity for a few hours after ingestion of coffee. The significance of coffee drinking and rinsing of the mouth with coffee for the health of the oral cavity is proposed.

KEYWORDS: Chlorogenic acid; coffee; 4,5-diaminofluorescein; human saliva; NO_2; N_2O_3

INTRODUCTION

Nitrate is ingested as a food component. The nitrate ingested is absorbed from intestine into the bloodstream and then secreted into the oral cavity as a component of saliva. The nitrate secreted is reduced to nitrite by certain bacteria, and the nitrite formed is reduced further to nitric oxide (NO) in the cavity (*1–3*). If NO is formed in the oral cavity, the compound can react with molecular oxygen producing NO_2 and N_2O_3.



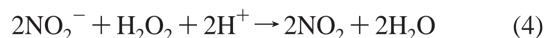
$$2NO + O_2 \rightarrow 2NO_2 \qquad k = (2.8\text{–}11.6) \times 10^6\ M^{-2}\ s^{-1} \quad (1)$$

$$NO_2 + NO \rightarrow N_2O_3 \qquad k = 1.1 \times 10^9\ M^{-1}\ s^{-1} \quad (2)$$

It is known that NO_2 is an oxidizing and nitrating agent and that N_2O_3 is a nitrosating agent (*4*). In addition to NO, O_2^- is also formed by bacteria and leukocytes in the human oral cavity (*5*). O_2^- can react with NO producing peroxynitrite ($ONOO^-$) ($pK_a = 6.8$) (*4*)

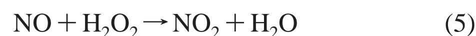
$$O_2^- + NO \rightarrow ONOO^- \qquad k = 9 \times 10^9\ M^{-1}\ s^{-1} \quad (3)$$

Peroxynitrite is also an oxidizing and nitrating agent (*4*). On the other hand, in human saliva, myeloperoxidase as well as salivary peroxidase is present (*5–8*). The peroxidases can catalyze the oxidation of nitrite, producing NO_2 or its equivalent in the presence of H_2O_2 (*9–13*) that is formed from O_2^-



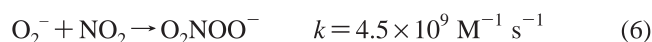
$$2NO_2^- + H_2O_2 + 2H^+ \rightarrow 2NO_2 + 2H_2O \quad (4)$$

In addition, a peroxidase can catalyze the oxidation of NO to NO_2 or its equivalent (*14*)



$$NO + H_2O_2 \rightarrow NO_2 + H_2O \quad (5)$$

When O_2^- is formed during the formation of NO_2, NO_2 reacts with O_2^-



$$O_2^- + NO_2 \rightarrow O_2NOO^- \qquad k = 4.5 \times 10^9\ M^{-1}\ s^{-1} \quad (6)$$

producing peroxynitrate (O_2NOO^-) ($pK_a = 5.9$) (*15, 16*).

It has been postulated that the production of reactive oxygen and nitrogen species in the human oral cavity is related to periodontal diseases like periodontitis and alveolar abscess (*17–22*).

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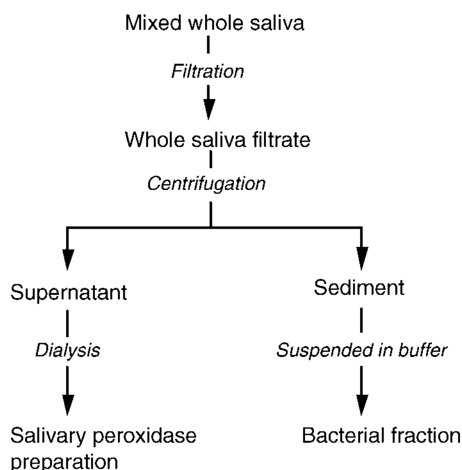

Figure 1. Preparation of various fractions from mixed whole saliva.

Therefore, suppression of the formation of reactive oxygen and nitrogen species and scavenging of the reactive species seem to be important to protect the oral cavity from periodontal diseases. In coffee, chlorogenic acid and its isomers are contained as major phenolics. Chlorogenic acid and its isomers are known to be antioxidants (*23, 24*). In addition to chlorogenic acid and its isomers, other antioxidants including melanoidins, Zn-binding substance, and hydroxycinnamic acids like caffeic, ferulic, and *p*-coumaric acids are also contained in coffee (*25–27*). If the antioxidants scavenge reactive nitrogen species in the oral cavity, coffee drinking may be helpful to prevent oral diseases, in which reactive nitrogen species participate.

It has been reported that N_2O_3 generated in the human oral cavity can react with 4,5-diaminofluorescein (DAF-2), transforming it to a fluorescent component triazolfluorescein (DAF-2T) using the whole saliva and the bacterial fraction (*28–30*). If NO_2 is scavenged by antioxidants, the formation of N_2O_3 by reaction 2 is inhibited in NO generating systems. The formation of N_2O_3 can also be inhibited by O_2^- because O_2^- reacts with NO and NO_2 (reactions 3 and 6). Therefore, the formation of N_2O_3 is enhanced when O_2^- is scavenged by superoxide dismutase (SOD). On the other hand, the formation of N_2O_3 is enhanced when peroxidases oxidize nitrite and NO to NO_2 by reactions 4 and 5, and the enhancement is suppressed when peroxidase-catalyzed formation of NO_2 or its equivalent is inhibited by a physiological substrate of salivary peroxidase SCN^-. In this paper, it is shown that chlorogenic acid and its isomers were participated in the coffee-dependent scavenging of NO_2 formed in mixed whole saliva.

MATERIALS AND METHODS

Reagents. DAF-2 and aminophenyl fluorescein (APF) were obtained from Daiichi Pure Chemicals (Tokyo, Japan). Chlorogenic acid, Griess-Romijn reagent for nitrite, SOD from bovine erythrocytes, and fluorescein were from Wako Pure Chem. Ind. (Osaka, Japan). (±)-(*E*)-4-Ethyl-2-[(*E*)-hydroxyimino]-5-nitrohexanamide (NOR 3) was from Dojin (Kumamoto, Japan). Instant coffee (freeze-dried) was obtained from a local market. Coffee was dissolved in hot water at concentrations of 1 or 10 mg of coffee/mL and used for experiments.

Preparation of Saliva. Mixed whole saliva (5 mL) was collected at 9–10 A.M. from three healthy volunteers for each experiment by chewing parafilm after their informed consent had been obtained, and the pH values ranged from 7.2 to 7.6. The saliva from each volunteer was 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 whole saliva filtrate (**Figure 1**). Concentrations of nitrite and SCN^- in whole saliva filtrates were determined as described previously using the Griess-Romijn reagent for nitrite and acidic Fe(III), respectively (*31*). The filtrate was centrifuged at 20000*g* for 5 min, and the sediment was suspended in 5 mL of 50 mM sodium phosphate buffer (pH 7.0). The suspension contained bacteria with some contamination of leukocytes when observed using a microscope and was used as bacterial fraction (**Figure 1**). The supernatant obtained by the centrifugation was dialyzed against 1 L of 10 mM sodium phosphate buffer (pH 7.0) for 1 night at 4 °C. After centrifugation of the dialyzed saliva at 20000*g* for 5 min, the supernatant was used as salivary peroxidase preparation (**Figure 1**) to investigate H_2O_2-induced oxidation of chlorogenic acid.

Bacterial fraction of saliva was also prepared from saliva that had been collected after the ingestion of 0.5 g of freeze-dried instant coffee dissolved in 100 mL of hot water. Coffee was ingested at 10 A.M., and mixed whole saliva (about 5 mL) was collected immediately and every hour after the ingestion by chewing parafilm. Bacterial fraction was prepared as described above. One hour before the coffee ingestion, saliva had been collected to prepare bacterial fraction to be used as control. The subjects did not take breakfast, and no food and beverages but water were taken during the experiments.

Measurements of Fluorescence. Transformation of DAF-2 to a fluorescent component DAF-2T was measured at about 25 °C using a spectrofluorometer (RF-550, Shimadzu, Kyoto, Japan), as described previously (*28, 32*). The reaction mixture contained 10 μM DAF-2 in 0.5 mL of bacterial fraction when bacterial fraction was used. When whole saliva filtrate was used, the reaction mixture contained 10 μM DAF-2 in the mixture of 0.25 mL of whole saliva filtrate and 0.25 mL of 0.1 M sodium phosphate buffer (pH 7.0). The reason that whole saliva filtrate was mixed with the buffer solution was to prevent the increase in pH due to the decrease in concentration of carbon dioxide and to decrease the viscosity. The excitation and emission wavelengths were 485 and 538 nm, respectively. 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). Coffee was added to the above reaction mixtures after incubation for about 12 min. The effect was calculated using rates of fluorescence increase before and after the addition of coffee.

Oxidation of APF was measured in the reaction mixture that contained 10 μM APF in 0.5 mL bacterial fraction with 0.2 mM $NaNO_2$. APF is oxidized by strong oxidants like $ONOO^-$, $^{\bullet}OH$, and OCl^- and horseradish peroxidase/H_2O_2 (*33*). Accompanying the oxidation, the aminophenyl group of APF was removed, generating fluorescein (*33*). The excitation and emission wavelengths were 490 and 515 nm, respectively. The excitation light was passed through two filters described above. After incubation of the above reaction mixture for ~12 min, coffee and other reagents were added to study their effects on the fluorescence increase. To identify the oxidation product of APF, 0.5 mL of methanol was added to the reaction mixture which was incubated for 12 min and then filtered with a cellulose acetate filter (0.45 μm; Advantec, Tokyo, Japan). The filtrate was analyzed by HPLC as described below.

Measurements of Concentrations of Chlorogenic Acid and Its Isomers. Whole saliva contained detached epithelial cells and bacteria of the oral cavity. To study whether chlorogenic acid and its isomers in coffee bind to the cells, various amounts of coffee (10 mg/mL) were added to 0.5 mL of mixed whole saliva that was not passed through nylon filter nets. After incubation for 10–15 s, the saliva was centrifuged at 6500*g* for 5 min. The pH of the supernatant was decreased to 3–3.5 by adding 1 M HCl to precipitate proteins and then filtered with a cellulose acetate filter (0.45 μm). An aliquot (0.05 mL) of the filtrate was applied to an HPLC column to quantify chlorogenic acid and its isomers. The sediment was suspended in 0.5 mL of methanol and then centrifuged at 6500*g* for 5 min. An aliquot (0.05 mL) of the supernatant was also applied to an HPLC column to determine the concentrations of chlorogenic acid and its isomers. The concentrations of chlorogenic acid and its isomers in the precipitate were calculated using the volume of the precipitate. HPLC was performed as described below.

Decreases in the concentrations of chlorogenic acid and its isomers in bacterial fraction were measured as follows. After incubation of bacterial fraction (0.5 mL), which contained coffee at the concentration of 2 μg/mL, for defined periods under various conditions, the reaction

mixture was filtered with the membrane filter (0.45 μm), and then an aliquot (0.05 mL) of the filtrate was applied to an HPLC column to quantify chlorogenic acid. The decreases in concentrations of chlorogenic acid and its isomers in whole saliva filtrate were measured as follows. The reaction mixture (0.5 mL) contained 1 μg of coffee and various concentrations of $NaNO_2$ in a mixture of 0.25 mL of saliva filtrate and 0.25 mL of 50 mM sodium phosphate buffer (pH 7.0). After incubation for 30 min, pH of the reaction mixture was adjusted to 3–3.5 by adding 1 M HCl. The acidic solution was filtrated using the membrane filter (0.45 μm), and an aliquot (0.05 mL) of the filtrate was used to quantify chlorogenic acid and its isomers by HPLC.

HPLC and Spectrophotometric Measurements. HPLC was performed using a Shim-pack CLC-ODS column (15 cm × 6 mm i.d.) (Shimadzu, Kyoto, Japan). Chlorogenic acid and its isomers separated by the column were detected at 320 nm using a spectrophotometric detector with a photodiode array (SPD-M10Avp, Shimadzu), and their concentrations were determined using their peak areas. The mobile phase used was a mixture of methanol and 25 mM KH_2PO_4 (1:4, v/v), and the flow rate was 1 mL/min.

DAF-2T formed from DAF-2 was separated by HPLC as reported previously (*28*). Fluorescein formed by the oxidation of APF was separated using a Shim-pack CLC-ODS column and detected with a RF-550 spectrofluorometer. The excitation and emission wavelengths were 490 and 515 nm, respectively. The mobile phase was a mixture of methanol and 10 mM sodium phosphate (pH 7.0) (1:2, v/v), and the flow rate was 1 mL/min.

Oxidation of chlorogenic acid by salivary peroxidase preparation was measured at 320 nm with a 557 spectrophotometer (Hitachi, Tokyo, Japan). The reaction mixture contained 0.1 mM H_2O_2 and 0.05 mM chlorogenic acid in the mixture of 0.1 mL of salivary peroxidase preparation and 0.9 mL of 50 mM sodium phosphate buffer (pH 7.0). When required, 0.25 mM $NaNO_2$ was added. Reactions were started by the addition of H_2O_2.

RESULTS

Inhibition of DAF-2T Formation by Coffee. Figure 2 (upper panel) shows typical time courses of changes in fluorescence intensity in bacterial fraction in the presence of DAF-2. No clear increase in fluorescence was observed when DAF-2 was added to bacterial fraction. The addition of nitrite in the presence of DAF-2 resulted in the increase in fluorescence due to the formation of DAF-2T, and its formation was confirmed by HPLC (*28*). The rate of the formation of DAF-2T ranged from 0.5 to 2.1 μM per 30 min (average = 1.25 μM) in the presence of 10 μM DAF-2 (*30*). The fluorescence increase was inhibited by coffee. Half-maximal inhibition was observed at about 2.5 μg of coffee/mL (**Figure 2**, lower panel). Almost all the fluorescence increase was inhibited at 20 μg of coffee/mL. Chlorogenic acid (0.5–4 μM) nearly completely inhibited the fluorescence increase, as reported previously (*29*). Glutathione (6.6 μM) inhibited the fluorescence increase about 40%. Such inhibition was also observed in the presence of 2 μg of coffee/mL.

Effects of coffee on the fluorescence increase were also studied in the presence of SOD and/or SCN^- (**Table 1**). Coffee inhibited the fluorescence increase independent of the presence and absence of SOD and/or SCN^-. SOD enhanced and SCN^- inhibited the fluorescence increase in the absence of coffee as reported previously (*29, 30*). The effect of SOD was not significant in the presence of SCN^-. In the presence of 2 and 10 μg of coffee/mL, SCN^- inhibited the fluorescence increase, but the effects of SOD were insignificant.

Oxidation of APF. APF can be oxidized by strong oxidants ($ONOO^-$, $^{\bullet}OH$, and OCl^- and horseradish peroxidase/H_2O_2) but not by O_2^-, NO, and N_2O_3 (*33*). **Figure 3** shows typical time courses of fluorescence increase due to the oxidation of APF in bacterial fraction. When APF was added to the fraction,

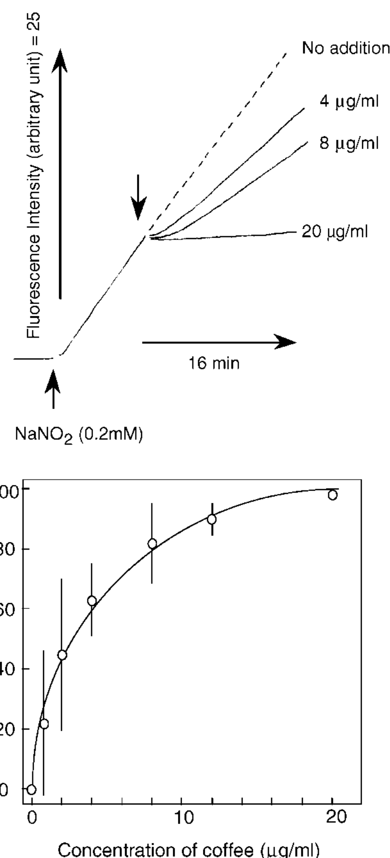

Figure 2. Inhibition of DAF-2T formation by coffee in bacterial fraction. Upper panel: time courses of fluorescence increase. Upward arrow, addition of 0.2 mM $NaNO_2$; downward arrow, addition of various amounts of coffee as indicated in the figure. Lower panel: inhibition of DAF-2T formation as a function of concentration of coffee. Values are means ± SDs ($n = 4$; one or two saliva preparations from three persons).

Table 1. Inhibition of Fluorescence Increase by Coffee in the Presence of SOD and/or SCN^- [a]

addition	relative rate of fluorescence increase		
	0	2	10 (μg of coffee/mL)
no addition	100	42 ± 19	6 ± 3
SOD (34 units/mL)	183 ± 56[b]	51 ± 11	10 ± 6
NaSCN (1 mM)	29 ± 9[b,c]	4 ± 5[b,c]	2 ± 2[b,c]
SOD + NaSCN	40 ± 20[b,c]	4 ± 2[b,c]	1 ± 1[b,c]

[a] Reactions were started by the addition of 0.2 mM $NaNO_2$ in the presence of 10 μM DAF-2 in bacterial fraction. After incubation for about 10 min, SOD and/or NaSCN were added, and then 1 and 5 μg of coffee were added successively. Rates of fluorescence increases are indicated relative to those of fluorescence increase before the addition of reagents or coffee. Each data represents average ± SD ($n = 6$–8; three persons, two or three saliva preparations from each person). [b] $p < 0.05$, compared with no addition. [c] $p < 0.05$, compared with the addition of SOD (34 units/mL).

no significant fluorescence increase was observed (each trace before the addition of nitrite). The fluorescence increase was greatly enhanced by nitrite and fluorescein (retention time, 17 min) was detected as a sole fluorescent compound by HPLC. The oxidation of APF to fluorescein was also observed when 10 μM APF was incubated with 5–20 μM NOR 3, an NO generating reagent (*28, 30*), in 50 mM sodium phosphate buffer (pH 7.0), and the fluorescent product was fluorescein (data not shown). No fluorescence increase was observed when 10 μM APF and 0.2 mM nitrite were incubated in the above buffer.

SOD did not significantly affect the fluorescence increase (trace A), but SCN^- inhibited the increase (trace B). The

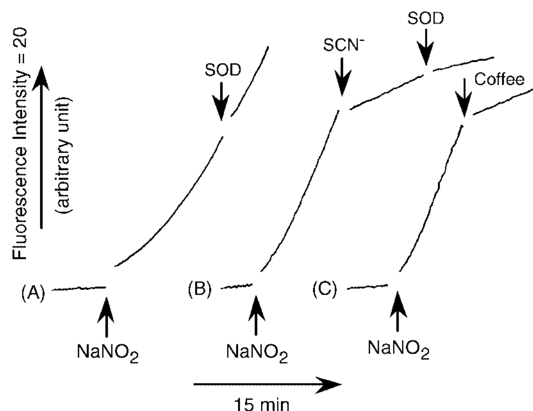

Figure 3. Oxidation of APF by bacterial fraction. Upward arrows, addition of 0.2 mM $NaNO_2$; downward arrows, addition of SOD (34 units/mL), SCN^- (1 mM), and coffee (20 μg/mL).

inhibitory effect of SOD became clear in the presence of SCN^- (trace B). Coffee (20 μg/mL) inhibited nitrite-induced oxidation of APF (trace C).

Content of Chlorogenic Acid and Its Isomers in Coffee. The amounts of chlorogenic acid and its isomers in instant coffee used in this study were determined by HPLC. Chlorogenic acid was detected at a retention time of about 6.5 min, and the absorption spectrum had peaks at 219, 235 (shoulder), and 324 nm in the mobile phase. The values were identical with those of standard chlorogenic acid. In addition, two other components with retention times of 5.9 and 10.6 min were also separated, and the absorption spectra of these components were identical with that of chlorogenic acid. We tentatively identified that the two components were isomers of chlorogenic acid (3- and 4-caffeoylquinic acids). The amount of chlorogenic acid in this instant coffee was estimated to be about 9.2 mg/g of coffee. Amounts of the isomers were estimated to be about 6.8 and 5.9 mg/g of coffee, assuming that the molar extinction coefficient of chlorogenic acid was the same as those of the isomers. The values were averages of two experiments and were in ranges that had been reported in the literature (*34*). From the amounts of chlorogenic acid and its isomers, molar concentration of chlorogenic acid plus its isomers in 10 μg of coffee/mL was calculated to be equivalent to about 0.6 μM chlorogenic acid.

Oxidation of Chlorogenic Acid. Changes in concentration of chlorogenic acid in coffee were measured in bacterial fraction (**Figure 4**). The concentration of chlorogenic acid decreased in bacterial fraction, and decrease in the concentration was inhibited by SCN^-. Nitrite (0.2 mM) enhanced the decrease in concentration and SCN^- also inhibited the decrease in the presence of nitrite. No clear effects of SOD (34 units/mL) were observed on the oxidation of chlorogenic acid independent of the presence and absence of nitrite (data not shown). Concentrations of isomers of chlorogenic acid also decreased as chlorogenic acid. Assuming that the antioxidative activity of chlorogenic acid was similar to those of the isomers (*35, 36*), the rate of the decrease in concentration of chlorogenic acid and its isomers was calculated using the data in **Figure 4**. The value in the presence of nitrite was equivalent to about 0.1 μM chlorogenic acid per 30 min in the presence of 2 μg of coffee/mL (equivalent to 0.12 μM chlorogenic acid).

Chlorogenic acid was oxidized by salivary peroxidase preparation (**Figure 5A**). Slow absorbance decrease was observed following initial rapid absorption decrease by the addition of H_2O_2 (trace 1). Although the effect was small, SCN^- (1 mM) inhibited the slow absorbance decrease (trace 2). The absorbance

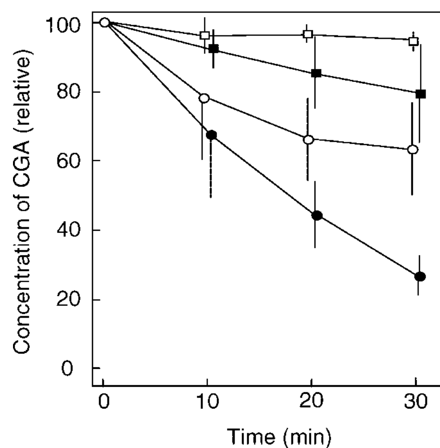

Figure 4. Time courses of decrease in concentration of chlorogenic acid in coffee in bacterial fraction: ■, no addition; ●, 0.2 mM $NaNO_2$; □, 1 mM NaSCN; ○, 0.2 mM $NaNO_2$ + 1 mM NaSCN. Values are means ± SDs (n = 3; three persons).

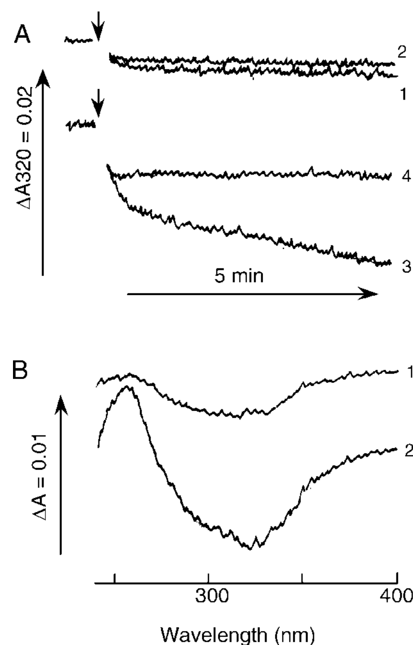

Figure 5. Oxidation of chlorogenic acid by the salivary peroxidase/H_2O_2 system. (**A**) Time courses of absorbance decrease at 320 nm. Trace 1, no addition; trace 2, 1 mM NaSCN; trace 3, 0.25 mM $NaNO_2$, trace 4, 0.25 mM $NaNO_2$ + 1 mM NaSCN. Downward arrows, addition of 0.1 mM H_2O_2. (**B**) Difference spectra. Trace 1, no addition; trace 2, 0.25 mM $NaNO_2$. Scanning was started 1 min after the addition of 0.1 mM H_2O_2 from 400 to 240 nm at a speed of 120 nm/min.

decrease induced by H_2O_2 was enhanced by nitrite (trace 3). SCN^- (1 mM) inhibited the initial rapid absorption decrease by about 50% and completely inhibited the slow absorbance decrease (trace 4). The difference spectra before and after the addition of H_2O_2 show that the absorbance decreased around 320 nm independent of the presence and absence of nitrite (**Figure 5B**).

We investigated the products formed from chlorogenic acid and its isomers under the conditions of **Figure 4**, but no quinones and no nitrated compounds of chlorogenic acid and its isomers (*37–39*) were detected by HPLC after 10–30 min of incubation. Neither quinone nor nitrated compound of chlorogenic acid was detected even when chlorogenic acid was incubated with nitrite in bacterial fraction. When changes in absorption spectrum of coffee were measured in bacterial

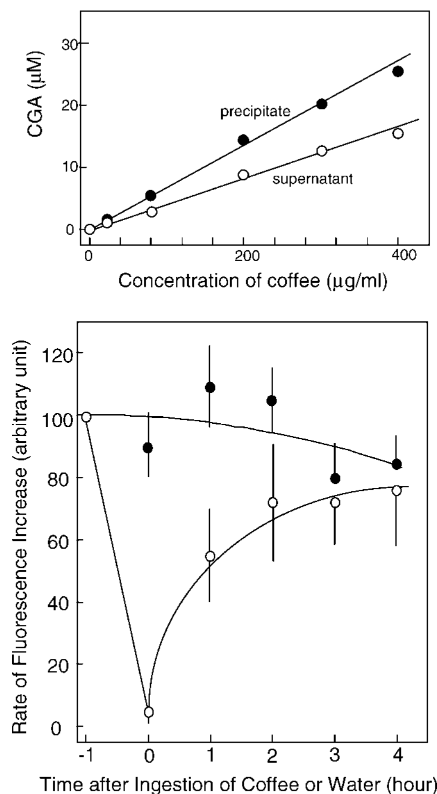

Figure 6. Binding of chlorogenic acid in coffee to cells in whole saliva and DAF-2T formation in bacterial fraction prepared after coffee drinking. Upper panel: Binding of CGA. ○, supernatant; ●, precipitate. Each data point represents averages of two experiments. Lower panel: DAF-2T formation. Bacterial fraction was prepared at the time indicated before and after coffee (○) or water (●) ingestion, and rate of DAF-2T formation was measured. –1, 1 h before coffee or water ingestion; 0, immediately after coffee or water ingestion. Each data point shows mean ± SE ($n = 6$; three persons, two saliva preparations from each person).

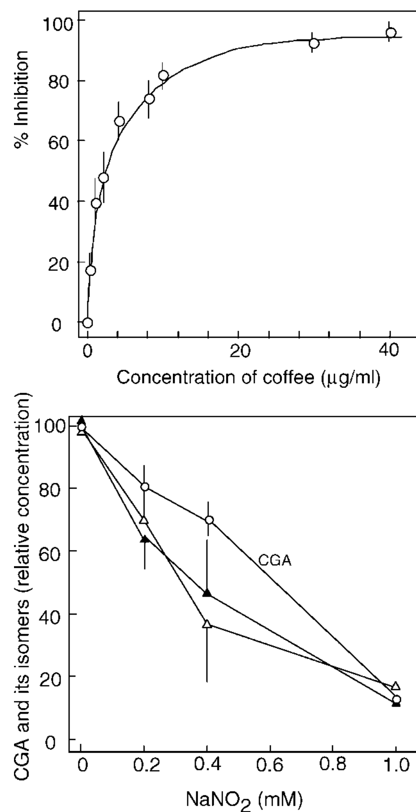

Figure 7. Inhibition of DAF-2T formation by coffee and oxidation of chlorogenic acid and its isomers in whole saliva filtrate. Upper panel: inhibition of fluorescence increase by coffee. Lower panel: oxidation of chlorogenic acid and its isomers in coffee. ○, chlorogenic acid; and △ and ▲, isomers of chlorogenic acid. The concentrations of nitrite and SCN^- contained in the saliva used in the upper and lower experiments were 0.15–0.25 and 0.5–1 mM, respectively. Each data point represents average ± SD ($n = 3$, three persons).

fraction in the presence of nitrite, absorption increased in the wavelength range from 400 to 600 nm accompanying the absorption decrease around 320 nm (data not shown). This result suggests that polymers or melanoidin-like compounds were formed from chlorogenic acid and its isomers during the incubation of coffee in bacterial fraction in the presence of nitrite.

Binding of Chlorogenic Acid and Its Isomers to Cells. The concentration of chlorogenic acid in the precipitate of whole saliva was about 1.5-fold of that in the supernatant independent of the amount of coffee added (**Figure 6**, upper panel). Isomers of chlorogenic acid also bound to the cells as chlorogenic acid did (data not shown).

Bacterial fraction was prepared as a function of time after coffee ingestion and nitrite-induced fluorescence increase was measured using the bacterial fraction in the presence of DAF-2 (**Figure 6**, lower panel). The rate of the fluorescence increase in bacterial fraction, which was prepared immediately after coffee ingestion, was quite slow. In bacterial fraction that was prepared 1 h after the ingestion of coffee, the rate of the fluorescence increase was about half of the fluorescence increase that was observed in bacterial fraction prepared before coffee ingestion. When 100 mL of water was ingested instead of coffee, no significant effects of water drinking were observed.

Inhibition of DAF-2T Formation by Coffee in Whole Saliva Filtrate. The rate of the fluorescence increase due to the formation of DAF-2T in whole saliva filtrate was 5–10% of that in bacterial fraction. The fluorescence increase was suppressed by coffee, and 50% inhibition was observed at about 3 μg of coffee/mL (**Figure 7**, upper panel). The concentrations of chlorogenic acid and its isomers decreased in the reaction mixture in the presence of 1 μg of coffee/mL (equivalent to 0.06 μM chlorogenic acid) (**Figure 7**, lower panel). The decrease was dependent on the concentration of nitrite added, and the decrease in chlorogenic acid plus its isomers in the presence of 1 mM $NaNO_2$ was calculated to be equivalent to about 0.054 μM chlorogenic acid per 30 min.

DISCUSSION

The fluorescence increase in bacterial fraction of saliva in the presence of DAF-2 is mainly due to N_2O_3-dependent formation of DAF-2T (*28–30*). As N_2O_3 is produced by reaction 2 in NO generating systems, coffee-dependent inhibition of the fluorescence increase can be explained by scavenging of NO, NO_2, or N_2O_3 by the components of coffee. Among the nitrogen oxides, NO_2 (NO_2/NO_2^-, $E^{\circ\prime} = 0.99$ V) is a candidate to be scavenged by the components of coffee. The scavenging of NO and N_2O_3 by the components of coffee might not be so significant since these nitrogen oxides are less oxidative than NO_2. As chlorogenic acid (0.2 μM) inhibits the formation of DAF-2T by about 80% in bacterial fraction (*29*), chlorogenic acid and its isomers in coffee may also inhibit the formation of DAF-2T by scavenging NO_2. Coffee (2.5 μg/mL), which was equivalent to about 0.15 μM chlorogenic acid, inhibited the

formation of DAF-2T by about 50% in bacterial fraction. The result suggests that chlorogenic acid and its isomers in coffee mainly participated in the scavenging of NO_2 to inhibit the formation of N_2O_3 in bacterial fraction. The main participation is deduced from the fact that chlorogenic acid and its isomers were the main phenolics in coffee. In addition to phenolics, contribution of melanoidins and other substances to the scavenging of NO_2 could not be excluded at present.

SOD enhanced the formation of DAF-2T (**Table 1**). The enhancement could be explained by scavenging of O_2^-, which reacted with NO, producing $ONOO^-$ (reaction 3) (*30, 40, 41*). The reaction between O_2^- and NO_2 (reaction 6) producing O_2NOO^- might also contribute to the SOD-dependent enhancement of the formation of DAF-2T. There was large deviation on the enhancement by SOD. The deviation can be explained by the deviation of rates of the formation of NO and O_2^-. The formation of O_2^- affects the formation of H_2O_2 and then formation of NO_2 by peroxidase/nitrite systems, successively.

SCN^- inhibited the formation of DAF-2T (**Table 1**). The inhibition could be explained by SCN^--dependent inhibition of the oxidation of nitrite and NO to NO_2 or its equivalent by peroxidases by reactions 4 and 5, respectively, as discussed previously (*30*). From the degree of the inhibition by SCN^- in the presence and absence of SOD, it could be estimated that 70–80% of the formation of DAF-2T was due to N_2O_3 that was produced by the reaction between NO generated by bacteria and NO_2 or its equivalent generated by peroxidase/H_2O_2/(nitrite plus NO) systems. The participation of salivary peroxidase in the formation of DAF-2T is supported by the facts (i) that SCN^- is a physiological substrate of peroxidases in saliva (*5*), (ii) that peroxidases in saliva bind to bacteria in the oral cavity (*5, 30*), and (iii) that bacterial fraction produces H_2O_2 (*30*).

SOD seemed to enhance the formation of DAF-2T in the presence of SCN^-, but its effect was not significant (**Table 1**). This result may suggest that the reaction between NO and O_2^- did not significantly affect the formation of N_2O_3. If one takes this assumption into consideration, it can be deduced that a large part of SOD-dependent enhancement of DAF-2T formation was due to the inhibition of the reaction between O_2^- and NO_2 or its equivalent. The inhibition of DAF-2T formation by coffee in the presence of SCN^- (**Table 1**) suggests that the antioxidants in coffee could also scavenge NO_2 or its equivalent formed by salivary peroxidase-independent reactions.

APF was oxidized quite slowly in bacterial fraction, and nitrite greatly enhanced the oxidation (**Figure 3**). The result suggests that direct oxidation of APF by salivary peroxidase using the H_2O_2 generated in bacterial fraction was very slow and that oxidants formed in the presence of nitrite oxidized APF. The oxidation of APF in bacterial fraction was inhibited by about 80% by 1 mM SCN^- (**Figure 3**). The inhibition suggests that NO_2 or its equivalent, which was formed by salivary peroxidase/H_2O_2/(nitrite and NO) systems, mainly participated in the oxidation of APF. The oxidation of APF by NO_2 is possible because $E^{\circ\prime}$ of HOCl/H_2O,Cl^- (1.1 V) is close to that of NO_2/NO_2^- (0.99 V). NOR 3-induced fluorescence increase in a buffer solution supports the participation of NO_2 in the oxidation of APF to fluorescein.

SOD did not clearly inhibit the oxidation of APF in the absence of SCN^- but inhibited it in the presence of SCN^-. The inhibition in the presence of SCN^- supports that $ONOO^-$ was formed slowly in the bacterial fraction in the presence of nitrite as discussed above. The slower formation of $ONOO^-$ compared with the formation of NO_2 might result in the failure of observation of the effect of SOD on the oxidation of APF in the absence of SCN^-. Although O_2NOO^- seemed to be produced in bacterial fraction, further studies were required to make clear whether O_2NOO^- participated in the oxidation of APF. O_2NOO^- is known to be in equilibrium with $NO_2 + O_2^-$ and to decompose to $NO_2^- + O_2$ (*15*). 20 μg of coffee/mL, which was equivalent to 1.2 μM chlorogenic acid, also inhibited the oxidation of APF by about 80%. This result may support the scavenging of NO_2 or its equivalent by chlorogenic acid and its isomers. In summary, the data in **Figures 2** and **3** and **Table 1** indicate (i) that NO_2 or its equivalent, which was formed by salivary peroxidase/H_2O_2/(nitrite and NO) systems, might be main reactive nitrogen species formed in the bacterial fraction, (ii) that N_2O_3 was mainly formed by the reaction between NO formed by bacteria and NO_2 formed by salivary peroxidase/H_2O_2/(nitrite and NO) systems, and (iii) that chlorogenic acid and its isomers in coffee could scavenge NO_2 or its equivalent even when SCN^- was present. Participation of $^{\bullet}OH$ and HOCl in the oxidation of APF was insignificant in this study because APF was not oxidized rapidly in the absence of nitrite and no Cl^- was added to the reaction mixture.

The decrease in concentration of chlorogenic acid in coffee was enhanced by nitrite and inhibited by SCN^- in bacterial fraction (**Figure 4**). The oxidation of chlorogenic acid by salivary peroxidase preparation in the presence of H_2O_2 was also enhanced by nitrite and inhibited by SCN^- (**Figure 5**). The enhancement and the inhibition suggest that NO_2 or its equivalent, which was formed by salivary peroxidase/H_2O_2/(nitrite and NO) systems, participated in the oxidation of chlorogenic acid. It has been reported that NO_2 can oxidize phenolic compounds (*30, 42–45*). In addition to NO_2, $ONOO^-$ is known to be able to oxidize phenolic compounds including chlorogenic acid (*23, 45–49*). The contribution of this oxidant to the oxidation of chlorogenic acid and its isomers in bacterial fraction seemed to be small because SOD did not affect nitrite-induced oxidation of chlorogenic acid in the fraction. Although it has been reported that O_2NOO^- cannot oxidize tyrosine (*16*), further studies are required on the reaction between O_2NOO^- and chlorogenic acid and its isomers.

A monophenol 4-hydroxyphenylacetic acid is nitrated by salivary peroxidase/H_2O_2/nitrite systems (*11*). No nitration of chlorogenic acid and its isomers was observed in this study. The failure of the detection may be explained by rapid transformation of the radicals of chlorogenic acid and its isomers to the *o*-quinones and the *o*-quinones to polymers that absorb visible light (*37*). It has been reported that *o*-quinones of phenolic compounds are unstable under neutral and alkaline conditions and stable under acidic conditions (*50, 51*).

When the formation of DAF-2T was measured using bacterial fraction prepared after coffee ingestion, the rate was faster in bacterial fraction that was prepared later after the ingestion (**Figure 6**, lower panel). This result suggests that antioxidants in coffee were present in bacterial fraction and that the antioxidants slowly removed from the oral cavity. Chlorogenic acid and its isomers are candidates for the antioxidants in the bacterial fraction as chlorogenic acid and its isomers could bind to cells in whole saliva (**Figure 6**, upper panel). If chlorogenic acid and its isomers in coffee remain in the oral cavity for a while after coffee drinking, the acids can function to scavenge NO_2 and $ONOO^-$. It has been reported that quercetin remains in the oral cavity for few hours (*42*).

In whole saliva filtrate, fluorescence increase due to the formation of DAF-2T was also observed although the rate was much slower than that in bacterial fraction. This result indicates that N_2O_3 was still formed in whole saliva filtrate. The slow

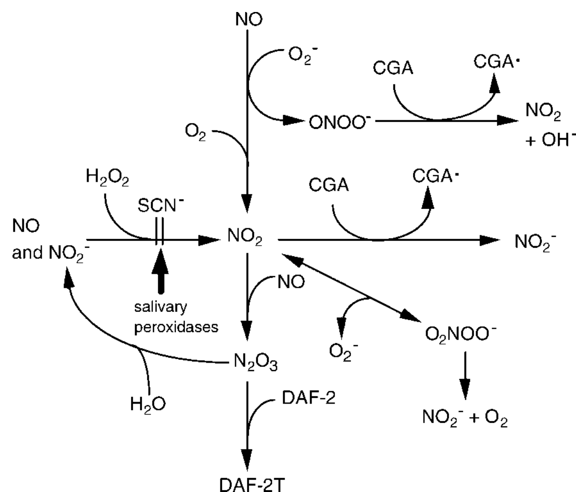

Figure 8. Possible reactions between chlorogenic acid and reactive nitrogen species. CGA, chlorogenic acid; CGA·, chlorogenic acid radical. ||, inhibition by SCN^-.

fluorescence increase might be due to the presence of SCN^- and other antioxidants in whole saliva filtrate (*28–30, 52*). Coffee inhibited the fluorescence increase, and chlorogenic acid and its isomers in coffee were oxidized in whole saliva filtrate (**Figure 7**). The concentration of coffee required for 50% inhibition was 3 μg of coffee/mL, and about 90% inhibition was observed at 20 μg of coffee/mL. This result suggests that chlorogenic acid and its isomers can also scavenge NO_2 and $ONOO^-$ in the oral cavity.

The present study suggests that chlorogenic acid and its isomers in coffee could function to scavenge NO_2 in the human oral cavity. Chlorogenic acid and its isomers seemed to be able to scavenge $ONOO^-$ if the oxidant was formed in the oral cavity. Possible reactions proceeding in bacterial fraction are summarized in **Figure 8**. The scavenging function of antioxidants may become important to protect the human oral cavity from oxidative damages when the production of NO_2 and $ONOO^-$ is enhanced. Antioxidative components of coffee, in which chlorogenic acid and its isomers are included, can remain in the oral cavity for a few hours after coffee ingestion (**Figure 6**), and chlorogenic acid can inhibit carcinogenesis in tongue (*53*). The result suggests that drinking of coffee or rinsing of the mouth with coffee may be helpful to protect oral tissues from oxidative damages induced by the reactive nitrogen species.

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