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Oxidation of Quercetin by Salivary Components II. Effects of Quercetin on Reactive Oxygen Metabolism by Salivary Polymorphonuclear Leukocytes

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Salivary polymorphonuclear leukocytes (SPMN) produce O_2^- and H_2O_2 reducing molecular oxygen, and quercetin is a phenolic compound found in vegetables and fruits. The aim of the present study was to elucidate the interaction between SPMN and quercetin. The oxygen consumption by SPMN, which was stimulated by phorbol myristate acetate (PMA), was suppressed by quercetin. Half-inhibition was observed at about 20 μM quercetin. During inhibition of the oxygen uptake, quercetin was oxidized; the oxidation was inhibited by superoxide dismutase and enhanced by horseradish peroxidase. Quercetin was taken up into SPMN and myeloperoxidase in SPMN could oxidize quercetin if H_2O_2 was provided. These results indicate that quercetin can inhibit the formation of O_2^- and H_2O_2 by PMA-stimulated SPMN and that the flavonol can scavenge O_2^- and H_2O_2 generated by the SPMN. Quercetin inhibited the growth of *Porphyromonas gingivalis* that might cause periodontal disease. According to the above results, quercetin seems able to suppress SPMN-dependent inflammation caused by reactive oxygen species inhibiting the growth of bacteria in the mouth.

Keywords: oxygen metabolism, quercetin, salivary polymorphonuclear leukocyte, scavenging of reactive oxygen species

We showed previously that when onion soup is ingested, quercetin glucosides in the soup are hydrolyzed to quercetin in the oral cavity to bind to the epithelial tissues, and that quercetin can be a hydrogen donor to peroxidase in saliva to scavenge H_2O_2 (Hirota *et al*., 2001). It was discussed then that the H_2O_2 scavenging function may be important when H_2O_2 production is enhanced. The enhancement of H_2O_2 production is possible by the stimulation of leukocytes in the oral cavity. H_2O_2 formation usually accompanies the formation of O_2^- and it is known that flavonol aglycones like kaempferol and quercetin reduce O_2^- to H_2O_2 (Takahama 1983, 1987; Bors *et al*., 1997).

Blackburn Jr. *et al*. (1987) reported that quercetin can inhibit O_2^- production, degranulation and phosphorylation of specific proteins, which are observed in plasma neutrophils when stimulated. The inhibition of O_2^- production may be in part due to inhibition of the NADPH oxidase system of leukocytes that is located on the cell membranes (Tauber *et al*., 1984). In addition, quercetin can also inhibit the activation of phospholipases that is induced on the stimulation of leukocytes (Takemura *et al*., 1997; Tordera *et al*., 1994). Berton *et al*. (1980) discussed that quercetin can regulate inflammatory processes taking account of quercetin-dependent inhibition of O_2^- production by stimulated leukocytes. It is known that some flavonoids including quercetin have an anti-inflammatory function (Pietta, 1997; Tordera *et al*., 1994). These reports using plasma leukocytes prompted us to study the effects of quercetin which can stay in the oral cavity for 4–5 h after ingestion of onion soup (Hirota *et al*., 2001), on the stimulation of salivary polymorphonuclear leukocytes (SPMN) and on the scavenging of O_2^- and H_2O_2 that are formed by SPMN. In addition, as some phenolics might decrease the risk of caries (Haslam, 1998), the effects of quercetin on bacteria which cause caries and periodontal diseases were also studied. The results obtained in this study are quercetin-dependent inactivation of SPMN, scavenging of O_2^- and H_2O_2 generated by SPMN and inhibition of the growth of *P. gingivalis*. These results suggest that quercetin might suppress the growth of *P. gingivalis* attenuating the production of reactive oxygen species by stimulated SPMN in the oral cavity and tissues.

Materials and Methods

Reagents Quercetin and 3,4-dihydroxybenzoic acid were obtained from Wako Pure Chem. Ind. (Osaka). Superoxide dismutase (SOD) from bovine erythrocytes, myeloperoxidase from human leukocytes, horseradish peroxidase and phorbol myristate acetate (PMA) were from Sigma-Aldrich Japan (Tokyo).

Preparation of SPMN SPMN was prepared according to a previous report (Yamamoto *et al*., 1991) with a slight modification. The oral cavity of each donor (four healthy volunteers) was thoroughly washed with 10 ml of Krebs-Ringer-phosphate (KRP) solution for 30 s. This washing procedure was repeated ten times. The pooled oral washings (about 100 ml per person) were passed through 380-mesh nylon sheets (32 μm, Sansho, Tokyo) and SPMN was collected by centrifugation at 300×*g* for 5 min. After a washing with Ca^{2+}-free KRP solution by centrifugation, the resultant pellet was suspended in 1 ml of Ca^{2+}-free KRP solution for counting. The value was 2–7×10^6 SPMN ml^{-1}. The number of contamination of detached epithelial cells was

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Abbreviations: HPLC, high performance liquid chromatography; KRP, Krebs-Ringer-phosphate; PMA, phorbol myristate acetate; SPMN, salivary polymorphonuclear leukocyte; SOD, superoxide dismutase.

less than 1%.

Oxygen uptake and oxidation of quercetin Oxygen uptake by SPMN was measured at 35°C using an oxygen electrode obtained from Rank Brothers (Cambridge, UK.). The reaction mixture (1 ml) contained 0.8 ml of Ca^{2+}-free KRP solution, 0.2 ml of SPMN preparation and various concentrations of quercetin. Oxidation of quercetin by SPMN was measured at 35°C using a double-beam spectrophotometer (557, Hitachi, Tokyo). The reaction mixture (1 ml) contained 0.9 ml of Ca^{2+}-free KRP solution, 0.1 ml of SPMN preparation and various concentrations of quercetin.

HPLC Oxidation products of quercetin were analyzed by HPLC. Oxidation of quercetin by SPMN was terminated by adding 10 μl of 2.5 M HCl to the reaction mixture (1 ml) for the spectrophotometric measurement. The acidic solution was extracted with 2 ml of ethyl acetate three times. The ethyl acetate extracts were combined and ethyl acetate was evaporated with a rotary evaporator. The residue was dissolved in 0.5 ml of the mixture of methanol and 25 mM KH_2PO_4 (1 : 1, v/v), and applied to an HPLC column. HPLC was performed using a Shim-pack CLC-ODS column (6 mm i.d. ×15 cm) (Shimadzu, Kyoto) combined with a spectrophotometric detector with a photodiode array (SPD-M10A, Shimadzu). Mobile phases used were mixtures of methanol and 25 mM KH_2PO_4 (1 : 3 and 3 : 2, v/v) and the flow rate was 1 ml min^{-1}.

Growth curve *Porphyromonas gingivalis* ATCC 33277, *Streptococcus sobrinus* AHT and *Streptococcus sanguis* ATCC 10558 were kindly provided by Dr. T. Koga, Kyushu University. *S. sobrinus* and *S. sanguis* were grown at 37°C in brain-heart infusion broth (Difco Laboratory, Detroit, MI) supplemented with yeast extract (0.5%). In the *P. gingivalis* strain, cultures were grown at 37°C in brain-heart infusion broth, which had been supplemented with hemin (5 mg ml^{-1}) and menadione (0.5 mg ml^{-1}), under anaerobic conditions (80% N_2, v/v; 10% H_2, v/v; 10% CO_2, v/v). Growth experiments were started by inoculating with a 10% inoculum from 18-h subculture in the brain-heart infusion broths for the respective bacterial strains. Growth was monitored by the absorbance increase at 660 nm.

Results and Discussion

Figure 1 (upper panel) shows a typical time course of oxygen uptake by SPMN; slow oxygen uptake was observed with the addition of SPMN. This uptake was slightly inhibited by 1 mM KCN (data not shown); the KCN-insensitive oxygen uptake may be due to the formation of O_2^- by SPMN. The formed O_2^- is transformed to H_2O_2 in aqueous solutions. It has already been reported that isolated SPMN produces O_2^- in the absence of a stimulus like PMA (Yamamoto *et al*., 1991). The oxygen uptake by SPMN was enhanced by PMA and the enhancement was suppressed by quercetin. This result suggests that quercetin can suppress the O_2^- producing system of SPMN as reported by Tauber *et al*. (1984). Rates of PMA-enhanced oxygen uptake were decreased as a function of quercetin concentration (Fig. 1, lower panel); half-inhibition was observed at 20–30 μM quercetin. This value was consistent with the reported values for the half-inhibition of plasma neutrophil-dependent O_2^- formation, which ranges from 10 to 100 μM quercetin (Blackburn *et al*., 1987; Busse *et al*., 1984). Half-inhibition of the degranulation (Blackburn Jr. *et al*., 1987; Tordera *et al*., 1994) and the phospholipase activity (Takemura *et al*., 1997; Tordera *et al*., 1994) of stimulated plasma neutrophils has also been reported in the above quercetin concentration range.

Quercetin did not significantly affect the oxygen uptake in the absence of PMA (data not shown). When O_2^- is reduced to H_2O_2 by a reductant, oxygen uptake accompanying the formation of O_2^- is stimulated (Allen & Hall, 1974). As unstimulated SPMN can also produce O_2^- (Yamamoto *et al*., 1991) and quercetin can reduce O_2^- to H_2O_2 (Bors *et al*., 1997), the flavonol should stimulate the oxygen uptake unless quercetin has not affected the formation of O_2^-. However, no significant effects of quercetin were observed on the oxygen uptake. This may be explained by inhibition of the NADPH oxidase of SPMN by quercetin, which can catalyze reduction of O_2 to O_2^-. Quercetin-dependent inhibition of the NADPH oxidase system was described above.

Since SPMN can produce O_2^- and H_2O_2 (Yamomoto *et al*., 1991) and quercetin can be oxidized by O_2^- (Bors *et al*., 1997) and peroxidase/H_2O_2 systems (Hirota *et al*., 1998, 2001; Takahama & Hirota, 2000), we studied the oxidation of quercetin by SPMN. Figure 2 (trace A) shows an absorption spectrum of a reaction mixture that contained SPMN and 20 μM quercetin. The absorption spectrum was essentially the same as that in the absence of SPMN (data not shown). When changes in the absorption spectrum of quercetin (oxidized quercetin minus quercetin) were measured in the absence of SPMN by repeat scan-

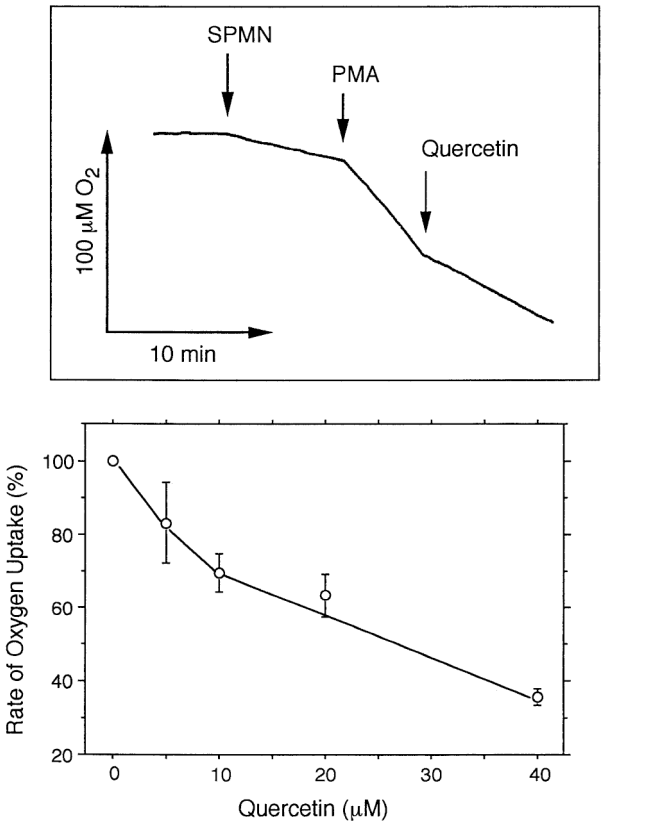

Fig. 1. Effects of quercetin on oxygen uptake by SPMN. The reaction mixture (1 ml) contained 2×10^6 SPMN in Ca^{2+}-free KRP solution. Upper panel: Time course of oxygen uptake, where indicated SPMN, PMA (5×10^{-8} M) and quercetin (40 μM) were added. Lower panel: Effects of quercetin concentration on oxygen uptake in the presence of 5×10^{-8} M PMA. Rate of oxygen uptake in the presence of PMA but absence of quercetin was 0.17±0.04 mmol 10^{-6} cell h^{-1}.

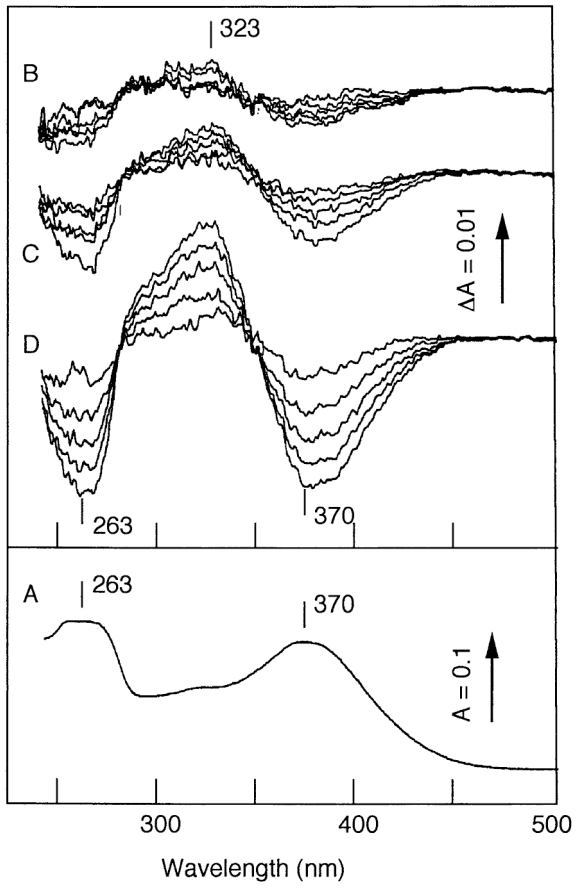

Fig. 2. Oxidation of quercetin by SPMN. Trace A: Absorption spectrum of the reaction mixture (1 ml) that contained 0.3×10^6 SPMN and 20 μM quercetin in Ca^{2+}-free KRP solution. Traces C-D: Difference spectra. Traces B, 20 μM quercetin in Ca^{2+}-free KRP solution; traces C, trace B plus 0.3×10^6 SPMN; traces D, traces C plus 5×10^{-8} M PMA. Measurements of difference spectra were started at 0, 2.4, 4.8, 7.2, 9.6 min after memorizing reference spectra. Scanning speed was 120 nm min^{-1}.

ning (traces B), slow changes were observed and there was a positive peak around 320 nm and negative peaks at about 260 and 370 nm. The changes in absorbance may be due to autooxidation of quercetin (Takahama & Hirota, 2000). SPMN enhanced the changes in absorbance about 2-fold (compare traces B and C) and the changes in absorbance in the presence of SPMN were enhanced further by PMA (compare traces C and D). These results indicate that quercetin was oxidized by not only stimulated but also unstimulated SPMN. Changes in absorption at about 320 and 370 nm have been observed during oxidation of quercetin (Hirota *et al.*, 2001; Hösel & Barz, 1972) and an increase in absorbance around 320 may be due to the formation of a 2,3,5,7,3′,4′-hexahydroxyflavanone-like compound according to Hösel & Barz (1972) and Schreier & Miller (1985).

Oxidation rates of quercetin were plotted as a function of quercetin concentration (Fig. 3). Rates of quercetin autooxidation increased nearly linearly as a function of the concentration. Whereas the oxidation rate of quercetin by SPMN increased as a function of quercetin concentration when that concentration was below 10 μM. As the concentration was increased above 10 μM, the increase in the oxidation rate was suppressed independent of the presence or absence of PMA. The suppression of quercetin oxidation may, in part, be due to quercetin-dependent inhibition of O_2^- formation by SPMN as shown in Fig. 1. It has been reported that quercetin can directly interact with NADPH-oxidase complex, which reduces O_2 to O_2^-, inhibiting the activity (Tauber *et al.*, 1984).

The effects of SOD and horseradish peroxidase on SPMN-dependent oxidation of quercetin were studied (Fig. 4). The oxidation was measured by the decrease in absorbance at 370 nm. The oxidation was inhibited 30–50% by SOD in the presence or absence of PMA indicating participation of O_2^- in the oxidation of quercetin. Horseradish peroxidase enhanced the oxidation of quercetin by about 10–30%. The enhancement of quercetin oxidation by horseradish peroxidase indicates that H_2O_2 as well as O_2^- was formed by SPMN with or without PMA and that H_2O_2, which was formed outside of or diffused out from SPMN, was used by horseradish peroxidase to oxidize quercetin.

The partial inhibition of SPMN-dependent oxidation of quercetin by SOD suggests that quercetin may also be oxidized by O_2^- at sites where added SOD cannot attain and/or by myeloperoxidase-dependent reactions inside and outside of SPMN. To examine the possibilities, we studied whether quercetin was taken up into SPMN. SPMN (3.4×10^6) was suspended in 1 ml of 20 μM quercetin dissolved in Ca^{2+}-free KRP solution for 10 s and the suspension was centrifuged at 2900×*g* for 5 min. The volume of the sediment, which mainly contained SPMN, was 12.9–15.3 μl (n=3). Quercetin in the sediment was extracted with 0.5 ml of 80% methanol and the amount of quercetin in the methanol was determined by HPLC. The amount of quercetin in the sediment was calculated to be 8.0–14.3 nmol. As 20 μM quercetin is equivalent to 20 nmol quercetin per ml, 40–70% of the added quercetin was estimated to be taken up into SPMN. The concentration of quercetin in SPMN was calculated to be 0.5–0.9 mM. This result suggests that quercetin taken up into SPMN can be oxidized by O_2^- if the radical is formed in and/or diffused into SPMN. In a reaction mixture that contained isolated SPMN and 20 μM quercetin, quercetin was rapidly oxidized by 0.1 μM H_2O_2. This result suggests that myeloperoxidase in SPMN can directly or indirectly oxidize quercetin that is taken up into SPMN. The direct oxidation of quercetin by myeloperoxidase is supported by the data that commercially obtained

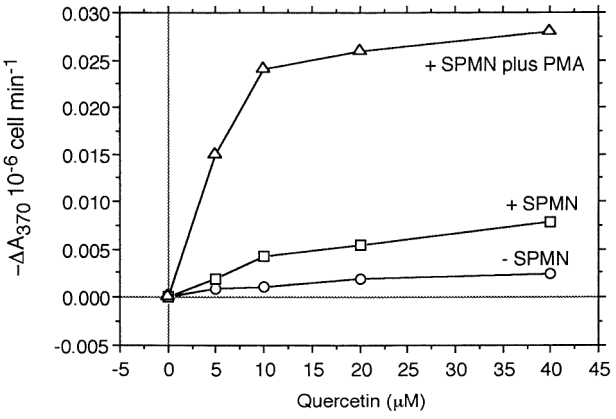

Fig. 3. Effects of quercetin concentration on its oxidation. The reaction mixture (1 ml) contained 0.3×10^6 SPMN and various concentrations of quercetin in Ca^{2+}-free KRP solution. Initial rates of quercetin oxidation were plotted as a function of quercetin concentration. Circles, without SPMN; triangles, SPMN; triangles, SPMN plus 5×10^{-8} PMA.

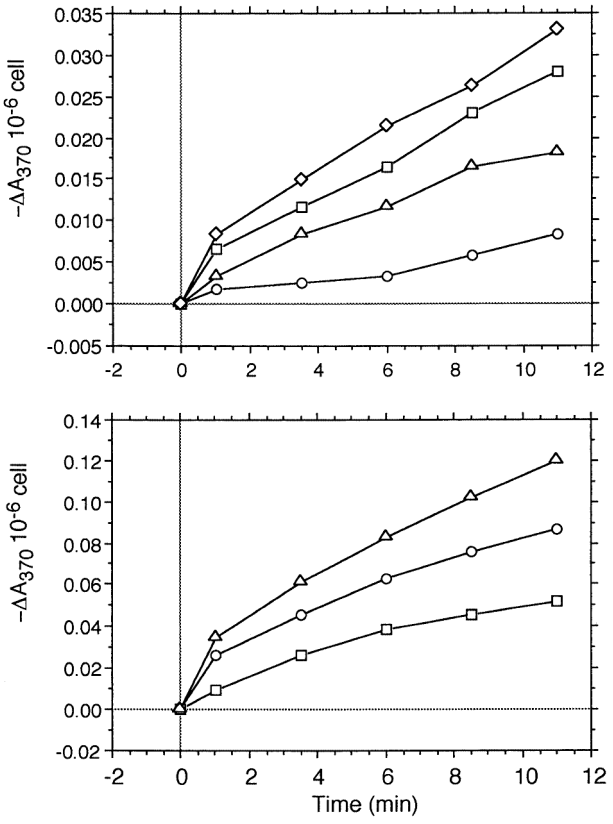

Fig. 4. Effects of SOD and horseradish peroxidase on the oxidation of quercetin. The reaction mixture (1 ml) contained 0.3×10^6 SPMN and 20 μM quercetin in Ca^{2+}-free KRP solution. Oxidation of quercetin was measured by absorption decrease at 370 nm. Upper panel, without PMA. Circles, whthout SPMN; squars, SPMN; triangles, SPMN+50 unit SOD; diamonds, SPMN+0.5 μg horseradish peroxidase. Lower panel, 5×10^{-8} M PMA. Circles, SPMN; squares, SPMN+50 unit SOD; triangles, SPMN+0.5 μg horseradish peroxidase.

Table 1. SPMN-dependent oxidation products of quercetin.

	Retention Time (min)	Absorption peaks in mobile phase (nm)
DHA	5.3[a]	204, 252, 289[a]
CX	17.4[a]	207, 294[a]
QX 1	18.5[b]	204, 303, 360[b]
QX 2	19.2[b]	204, 303, 360[b]

DHA, 3,4-dihydroxybenzoic acid; CX, 2,3,4,7,3′,4′-hexahydroxyflavanone-like compound; QXs 1 and 2, two isomers of 2,3-epoxy-2-(3,4-dihydroxyphenyl)-3-[4O-[2-(3-hydroxyphenyl)-3,5,7-]trihydroxy-4H-1-benzopyran-4-onyl]-5,7-dihydroxy-4H-1-benzopyran-4-one-like compounds.
[a]mobile phase [methanol : 25 mM KH_2PO_4=1 : 3 (v/v); flow rate, 1 ml min^{-1}].
[b]mobile phase [methanol : 25 mM KH_2PO_4=3 : 2 (v/v); flow rate, 1 ml min^{-1}].

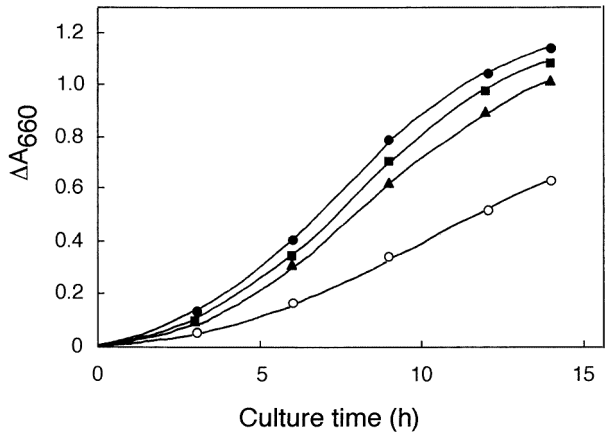

Fig. 5. Inhibition of growth of *P. gingivalis* by quercetin. Closed circles, 0 μM; squares, 8 μM; triangles, 16 μM; open circles, 64 μM quercetin.

myeloperoxidase oxidized quercetin in the absence of Cl^- when H_2O_2 was added (data not shown). The indirect oxidation of quercetin may be mediated by OCl^- produced by myeloperoxidase-dependent oxidation of Cl^-. Pincemail *et al.* (1988) reported chemical oxidation of quercetin by OCl^-. From these results, it is suggested that quercetin could be oxidized by not only O_2^- but also the H_2O_2/myeloperoxidase system of SPMN in SPMN suspensions.

SPMN-dependent oxidation products of quercetin were analyzed by HPLC. The main products were essentially the same as those formed by oxidation of quercetin by salivary peroxidase (Takahama *et al.*, 2002). The result is summarized in Table 1. 3,4-Dihydroxybenzoic acid has repeatedly been reported as one of the oxidase- and peroxidase-dependent degradation products of quercetin (Barz *et al.*, 1985; Schreier & Miller, 1985; Takahama *et al.*, 2002). 2,3,5,7,3′,4′-Hexahydroxyflavanone is an oxidation product of quercetin that still has a flavonoid structure (Barz *et al.*, 1985; Schreier & Miller, 1985). A compound similar to the flavanone has been detected in the salivary peroxidase-dependent oxidation product of quercetin (Takahama *et al.*, 2002). Two isomers of 2,3-epoxy-2-(3,4-dihydroxyphenyl)-3-[4O-[2-(3-hydroxyphenyl)-3,5,7-]trihydroxy-4H-1-benzopyran-4-onyl]-5,7-dihydroxy-4H-1-benzopyran-4-one are horseradish peroxidase-dependent oxidation products of quercetin (Schreier & Miller, 1985). Compounds, the characteristics of which are similar to the isomers, have been reported as the salivary peroxidase-dependent oxidation products of quercetin (Takahama *et al.*, 2002). Further studies are required to elucidate what compounds are formed by O_2^-- and myeloperoxidase-dependent oxidation of quercetin.

Results in Figs. 1–4 suggest that quercetin not only inhibits the formation of reactive oxygen species by SPMN but also scavenges O_2^- generated by SPMN. It is also possible that quercetin can be a substrate for myeloperoxidase to scavenge H_2O_2 that is formed by disproportionation of O_2^- and by quercetin-dependent reduction of O_2^-. If quercetin is a substrate for myeloperoxidase, the peroxidase-dependent formation of OCl^- is inhibited. Based on the results and discussion, it is deduced that if quercetin accesses tissues like gingivals which are inflamed, the flavonol can decrease the concentrations of O_2^-, H_2O_2 and OCl^- by inactivating SPMN and by scavenging the oxidants. Since quercetin suppresses lipid peroxidation (Sorata *et al.*, 1984; Terao & Piskula, 1997), the flavonol can also inhibit the peroxidation of low-density lipoprotein which is induced by macrophages in inflamed tissues (Leake, 1997). In addition to myeloperoxidase, peroxidase in saliva may also participate in the scavenging of H_2O_2 if this oxidant is formed in or diffused into saliva. Quercetin as well as thiocyanate is a substrate of peroxidase in saliva (Hirota *et al.*, 2001).

An oxidation product of quercetin was 3,4-dihydroxybenzoic acid. This phenolic acid (Walker & Stahman, 1955) as well as

quercetin itself (Reichling, 1999) can be antimicrobial. Therefore, it is expected that these phenolics suppress the growth of bacteria found in the oral cavity. Growth of *P. gingivalis*, which might cause periodontal inflammation and disease (Haffajee & Socransky, 1994), was inhibited by quercetin in the concentration range for the inhibition of SPMN (Fig. 5), but 3,4-dihydroxybenzoic acid did not inhibit the growth of *P. gingivalis* in the concentration up to 0.1 mM. The effects of quercetin (0–64 μM) were small on the growth of *S. sanguis* which causes dental caries (Hamada & Slade, 1980; Loeshe, 1986) and on the growth of *S. sobrinus* which is found in normal bacterial flora in the oral cavity. No effects of 3,4-dihydroxybenzoic acid were observed on the growth of the above two bacteria. These effects of quercetin on SPMN and *P. gingivalis* may imply that the suppression of reactive oxygen formation, which is related to the antimicrobial function of SPMN, is balanced or compensated for by the suppression of growth of *P. gingivalis*. Further studies are required to elucidate functions of quercetin from the standpoints of antimicrobial and anti-inflammation in the oral cavity and tissues.

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