Inhibitory Effects of Flavonoids on Free Radical-Induced Hemolysis and Their Oxidative Effects on Hemoglobin

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To investigate the effects of flavonoids on free radical-mediated biological membrane damage and the interaction of flavonoids with heme proteins, we studied the effects of quercetin, its glycosides (rutin and quercetin-3-*O*-glucoside), morin and (−)epicatechin on the hemolysis of the bovine erythrocytes induced by the hydrophilic free radical initiator, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), and their interaction with hemoglobin. These flavonoids retarded the onset of the hemolysis dose-dependently. The effects of quercetin and other flavonoids were much greater than those of dihydric-phenols studied previously. The inhibitory effects of quercetin and its glycosides were stronger than those of morin and (−)epicatechin. In the absence of AAPH, the relatively hydrophobic flavonoids quercetin and morin induced the oxidation of oxyhemoglobin to methemoglobin. Oxidation by quercetin was especially marked. However, this oxidation did not induce hemolysis. These findings indicate that relatively hydrophobic flavonoids penetrate the cytoplasm of erythrocytes, interact with hemoglobin, and oxidize the heme iron.

Key words flavonoid; hemolysis; hemoglobin; methemoglobin; free radical

Flavonoids, which are phytochemicals produced by various plants in high quantities,[1] have been revealed to protect biological membranes against free radical-induced oxidative damage. They scavenge reactive oxygen species,[2,3] inhibit free-radical-induced membrane lipid oxidation[4] and inhibit the oxidation of low-density lipoproteins.[5] However, in some cases they have also been suggested to work as prooxidants.[6,7] Their interaction with cellular proteins, especially with heme proteins which exert their physiological functions by the oxidation and reduction of heme iron, requires elucidation. In this study, we investigated the effects of various flavonoids on the oxidative state of heme proteins as well as free radical-induced membrane damage, using erythrocytes as a model membrane system. We observed the effects of flavonoids on hemolysis induced by the hydrophilic free radical initiator, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH). We also examined the oxidation of hemoglobin inside the erythrocytes induced by flavonoids in the absence of AAPH. As flavonoids, we studied the effects of two flavonols (quercetin and morin), two glycosides of quercetin (quercetin-3-*O*-glucoside and rutin) and one relatively hydrophilic catechin ((−)epicatechin).

Experimental

Materials Quercetin, morin and rutin were purchased from Nacalai Tesque (Kyoto, Japan). (−)Epicatechin was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Quercetin-3-*O*-glucoside was from Funakoshi Co. (Tokyo, Japan). 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) and other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Measurement of Hemolysis Holstein bovine blood was obtained from Niigata Municipal Meat Center. Blood containing 10% volume of ACD anticoagulant solution (2.2% sodium citrate, 0.8% citric acid and 2.2% glucose) was centrifuged at 2000×***g*** for 5 min at 4 °C to remove plasma and the buffy coat, and the erythrocyte suspension (hematocrit, 10%) in phosphate buffered saline (PBS, pH 7.4) was prepared as described previously.[8] The erythrocyte suspension was incubated in air in a shaking water bath at 37 °C in the presence of 100 mM AAPH, either with or without flavonoids. An aliquot (0.4 ml) of this mixture was taken out periodically to measure the extent of hemolysis spectrophotometrically by the cyanmethemoglobin method.[9]

Measurement of Hemoglobin Oxidation Oxidation of hemoglobin inside the erythrocytes in the presence of flavonoids was observed by measuring the absorption spectra of hemoglobin.[8] The erythrocyte suspension (hematocrit, 10%) described above was incubated in air in the presence of flavonoids without addition of AAPH. An aliquot (100 μl) of the erythrocyte suspension was taken out periodically, washed with ice-cold PBS, lysed in 4 ml hypotonic phosphate buffer (pH 7.4), and the hemoglobin spectrum was measured.

Measurement of *n*-Octanol/PBS Partition Coefficients To PBS containing the flavonoids (0.2—0.4 mM for quercetin and morin, 0.4—1.0 mM for the other flavonoids) presaturated with *n*-octanol, 2 ml *n*-octanol presaturated with PBS was added at a volume ratio of 1 : 1 or 3 : 1. The PBS and *n*-octanol were deoxygenized with a nitrogen gas stream before the experiment. Light was shut out by wrapping the tubes with aluminum foil. Both phases were equilibrated at 37 °C by gentle shaking for 18 h after vigorous mixing every hour for the first 5 h. After stopping the shaking, the incubation continued for another 1 h. Aliquots of the samples were collected from both phases and analyzed by HPLC (L-6000; Hitachi, Tokyo, Japan) with an L-4000 UV detector (Hitachi). Separation was achieved on a reversed-phase column (Mightysil RP-18GP, 4.6 mm i.d., 250 mm) using a mobile phase consisting of methanol, water and phosphoric acid (100 : 100 : 1) at a flow rate of 0.7 ml/min. Analyzed wavelengths were 360 nm for quercetin, 353 nm for morin, 356 nm for rutin, 355 nm for quercetin-3-*O*-glucoside and 280 nm for (−)epicatechin. Ferulic acid was used as an internal standard for quercetin, morin, and (−)epicatechin. Quercetin was also used as an internal standard for quercetin-3-*O*-glucoside and rutin.

Statistical Analysis Mann-Whitney's *U*-test was used to analyze differences between sets of data. The level of significance was adjusted by Bonferroni's method. A *p*-value less than 0.05 was considered significant.

Results and Discussion

Effects of Flavonoids on AAPH-Induced Hemolysis We first investigated the effects of flavonoids shown in Fig. 1 on AAPH-induced hemolysis. As shown in Fig. 2 for the effects of quercetin, hemolysis was retarded in a dose-dependent manner in the presence of flavonoids. From the hemolysis curves, the times at which 50% of the erythrocytes caused hemolysis were determined and the concentration of flavonoids causing a two-fold increase in that time, IC_2, was obtained as a measure of their inhibitory activities. The results are shown in Table 1. The inhibitory effects of the flavonoids examined were much greater than that of dihydric-phenol pyrocatechol. The inhibitory effects of quercetin and

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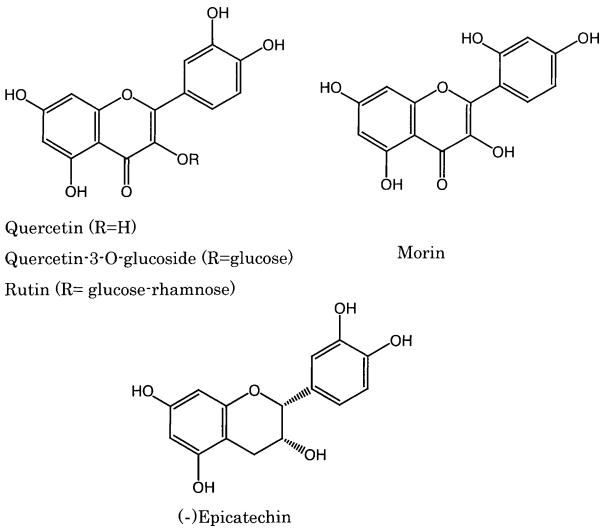

Fig. 1. Structure of Flavonoids Tested in This Study

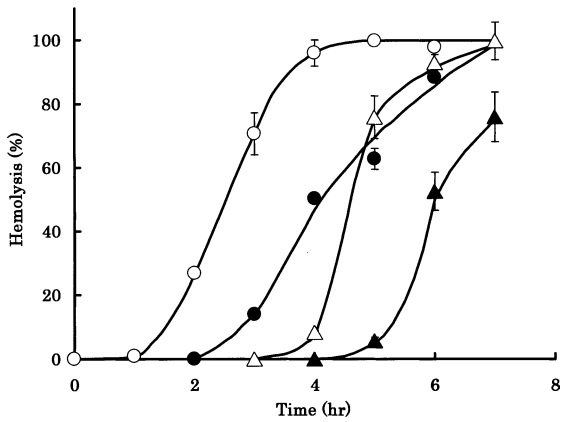

Fig. 2. Effect of Quercetin on 100 mM AAPH-Induced Hemolysis

○, control; ●, 15 μM; △, 30 μM; ▲, 50 μM. Data are means±S.D. of three experiments.

its glycosides were greater than those of morin and (−)epicatechin, possibly due to their strong electron donor activities.[10] However, the order of the inhibitory effects of the flavonoids and pyrocatechol was slightly different from that of their scavenging activities against superoxide anion radicals, which we previously reported.[2] That is, the scavenging activity of quercetin was much stronger than that of the others. However, that of (−)epicatechin was similar to that of pyrocatechol, and that of morin was much weaker than that of the others. These differences may be due to the contribution of the interaction of the flavonoids with the erythrocyte membrane and the cytoplasmic components to the preventing reaction against free radical-induced membrane damage.

Effects of Polyphenols on Oxidation of Hemoglobin Inside Erythrocytes We have already reported that pyrocatechol and its non-ionic mono-substituents oxidized hemoglobin after penetration into the cytoplasm of the erythrocytes at relatively high concentrations (more than 200 μM). Therefore, we also examined the effects of these flavonoids and tried to clarify the relationship of their effects with their hydrophobicity. As shown in Fig. 3 and Table 2 for the effects of 100 μM flavonoids, oxidation of oxyhemoglobin to methemoglobin was especially observed in the presence of quercetin and morin, whose logarithm values of *n*-octanol/PBS partition coefficients, $\log P_{oct}$, were larger than 1.0. The oxidative effect of quercetin, whose $\log P_{oct}$ value was larger than that of morin as well as its electron donor activity,[10] was more marked than the effect of morin. On the other hand, only slight oxidation was observed in the presence of the hydrophilic flavonoids, rutin, quercetin-3-*O*-glucoside and (−)epicatechin. We further examined the possibility that the hemoglobin oxidation observed here might induce hemolysis. However, the oxidation by quercetin did not induce hemolysis even after a 24-h incubation (the mean percentage hemolysis after a 24-h incubation with or without 100 μM quercetin at 37 °C for four experiments was 1.1±0.1

Table 1. Concentrations of Flavonoids Producing a Two-Fold Increase in the Time to Cause 50% Hemolysis of Bovine Erythrocytes Induced by AAPH, IC_2

Flavonoid	IC_2 (μM)
Pyrocatechol	229±46[a]
Quercetin	31±9
Rutin	37±5
Quercetin-3-*O*-glucoside	47±4
Morin	87±3
(−)Epicatechin	68±10

Values represent means±S.D. of data from triplicate experiments on three different preparations. *a*) Cited from ref. 8.

Table 2. Oxidation of Hemoglobin Induced by 100 μM Flavonoids after a 7-h Incubation and Their *n*-Octanol/PBS Partition Coefficients, P_{oct}, at 37 °C

Flavonoid	Relative absorbance at 541 nm	$\log P_{oct}$
Control	1.00	—
Quercetin	0.618±0.018***	2.74±0.14
Rutin	0.974±0.008*	−0.71±0.09
Quercetin-3-*O*-glucoside	0.958±0.032	−0.076±0.028
Morin	0.874±0.028**	1.27±0.07
(−)Epicatechin	0.982±0.023	−0.079±0.026

Data are means±S.D. of three experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to control.

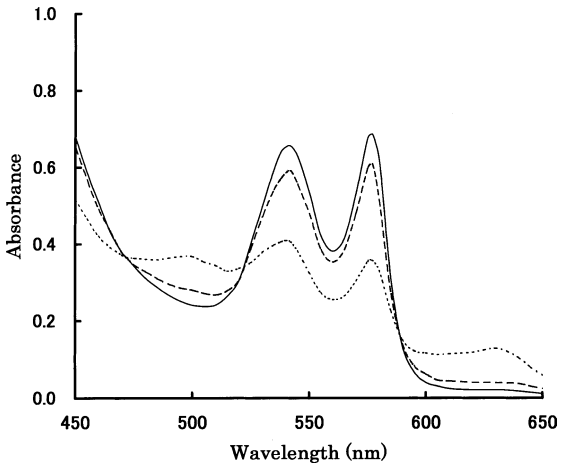

Fig. 3. Effects of 100 μM Quercetin and Morin on Absorption Spectrum of Oxyhemoglobin after a 7 h-Incubation at 37 °C

——, control; ·····, quercetin; - - - - -, morin.

and 1.2±0.2, respectively).

According to the study on the reaction between oxyhemoglobin and *p*-hydroxyanisole, it has been suggested that methemoglobin ($MetHb^{3+}$) is formed due to the following reaction by the interaction of oxyhemoglobin ($[Hb^{2+}O_2]$) with phenolic compounds (ArOH), because formation of phenoxyl radicals ($ArO\cdot$) was confirmed by an electron spin resonance study.[11)]

$$[Hb^{2+}O_2]+ArOH\rightarrow[MetHb^{3+}-O_2{}^{2-}]+ArO\cdot+H^+ \quad (1)$$

$$[MetHb^{3+}-O_2{}^{2-}]+2ArOH\rightarrow 2ArO\cdot+MetHb^{3+}+2OH^- \quad (2)$$

Here, $[MetHb^{3+}-O_2{}^{2-}]$ represents the perferryl species of hemoglobin, which is assumed to be formed as a reaction intermediate.[11,12)] Flavonoids such as quercetin and morin seem to form methemoglobin by a similar mechanism. However, since significant hemolysis during methemoglobin formation was not observed in this study, phenoxyl radicals did not seem to induce further oxidative reaction that causes membrane damage.

Relatively lipophilic polyphenols such as quercetin and morin, which easily penetrate the cytoplasm of erythrocytes, seem to react with hemoglobin. Since quercetin-3-*O*-glucoside had only small effects, the glucoside may not permeate through the glucose transporter (GLUT1) in the erythrocytes.[13)] In various cells there are many kinds of heme proteins. Flavonoids possessing hydroxyl groups have been revealed to inhibit the activities of heme enzymes such as cytochrome P450[14)] and cyclooxygenase.[15)] Therefore, it is likely that lipophilic polyphenols penetrate through the plasma membrane, oxidize various cytoplasmic heme proteins and inhibit their enzymatic reactions.

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