Active Oxygens Generation by Flavonoids

Yukiko H. MIURA,^{*,a} Isao TOMITA,^a Tetsushi WATANABE,^b Teruhisa HIRAYAMA,^b and Shozo FUKUI^b

School of Pharmaceutical Sciences, University of Shizuoka,^a 52-1 Yada, Shizuoka 422, Japan and Kyoto Pharmaceutical University,^b 5, Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607, Japan.

Received August 4, 1997; accepted November 1, 1997

The hydrogen peroxide (H₂O₂)-generating effects of 14 flavonoids were investigated. Seven out of 14 flavonoids tested were found to generate H₂O₂ in an acetate buffer of pH 7.4. The H₂O₂-generating abilities of flavonoids decrease in the order of myricetin>baicalein>quercetin>(-)-epicatechin>(+) -catechin>fisetin=7,8-dihydroxy flavone. This ability was observed in flavonoids with either a pyrogallol or catechol structure, and the pyrogallol-type flavonoids generated more H₂O₂ than the catechol-types. The amount of H₂O₂ generated by myricetin (pyrogallol-type flavonoid) was proportional to its concentration and to the reaction time until about 4 h. In addition, H₂O₂ generation by myricetin was dependent on the amount of dissolved oxygen in the buffer, and it was inhibited by the addition of superoxide dismutase.

These results suggest that the flavonoids generate H₂O₂ by donating a hydrogen from their pyrogallol or catechol structure to oxygen, through a superoxide anion radical.

It was also found that flavonoids which generated more H₂O₂ were more powerful antioxidants in the NADPH-dependent lipid peroxidation of rat microsomes.

Key words flavonoid; antioxidant; prooxidant; hydrogen-donation; free radical

Flavonoids, which are widely distributed in the plant kingdom, have been reported to have various biological effects.^{1–10} Their strong antioxidative and free radical scavenging effects are attracting attention, as various age-related diseases and the aging process have been considered to be basically due to the free radical-mediated degeneration of cell components.¹¹ It has been indicated that there are at least three structural criteria for flavonoids to be effective as radical scavengers.¹² 1. An *o*-dihydroxy structure in the B ring. 2. A 2, 3 double bond in conjugation with a 4-oxo group in the C ring. 3. And 3- and 5-OH groups with a 4-oxo group in the A and C rings, respectively. The flavonoids which fill these criteria in their structures suppress lipid peroxidation by hydrogen-donating¹³ as well as by their metal chelating ability.¹⁴

In our earlier study, we reported that there is a correlation between active oxygen scavenging potency and the antioxidative effects of flavonoids.¹⁵ Several flavonoids, such as myricetin and baicalein, promoted •OH generation in the Fenton reaction with Fe²⁺ (H₂O₂+Fe²⁺→•OH+OH⁻+Fe³⁺), while they were very strong suppressors of lipid peroxidation, presumably by trapping Fe²⁺ in this reaction system. Cao *et al.*¹⁶ reported that some flavonoids act as prooxidants in the presence of metals such as Cu²⁺, yet they were the peroxy and hydroxy radical scavengers.

In the present study, we attempted to determine the H₂O₂ generation by 14 flavonoids and investigated the effects of these flavonoids on lipid peroxidation in rat liver microsomes with adenosine diphosphate (ADP)-Fe³⁺. There appear to be no comprehensive studies in which the H₂O₂ generation and suppression of lipid peroxidation by a series of flavonoids have been systematically compared.

MATERIALS AND METHODS

Materials All of the flavonoids were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Peroxidase (from horseradish, E.C. 1.11.1.7), superoxide dismutase

(SOD; from bovine erythrocytes, E.C. 1.15.1.1) and catalase (from bovine liver, E.C. 1.11.1.6) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *p*-Hydroxyphenyl acetic acid, from Nacalai Tesque, Inc. (Kyoto, Japan), was recrystallized from water prior to use. H₂O₂, pyrogallol and catechol were obtained from Wako Pure Chemical Co. (Tokyo, Japan). All other reagents were guaranteed grade and were used as received without further purification.

Hydrogen Peroxide Assay¹⁷ The flavonoid solutions were prepared by sonicating their powders with 50 mM acetate buffer (pH 7.4) for 1 min. They were allowed to stand at 21 °C for 5 h. To 1.0 ml of flavonoid solution, 0.1 ml of 0.1 M acetic acid solution was added, and then the mixture was passed through a Sep-pak C₁₈ cartridge (Waters Associates, Millipore Co., Millford, MA, U.S.A.) to remove the flavonoids. To the effluent, 0.2 ml of 0.84 mM EDTA-2Na in 0.35 M phthalate buffer (pH 7.0) and 0.2 ml of 8 mM *p*-hydroxyphenyl acetic acid in 0.35 M phthalate buffer (pH 7.0) containing peroxidase (4 units/ml) were added, then 0.2 ml of 0.4 N NaOH solution was added. The fluorescence of the solution was measured at ex. 322 nm and em. 406 nm. As a negative control, to the effluent, 0.2 ml of 0.84 mM EDTA-2Na in 0.35 M phthalate buffer (pH 7.0) containing catalase (400 units/ml) was added instead of the EDTA-2Na solution. The standard curve was plotted with H₂O₂ solution.

Determination of the Antioxidative Effect of Flavonoids on NADPH-Dependent Lipid Peroxidation of Rat Liver Microsomes Liver microsomes were isolated from male Wistar rats weighing about 200 g. The rats were anesthetized with diethyl ether and the liver was perfused with 0.15 M KCl solution. Liver homogenates were centrifuged at 9000×*g* to obtain the supernatant. Microsomes were isolated by further centrifugation at 105000×*g*, and then the pellets were resuspended in 20% glycerol solution. Protein was determined by the method of Lowry.¹⁸ Microsomes were stored at -80 °C. One milliliter of 50 mM Tris HCl buffer (pH 7.4) containing 1.0 mg of rat liver microsomes was incubated with 0.2 ml of 30 μM ADP, 0.2 ml of 1.5 μM FeCl₃ and 10 μl of flavonoid di-

* To whom correspondence should be addressed.

methyl sulfoxide solution at 37 °C for 10 min. To the mixture, 0.2 ml of 5 μ M NADPH was added, and the new mixture was incubated at 37 °C for 60 min. Then, 0.2 ml of 2% butylated hydroxytoluene, 0.2 ml of 40 mM EDTA-2Na and 7.6 ml of 0.35% thiobarbituric acid acetate solution were added, and the mixture was kept at 100 °C in a boiling water bath for 30 min. After cooling, its absorbance was measured at 532 nm.

RESULTS AND DISCUSSION

Hydrogen Peroxide Generation by Flavonoids A series of fourteen flavonoids was employed in a structure activity study to assess their ability to generate H_2O_2 in a pH 7.4 buffer solution. The results are shown in Table 1. Seven of the fourteen flavonoids tested exhibited H_2O_2 generating ability with the following order of potency; myricetin > baicalein > quercetin > (-)-epicatechin > (+)-catechin > fisetin = 7,8-dihydroxy flavone. Flavonoids possessing adjacent trihydroxy (pyrogallol) and dihydroxy (catechol) configurations in the B ring showed potent ability. Pyrogallol and catechol, reference compounds of flavonoids, also showed pronounced H_2O_2 generating ability, and the activity of pyrogallol was about 10-fold that of catechol, as shown in Table 2. Pyrogallol (trihydroxy benzene) may have more opportunities to react than catechol (dihydroxy benzene). We used myricetin, a flavonoid with a pyrogallol moiety in the B ring, as a model compound for further experiments. The H_2O_2 generation by myricetin was always accompanied by the con-

sumption of dissolved oxygen in the buffer, and aeration of the reaction mixtures accelerated the H_2O_2 generation (Table 3). The time course of H_2O_2 generation by myricetin is shown in Fig. 1. It reached a maximum at 4 h, regardless of the concentration of myricetin (10 or 50 μ M). These results suggest that the myricetin is gradually auto-oxidized in the presence of dissolved oxygen. Through the reaction, electrons and H^+ were donated to oxygen from myricetin to yield H_2O_2 , and the overall reactions ceased after all the myricetin was converted to its oxidized form (Chart 1: Eq. 1).

Figure 2 shows the effect of SOD on H_2O_2 generation by myricetin. The addition of SOD inhibited H_2O_2 generation by myricetin. Hodnick *et al.*¹⁹⁾ reported that the oxygen consumption of myricetin could be inhibited by the addition of SOD, and therefore, a superoxide anion radical might be involved in the reaction. Our results are in accord with their experimental data, and support their suggestion that the H_2O_2 generating reaction of myricetin would involve the formation of a superoxide anion radical as an intermediate (Chart 1: Eq. 2). SOD was more effective on the inhibition, contrary to the theory. It was considered that the decomposition of H_2O_2 was promoted by the SOD-induced change in the H_2O_2 generating rate. In fact, H_2O_2 easily decomposed under this experimental condition (Fig. 1).

Inhibition of NADPH-Dependent Microsomal Lipid Peroxidation by Flavonoids An inhibitory study of NADPH-dependent microsomal lipid peroxidation by 14

Table 1. Generation of Hydrogen Peroxide by Flavonoids

Flavonoids	Structures	H_2O_2 generation (μ M)
Myricetin	(3,3',4',5,5',7-Hexahydroxy)	10.79 $\pm$ 0.90
Baicalein	(5,6,7-Trihydroxy)	6.89 $\pm$ 0.39
Quercetin	(3,3',4',5,7-Pentahydroxy)	1.67 $\pm$ 0.13
(-)-Epicatechin	(3,3',4',5,7-Pentahydroxy)	1.18 $\pm$ 0.11
(+)-Catechin	(3,3',4',5,7-Pentahydroxy)	0.94 $\pm$ 0.09
Fisetin	(3,3',4',7-Tetrahydroxy)	0.44 $\pm$ 0.10
7,8-Dihydroxy flavone		0.44 $\pm$ 0.00
3-Hydroxy flavone		ND
7-Hydroxy flavone		ND
Chrysin	(5,7-Dihydroxy)	ND
Apigenin	(4',5,7-Trihydroxy)	ND
Galangin	(3,5,7-Trihydroxy)	ND
Kaempferol	(3,4',5,7-Tetrahydroxy)	ND
Morin	(2',3,4',5,7-Pentahydroxy)	ND

Samples containing 50 μ M flavonoid in 50 mM acetate buffer (pH 7.4) were allowed to stand for 5 h at 21 °C. H_2O_2 was measured as described in Materials and Methods. ND: not detected. Values are mean $\pm$ S.D. $n=5$.

Table 2. Generation of Hydrogen Peroxide by Pyrogallol and Catechol

Compounds	Conc. (μ M)	H_2O_2 generation (μ M)
Pyrogallol	50	1.50 $\pm$ 0.50
	100	1.84 $\pm$ 0.22
Catechol	50	0.08 $\pm$ 0.01
	100	0.14 $\pm$ 0.03

Samples in 50 mM acetate buffer solution (pH 7.4) were allowed to stand for 1 h at 21 °C. H_2O_2 was measured as described in Materials and Methods. Values are mean $\pm$ S.D. $n=5$.

Table 3. Effect of Aeration on the Generation of Hydrogen Peroxide by Myricetin

Myricetin conc. (μ M)	H_2O_2 generation (μ M)	
	With aeration	Without aeration
1	0.37 $\pm$ 0.07	0.00
10	8.41 $\pm$ 0.24	6.23 $\pm$ 0.16
100	62.52 $\pm$ 3.43	29.62 $\pm$ 0.01

Samples containing myricetin in 50 mM acetate buffer (pH 7.4) were allowed to stand for 5 h at 21 °C. H_2O_2 was measured as described in Materials and Methods. Values are mean $\pm$ S.D. $n=5$.

Fig. 1. Time Course of the Generation of Hydrogen Peroxide by Myricetin

Samples containing 10 μ M (○) or 50 μ M (●) myricetin in 50 mM acetate buffer (pH 7.4) were allowed to stand at 21 °C.

Chart 1. The Reaction of a Catechol Type Flavonoid with Oxygen

Fig. 2. Effect of SOD on the Generation of Hydrogen Peroxide by Myricetin.

Samples containing SOD and 50 μM myricetin in 50 mM acetate buffer (pH 7.4) were allowed to stand for 5 h at 21 $^{\circ}\text{C}$.

Table 4. Inhibitory Effect of Flavonoids on Lipid Peroxidation of Rat Liver Microsomes with ADP- Fe^{3+}

Flavonoids	IC ₅₀ (μM)
Baicalein	3.79 $\pm$ 0.01
Myricetin	4.27 $\pm$ 0.17
	(11.31 $\pm$ 0.01) ^{a)}
Quercetin	5.20 $\pm$ 0.26
7,8-Dihydroxy flavone	5.60 $\pm$ 0.01
Fisetin	8.89 $\pm$ 0.01
(-)-Epicatechin	17.44 $\pm$ 1.42
(+)-Catechin	25.82 $\pm$ 0.57
Kaempferol	46.53 $\pm$ 0.05
Galangin	>200
Morin	>200

The experimental condition is as described in Materials and Methods. a) Myricetin which was allowed to stand for 5 h in 50 mM Tris HCl buffer (pH 7.4) was used. Values are mean $\pm$ S.D. $n=5$.

flavonoids was conducted, and the IC₅₀ values obtained are shown in Table 4. The following order of potency was observed: bicaicin>myricetin>quercetin>7,8-dihydroxy flavone>fisetin>(-)-epicatechin>(+) -catechin>kaempferol>galangin>morin. Flavonoids possessing a pyrogallol or catechol moiety in their structures were potent inhibitors of microsomal lipid peroxidation. Flavonoids lacking these moieties, for example, 3-hydroxy flavone, 7-hydroxy flavone, chrysin and apigenin, showed no activity. Those flavonoids which have H₂O₂ generating ability, were able to inhibit lipid peroxidation in the iron-recycle system at pH 7.4. This result suggests that the flavonoids act as antioxidants *in vivo*.

Flavonoids have been known to inhibit various enzymes, such as human neutrophil NADPH oxidase,²⁰⁾ mitochondrial succinoxidase, and NADH oxidase²¹⁾ etc. It has been assumed that one of the reasons they inhibit lipid peroxidation is that they inhibit a microsomal cytochrome p-450 (Fe^{2+}) dependent enzymatic reaction, since flavonoids with a pyrogallol and catechol moiety may serve as strong chelating agents of Fe^{2+} . The electron and H^+ donating activity of flavonoids may also contribute to terminating the lipid peroxidation chain reactions. Thus, the flavonoids act as powerful reducing agents.²²⁾

The flavonoids, as powerful reducing agents, can act as

both antioxidants and prooxidants according to the conditions. They act as prooxidants in the absence of the radicals. α -Tocopherol, a typical antioxidant, is also known to act as both anti- and pro-oxidants according to the reaction condition.²³⁾

In conclusion, flavonoids which possess pyrogallol and catechol moieties in their structures showed strong H₂O₂-generating activity *via* a superoxide anion radical. They also showed inhibitory activities in rat liver microsomal lipid peroxidation. These conflicting effects of the flavonoids may result from their reducing abilities. It was suggested that flavonoids which generate H₂O₂ can scavenge free radicals. It was thus considered that flavonoids act as antioxidants not only by free radical scavenging but also by the chelation of metals and the inhibition of enzymes. However, it was not determined whether the antioxidative effects of the flavonoids resulted from the free radical scavenging and other reactions, or from the superior chelating effect, in spite of the active oxygen species generating effect in this experimental condition.

It is suggested that careful attention must be paid to evaluate the activities of flavonoids, depending on the assay systems, as they often exhibit contrary effects.

Acknowledgements We thank Mr. Jun-ichiro Higuchi, Ms. Noriko Matsuda, Ms. Hiroko Hayashi and Ms. Tomoko Yamamura for their skillful technical assistance.

REFERENCES

- 1) Kootstra A., *Plant Mol. Biol.*, **26**, 771—774 (1994).
- 2) Heo M. Y., Lee S. J., Kwon C. H., Kim S. W., Sohn D. H., Au W. W., *Mutat. Res.*, **311**, 225—229 (1994).
- 3) Middleton E., Kandaswami C., *Biochem. Pharmacol.*, **43**, 1167—1179 (1992).
- 4) Pattichis K., Louca L. L., Jarman J., Sandler M., Glover V., *Eur. J. Pharmacol.*, **292**, 173—177 (1995).
- 5) Sahu S. C., Gray G. C., *Cancer Lett.*, **85**, 159—164 (1994).
- 6) Shimoi K., Masuda S., Furugori M., Esaki S., Kinae N., *Carcinogenesis*, **15**, 2669—2672 (1994).
- 7) Singhal R. L., Yeh Y. A., Praja N., Olah E., Sledge G., Weber G., *Biochem. Biophys. Res. Commun.*, **208**, 425—431 (1995).
- 8) Viana M., Barbas C., Bonet B., Bonet M. V., Castro M., Fraile M. V., Herrera E., *Atherosclerosis*, **123**, 83—91 (1996).
- 9) Wolle J., Hill R. R., Ferguson E., Devall L. J., Trivedi B. K., Newton R. S., Saxena U., *Arterioscler. Thromb. Vasc. Biol.*, **16**, 1501—1508 (1996).
- 10) Pannala A., Rice-Evans C. A., Halliwell B., Singh S., *Biochem. Biophys. Res. Commun.*, **232**, 164—168 (1997).
- 11) Halliwell B., *Lancet*, **344**, 721—724 (1994).
- 12) Bors W., Heller W., Michel C., Saran M., "Methods in Enzymology," Vol. 186, ed. by Packer L., Glazer A. N., Academic Press, Orlando, 1990, pp. 343—355.
- 13) Rice-Evans C. A., Miller N. J., Paganga G., *Free Radic. Biol. Med.*, **20**, 933—956 (1996).
- 14) Cook N. C., Samman S., *J. Nutr. Biochem.*, **7**, 66—76 (1995).
- 15) Hanasaki Y., Ogawa S., Fukui S., *Free Radic. Biol. Med.*, **16**, 845—850 (1994).
- 16) Cao G., Sofic E., Prior R. L., *Free Radic. Biol. Med.*, **22**, 749—760 (1997).
- 17) Lazrus A. L., Kok G. L., Gitlin S. N., Lind J. A., *Anal. Chem.*, **57**, 917—922 (1985).
- 18) Lowry O. H., Rossebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
- 19) Hodnick W. F., Kung F. S., Roettger W. J., Bohmont C. W., Pardini R. S., *Biochem. Pharmacol.*, **35**, 2345—2357 (1986).
- 20) Tauber A. I., Fay J. R., Marletta M. A., *Biochem. Pharmacol.*, **33**, 1367—1369 (1984).
- 21) Hodnick W. F., Duval D. L., Pardini R. S., *Biochem. Pharmacol.*, **47**, 573—580 (1994).
- 22) Acker S. A. B. E., Berg D. J., Tromp M. N. J. L., Griffioen D. H., Bennekom W. P., Vijgh W. J. F., Bast A., *Free Radic. Biol. Med.*, **20**, 331—342 (1996).
- 23) Kontush A., Finckh B., Karten B., Kohlschutter A., Beisiegel U., *J. Lipid Res.*, **37**, 1436—1448 (1996).