PROCEEDING

Antioxidant capacity of albumin-bound quercetin metabolites after onion consumption in humans

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Abstract : Quercetin is a major dietary flavonoid found in onions and other vegetables. It is known that dietary quercetin is metabolized in the intestinal mucosa and the liver and is present as its glucuronide/sulfate conjugates with or without methylation. Although quercetin is known to possess strong antioxidant activity, there are only limited reports on the antioxidant activity of its metabolites. In this study, the antioxidant capacity of quercetin metabolites under physiological conditions was investigated. After consumption of cooked onion, more than 80% of quercetin metabolites were localized in the human plasma fraction containing concentrated serum albumin. Other lipoprotein fractions contained only small amounts of quercetin metabolites. Addition of quercetin 3-*O*-β-glucuronide to the lipoprotein-eliminated plasma fraction generated antioxidant activity against LDL oxidation in a dose-dependent manner. However, onion consumption failed to enhance the antioxidant activity of the lipoprotein-eliminated plasma fraction against LDL oxidation, probably because the amount of quercetin metabolites bound to albumin was less than the effective level in an ex vivo study. The physiological role of plasma albumin in retaining quercetin metabolites needs to be further clarified. J. Med. Invest. 54 : 370-374, August, 2007

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INTRODUCTION

Dietary flavonoids have attracted much attention because of their potentially beneficial effects in preventing various diseases, especially coronary heart disease. Quercetin is a major flavonol-type flavonoid commonly found in vegetables and fruits, and in especially high amounts in onions. The bioavailability of quercetin in humans has been studied by many research groups (1). Quercetin metabolites have been detected in human plasma as the glucuronide and/or sulfate with or without additional methylation (2). One of the major quercetin metabolites, quercetin 3-*O*-β-D-glucuronide, has been shown to protect low-density lipoprotein (LDL) from copper ion-induced oxidative damage *in vitro* (3). However, it is not known whether quercetin metabolites present in human plasma following consumption of a flavonoid-

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rich diet can act as a part of biological antioxidant system for protection of plasma LDL from harmful oxidative stress.

We have previously reported that short-term ingestion of onions by female volunteers resulted in accumulation of quercetin metabolites in the plasma (4). However, these metabolites were only detected in the LDL fraction at a very low level, and failed to improve its resistance to copper ion-induced oxidation following the trial. McAnlis, *et al.* (5) reported that quercetin metabolites were not detected in the LDL fraction after onion consumption by 5 healthy volunteers and that no direct protective effect was provided against LDL oxidation. On the other hand, they detected quercetin metabolites in the HDL fraction, although this fraction contained other proteins including albumin.

The aim of this study was to clarify the distribution of plasma quercetin metabolites after single onion consumption and to investigate their potential antioxidative effect against LDL oxidation ex vivo.

MATERIALS AND METHODS

Chemicals

Quercetin-3-*O*-β-*D*-glucuronide (quercetin-3-glucuronide) was chemically synthesized as previously described (6). β-Glucuronidase H-5 (from *Helix pomatina*) was obtained from Sigma Chemical Co. (St. Louis, MO). Human serum albumin (essentially fatty acid free), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 3-morpholinosydnonimine hydrochloride (SIN-1) were purchased from Wako Pure Chemical Ind. (Tokyo, Japan). All other chemicals were of analytical grade.

Study design

Three healthy male volunteers aged 30-55 years were recruited for this study. Each subject ingested 500 g of fried onions (Kagome Co., Japan), equivalent to 150 mg quercetin aglycone, after fasting overnight. Peripheral venous blood was collected into a heparinized tube 0.5 h before and 1.5 h after consumption. The protocol was approved by the Ethical Committee of the University of Tokushima, Japan.

Isolation of lipoprotein fraction

Plasma was obtained from heparinized blood samples by centrifugation at 3,000 rpm for 10 min at 4°C. To obtain lipoprotein fractions, plasma was further subjected to density-gradient centrifugation. Briefly, plasma was adjusted to d=1.21 with KBr and overlaid with saline (d=1.008), and centrifugation was performed at 600,000g for 40 min at 4°C. After collecting the fractions containing VLDL/chylomicrons (CM) and LDL, further centrifugation was performed for 6 hr, and HDL was separated from the plasma (the lipoprotein-eliminated plasma fraction). The purity of each lipoprotein fraction was confirmed by SDS-PAGE (data not shown).

Analysis of plasma quercetin metabolites

Plasma and lipoprotein fractions were incubated with 50 U of β-glucuronidase type H-5 (from *H. pomatia*), which possesses both β-glucuronidase and sulfatase activity, in 0.1 M sodium acetate buffer, pH 5.0 for 60 min at 37°C. The liberated quercetin aglycone was extracted with ethyl acetate. Quercetin metabolites in the plasma and lipoprotein fractions were determined using an amperometric detector-HPLC system as previously described (4).

Binding of a quercetin conjugate to human serum albumin

The binding study was performed as previously reported by Janisch, *et al.* (7). Briefly, a human serum albumin (essentially fatty acid free) solution (5 μM) was mixed with quercetin-3-glucuronide (1.0 to 10.0 μM) and the tryptophan-derived fluorescence spectrum was measured (Ex 298 nm, Em 285-500 nm).

Antioxidant properties of the lipoprotein-eliminated plasma fraction vs LDL oxidation

The LDL fraction isolated from fasting plasma (0.2 mg protein/ml) was oxidized with the lipoprotein-eliminated plasma fraction (2.0 mg protein/ml) obtained from fasting and post-prandial plasma samples. AAPH was used as a pro-oxidant. The reaction mixture was incubated at 37°C with continuous shaking for 6 hr. To estimate the effect of quercetin metabolites bound to plasma albumin, the lipoprotein-eliminated fasting plasma fraction was incubated with quercetin-3-glucuronide to mimic the albumin-bound quercetin conjugates. Either $CuSO_4$, AAPH or SIN-1 was added to this model solution and reacted similarly. The time-dependent production of cholesteryl ester hydroperoxide (CEOOH) was determined as a marker of lipid peroxidation level, by UV-HPLC analysis at 235 nm using an Octyl-80Ts (TSK-gel, 4.6 × 150 mm, Tosoh) column and a mobile phase of 97% methanol at a flow rate of 1.0 ml/min.

Statistics

Results are presented as means ± SD (n=3). Significant differences between values obtained under fasting and post-prandial conditions were analyzed by a paired t-test. Data were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Plasma distribution of quercetin metabolites after onion consumption in humans

Following onion consumption, the plasma concentration of quercetin metabolites was significantly increased compared to that under fasting conditions (Table 1). On the other hand, the amount of quercetin metabolites detected in the pellet or the red blood cell fraction, was approximately 10% of the total quercetin content in the blood and not different before and after onion consumption (0.170 ± 0.058 and 0.214 ± 0.040 pmol/mg protein, respectively). In the plasma, 80% of quercetin metabolites remained in the lipoprotein-eliminated plasma fraction (Table 1). On the other hand, a relatively small proportion of the quercetin metabolites were distributed in the lipoprotein fractions. However, while these amounts were increased in the LDL and HDL fractions after onion consumption, the difference in content before and after consumption was not statistically significant because of the limited numbers of subjects ($p=0.192$ and 0.112, respectively).

Binding of a quercetin conjugate to human serum albumin

As shown in Table 1, onion-derived quercetin metabolites were exclusively localized in the lipoprotein-eliminated plasma fraction. Albumin was the most abundant protein in this fraction and quercetin metabolites were considered to associate with serum albumin. Thus, the binding ability of quercetin-3-glucuronide, one of the major plasma metabolites, to human serum albumin was examined by measuring the quenching of fluorescence derived from tryptophan present in several binding subdomains (7). The addition of quercetin-3-glucuronide to serum albumin reduced the fluorescence intensity of the emission maximum at 343 nm in a dose-dependent manner (Fig. 1). These data are consistent with those previously reported (7). Thus, it is likely that plasma quercetin metabolites are mostly bound to albumin.

Table 1 Plasma distribution of quercetin metabolites after onion consumption by human volunteers

	Plasma concentration (μM)	CM/VLDL	LDL	HDL	Lipoprotein-eliminated plasma fraction
		Upper : pmol/mg protein (Lower : % of plasma)			
Fasting	0.263 ± 0.042	143.7 ± 90.7	18.8 ± 12.5	8.74 ± 4.50	2.97 ± 1.55
		(5.08 ± 0.26)	(3.60 ± 0.76)	(4.62 ± 0.93)	(79.7 ± 1.94)
1.5 h after onion consumption	1.031 ± 0.180[a]	149.3 ± 49.9	35.4 ± 14.2	35.7 ± 14.8	13.99 ± 2.49[a]
		(1.36 ± 0.24)	(2.20 ± 0.68)	(3.78 ± 1.32)	(83.9 ± 3.25)

Values are means ± SD, n=3. CM, chylomicrons ; VLDL, very low density lipoprotein ; LDL, low density lipoprotein ; HDL, high density lipoprotein. [a] $P<0.05$ vs. Fasting.

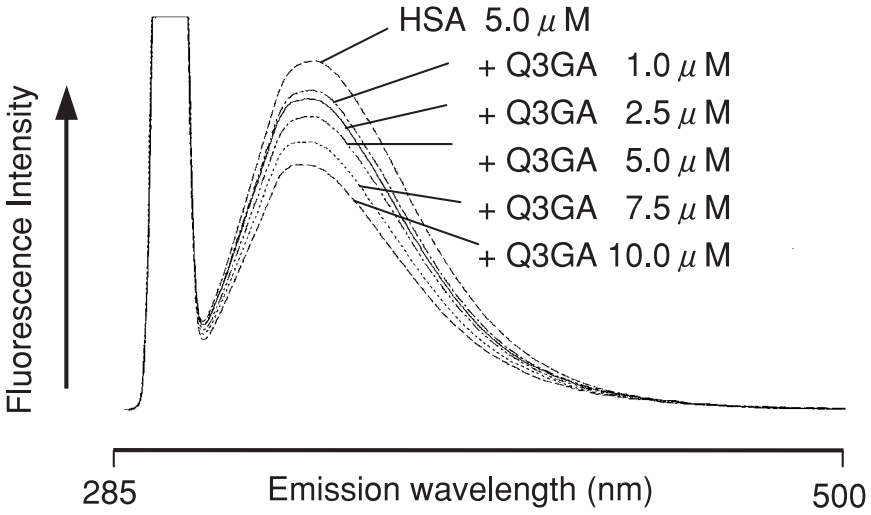

Fig. 1 Effect of quercetin-3-glucuronide on fluorescence quenching of human serum albumin.

Protective effect of albumin-bound quercetin metabolites against LDL oxidation

Oxidized LDL plays a major role in the development of atherosclerosis. Consumption of dietary antioxidants, such as quercetin and other flavonoids, is thought to prevent LDL from oxidative stress *in vivo*. We previously reported that quercetin metabolites inhibited LDL oxidation *in vitro* when incubated directly with LDL (3). However, the quercetin metabolites found in LDL after onion consumption were not sufficient to protect LDL, because the susceptibility of fasting and post-prandial LDL to oxidation did not change (data not shown), consistent with previous reports (4, 5). The antioxidant capacity of quercetin metabolites bound to albumin was next investigated. Fasting LDL was oxidized by the azo-radical generator AAPH, in the presence of the lipoprotein-eliminated plasma fractions from fasting and post-prandial plasma. Onion-derived quercetin metabolites present in the post-prandial plasma did not show significant protective effects against AAPH-induced lipid peroxidation (Fig. 2). In addition, when copper ion was used as a pro-oxidant, no protective effect emerged (data not shown). It has previously been reported that albumin-bound quercetin conjugates exhibited a protective effect against copper ion-induced LDL oxidation (7). Therefore, a possible antioxidant effect of quercetin-3-glucuronide incubated with the lipoprotein-eliminated plasma fraction against LDL oxidation was examined. The quercetin-3-glucuronide-bound albumin fraction inhibited LDL oxidation induced by three different pro-oxidants, AAPH, copper ion and SIN-1 (a peroxynitrite generator), in a dose-dependent manner (Fig. 3). In the case of copper ion-induced oxidation, the low dose of the quercetin conjugate enhanced LDL oxidation (Fig. 3B). Janisch, *et al.* have suggested that binding of quercetin metabolites to albumin could reduce the chelating ability of the albumin molecule (7). When AAPH was used as a pro-oxidant, the lowest dose (100 pmol quercetin-3-glucuronide per mg protein of albumin fraction) failed to protect LDL from oxidation (Fig. 3A). This may explain why the post-prandial albumin fraction did not prevent LDL oxidation ex vivo in this case, because the plasma concentration of quercetin metabolites after onion consumption was relatively low (approx. 1 μM). From these results and other previous reports (5, 7), it is suggested that quercetin metabolites may accumulate in the plasma after consumption of a quercetin-rich diet in the form of albumin-bound conjugates and possibly play a role in plasma antioxidant activity against LDL oxidation. However, since albu-

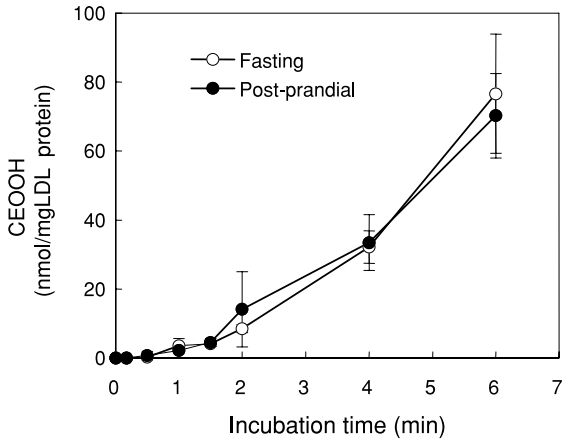

Fig. 2 Effect of the lipoprotein-eliminated plasma fraction on AAPH-induced LDL oxidation. The lipoprotein-eliminated plasma fraction was obtained either from fasting subjects or 1.5 hr after onion consumption. The reaction mixture contained the lipoprotein-eliminated plasma fraction and the fasting LDL fraction as a protein concentration ratio of 10 : 1. The concentration of AAPH used was 0.5 mM. Values presented are the means ± SD, n=3.

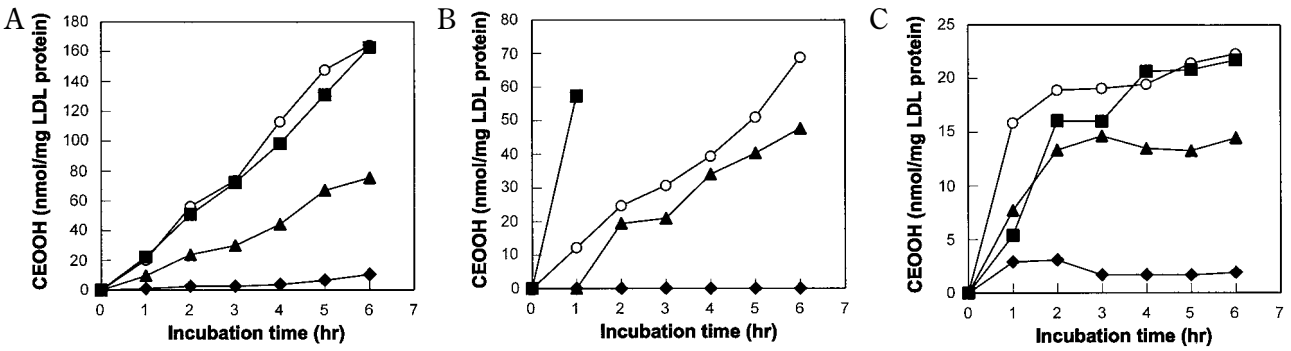

Fig. 3 Effect of free and albumin-bound quercetin-3-glucuronide on LDL oxidation. Oxidants used were 5 mM AAPH (A), 20 μM $CuSO_4$ (B), and 0.075 μM SIN-1 (C). The protein concentrations of the lipoprotein-eliminated plasma fraction (containing concentrated albumin) and LDL fraction used were 2.0 mg/ml and 0.2 mg/ml respectively, and final concentrations of quercetin-3-glucuronide were 0.2, 2 and 20 μM. The results shown are representative. ○, control (without quercetin conjugate) ; ■, 0.2 μM quercetin conjugate ; ▲, 2.0 μM quercetin conjugate ; ◆, 20.0 μM quercetin conjugate.

min itself is an effective antioxidant in the plasma, the contribution of quercetin metabolites may be rather insignificant. The significance of quercetin metabolites bound to plasma albumin may *not* be an involvement in plasma antioxidant defense *but* may relate to a delay of their clearance from plasma, thus allowing their efficient distribution to target organs.

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