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Attenuation of lipid peroxidation and hyperlipidemia by quercetin glucoside in the aorta of high cholesterol-fed rabbit

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Abstract

Antioxidative activity of dietary flavonoids is suggested to be, at least partly, responsible for a wide variety of their biological effects relating to anti-atherosclerosis. However, it is not known whether dietary flavonoids reach to the target site and act as antioxidants. In this study, we tried to evaluate the antioxidative effect of quercetin 3-*O*-β-D-glucoside (Q3G), a typical flavonoid present in vegetables, in rabbit aorta. New Zealand White rabbits were fed a control diet (control group), 2.0% cholesterol diet (HC group) and 2.0% cholesterol plus 0.1% Q3G (HC + Q3G group) for one month. The amounts of total cholesterol, triacylglycerol and total fatty acids in both the plasma and aorta were significantly lower in the HC + Q3G group as compared with the HC group. Quercetin was detected in the aorta of the HC + Q3G group after enzymatic deconjugation, indicating that quercetin accumulated as conjugated metabolites in the aorta. The contents of TBA-reacting substances (TBARS) and cholesteryl ester hydroperoxides (CEOOH) in the aorta of the HC + Q3G group were significantly lower than those in the HC group. The aorta of HC + Q3G group was more resistant than that of HC group in copper ion-induced lipid peroxidation *ex vivo*. HC + Q3G group accumulated a higher amount of vitamin E per total cholesterol than HC group in the aorta. These results strongly suggest that quercetin glucosides accumulate in the aorta as their metabolites and attenuate lipid peroxidation occurring in the aorta, along with the attenuation of hyperlipidemia.

Keywords: *Quercetin, atherosclerosis, lipid peroxidation, antioxidant, hyperlipidemia*

Introduction

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a typical flavonol-type flavonoid distributed ubiquitously in fruits and vegetables [1]. Dietary intake of quercetin and other flavonol-type flavonoids, kaempferol (3,3′,5,7-tetrahydroxyflavone) and myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone), by humans is estimated to be about 23 mg in the population of the Netherlands [2]. However, total intake of flavonoids from plant food may reach several hundred mg/day [3]. Quercetin is recognized as a powerful antioxidant by acting as a free radical scavenger as well as a chelator of transition metal ion, responsible for the generation of reactive oxygen species (ROS) [4,5]. Much attention has been paid to the antioxidative activity of dietary quercetin and its related flavonoids in connection with their physiological function [6], as the oxidative process is known to contribute to the pathogenesis of vascular diseases [7].

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Oxidative modification of low-density lipoprotein (LDL) has been implied in the initial event of atherosclerosis [8–9], although arguments have emerged as to its inevitability in atherosclerosis [10,11]. More recently, it is indicated that oxidative stress and vascular diseases involve the cellular production of ROS, which represent an important means of cellular signaling [12]. Several epidemiological studies have demonstrated that the high intake of certain flavonoids correlate with a decreased risk of atherosclerotic vascular diseases [13–15]. Thus, quercetin intake may be helpful in the prevention of atherosclerosis by inhibiting the oxidative modification of LDL [16] or other cellular oxidative events. In fact, Hayek et al. [17] demonstrated that consumption of quercetin suppressed the progression of atherosclerotic lesions in apolipoprotein E-deficient mice. They also found that quercetin intake was associated with reduced susceptibility of LDL to oxidation. Nevertheless, the behavior of dietary quercetin and its efficacy as an antioxidant in blood vessels is still obscure, although blood vessels are the target where LDL is oxidized and cellular oxidative events happen.

Flavonoids are mostly present as their glycosides in plant foods [1]. In general, quercetin glycosides containing a sugar group at the 3 position, such as isoquercitrin (quercetin 3-*O*-β-D-glucoside; Q3G) and quercitrin (quercetin 3-*O*-β-D-rhamnoside), are commonly distributed in a wide variety of vegetables, except for quercetin 4′-*O*-β-D-glucoside which is characteristically present in onion [18]. In recent years, intestinal absorption and metabolism of quercetin glucosides have been extensively investigated from the view of their bioavailability [19,20]. Quercetin glucosides are subjected to hydrolysis and subsequent conversion into conjugated glucuronides and/or sulfates in the process of intestinal absorption [21]. A variety of metabolites circulating in the blood stream were identified [22,23] and some of them were found to possess a substantial antioxidant activity [24,25]. We already demonstrated that one of the metabolites, quercetin-3-*O*-β-D-glucuronide (Q3GA), is capable of inhibiting LDL oxidation *in vitro* [26,27] and suppressing hypertrophy of smooth muscle cell via the inhibition of mitogen-activated protein (MAP) kinase activation [28].

The aim of this study is to determine whether or not dietary quercetin accumulates in the aorta, a target site for its anti-atherosclerotic effect, and exert an antioxidant activity in this site. We used high cholesterol diet-fed rabbits as a model animal of atherosclerosis and selected Q3G as a representative of quercetin glycosides for the additive to a high cholesterol diet. The results indicate Q3G intake induces the accumulation of quercetin metabolites and the attenuation of lipid peroxidation in the aorta, along with the attenuation of hyperlipidemia.

Materials and methods

Materials

Quercetin (3,3′,4′,5,7-pentahydroxyflavone;Q) and sulfatase H-5 from *Herix Pomatia* (EC 3.1.6.1) were obtained from Sigma Chemical Company (St. Louis. MO). Isoquercitrin (quercetin 3-*O*-β-D-glucopyranoside; Q3G) was purchased from Extrasynthese S.A. (Genay, France). Isorhamnetin (3,4′,5,7-tetrahydroxy-3′-methoxyflavone) and diosmetin (3′,5,7-trihydroxyflavone) were also from Extrasynthese S.A. Standard 4-hydroxynonenal (4-HNE) and hexanal were obtained from Cayman Chemical (Ann Arbor, MI) and Sigma Chemical Company, respectively. Cholesteryl ester hydroperoxides (CEOOH) for a standard compound were prepared by the method described by Arai et al. [29]. All other reagents are of analytical grade and with no purification.

Animals and diet administration

New Zealand White rabbits (male, 8 weeks old, 1.2–1.5 kg body weight) purchased from SLC Japan (Hamamatsu, Japan) were fed RC-4 rabbit chow (Oriental Yeast, Tokyo, Japan) for one week. These rabbits were randomly divided into three groups, each group of rabbit (four animals per group) being fed on the control diet (RC-4 diet; Oriental Yeast Co. Ltd, Kyoto, Japan; *Control group*), high-cholesterol diet (RC-4 diet containing 2% (w/w) cholesterol; *HC group*) or high-cholesterol diet containing Q3G (RC-4 diet containing 2% (w/w) cholesterol and 0.1% (w/w) Q3G; *HC + Q3G group*) for one month *ad libitum*. Contents of the components related to this experiment in RC-4 diet (per 100 g diet) were described as follows; water, 7.8 g; protein, 17.9 g; fat, 3.3 g; fiber, 8.6 g; energy, 291 kcal, cholesterol, 1 mg; vitamin E, 9.1 mg. The fatty acid composition of the diet was described as follows; 16:0, 15.9%; 18:0, 2.2%; 18:1, 13.5%; 18:2, 40.7%; 18:3, 18.7%, and others (less than 1.0%).

The animals were housed under standard laboratory conditions (25°C, 60%, 12 h light and 12 h dark cycle). The experiments were performed in accordance with the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science of 1987). At the end of the study, rabbits were anesthetized with sodium pentobarbital (50 mg/kg) after fasting for 12 h and killed by exsanguinations from the carotid artery. Plasma was immediately obtained from each animal by centrifuging heparinized blood at 2400 g for 20 min at 4°C. The aorta was stripped of excess adventitial tissue and opened longitudinally along the anterior wall. Plasma samples and aorta samples were stored at −85°C with nitrogen gas until required for analysis.

Preparation of aorta homogenate

Aortas were cut into small pieces and immediately added to 2.97 ml PBS containing 1 mM EDTA and 0.03 mL of 10 mM butyl hydroxytoluene (BHT) in ethanol (final concentration; 0.1 mM). In the experiment of $CuSO_4$-induced lipid peroxidation, 3.0 mL PBS containing no EDTA was used instead of 2.97 ml PBS containing EDTA and 0.03 mL of BHT solution. Aortas were then homogenized with Polytron homogenizer (KINEMATICA, AG, Co. Littau-Lucerne, Switzerland). After centrifugation (2500 rpm at 4°C for 10 min), resulting supernatants were obtained as aorta homogenate. Protein concentration was determined using Bradford assay, and bovine serum albumin was used as a standard [30]. All procedures for the homogenate preparation were performed in an ice bath and the homogenates were stored at −30°C until use.

Determination of cholesterol and triacylglycerol in plasma and aorta homogenate

Total cholesterol, free cholesterol and triacylglycerol in plasma and aorta homogenate were determined with respective enzymatic assay kits (Wako Pure Chemical Industries, Osaka Japan). The content of cholesteryl ester was calculated by subtracting free cholesterol from total cholesterol.

Determination of total quercetin from plasma and aorta homogenate

Rabbit plasma (100 μl) was mixed with 50 μl of sulfatase type H-5 (25 units from *H. Pomatia*) solution in 1.0 M sodium acetate buffer (pH 5.0). The mixture was incubated at 37°C for 50 min to liberate quercetin aglycone from its conjugates [25,31]. In the case of aorta homogenate, 75 μl of sulfatase type H-5 solution (37.5 units) in 1.0 M sodium acetate buffer (pH 5.0) was added to 300 μl of homogenate and incubated at 37°C for 50 min. In each case, the mixture was then added to 1.1 ml of methanol/acetic acid (100:5, v/v) containing diosmetin as an internal standard, followed by sonication for 30 s. The mixture was centrifuged at 7500 g for 5 min at 4°C. The supernatant was concentrated by evaporation with nitrogen gas and dissolved in the solution of methanol/acetic acid/H_2O (20:1:21, v/v/v). A portion of the resulting solution was subjected to HPLC column (TSK gel ODS-80 S, 5 μm, 150 × 4.6 mm, Tosoh). The mobile phase was composed of methanol/water/acetic acid (41:57:2, v/v/v) containing 50 mM lithium acetate for the analysis of the aorta and methanol/water/acetic acid (38:60:2, v/v/v) containing 50 mM lithium acetate for that of plasma. The flow rate was 1.0 ml/min and elution was monitored with an amperometric electrochemical detector (ICA-5212, TOA Electrics Co., Japan) with a working potential of +800 mV. For the identification of the peak appearing in the chromatograms, LC/ESI-MS analysis was performed using an HP1100 series HPLC (Agilent, Palo Alto, CA). The sample was injected to the column of Zorbax SB-C18 (3 × 150 mm, 3 μm, Agilent) at 40°C and eluted with a mixture of (A) 20% methanol containing 0.1% acetic acid and (B) 80% methanol containing 0.1% acetic acid as the mobile phase. Separation was done using 0 to 60% linear gradient of B in A for 30 min at the flow rate of 0.15 ml/min. The conditions for MS analysis in the negative-ion mode (LCQ, Thermoelectron) were a capillary voltage of 4500 V, spray voltage of 3 V, and a capillary temperature of 260°C.

Measurement of thiobarbituric acid-reacting substances (TBARS) and CEOOH in plasma and aorta homogenate

For TBARS assay, 30 μl of plasma diluted with 70 μl of PBS (pH 7.4) or 100 μl of aorta homogenate was mixed with 500 μl of 30% TCA containing 0.5N HCl and 10 mM BHT in ethanol 50 μl. Then the mixed solution was heated for 15 min. After cooling down, 1.5 ml of *n*-butanol was added to the solution and centrifuged (1750 g, 4°C for 5 min). The upper layer was taken out and its fluorescence (excitation 515 nm; emission 553 nm) was measured [32]. For the measurement of CEOOH, 100 μl of aorta homogenate or plasma which was 4 times diluted with PBS (pH 7.4) was added to 3 ml of methanol containing 2.5 mM BHT and 3 ml of hexane. After vigorous mixing, the hexane layer was obtained and evaporated *in vacuo*. The residue was mixed with the solution of methanol/chloroform (95:5, v/v) and subjected to HPLC analysis for the determination of CEOOH [33]. HPLC was performed with a column of Octyl-80Ts (TSK-gel, 4.6 × 150 mm, Tosoh) and a mobile phase of 97% methanol at a flow rate of 1.0 ml/min. CEOOH was detected at 235 nm absorption.

Copper ion-induced lipid peroxidation of the aorta homogenate and measurement of the lipid peroxidation level

$CuSO_4$ was added to an aliquot (300 μl) of aorta homogenate at the final concentration of 1.0 mM and incubated at 37°C with continuous shaking. The peroxidation level after 4 h incubation was monitored with TBARS-fluorescence assay and CE-OOH contents as described above. In addition, hexanal and 4-HNE were measured as additional biomarkers of lipid peroxidation [34]. In brief, an aliquot of the mixture (300 μl) was added to 10 μl of ethanol solution containing 10 mM BHT and 10 μl of 10 mM Desferal in Tris-HCl buffer (pH 7.4, 0.1 M). Then, 70 μl of freshly prepared dinitrophenylhydrazine reagent (0.35 mg/mL in 1 M HCl) was added and incubated in the dark for 2 h at room temperature.

The reaction mixture was extracted with chloroform/methanol (1:2, v/v) and subjected to centrifugation. The chloroform layer was collected and evaporated *in vacuo*. The residue was then dissolved in methanol and injected into a Shimadzu HPLC LC-10A equipped with an ODS-80Ts column (4.6 × 150 mm. Tosoh). The mixture of methanol/water (31:9,v/v) was used as the eluting solvent with a flow rate of 1.0 ml/min. Eluate was detected at the absorption at 378 nm. The peak assignment of 4-HNE and hexanal and their quantification were done in comparison with their respective standard compounds.

Determination of total fatty acid contents of plasma and aorta homogenate

The fatty acid compositions of total lipids in the plasma and aorta were determined by gas chromatographic analysis after transmethylation. Methyl margarate was added to the homogenate or plasma as an internal standard, and then total lipids were extracted by the method of Bligh and Dyer [35]. Then, the extracted lipids were added to 5.0% HCl in methanol. After the mixture was heated for 100°C for 2 h, 250 μl of hexane and 250 μl of H_2O were added and centrifuged at 1750 rpm for 10 min at 4°C. Hexane layer obtained were concentrated and subjected to GLC analysis using Shimadzu GC-18 Gas Chromatograph with a SP-2330 fused silica capillary column (30 m × 0.25 mm 0.2 μ, film, Superco, PA, USA). The operation column temperature was kept at 190°C. Total fatty acid contents were obtained from the sum of each fatty acid content calculated by the peak ratio of each fatty acid to methyl margarate in the chromatogram.

Determination of α-tocopherol in plasma and aorta homogenate

Aorta homogenate (200 μl) was mixed with 0.5 ml of 6% pyrogallol in ethanol and incubated at 70°C for 2 min. Then, the sample was added with 100 μl KOH (60%) and stood for 30 min at 70°C. After cooling, the sample was mixed with 2.25 ml of 1% NaCl and 1.5 ml of ethyl acetate/hexane (1:9, v/v). The hexane layer was obtained by centrifugation at 1750 g at 4°C for 5 min and evaporated under nitrogen gas. The residue was dissolved with 50 μl of methanol/ethanol (1:1, v/v). In the case of plasma, ethanol 250 μl and hexane 250 μl were added to the plasma (50 μl) and mixed vigorously. After centrifugation, the hexane layer was evaporated under nitrogen gas and the residue was dissolved with 50 μl of methanol/ethanol (1:1, v/v). In both cases, an aliquot was subject to HPLC analysis with a TSK gel ODS -80Ts (5 μm, 4,6 × 150 mm, Tosoh) using methanol/H_2O (93:7, v/v) as the eluting solvent at a flow rate of 1.0 ml/min. The eluate was monitored by fluorescence (excitation 295 nm; emission 325 nm). δ-Tocopherol was used as an internal standard for the calculation of α-tocopherol content.

Statistical analysis

Results were presented as the means ± SD of four animals. Statistical analysis was evaluated by one factor analysis of variance (ANOVA) followed by Bonferroni/Dunn posthoc multiple comparison using Stat-View software (ver.5, SAS institute Inc.) Differences of $p < 0.05$ were considered significant.

Results

Effect of Q3G on the growth, food intake and organ weights

Table I shows the average food intake during the feeding period, the body weight gain and the organ weights after the feeding period. No significant differences were observed in the body weight gain and average food intake among the three groups. In organ weights, significant differences were found in the liver and spleen. In particular, the liver weight increased in the order of control group < HC group < HC + Q3G group. No differences were observed among the three groups in the other organs including the aorta.

Effect of Q3G on cholesterol, triacylglycerol and total fatty acids in blood plasma and aorta homogenate

The contents of cholesterol, triacylglycerol and total fatty acids in blood plasma and the aorta after the feeding period were described in Table II. All contents were markedly increased by feeding the high cholesterol diet as shown in their higher concentrations in HC group than in the control group. In blood plasma, Q3G treatment for high

Table I. Average food intake, body weight gain and organ weights in NZW rabbits at the end of the study.

Group	Control	HC	HC + Q3G
Average food intake (g/day)	125 ± 1	131 ± 8	131 ± 7
Body weight gain (kg)	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.2
Organ weight at the end of study (g)			
Heart	6.3 ± 0.4	6.4 ± 0.7	6.5 ± 0.5
Aorta	0.78 ± 0.05	0.89 ± 0.21	0.81 ± 0.11
Liver	63 ± 7[a]	80 ± 6[b]	95 ± 4[c]
Lung	9.2 ± 2.0	11.3 ± 2.1	10.6 ± 2.9
Kidney	16 ± 1	15 ± 3	17 ± 2
Adrenal	0.27 ± 0.08	0.45 ± 0.10	0.42 ± 0.14
Spleen	0.83 ± 0.25[a]	1.53 ± 0.57[b]	1.20 ± 0.29[ab]
Brain	9.1 ± 1.2	8.1 ± 1.0	8.2 ± 0.6

Values are means ± SD ($n = 4$). Values with different superscripts are significantly different ($p < 0.05$).

Table II. Concentrations of cholesterol, triacylglycerol and total fatty acids in the plasma (A) and aorta (B) of NZW rabbits at the end of the study.

Group	Control	HC	HC + Q3G
(A) Plasma			
Total cholesterol (mg/dl)	31 ± 10[a]	2570 ± 340[b]	1940 ± 40[c]
Free cholesterol (mg/dl)	13 ± 2[a]	930 ± 190[b]	620 ± 150[c]
Esterified cholesterol (mg/dl)	19 ± 8[a]	1640 ± 170[b]	1320 ± 280[c]
Triacylglycerol (mg/dl)	67 ± 17[a]	580 ± 120[b]	400 ± 130[c]
Total fatty acids (mg/dl)	63 ± 14[a]	1040 ± 150[b]	760 ± 220[c]
(B) Aorta			
Total cholesterol (mg/mg protein)	0.12 ± 0.04[a]	0.68 ± 0.06[b]	0.36 ± 0.06[c]
Free cholesterol (mg/mg protein)	0.07 ± 0.02[a]	0.22 ± 0.08[b]	0.12 ± 0.02[a]
Esterified cholesterol (mg/mg protein)	0.05 ± 0.02[a]	0.46 ± 0.09[b]	0.23 ± 0.06[c]
Triacylglycerol (mg/mg protein)	0.45 ± 0.22[a]	1.90 ± 0.70[b]	0.71 ± 0.16[a]
Total fatty acids (mg/g tissue)	3.8 ± 0.9[a]	7.5 ± 0.5[b]	5.7 ± 0.7[c]

Values are means ± SD ($n = 4$). Values with different superscripts are significantly different ($p < 0.05$).

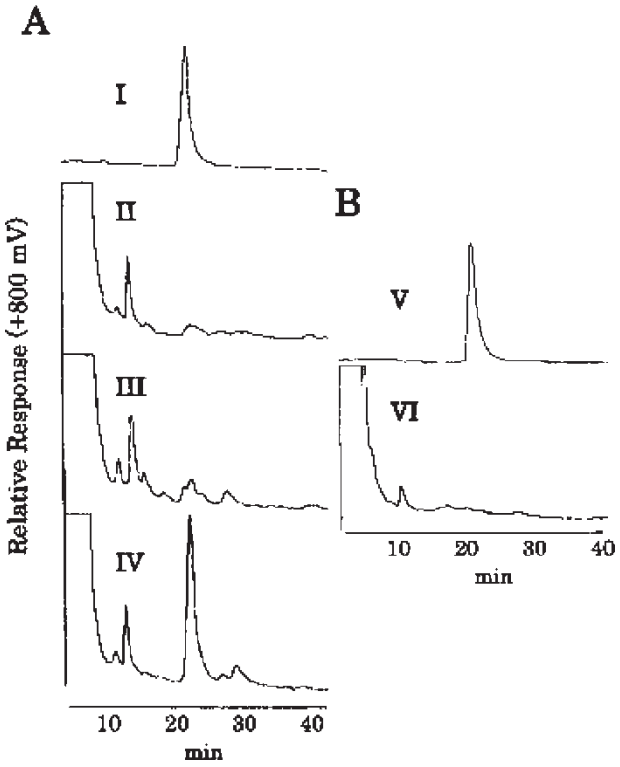

Figure 1. HPLC chromatogram of the extract from aorta homogenate. with the treatment of sulfatase H-5 (A). I: standard quercetin, II: control group, III: HC group, IV: HC + Q3G group. (B) HC + Q3G group without the treatment of sulfatase H-5 (V: standard of quercetin, VI: HC + Q3G group)

cholesterol-loaded rabbits decreased the plasma cholesterol levels including total cholesterol, free cholesterol, esterified cholesterol, and the triacylglycerol level as well as the total fatty acid level, as their concentrations were significantly lower in HC + Q3G group as compared with HC group (Table IIA). Similarly accumulation of cholesterol, triacylglycerol and total fatty acids was found to be suppressed by the treatment of Q3G in the aorta, as their content in the aorta of HC + Q3G group was significantly lower than those of HC group (Table IIB).

Accumulation of quercetin metabolites in the aorta

Figure 1A shows the typical HPLC chromatograms for the measurement of quercetin after sulfatase H-5 treatment. In either control group or HC group, no prominent peak corresponding to quercetin aglycone appeared in the chromatogram. However, a distinct peak corresponding to quercetin aglycone emerged in the chromatogram of HC + Q3G group, whereas no peak appeared in the chromatogram of HC + Q3G group without sulfatase H-5 treatment (Figure 1B). In LC-MS analysis, this peak was identified as quercetin aglycone because its mass spectrum gave a characteristic ion peak at m/z 301, which corresponded to $[M-H]^-$ for quercetin aglycone (data are not shown here). Sulfatase H-5 is known to deliver quercetin aglycone from its conjugated metabolites. It is therefore obvious that conjugated quercetin metabolites accumulated at the aorta tissue and liberated its aglycone by the enzymatic hydrolysis. Its content was calculated to be 0.70 ± 0.20 nmol/mg protein by using the standard curve of quercetin aglycone. At the same time, quercetin aglycone was also detected in the plasma after sulfatase H-5 treatment and its concentration was calculated as 95 ± 7 nM.

Effect of Q3G on TBARS and CE-OOH levels in the plasma and aorta

HC group showed higher plasma TBARS and CEOOH levels than control group, indicating that the feeding of the high cholesterol diet accelerated lipid peroxidation in the plasma (Table III). In contrast, both lipid peroxidation biomarkers were kept at a lower level in HG + Q3G group as compared with HC group, although the level of CEOOH per esterified cholesterol (cholesteryl ester; CE) was not significantly different between HC group and HC + Q3G group. In the aorta, both TBARS and CEOOH levels in HC group were also significantly higher than control groups. In all cases, HC + Q3G group exhibited apparently lower values than HC group in TBARS and CEOOH levels. This indicates that the Q3G intake with the high cholesterol diet is effective in lowering lipid peroxidation occurring in the aorta.

Table III. TBARS and CEOOH levels in the plasma (A) and aorta (B) of NZW rabbits at the end of the study.

Group	Control	HC	HC + Q3G
(A) plasma			
TBARS (μM)	0.21 ± 0.16[a]	0.85 ± 0.26[b]	0.28 ± 0.14[a]
CEOOH (μM)	0.003 ± 0.003[a]	0.85 ± 0.35[b]	0.54 ± 0.39[c]
CEOOH (μmol/mol CE)	8.4 ± 16.8[a]	33.7 ± 13.5[b]	25.2 ± 12.5[a,b]
(B) Aorta			
TBARS (nmol/mg protein)	0.29 ± 0.08[a]	2.02 ± 0.64[b]	0.72 ± 0.38[a]
TBARS (nmol/mg lipid)	0.56 ± 0.18[a]	2.34 ± 0.54[b]	1.10 ± 0.70[a]
CEOOH (nmol/mg protein)	0.02 ± 0.02[a]	1.25 ± 0.18[b]	0.14 ± 0.06[a]
CEOOH (nmol/mol CE)	0.13 ± 0.09[a]	1.85 ± 0.54[b]	0.47 ± 0.13[a]

Values are means ± SD ($n = 4$). Values with different superscripts are significantly different ($p < 0.05$).

Effect of Q3G on α-tocopherol level in the plasma and aorta

In the plasma, α-tocopherol concentrations in HC group and HC + Q3G group were much higher than that in control group (Table IV). Nevertheless, no significant difference was observed in α-tocopherol concentrations between HC group and HC + Q3G group. In the aorta, α-tocopherol levels were high in both HC and HC + Q3G group when the concentration was calculated based on the unit of nmol per mg protein. On the other hand, HC group, *but not* HC + Q3G group, gave a significantly lower level of α-tocopherol when it was expressed by the unit of nmol per mg cholesterol. This means that the feeding of high cholesterol diet lowered the tocopherol concentration per cholesterol in the aorta and its decrease could be compensated by the intake of Q3G.

Effect of Q3G on copper ion-induced lipid peroxidation of plasma and aorta ex vivo

Figure 2 shows TBARS and CEOOH levels in the plasma of three groups after 6 h incubation with copper ion increased in the order of control group < HC + Q3G group < HC group in all cases. This figure clearly demonstrated that feeding of high cholesterol diet enhanced copper ion-induced lipid peroxidation *ex vivo* and the intake of Q3G suppressed this oxidative susceptibility. In the aorta, this order of increase was found in TBARS with either calculation based on protein or lipid as shown in Figure 3. No significant difference was, however, observed in the CEOOH level between HC group and HC + Q3G group, when it was calculated by CE base. In 4-HNE and hexanal analysis, neither carbonyl compounds appeared in the control group after the incubation. These two carbonyls emerged in the groups on the high cholesterol diet, that is, HC and HC + Q3G groups. Thus it is obvious that feeding of high cholesterol diet remarkably enhanced the susceptibility toward lipid peroxidation and subsequent formation of carbonyl compounds in the aorta. Hexanal level was lowered in HC + Q3G group in either calculation based on proteins or lipids. However, it cannot be judged whether or not the intake of Q3G lowered the susceptibility to lipid peroxidation and carbonyl formation in thez aorta, because no difference was observed in the 4-NHE level based on lipids between HC and HC + Q3G groups. It is therefore likely that high cholesterol diet enhances the oxidative susceptibility in the plasma and aorta. Although the effect of dietary Q3G on the enhancement lipid peroxidation *ex vivo* due to cholesterol diet is not definitive, some inhibitory effects may be observed in a series of lipid peroxidation reactions.

Table IV. α-Tocopherol concentration in the plasma (A) and aorta (B) in NZW rabbits at the end of the study.

	Control	HC	HC + Q3G
(A) plasma			
(μM)	2.1 ± 0.6[a]	34.3 ± 9.5[b]	34.2 ± 5.36[b]
(nmol/mg total cholesterol)	6.82 ± 1.52[a]	1.46 ± 0.24[b]	1.80 ± 0.27[b]
(B) aorta			
(nmol/mg protein)	0.21 ± 0.01[a]	0.58 ± 0.06[b]	0.68 ± 0.06[c]
(nmol/mg total cholesterol)	2.02 ± 0.12[a]	0.93 ± 0.15[b]	2.03 ± 0.78[a]

Values are means ± SD ($n = 4$). Values with different superscripts are significantly different ($p < 0.05$).

Discussion

It is of necessity for estimating the biological activities of dietary components to elucidate their transport into target sites and their action mechanism. In the case of quercetin and its related flavonoids, considerable works have already clarified the process of their intestinal absorption and enterohepatic circulation [21]. The presence of a variety of conjugated metabolites in blood plasma was also confirmed in both human and animal studies [21]. However, it is essential to know the behavior of dietary quercetin in aorta, the target site of atherosclerosis, in evaluating its anti-atherosclerotic effect. In this study, we first demonstrated that the intake of Q3G, a common form of quercetin in plant foods, results in the accumulation of its conjugated metabolites in the aorta. The amount of quercetin metabolites in the aorta, 0.70 ± 0.20 nmol/mgprotein, is comparable to that of α-tocopherol, a representative antioxidant from food (0.68 ± 0.06 nmol/mg protein, shown in Table IV).

Quercetin metabolites are transiently released into plasma at the level of $\sim 10^{-6}$ M and thereafter excreted into the urine, when quercetin is incorporated into the body from food [24]. We previously detected quercetin metabolites in human plasma at

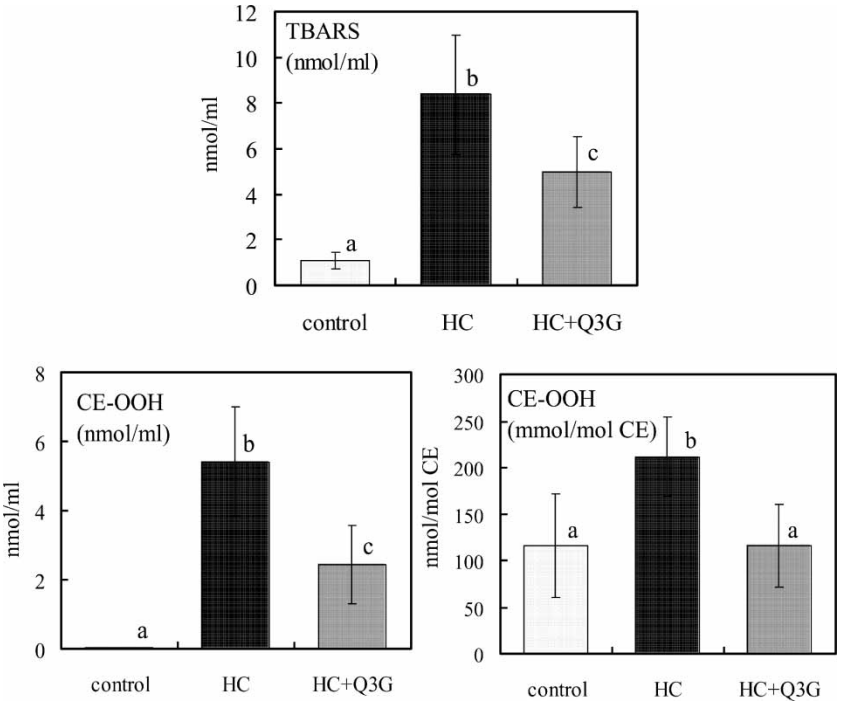

Figure 2. Copper ion–induced lipid peroxidation in the plasma *ex vivo*. Values are means ± SD ($n = 4$). Values with different superscripts are significantly different ($p < 0.05$).

10^{-7} M level after continuous ingestion of quercetin glucoside-rich foods for one week and fasting overnight [31]. In this study, conjugated metabolites at a similar concentration $0.95 \pm 0.7 \times 10^{-7}$ M were obtained from rabbit plasma after fasting. Thus, it is hard for dietary quercetin to accumulate gradually in blood plasma. In contrast, quercetin metabolites obviously accumulate in the aorta by the continuous intake of quercetin glucosides.

It is well documented that ROS is tightly correlated with the atherosclerotic events, such as the formation of oxidatively modified LDL and its onset to endothelial cells and smooth muscle cells [8,9]. Furthermore, it should be noted that NADPH oxidase in vascular cells, which generates $O_2^{\bullet -}$ by stimulation with growth factors and cytokines, is linked to atherosclerosis [36]. Interestingly, Li et al. [37] recently reported that lipid hydroperoxides increase $O_2^{\bullet -}$ production in smooth muscle cells by activating cellular NADPH oxidase. Therefore, it seems likely that primary products of lipid peroxidation enhance ROS-induced damage in the aorta by activating NADPH oxidase in vascular cells. It is noteworthy that hypercholesterolemia increase endothelial $O_2^{\bullet -}$ production via xanthine oxidase [38] or NADPH oxidase activity [39], contributing to the early atherosclerotic process. In our experiment, the aorta from high cholesterol diet-fed rabbits (HC group) seems to be exposed to severe oxidative stress by vascular $O_2^{\bullet -}$ production. In fact, biomarkers of lipid peroxidation, TBARS and CE-OOH increased in HC group as compared with control group (Table III). It should be pointed out that the level of CEOOH per cholesteryl ester (CE) in the aorta was also higher in HC group as compared with control group, although much higher amounts of CE accumulated in HC group than control group. Thus, the elevation of CEOOH in the aorta reflects, not the apparent increase of CE, but the peroxidizability of CE in blood vessels. The result of copper ion inducing lipid peroxidation of aorta *ex vivo* (Figure 3) also exhibited the increase of the biomarkers of lipid peroxidation in HC group, indicating that a high cholesterol diet increases the susceptibility of aorta to lipid peroxidation, similar to plasma (Figure 2).

Antioxidants are likely to prevent the early atherosclerotic event in hypercholesterolemia by not only inhibiting the formation of oxidatively modified LDL but also suppressing vascular NADPH oxidase-linked ROS formation and subsequent oxidative damage or signaling in vascular cells. It has been shown that quercetin and its metabolites can suppress lipoxygenase-induced [33] and peroxynitrite-induced [27] modification of human LDL. Quercetin metabolites accumulated in the aorta seem be included in vascular antioxidant defense, as Q3GA is found to be permeable into smooth muscle through endothelium by the stimulation with inflammatory cytokines [40]. Actually, this study showed that biomarkers of lipid peroxidation, including the level of CE-OOH per CE, from HG + Q3G group are significantly lower than those from HC group (Table III). It is therefore apparent that simultaneous intake of Q3G with cholesterol attenuates lipid peroxidation in the aorta

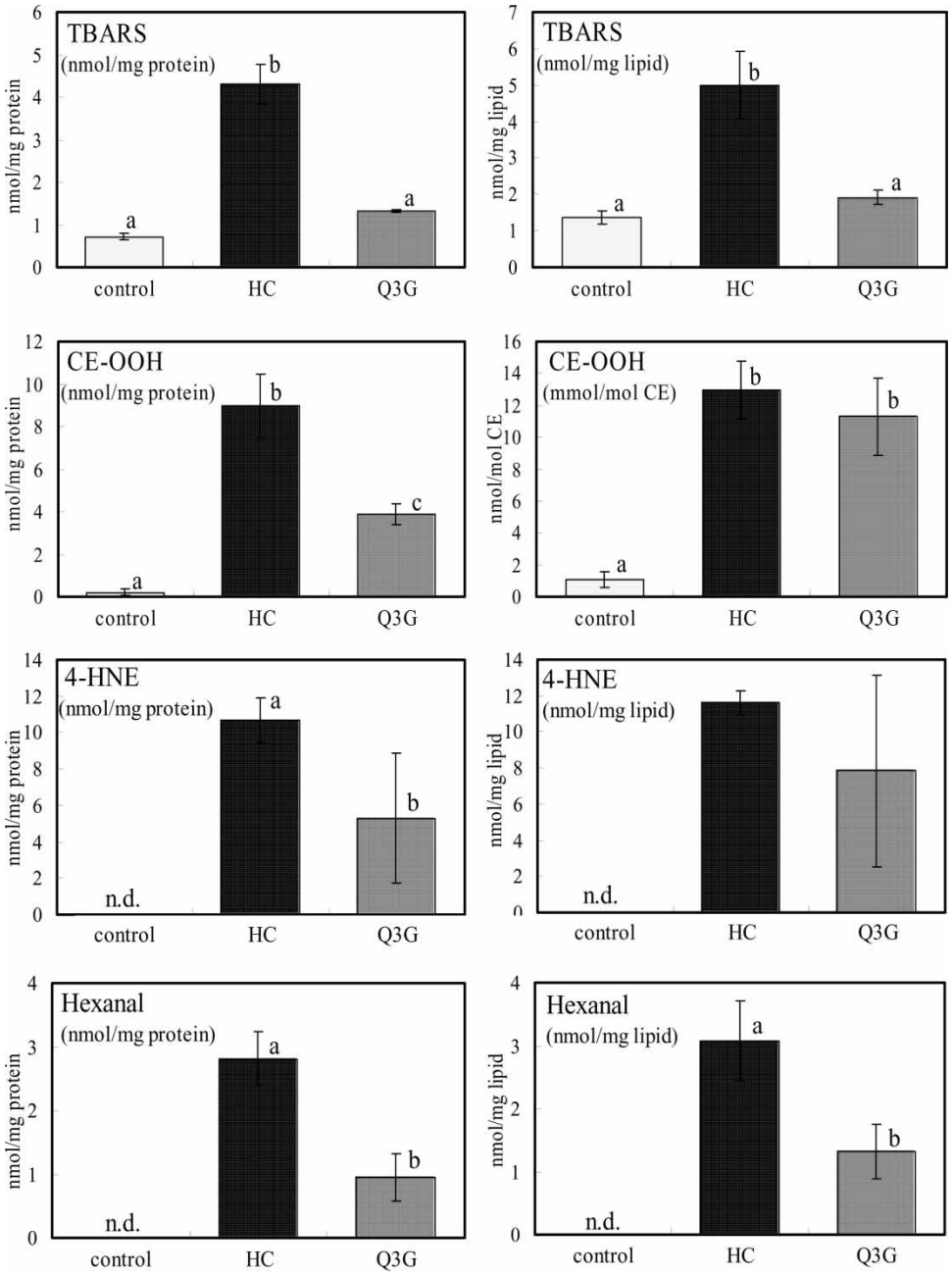

Figure 3. Copper ion-induced lipid peroxidation in the aorta homogenate *ex vivo*. Values are means ± SD ($n = 4$). Values with different superscripts are significantly different ($p < 0.05$). n.d.: not detected.

induced by the high cholesterol diet. However, it is unclear whether or not attenuation of lipid peroxidation is derived from quercetin metabolites directly. Antioxidant activity of conjugated metabolites of quercetin including free radical scavenging activity, lipoxygenase inhibiting activity and xanthin oxidase inhibiting activity are different, depending on the site of conjugation in which the sulfate/glucuronide are attached [26,41]. Here it is difficult to judge the direct contribution of the metabolites, because no information was obtained on the detailed structure and distribution of each metabolite. On the other hand, the phenomenon of the elevation of α-tocopherol level per aorta cholesterol by the intake of Q3G (Table IV) may suggest that simultaneous intake of Q3G with cholesterol compensates for the decrease of α-tocopherol in a high cholesterol diet or facilitates the incorporation of α-tocopherol into the aorta. Increase of α-tocopherol level seems to be linked to the enhancement of antioxidative defense to lipid peroxidation in the aorta.

An alternative idea that explains the effect of dietary quercetin in a high cholesterol diet involves the inhibition of intestinal absorption of cholesterol and the inhibition of cholesterol synthesis in the liver. Ikeda et al. [42] indicated that tea catechins, a series of

flavanol type-flavonoids, suppress intestinal absorption of cholesterol in rats by decreasing its micellar solubility. It is also reported that dietary quercetin enhance the excretion of cholesterol into feces in high cholesterol-fed rats [43]. Furthermore, it is also clarified that the activity of hepatic HMG-CoA reductase, a key enzyme for cholesterol synthesis, was lowered by the intake of quercetin in high cholesterol-fed rats [44]. Intake of Q3G may therefore inhibit cholesterol synthesis resulting in the lower secretion of lipoprotein into plasma and decreases of the plasma cholesterol level. Our results also indicate that Q3G intake affects lipid metabolism as shown in the lower level of triacylglycerol in the plasma and aorta of HC + Q3G group as compared with those of HC group (Table II). Intake of quercetin may elevate the enzyme activity of β-oxidation in lipid metabolism such as acyl-CoA oxidase as indicated by Murase et al. [44] in the case of the intake of catechin by mice. Thus, the hypolipidemic effect of quercetin glucoside in a high cholesterol diet, due to the inhibition of cholesterol absorption in the intestine and the modification of lipid metabolism in the liver, is likely to contribute to anti-atherosclerotic factors of plant foods.

This study suggests that the intake of quercetin from food is expected to attenuate the oxidative stress in the aorta originating from hypercholesterolemia. The combination of antioxidant activity and hypolipidemic effect in quercetin metabolites may be included in the mechanism of dietary quercetin for acting as anti-atherosclerotic reagent. However, it should be noted that the intake of Q3G increases the liver weight in spite of there being no change in total body weight (Table I). The intake of Q3G for each rabbit in this experiment can be calculated to be approximately 50 mg/kg body weight/day. This level is rather high, although it is not extremely beyond the physiologically relevant dose. Further studies using lower dose of quercetin glucosides may be required to assess their adequate intake amounts for exerting their antioxidant effect without any disorders.

In conclusion, dietary quercetin glucoside surely accumulates in the aorta as its conjugated metabolites and suppresses the formation of primary products and secondary products of lipid peroxidation, which seem to be responsible for the generation of ROS and the oxidative damage of vascular cells. A report on a human study shows that the intake of quercetin does not affect the risk factors for atherosclerosis in blood plasma and platelets [45]. However, the results of this study warrant further studies on the effect of quercetin-rich food intake in the prevention of atherosclerosis.

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