Quercetin metabolites inhibit copper ion-induced lipid peroxidation in rat plasma

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Abstract The oxidative susceptibility of plasma obtained from rats after intragastric administration of quercetin was studied to know whether or not quercetin acts as an in vivo antioxidant after metabolic conversion. Quercetin was raised in the rat blood plasma essentially as glucuronide and/or sulfate conjugates. The plasma obtained from rats after quercetin administration was more resistant against copper sulfate-induced lipid peroxidation than the control plasma on the basis of the accumulation of cholesteryl ester hydroperoxides and the consumption of α-tocopherol. The results strongly suggest that some conjugated metabolites of quercetin act as effective antioxidants when plasma is subject to metal ion-induced lipid peroxidation.

Key words: Flavonoid; Quercetin; Conjugated metabolite; Antioxidant; Lipid peroxidation

1. Introduction

Flavonoids are polyphenolic compounds that occur in a variety of foods and beverages from vegetable origin [1,2]. Among flavonoids, quercetin is the major representative of the flavonol subclass. In vitro, it has been shown that quercetin is a strong antioxidant with peroxyl radical-scavenging activity [3–5] and suppresses oxidation of low-density lipoprotein (LDL) [6,7]. Much evidence has implicated the role of oxidized LDL in atherogenesis [8,9]. Epidemiological studies showed that consumption of certain flavonoids including quercetin was correlated with decreasing risk of coronary artery disease mortality [10–12].

After some negative early reports about uptake of quercetin in man [13,14], recent studies have shown good absorption of quercetin from onions [15,16] and from normal diet [17] by human beings. In addition, Manach et al. [18–20] have demonstrated the uptake of quercetin and its metabolic conversion to conjugated and methylated derivatives in rats. In the preceding study, we succeeded in the determination of conjugated metabolites of quercetin in rat plasma by using a combination of enzymatic hydrolysis and HPLC analysis (Piskula, M. and Terao, J., unpublished). However, there is little evidence on the antioxidative ability of conjugated metabolites of quercetin. The present study was designed as an attempt to clarify this topic by measuring the susceptibility to lipid peroxidation of plasma obtained from rats after intragastric administration of quercetin.

2. Materials and methods

2.1. Materials

Quercetin (3,3′,4′,5,7-pentahydroxyflavone), β-glucuronidase type VII-A, sulfatase type VIII and sulfatase type H-5 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals Co., (Tokyo, Japan). All solvents used were of HPLC grade (Nacalai Tesque, Inc., Kyoto, Japan).

2.2. Animals and quercetin administration

Twenty-four male Wistar rats, weighing 195–210 g were obtained from Charles River Japan Inc. (Atsugi, Japan). The animals were randomly divided into three groups of eight rats. All groups of animals were maintained in a room at 23°C under 12 h dark/light cycling. Water and regular chow were supplied ad libitum. Quercetin (2 or 10 mg/200 g weight body) was dissolved in 2 ml of propylene glycol and then administered intragastrically by stomach intubation to two groups of 12–15 h fasting rats. Control rats (n=8) were administered with only vehicle (propylene glycol). No side effects were observed during the experimental time period. One h and 6 h after administration, four rats from each group were anesthesized by diethyl ether, the abdomen wall was opened and blood was collected from the abdominal aorta into heparinized tubes. Plasma was isolated by centrifugation at 4°C (1000×g for 20 min) and used immediately for experiments or stored at −20°C for no more than one week.

2.3. Determination of quercetin conjugated metabolites in rat plasma

For the determination of quercetin glucuronides, plasma aliquots (50 μl) from rats administered with 2 or 10 mg quercetin were mixed with 50 units of β-glucuronidase solution in 0.2 M acetate buffer (pH 5.0) and incubated at 37°C under agitation for 2 h. Plasma was mixed with sulfatase type VIII (25 U) in 0.2 M acetate buffer (pH 5.0) at 37°C for 4 h for the determination of sulfate conjugates. To inhibit the β-glucuronidase contaminant activity present in the sulfatase preparation (< 3 U), D-saccharic acid 1,4-lactone (4 mg/ml) was added. For sulfoglucuronide conjugates, plasma was incubated with 25 U sulfatase type H-5 in 0.2 M acetate buffer pH 5.0 at 37°C in water shaking bath for 45 min. This sulfatase preparation revealed substantial β-glucuronidase activity (500 U of enzyme). Quercetin containing plasma released by enzymatic hydrolysis (50 μl) was extracted with 50 μl of 0.2 M acetate buffer and 950 μl of methanol:acetic acid (100:5, v/v). The mixture was vortexed and sonicated for 30 s and centrifuged for 5 min at 5000×g. The supernatant was diluted with equivalent volume of water and a resulting aliquot was injected on an HPLC column (TSK gel ODS-80TS, 5 μm, 150×4.6 mm, TOSOH, Japan). The flow rate of the mobile phase, composed of methanol/water/acetic acid (55/43/2, v/v/v) containing 50 mM lithium acetate, was 0.9 ml/min. Elute was monitored with amperometric electrochemical detector (ICA-3062, TOA, Japan) set at 6.4 nA/F.S and working potential at +800 mV. Experiments with quercetin spiked plasma

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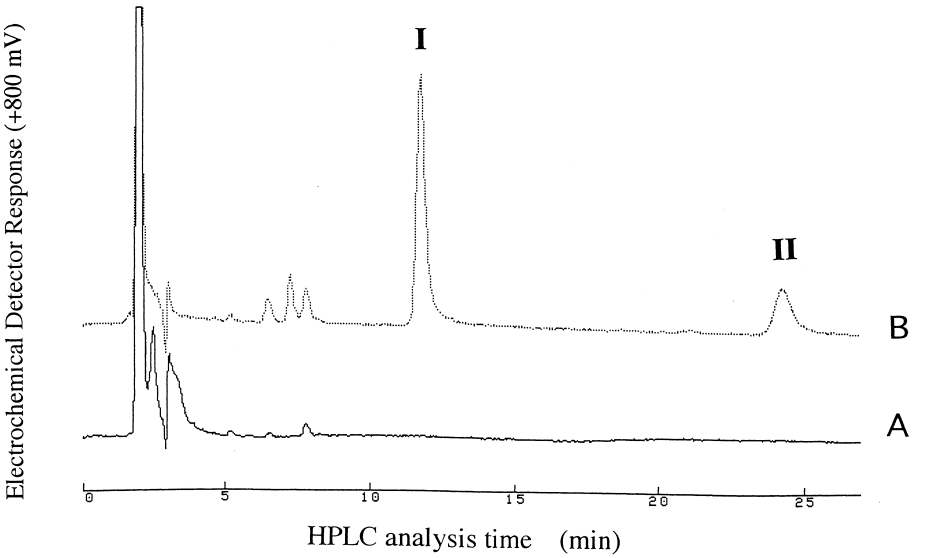

Fig. 1. Typical chromatogram of HPLC analysis of rat plasma extracts after oral administration of quercetin. A: The extracts without enzymatic hydrolysis. B: The extracts with hydrolysis by sulfatase H-5. Peaks I and II were identified as quercetin and isorhamnetin, respectively. The plasma extracts were obtained from the 2 h after oral administration of propylene glycol solution of quercetin (2 mg/200 g body weight). The eluting solvent for the HPLC analysis was composed of methanol/water/acetic acid (45/53/2, v/v/v). Other analytical conditions were the same as those described in Section 2.

showed that this procedure ensures approximately 95% of quercetin recovery. Quantitative determination was done by external standard method. It was verified that the detector response was linear with standards up to 20 μM. When necessary, samples were diluted with chloroform/water/acetic acid (50/50/3, v/v/v) prior to HPLC injection.

2.4. Isolation and mass spectrometry of quercetin metabolites

Plasma (4.0 ml) was obtained from four rats after 2 h oral administration of quercetin (10 mg/200 g body weight in 2.0 ml propylene glycol) by the same procedure as described above, and then mixed with 16 ml of methanol vigorously. The mixture was then centrifuged at 2000×*g* for 20 min to obtain methanol extract. The extract was washed with 32 ml of water and 24 ml of chloroform. After centrifugation, the methanol-water layer was evaporated in a rotary evaporator and redissolved with 4.0 ml of water. Water fraction was then applied to solid-phase-extract cartridge (Mega Bond Elut C18, Varian, CA, USA). The cartridge was treated with 6.0 ml of methanol and 6.0 ml of water. After the sample application and washing with 6.0 ml of water, quercetin metabolites were eluted with 6.0 ml of 30% methanol. After evaporation, the metabolites were hydrolyzed by 4.0 ml of 2 N HCl at 90°C for 2 h. The solution was neutralized by NaOH and was applied to solid-phase-extract cartridge with the same procedure as described above, except for 90% methanol instead of 30% methanol for the elution of hydrolyzed metabolites. The elute was concentrated in vacuo and then subject to LC-MS analysis (M-1200AP Mass spectrometer, Hitachi, Japan), which was connected to L-7000 HPLC system (Hitachi). The column, YMC-Pack A-312 S-5 ODS, 6×150 mm (YMC, Japan) was used and eluted with methanol/water/acetic acid (60:40:0.2, v/v/v) at flow rate of 0.9 ml/min. The LC-MS conditions were as follows; nebulizer temperature at 200°C, dissolver temperature at 400°C, apparatus-1 and -2 temperature of 120°C.

2.5. Oxidation of rat plasma pool induced by copper sulfate

Plasma from propylene glycol (control) or quercetin solution administered rats was diluted four times with PBS (pH 7.4). The oxidation was initiated by the addition of 100 μM $CuSO_4$. This condition was found to be suitable to oxidize the plasma within the time period of 6 h. In another set of experiments, quercetin at concentrations given in the figure legend was added externally to control plasma 5 min before initiation of the oxidation with copper ion. The progress of plasma oxidation was monitored by the measurement of cholesteryl ester hydroperoxides (CE-OOH) and consumption of plasma α-tocopherol. The procedures for the determination of CE-OOH and α-tocopherol in the plasma were described elsewhere [21].

Table 1
Micromolar concentration of quercetin metabolites in rat plasma after intragastric adminstration of quercetin at two dose levels to rats

	Time after adminstration			
Conjugates	1 h		6 h	
Dose 2 mg[a]	Quercetin (μM)	Isorhamnetin (μM)	Quercetin (μM)	Isorhamnetin (μM)
Free	0	0	0	0
Sulfate	0.38	0	0	0
Glucuronide	6.14	0	1.33	0
Sulfoglucuronide	3.13	4.22	2.88	4.88
Total	9.64	4.22	4.21	4.88
Dose 10 mg[a]	Quercetin (μM)	Isorhamnetin (μM)	Quercetin (μM)	Isorhamnetin (μM)
Free	0	0	0	0
Sulfate	10.4	0	0.4	0
Glucuronide	14.0	0	1.03	2.44
Sulfoglucuronide	44.6	20.4	18.0	13.56
Total	69.0	20.4	19.3	16.00

[a]Dose: mg of free quercetin/200 g of rat body weight.

3. Results and discussion

Quercetin dissolved in propylene glycol was administered by intragastric intubation to fasted Wistar male rats at two different dose levels, that is, 2 and 10 mg/200 g of body weight. Fig. 1 shows the typical chromatogram of the plasma extracts obtained from quercetin administered rats. After enzymatic hydrolysis, two new peaks appeared in the chromatogram and were verified to be free quercetin and its 3′-*O*-methylated form (isorhamnetin) by comparison with the retention time of respective external standards. In addition, mass spectrometry showed an $[M+1]^+$ at m/z 303 for the first peak in the chromatogram, corresponding to the $[M+1]^+$ of standard quercetin, and an $[M+1]^+$ at 317 for the second peak, corresponding to the $[M+1]^+$ of standard isorhamnetin, respectively.

Plasma from four rats of each group was pooled in order to minimize the variation in the metabolite levels due to individual differences of absorption. Quercetin or isorhamnetin content after incubation with glucuronidase and sulfatase type VIII was assumed to be the amount of glucuronide conjugates and sulfate conjugates, respectively. The amount released by the incubation with sulfatase type H-5 minus the amount released by the glucuronidase and sulfatase type VIII treatment was defined as the amount of sulfoglucuronide conjugates. Table 1 shows the pooled plasma level of quercetin metabolites determined 1 and 6 h after administration. No free quercetin nor free isorhamnetin could be observed. One h after administration of 2 mg quercetin, 13.86 μM of total metabolites were found in the plasma pool, in which 70% (9.64 μM) distributed as non-methylated quercetin metabolites. The level of total metabolites decreased to 9.09 μM after 6 h, while the level of methylated form was unchanged. Administration of 10 mg quercetin enhanced the total metabolites level to 89.4 and 35.3 μM after 1 and 6 h respectively (6.4- and 3.9-fold compared to 2 mg administration). No

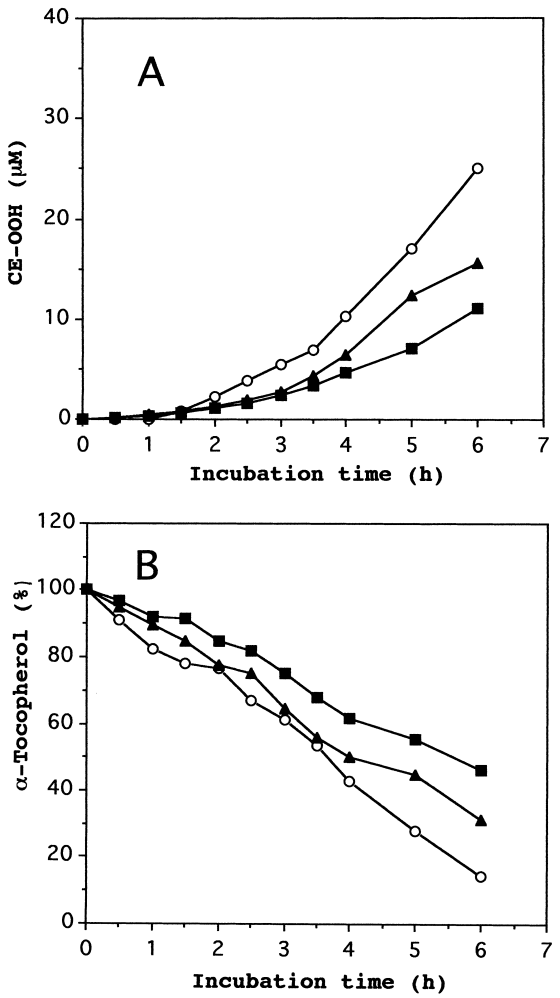

Fig. 2. Copper ion-induced lipid peroxidation of rat plasma obtained from 1 h after administration of propylene glycol (control; open symbols) or quercetin solution (closed symbols), 2 mg (▲) or 10 mg (■)/200 g body weight. Accumulation of CE-OOH (A) and consumption of α-tocopherol (B). Plasma from four rats of each group was pooled, diluted four times with PBS and incubated with 100 μM $CuSO_4$ at 37°C for up to 6 h. The concentration of α-tocopherol in the incubation system was 4.35 μM and data were percentages of the initial concentration.

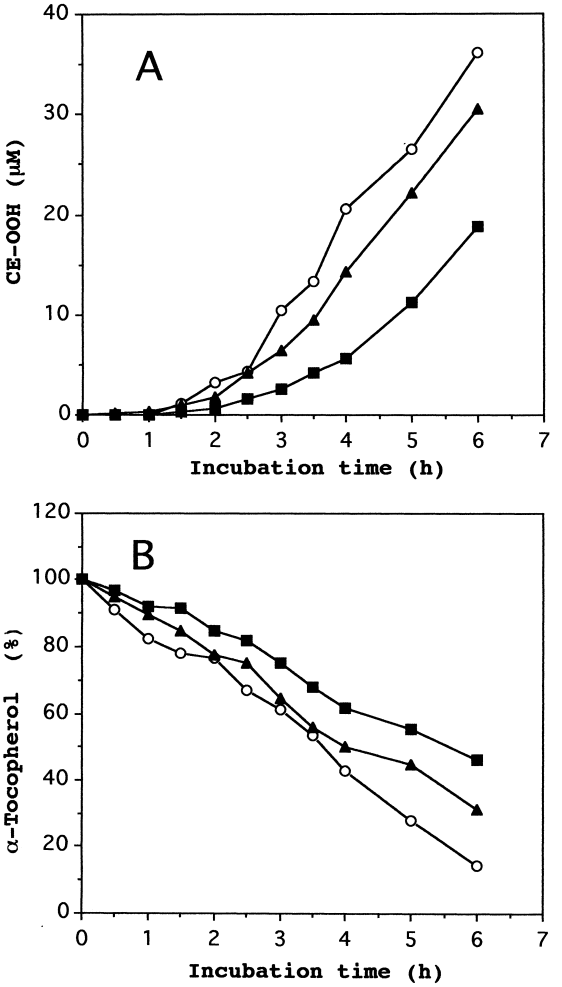

Fig. 3. Copper ion-induced lipid peroxidation of rat plasma obtained from 6 h after administration of propylene glycol (control; open symbols) or quercetin solution (closed symbols), 2 mg (▲) or 10 mg (■)/200 g body weight. Accumulation of CE-OOH (A) and consumption of α-tocopherol (B). Plasma from four rats of each group was pooled, diluted four times with PBS and incubated with 100 μM $CuSO_4$ at 37°C for up to 6 h. The concentration of α-tocopherol in the incubation system was 4.35 μM and data were percentages of the initial concentration.

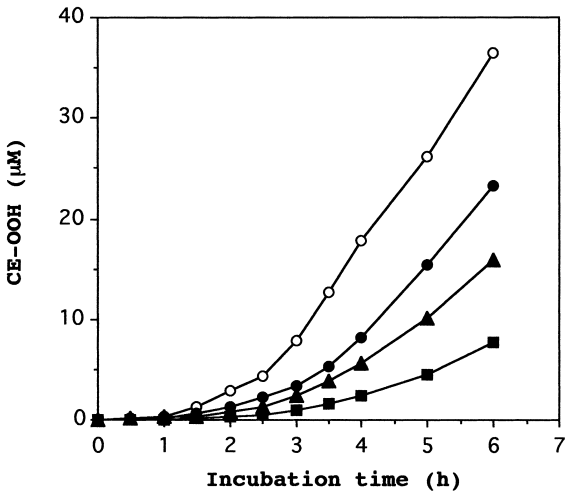

Fig. 4. Effect of in vitro addition of quercetin at different concentrations on the accumulation of CE-OOH in copper ion-induced lipid peroxidation of rat plasma. Control plasma pool after 6 h administration of propylene glycol was diluted four times with PBS and oxidized with 100 μM $CuSO_4$ at 37°C for up to 6 h. Concentration of quercetin added in the pooled plasma was as follows; ○, none; ●, 8 μM; ▲, 40 μM; ■, 80 μM.

quercetin metabolites were detected in the plasma from control rats.

The antioxidative activity of quercetin metabolites was examined by ex vivo incubation of the plasma with copper ion. Fig. 2 shows the accumulation of CE-OOH (Fig. 2A) and consumption of plasma α-tocopherol (Fig. 2B) in the plasma from rat 1 h after administration of quercetin. An apparent lag phase was observed in the first 2 h of incubation in the case of CE-OOH accumulation. Both plasma after administration of 2 mg quercetin and 10 mg quercetin showed a slight but consistent lower level of CE-OOH and lower consumption of α-tocopherol than control plasma. Quercetin administered at 10 mg to rats increased the plasma resistance against copper ion-induced oxidation as compared with the case of 2 mg administration. As the final metabolite levels in the pooled plasma were 13.9 and 89.4 μM for 2 mg quercetin and 10 mg quercetin administration, a dose-dependent inhibition was observed in the amount of administered quercetin.

The same phenomena were observed in the oxidative resistance of the rat plasma obtained after 6 h administration of quercetin (Fig. 3A, B). The final metabolite levels in the pooled plasma after 6 h administration was 9.1 μM and 35.3 μM for 2 mg and 10 mg quercetin administration, respectively. The oxidation rate of plasma after 6 h of propylene glycol (control), indicated by the increase of CE-OOH level, was higher than that after 1 h. Since the animals were already 12–15 h fasted, additional 6 h of experimental period might be significant to modify the level of other metabolites in plasma. It is well known that the circulating levels of ketone bodies (acetoacetate and β-hydroxybutyrate) and free fatty acids rise during prolonged starvation [22]. Thus, the enhanced level of free fatty acids may be responsible for increasing plasma oxidizability, but an influence of other components cannot be excluded.

The antioxidant activity of free quercetin was confirmed in our experimental model. In vitro addition of free quercetin to the control plasma after 6 h administration of propylene glycol inhibited the copper ion-induced CE-OOH formation (Fig. 4) in a dose-dependent manner from 4 to 80 μM. By comparing this result with those from the result of ex vivo experiment (Fig. 3A), it can be concluded that some conjugated metabolites of quercetin can inhibit copper ion-induced plasma peroxidation, similarly to free quercetin. Antioxidant activity of quercetin is attributed to chelating and/or free radical-scavenging property of phenolic hydrogens in its structure [5]. Some phenolic hydrogens responsible for the antioxidant activity is likely to remain during the process of metabolic conversion. Thus, quercetin possesses an antioxidative activity against copper ion-induced peroxidation of plasma lipids even after absorption and metabolic conversion.

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