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Original Contribution

Accumulation of orally administered quercetin in brain tissue and its antioxidative effects in rats

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ABSTRACT

Quercetin is widely distributed in vegetables and herbs and has been suggested to act as a neuroprotective agent. Here, we demonstrate that quercetin can accumulate enough to exert biological activity in rat brain tissues. Homogenates of perfused rat brain without detectable hemoglobin contaminants were treated with β-glucuronidase/sulfatase and the released quercetin and its methylated form were analyzed using high-performance liquid chromatography (HPLC) with three different detection methods. Both quercetin and the methylated form were detected in the brain of quercetin-administered rats using HPLC-UV and HPLC with electrochemical detection and were further identified using HPLC–tandem mass spectrometry. Oral administration of quercetin (50 mg/kg body wt) attenuated the increased oxidative stress in the hippocampus and striatum of rats exposed to chronic forced swimming. The possible transport of quercetin derivatives into the brain tissue was reproduced in vitro by using a rat brain capillary endothelial cell line, a model of the blood–brain barrier. These results show that quercetin could be a potent nutrient that can access the brain and protect it from disorders associated with oxidative stress.

The flavonoid quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a typical polyphenolic compound found in a variety of vegetables and fruits that are regularly consumed by humans [1]. Herbs such as *Ginkgo biloba,* which is a traditional Chinese herbal medicine [2], also contain a high quercetin content. Quercetin is mostly present as its glycosides, which are particularly abundant in onion (0.3 mg/g fresh wt) and tea (10–25 mg/L) [1]. Dietary glycosides are hydrolyzed to quercetin aglycone and rapidly converted into its conjugated metabolites during intestinal absorption [3,4]. Part of the quercetin metabolites are further methylated by catechol-*O*-methyltransferase during absorption and circulation. Thus, neither quercetin aglycone nor quercetin glycosides are present in human and rat plasma [5].

Quercetin has attracted much attention for its potential to prevent cardiovascular [6], neoplastic [7], and neurodegenerative [8] diseases. Because oxidative stress has been implicated in the pathogenesis of these diseases, the antioxidant activity of quercetin (and other flavonoids) may be at least partly responsible for its mode of action. The mechanism of the antioxidant activity of flavonoids is characterized by direct scavenging or quenching of oxygen free radicals or excited oxygen species. Our previous studies have shown that one of the major quercetin metabolites in rat plasma, quercetin-3-*O*-β-D-glucuronide (Q3GA), retains appreciable antioxidant activity because the *o*-dihydroxyl (catechol) structure in the B ring remains unconjugated [9]. We have also shown that Q3GA significantly suppresses reactive oxygen species formation in a mouse fibroblast cell line [10]. These observations suggest that quercetin, even after conjugation metabolism, may exert antioxidative activity in plasma and tissues.

Abbreviations: HPLC, high-performance liquid chromatography; UV, ultraviolet; Q3GA, quercetin-3-*O*-β-D-glucuronide; HPLC-ECD, HPLC–electrochemical detection; LC-MS/MS, HPLC–tandem mass spectrometry; SRM, selected reaction monitoring; TBARS, thiobarbituric acid-reactive substances; GSH, reduced glutathione; BBB, blood–brain barrier.

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Recent studies using HPLC analysis have shown that quercetin metabolites accumulated in a wide variety of tissues in pigs and rats after oral administration of quercetin [11,12]. However, it is still unclear whether quercetin can accumulate and exert biological activity in tissues. In particular, there is still controversy over the accumulation of flavonoids in brain because the blood–brain barrier (BBB) blocks many drugs and chemicals from entering the brain. It has been shown that onions and herbs containing high amounts of quercetin glycosides possess antidepressant-like activity in a rat forced-swimming test and an animal model for assessing the efficacy of antidepressant drugs [13,14]. In addition, Q3GA significantly suppressed reactive oxygen species formation in differentiated PC-12 cells, a model for neuron cells [15]. These observations indicate that orally administered quercetin can exhibit antioxidative activity in brain, a potent target tissue for treatment of mental stress and depression. The aim of this study was to clarify the accumulation of orally administered quercetin in rat brain tissue using HPLC with three different detection methods (UV, electrochemical, and tandem mass spectrometry) and to investigate whether quercetin exerts biological activity in brain tissues.

Materials and methods

Chemicals

Quercetin dehydrate and sulfatase H-1 were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Isorhamnetin (3′-methylquercetin) was obtained from Extrasynthese (Genay, France). Q3GA was chemically synthesized as previously reported [9].

Animals

Wistar rats (male, 120–140 g, 6 weeks of age; Japan SLC, Shizuoka, Japan) or Crlj:CD (SD) rats (male, 220–250 g, 8 weeks of age; Charles River Japan, Yokohama, Japan) were housed in a controlled room (temperature, 23 ± 1 °C; humidity, 45–50%; light–dark cycle, 12 h each; lights on, 8:00 AM). The rats were given free access to laboratory feed (MF; Oriental Yeast Co., Tokyo, Japan) and tap water for 5 days before experiments were started. This study was performed according to the guidelines for the care and use of laboratory animals of The University of Tokushima Graduate School, Institute of Health Biosciences. All efforts were made to minimize animal suffering and to reduce the number of animals used. In this study, Crlj:CD (SD) rats were specifically used for forced-swimming experiments according to previous papers [13,14]. It was preliminarily confirmed that the absorption and accumulation of orally administered quercetin in plasma and brain tissues were not different between Wistar and Crlj:CD (SD) rats.

Administration of quercetin to the rats

For intragastric administration, the rats ($n = 4$ for each group) were starved overnight and then given quercetin (50 or 200 mg/kg of body weight) dissolved in 1 ml of propylene glycol by using an oral Zonde needle connected to a 1-ml syringe. For a free-access experiment, the rats were given free access to a commercial solid diet (MF; Oriental Yeast) or a purified diet [corn starch 40, casein 20, sucrose 20, corn oil 5, lard 5, cellulose 5, mineral mixture (oriental mixture) 3.5, vitamin mixture (oriental mixture) 1, D,L-methionine 0.3, choline chloride 0.2 g/100 g diet] containing 1% (w/w) quercetin for 1 to 4 weeks until scarification. The average intake of the diet was 21.3 g (i.e., 213 mg of quercetin) a day. After anesthesia (pentobarbital sodium injection), rats were perfused with 200 ml saline. At the same time, blood samples were immediately collected into heparinized tubes, and the plasma was obtained by centrifugation. The brain tissues obtained were immediately stored at −80 °C until further preparation.

Analysis of quercetin metabolites in the brain

Each portion of brain tissue was homogenized using a Teflon homogenizer in 2 ml of PBS. For analysis of total quercetin (conjugated + nonconjugated) in brain, 2 ml of brain homogenates was mixed with 100 pmol myricetin (internal standard) and 1 ml of 4 mg/ml sulfatase H-1 (from *Helix pomatia,* 14 units of sulfatase, and 300 units of β-glucuronidase activity/mg of enzyme) in a 50 mM sodium phosphate buffer (pH 5.0) and incubated at 37 °C for 90 min. The hydrolysates were extracted with the same volume of ethyl acetate twice and evaporated under an N_2 stream. The extract was dissolved in 300 μl of 20% aqueous acetonitrile containing 0.5% phosphoric acid. A total of 100 μl of the sample was injected into the HPLC-UV detection system (Jasco Gulliver 900 series; Jasco Corp., Tokyo, Japan) or 10 μl of the sample was injected into the HPLC–electrochemical detection (ECD) system (Model 5600 A Coul-Array Detector; ESA, Cambridge, MA, USA) equipped with a TSK-gel ODS-80Ts column (4.6 × 150 mm; Tosoh, Tokyo, Japan). The separation of the compounds was carried out by gradient elution. For the HPLC-UV detection system, solvent A was 0.5% phosphoric acid, and solvent B was 100% acetonitrile. The gradient program was as follows: 0–2 min, 15% B; 2–22 min, linear gradient to 40% B; 22–24 min, 40% B hold; 24–32 min, linear gradient to 15% B; flow rate, 0.8 ml/min. UV detection was performed at 370 nm.

For the HPLC-ECD detection, two gradient systems were used. For system 1, solvent A was 20% acetonitrile, 0.5% phosphoric acid, and solvent B was 100% acetonitrile containing 0.5% phosphoric acid. The gradient program was as follows: 0 min, 5% B; 0–20 min, linear gradient to 25% B; 20–25 min, linear gradient to 5% B; 25–30 min, 5% B hold; flow rate, 0.8 ml/min. For system 2, solvent A was 0.5% phosphoric acid, and solvent B was 100% acetonitrile containing 0.5% phosphoric acid. The gradient program was as follows: 0–10 min, 21% B; 10–20 min, linear gradient to 44% B; 20–25 min, linear gradient to 21% B; 25–30 min, 21% B hold; flow rate, 0.8 ml/min. Electrochemical detection was performed with a coulometric electrode at 150 mV (for channel 1) and 200 mV (for channel 2). Analysis of nonconjugated quercetins in brain was performed without sulfatase H-1 treatment.

For analysis using HPLC–tandem mass spectrometry (LC-MS/MS), the quercetin hydrolysates were dissolved in 0.1% acetic acid and injected into an API3000 spectrometer (Applied Biosystems). The mass spectrometer was operated in the negative-ion ESI mode with selected reaction monitoring (SRM) for all analytes by LC-MS/MS. The instrumental parameters were optimized for maximal generation of the deprotonated analyte molecules $[M-H]^-$ and characteristic fragment ions. Samples were separated by HPLC (Agilent 1100) using a Develosil ODS-SR-5 (2 × 150 mm; Nomura Chemicals, Aichi, Japan) column. Gradient elution was performed using a two-solvent system (solvent A, 0.1% acetic acid; solvent B, acetonitrile containing 0.1% acetic acid) at a flow rate of 0.2 ml/min. The gradient elution program was as follows: initial, A 80%; 15 min, A 20%; 15–16 min, A 20% (hold); 16.1 min, A 80%; 16.1–25 min, A80% (hold).

Analysis of trace amounts of quercetin in animal diets

The quercetin derivatives in the diet (3 g) were extracted repeatedly with methanol. After centrifugation, methanolic fractions were evaporated and the extracts were dissolved in methanol containing 0.1 N HCl and boiled for 1 h for acidic hydrolysis of glycosylated quercetins. The hydrolysates were extracted with ethyl acetate three times, evaporated, and dissolved in 20% aqueous acetonitrile containing 0.5% phosphoric acid. The 50 μl was analyzed by HPLC-ECD (system 2) as described above.

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Forced-swimming procedure

Crlj:CD (SD) rats were forced to swim in an acrylic cylinder (450 × 192 mm i.d.) containing water at 25 ± 1 °C to a depth of 17 cm. On the first day, the rats were forced to swim for 15 min. After the second day, the rats were forced to swim for 10 min. After completion of the swim procedure on the fifth day, the rats were immediately perfused, and blood and brain tissue were collected. Thirty minutes before every swim procedure, the rats were given quercetin (50 mg/kg body wt) dissolved in 1 ml of propylene glycol by using an oral Zonde needle connected to a 1-ml syringe. Control groups were given propylene glycol alone.

Measurement of corticosterone in the plasma

The blood samples were centrifuged and the plasma was obtained. The plasma corticosterone level was determined in duplicate using an EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA).

Measurement of thiobarbituric acid-reactive substances (TBARS) and reduced glutathione (GSH) levels in the brain

The rat brains were separated into several regions. TBARS were determined according to the method of Buege and Aust [16]. Briefly, the supernatant of brain homogenate samples (50 µl) was mixed with 0.5 ml of a mixture containing 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 M HCl and 10 µl of 10 mM 2,6-di-*t*-butyl-4-hydroxytoluene. The mixture was heated in boiling water for 15 min, cooled, and then mixed by vortex with 0.5 ml of *n*-butanol. The *n*-butanol layer was collected after centrifugation and the fluorescence intensity was measured using a spectrofluorimeter (MTP-32 microplate reader; Corona Electric Co., Japan) at 515 nm excitation and at 553 nm emission.

GSH was analyzed by *o*-phthalaldehyde reaction according to the method of Hissin and Hilf [17]. Briefly, the supernatant of brain homogenate samples (400 µl) was mixed with 100 µl of 25% metaphosphoric acid containing 5 mM ethylenediaminetetraacetic acid. The mixture was centrifuged at 105,000 *g* for 30 min and the upper layer (100 µl) was mixed with 1.8 ml of 0.1 M sodium phosphate buffer (0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 5 mM ethylenediaminetetraacetic acid, pH 8) and 100 µl of *o*-phthalaldehyde (1 mg/ml in MeOH) for 15 min. The *o*-phthalaldehyde-derived fluorescence was measured at 350 nm excitation and 420 nm emission.

Cell culture

A rat brain endothelial cell line (RBEC1, established by Kido et al. [18]) was cultured in Dulbecco's modified Eagle's medium (Sigma) with 5% horse serum (Sigma), 5% fetal bovine serum (Sigma), 100 µg/ml penicillin, and 100 units/ml streptomycin. Cells were cultured in an atmosphere containing 5% CO_2 at 37 °C.

Analysis of cellular uptake of quercetin metabolites

RBEC1 cells (2×10^6 cells) were seeded on a 60-mm dish and cultured for 3 days. When the cells had reached confluence, the medium was replaced with serum-free Dulbecco's modified Eagle's medium containing Q3GA. After incubation, the cells were washed three times with Hanks' balanced salt solution and scraped from the dish. After centrifugation, Q3GA and its cellular metabolites were extracted in 200 µl of methanol/acetic acid (100/1) by sonication for 1 min using an Astrason XL2020 ultrasonic processor (Heat Systems–Ultrasonic, Farmingdale, NY, USA) at level 10. After centrifugation, the supernatants were collected, evaporated under an N_2 stream, and dissolved in 20% aqueous acetonitrile containing 0.5% phosphoric acid. The samples were injected into the HPLC-ECD system as described.

Results

In vivo accumulation of quercetin in rat brain

We first examined blood contamination in the perfused rat brain tissues by measuring hemoglobin content according to the method by Dahlberg [19] and confirmed that the brain homogenates contained no detectable hemoglobin, showing the almost complete removal of blood from the brain tissues. The brain homogenates were treated with β-glucuronidase/sulfatase and the released quercetin aglycone and its methylated forms (3′- or 4′-methylated) were analyzed using a HPLC-UV system at 370 nm, the maximal absorption of quercetin. To compensate for the extraction efficiency, myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) was added to the homogenates as an internal standard. Calibration curves for quercetin and a methylquercetin (isorhamnetin; 3′-methylquercetin) were obtained by plotting the relative ratio of these quercetins to myricetin as shown in Fig. 1A. Because isorhamnetin cannot be clearly separated from tamarixetin (4′-methylquercetin) under our HPLC conditions, they were measured as a mixture of two methylquercetins. As shown in Fig. 1B, peaks for quercetin and methylquercetins, the retention times of which corresponded to those exhibited by authentic standards, were detected in the brain samples of quercetin-fed rats. These peak areas increased depending on the period of quercetin administration. After 1 month of administration, the concentrations of quercetin and methylquercetins were 40.1 ± 11.1 and 47.7 ± 17.1 (pmol/g tissue), respectively (Fig. 1C). These concentrations were comparable to those at 1 week of administration, suggesting that the brain quercetin concentrations have reached plateau within 1 week of administration. It is of interest that the ratio of methylquercetins to quercetin in the intragastric administration group (after 1 h) was much lower than that of long-term/free-access groups. In addition, quercetin, but no methylquercetins, was also detected in the control brain samples. These observations suggest that the increased ratio of methylated quercetin metabolites may reflect the chronic/higher amounts of quercetin intake.

The detectable amounts of quercetin in the control brain samples could be explained by the presence of a trace amount of quercetin (16.4 ± 3.3 µg aglycone equivalent/100 g) detected in the control diet (a commercially available nonpurified diet).

Identification of quercetin and methylquercetins in rat brain samples

To identify the peaks for quercetin and methylquercetins in the brain samples, the hydrolysates were also analyzed by HPLC-ECD. We used a coulometric multielectrode cell system, which provides a unique electrochemical response for each compound between independent working cells with different voltages. As shown in Fig. 2A, authentic quercetin and a methylquercetin (isorhamnetin) were significantly oxidized at the first channel (ch-1, 150 mV) and to a lesser extent at the second channel (ch-2, 200 mV). Analyses of the brain samples of quercetin-fed rats gave two peaks with retention times and electrochemical responses similar to those of the standards (Fig. 2B). When different voltages were charged on ch-1 and ch-2, similar electrochemical responses were also reproduced between authentic standards and brain samples (data not shown). It should be noted that feeding rats a purified diet (containing less quercetin; 2.9 ± 0.12 µg aglycone equivalent/100 g diet) for 5 weeks did not wash out the accumulated quercetin in brain (Fig. 2C), whereas no quercetin conjugates were detected in plasma (data not shown). Without β-glucuronidase/sulfatase treatment, nonconjugated quercetin and methylquercetins were undetectable (data not shown), indicating

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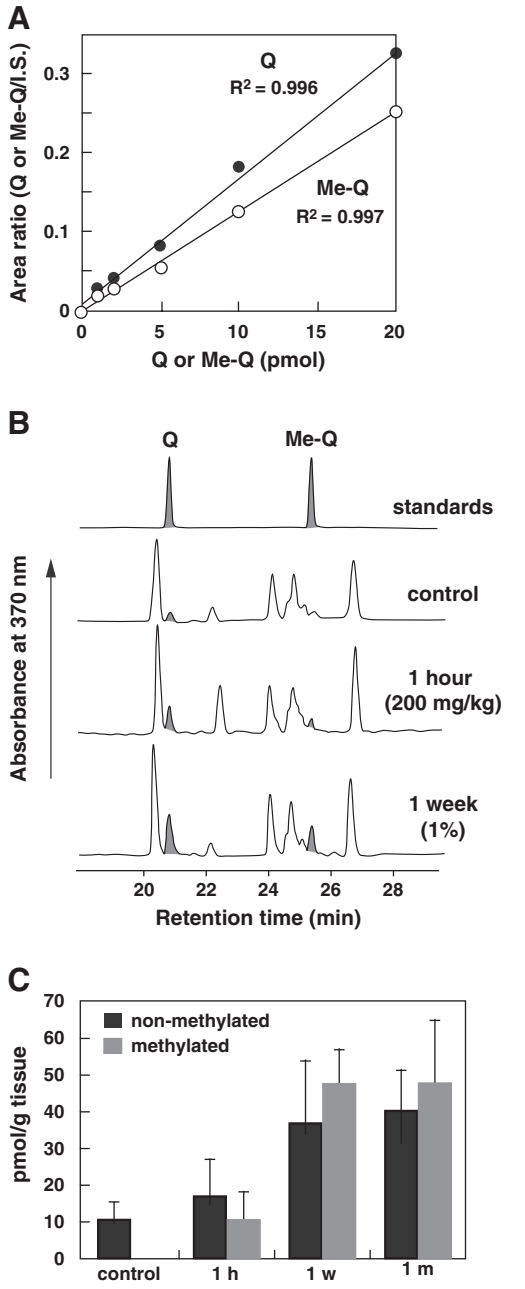

Fig. 1. HPLC-UV (370 nm) analysis for the detection of quercetin and methylquercetins in brain tissue. (A) Calibration curves for quercetin (Q) and a methylquercetin (Me-Q; isorhamnetin) using an internal standard myricetin. (B) HPLC-UV profiles of quercetin and methylquercetins in rat brain. Standards, authentic quercetin (Q) and isorhamnetin (Me-Q); control, free access to a commercial solid diet; 1 hour, intragastric administration of quercetin (200 mg/kg body wt) for 1 h; 1 week, free access to 1% quercetin for 1 week. (C) Brain tissue concentrations of Q and Me-Q determined by HPLC-UV analysis. Samples were analyzed after treatment with sulfatase/β-glucuronidase. Each value is presented as the mean ± SE ($n = 4$).

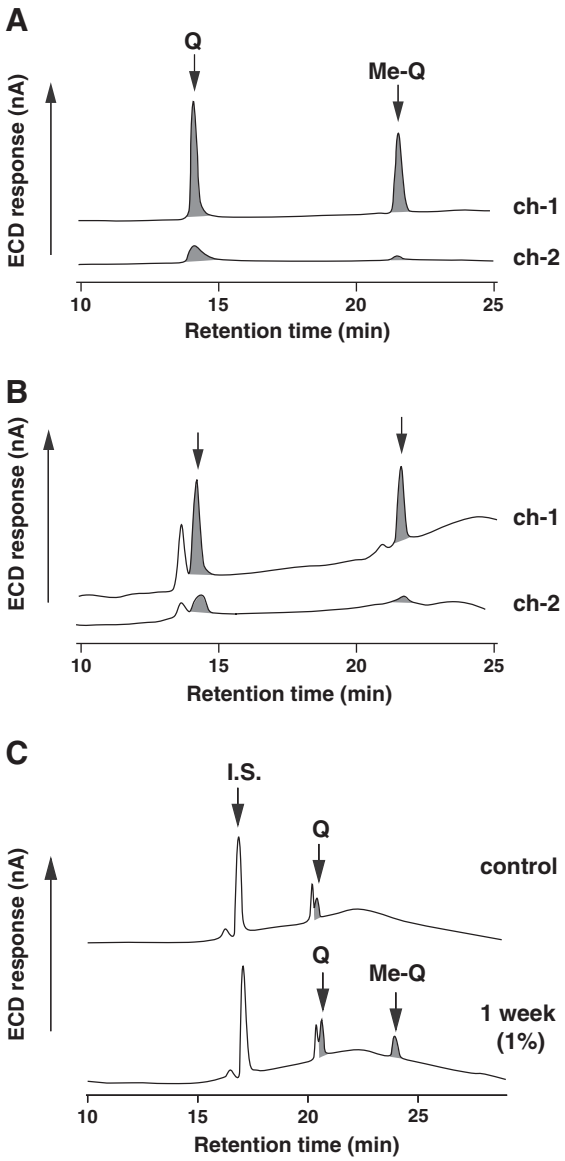

Fig. 2. HPLC-ECD analysis for the detection of quercetin and methylquercetins in brain tissue. Two coulometric channels (ch-1 and ch-2) were set at 150 and 200 mV, respectively. HPLC separation was carried out using (A and B) system 1 or (C) system 2 as described under Materials and methods. (A) HPLC-ECD profiles of authentic standards of quercetin (Q) and isorhamnetin (Me-Q). (B) HPLC-ECD profiles of quercetin and methylquercetins in the brain of rats fed 1% quercetin for 1 month. (C) HPLC-ECD profiles of quercetin and methylquercetins in the brain of rats fed the purified diet with or without 1% quercetin for 1 week.

that orally administered quercetin accumulated in the brain as its conjugated forms.

Identification of brain quercetins was finally completed by using LC-MS/MS, which provides specific fragmentation patterns for each parent compound. Authentic quercetin and isorhamnetin showed molecular ions at *m/z* 301.0 and *m/z* 315.0, respectively. The MS/MS fragmentation of quercetin (*m/z* 301.0→) and isorhamnetin (315.0→) exhibited several product ions (Fig. 3). Among them, the major precursor-to-product ion pairs, *m/z* 301.0→151.1 and *m/z* 315.0→301.0, were used for SRM of quercetin and methylquercetins, respectively. The SRM analyses of brain samples of quercetin-fed rats gave the two peaks for quercetin (*m/z* 301.0→151.1) and methylquercetins (*m/z* 315.0→301.0), the retention times of which corresponded to those exhibited by the authentic standards. The mass intensities were increased depending on the period elapsed after administration of quercetin. As well as the HPLC-UV and -ECD analyses, quercetin and trace amounts of methylquercetin(s) were also detected in brain samples of rats fed the control diet (Fig. 4). Collectively, these results obtained from three independent chromatographic methods (HPLC-UV, ECD, and LC-MS/MS), showed that the orally administered quercetin certainly accumulated in the brain tissue of quercetin-fed rats.

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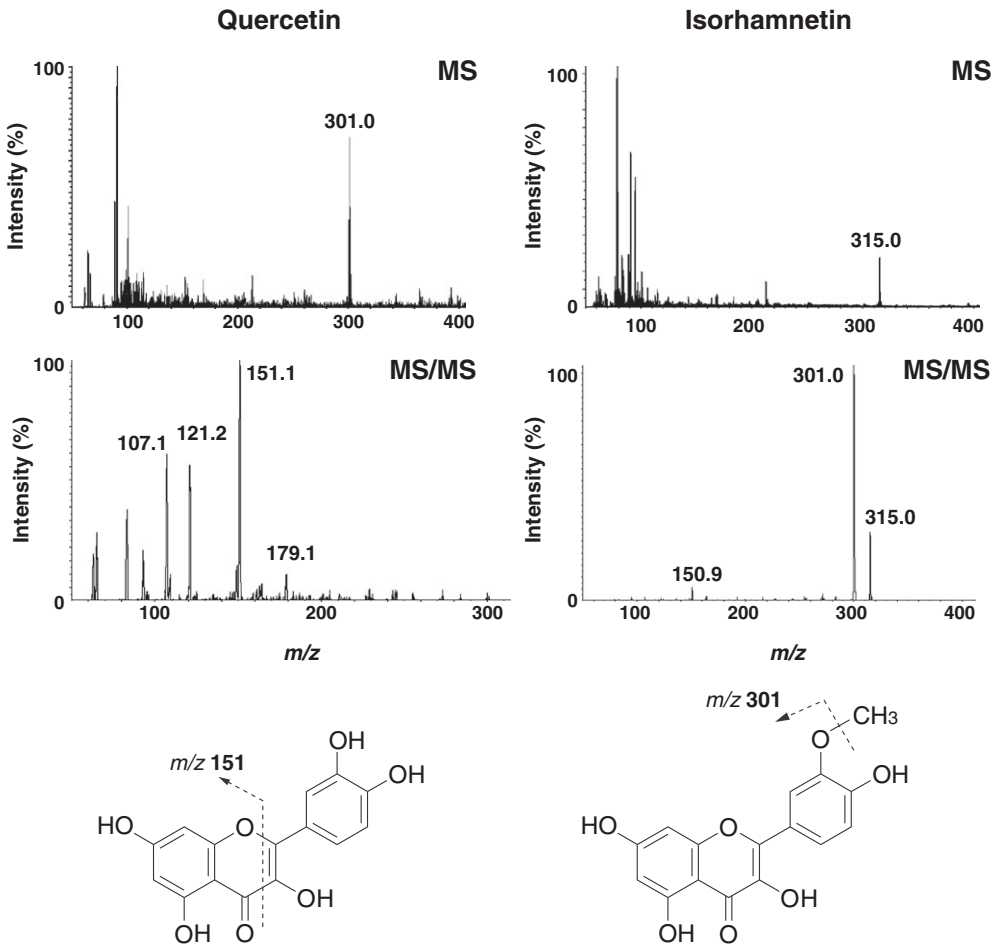

Fig. 3. Mass spectrometric characterization of authentic quercetin and methylquercetin. (Left) Quercetin showed the molecular ion at *m/z* 301.0 (top). The MS/MS fragmentation of quercetin (*m/z* 301.0→) exhibited the product ions at *m/z* 179.1, *m/z* 151.1, *m/z* 121.2, and *m/z* 107.1 (middle). (Right) Isorhamnetin showed the molecular ion at *m/z* 315.0 (top). The MS/MS fragmentation of isorhamnetin (315.0→) exhibited the product ions at *m/z* 315.0, *m/z* 301.0, and *m/z* 150.9 (middle). Proposed fragmentation patterns of quercetin and methylquercetin for their major product ions are shown at the bottom.

Effects of quercetin administration on brain stress levels

The potential roles of accumulated quercetin in rat brain were examined under stress conditions induced by chronic forced swimming. Levels of plasma corticosterone, a well-known stress marker, were significantly increased by the forced swimming compared with those of the control group (Table 1). Administration of quercetin (50 mg/kg body wt) before the forced swimming significantly suppressed the increased levels of corticosterone (Table 1), showing the inhibitory effect of orally administered quercetin on the forced-swimming-induced stress response.

It has been reported that chronic forced-swimming-induced stress responses are closely associated with brain oxidative stress [20]. We therefore examined the antioxidative effects of quercetin in the brain of rats exposed to chronic forced swimming. We analyzed oxidative stress levels of several brain regions and found that the hippocampus and striatum are more sensitive to oxidative stress (data not shown). TBARS levels were elevated in the hippocampus (statistically significantly) and striatum (though not statistically significantly) in the forced-swimming group compared with the control group (Table 1). Administration of quercetin before forced swimming attenuated the increased levels of TBARS in the hippocampus and striatum (Table 1). Similarly, GSH levels were significantly decreased in the hippocampus and striatum by the forced swimming. Administration of quercetin suppressed the decreased levels of GSH in both regions, with a statistically significant suppression in the striatum (in the hippocampus, $p = 0.106$). These results show that oral administration of quercetin can act as an antioxidant in the brain under oxidative stress conditions induced by forced swimming.

In vitro accumulation of Q3GA in RBEC1 cells

For quercetin metabolites to enter into the brain, they need to pass through the BBB. We next examined the accumulation of Q3GA, the major quercetin conjugate in rat and human plasma [5,9], in a BBB model using RBEC1 cells. HPLC-ECD analysis of cellular extracts of Q3GA-treated RBEC1 showed that Q3GA accumulated in the cells in a dose- and time-dependent manner (Fig. 5), suggesting the possible interaction of quercetin metabolites with the BBB.

Discussion

We demonstrated in this study that orally administered quercetin accumulated in the brain tissue of rats by using HPLC with three different detection methods (UV, electrochemical, and tandem mass spectrometry). Because HPLC-UV analysis is a general-purpose method to detect various flavonoids, numerous experimental studies have used HPLC-UV analysis for the quantitative or qualitative determination of flavonoids. HPLC-ECD analysis can be used as a more specific and sensitive assay for phenolic compounds than HPLC-UV analysis. We confirmed the presence of two peaks with similar retention times and electrochemical responses to authentic quercetin

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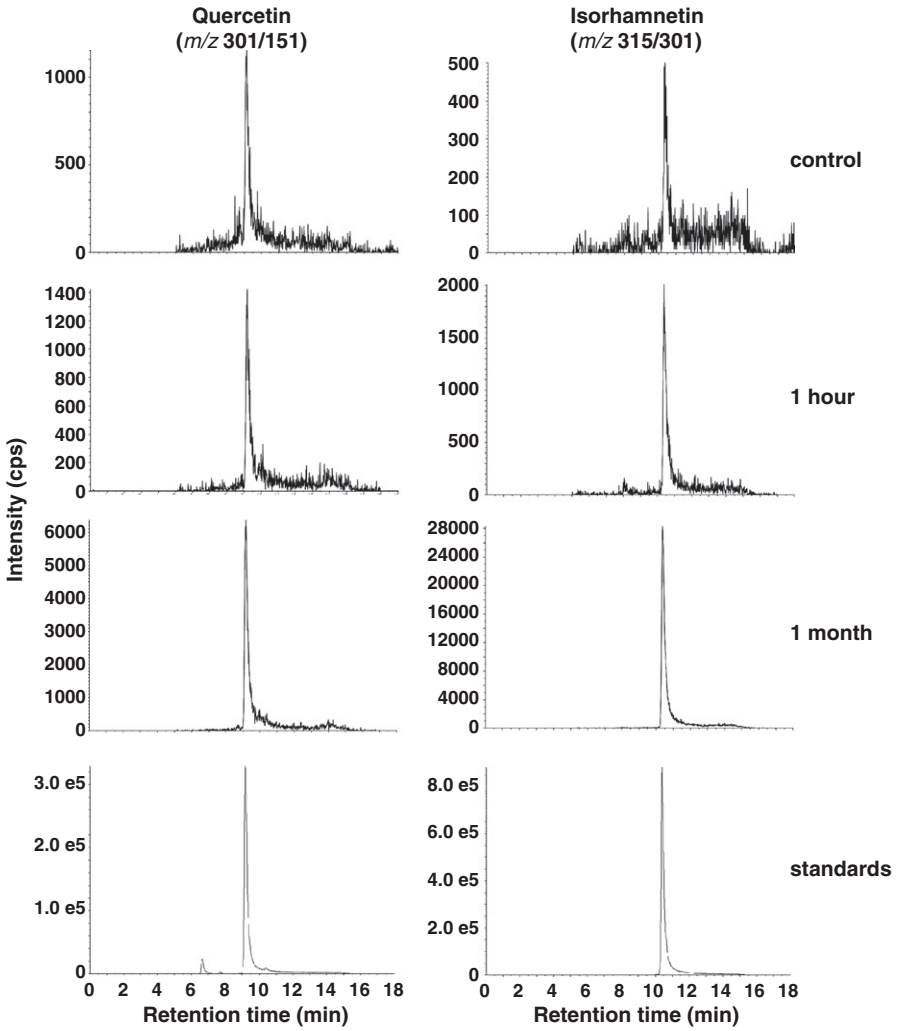

Fig. 4. SRM chromatograms of quercetin and methylquercetins in brain tissues. The typical chromatograms were monitored at the major precursor-to-product ion pairs, *m/z* 301.0 → 151.1 (quercetin, left) and *m/z* 315.0 → 301.0 (isorhamnetin, right). The samples are the same as those in Fig. 1.

and methylquercetin in the deconjugated brain samples, but these data were still insufficient to identify these flavonoids. These two peaks were successfully identified using LC-MS/MS by matching retention times and specific fragmentation patterns to authentic standards. These results showed that the orally administered quercetin was certainly accumulated in the brain tissue of quercetin-fed rats.

Table 1
Effects of quercetin administration on plasma (corticosterone) and brain stress levels.

Substance measured	Plasma	Brain tissue	
		Hippocampus	Striatum
Corticosterone (ng/ml)			
Control	15.35 ± 7.50^{a}		
Stress	51.88 ± 14.50^{b}		
Q + stress	29.35 ± 11.07^{a}		
TBARS (nmol/mg protein)			
Control		12.07 ± 5.56^{a}	13.60 ± 2.75^{a}
Stress		20.91 ± 1.43^{b}	16.28 ± 1.55^{a}
Q + stress		18.66 ± 2.00^{b}	14.66 ± 2.37^{a}
GSH (nmol/mg protein)			
Control		7.69 ± 1.75^{a}	11.26 ± 1.20^{a}
Stress		6.12 ± 1.32^{b}	7.91 ± 1.63^{b}
Q + stress		7.59 ± 2.01^{b}	9.70 ± 1.23^{a}

Q, quercetin. Stress conditions were induced by a chronic forced-swimming procedure. Concentration values of corticosterone in plasma are presented as the mean ± SE ($n = 8$). Values of thiobarbituric acid-reactive substances (TBARS) and reduced glutathione (GSH) in the brain tissues are presented as the mean ± SE ($n = 8$). Values with different superscripts are significantly different ($p < 0.05$).

Some flavonoids were shown to enter the brain after intravenous or oral administration to animals. Naringenin [21], puerarin [22], tangeretin [23], genistein [24,25], and epicatechin [26] were detected in brain tissues of rats. However, most of these studies did not correct for residual blood in the brain. In our study, we almost completely removed the blood by perfusion and confirmed that no detectable hemoglobin existed in the perfused brain. Thus, the quercetin and its methylated metabolites that were detected in the brain were thought to be located intracellularly and/or interstitially, but not intravascularly. De Boer et al. [11] have shown using HPLC analysis that quercetin metabolites accumulated in a wide variety of organs in rats after oral administration of a quercetin diet. Rats fed a 0.1 or 1% quercetin diet for 11 weeks accumulated quercetin metabolites in the brain at concentrations of 330 and 680 pmol/g tissue, respectively. The present study indicated lower concentrations of quercetin in the rat brain after a 1-month administration of 1% quercetin. This difference may be due to not only residual blood but also the different feeding and analytical conditions. We used myricetin as an internal standard for quantitative analysis by HPLC-UV, but myricetin eluted earlier than quercetin and methylquercetins under our conditions. More specific internal standards, such as stable isotope derivatives of

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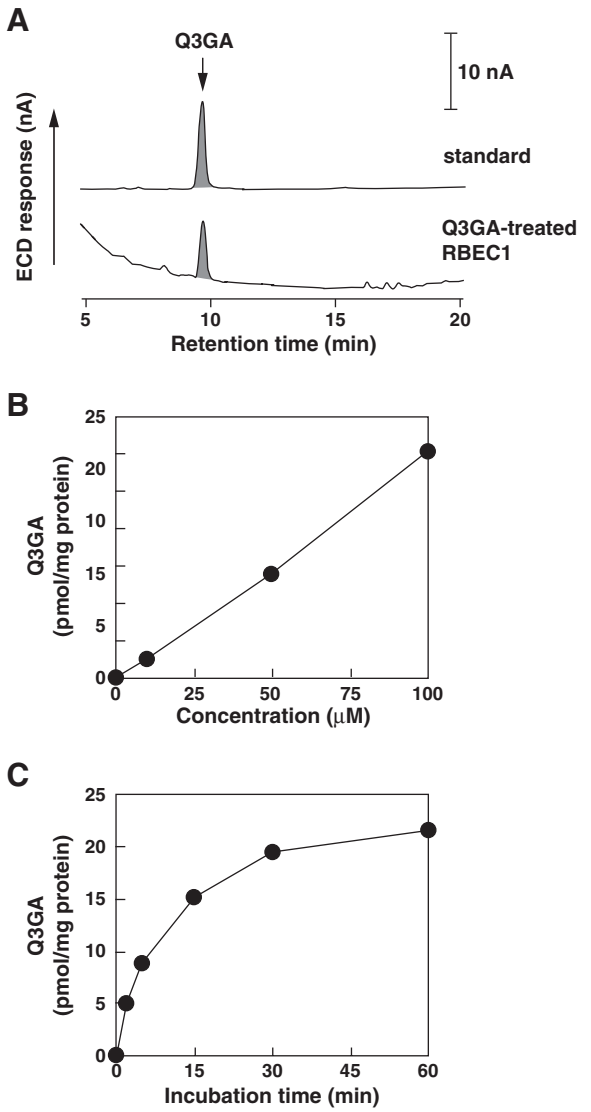

Fig. 5. Cellular accumulation of quercetin metabolites in a rat brain endothelial cell line (RBEC1). Cells were cultured in the presence of Q3GA in the serum-free culture medium and the extracts were analyzed by HPLC-ECD. (A) Typical HPLC-ECD profiles of authentic Q3GA (top) and the cellular extracts of RBEC1 treated with Q3GA for 15 min (bottom). (B) Dose-dependent accumulation of Q3GA in RBEC1 cells (treatment for 15 min). (C) Time-dependent accumulation of Q3GA in RBEC1 cells (treatment with 50 μM Q3GA).

quercetin and methylquercetins for mass spectrometry, are required for future studies. Our results showed that the concentrations of quercetin and methylquercetins almost reached a plateau within 1 week of administration and remained at constant levels after 1 month of administration (Fig. 1C). De Boer et al. [11] have also demonstrated that plasma and tissue quercetin levels were not linearly dependent on dose. These observations indicate the limited capacity of brain tissue to accumulate flavonoid compounds.

It is of interest that quercetin, but not methylquercetins, was detected in the brain samples of rats fed a control diet (Fig. 1C). A trace amount of quercetin was still detected in the brain of rats fed for 5 weeks with the purified diet, which still contained a trace amount of quercetin (2.9 ± 0.12 μg aglycone equivalent/100 g diet; Fig. 2C). Because quercetin is widely distributed in plant foods, it was difficult to prepare the quercetin-free diet. The fact that no methylquercetin was detected in the control brain samples indicates the different accumulation profiles between quercetin and methylquercetins in brain. Methylation of the catechol moiety of quercetin by catechol-*O*-methyltransferase results in the detoxification of its strong bioactivity; therefore, if the total quercetin level is lower, the extent of methylation may be attenuated. Alternatively, there may be the possibility that methylquercetin metabolites are more rapidly excreted than quercetin metabolites from the brain tissue. In contrast, our results further demonstrated that methylquercetin metabolites in brain increased in a fashion dependent on the administration period (Fig. 1C), suggesting that increased amounts of methylquercetin metabolites may reflect the potent effects of oral quercetin intake on brain functions. Although methylation of quercetin attenuates it antioxidative activity, we have previously shown that methylquercetins play much more important roles than quercetin in the inhibitory effects on the mRNA expression of a macrophage scavenger receptor [27].

Central nervous disorders such as dementia and Alzheimer disease, as well as decline in cognitive function, have been closely associated with oxidative stress in nerve cells. Quercetin intake has been expected to exert a neuroprotective effect, resulting in a lower risk of central nervous disorders [8]. In this study, we have found that oxidative stress levels were notably increased in the hippocampus and striatum by force swimming, but not changed in whole brain and other regions (data not shown), and that quercetin attenuated the increased oxidative stress in these specific regions (Table 1), suggesting the different redox sensitivity between each brain region. In addition, we have preliminarily found that the immunoreactivity of anti-Q3GA monoclonal antibody was mainly localized in hippocampus and striatum in the quercetin-fed rat brain sections (unpublished results), suggesting that quercetin metabolites could interact differently with each region in the brain. These observations may explain why lower concentrations (pmol/g whole brain) of quercetin metabolites could attenuate the oxidative stress in rat brain. As the flavonoids are oxidatively conjugated with thiols such as GSH [28,29], it might also be important to analyze quercetin–SG adducts as alternative metabolites in vivo. We have not yet determined the quercetin levels in each brain region; because each region was 2–5% of the whole brain, it was difficult to analyze using HPLC. More sensitive LC-MS/MS analysis is required to elucidate such specific accumulation of quercetin in each brain region.

We have also demonstrated that orally administered quercetin suppressed plasma corticosterone levels, as well as brain oxidative stress, of rats exposed to chronic forced swimming (Table 1). It is still unclear whether accumulated quercetin could directly or indirectly attenuate oxidative stress in brain. Sato et al. have recently shown that injection of corticosterone induced oxidative stress in hippocampus of rats [30]. Corticosterone is also implicated in hippocampal atrophy when large amounts of it are secreted by strong stress [31,32]. These observations suggest that the increase in corticosterone might induce oxidative stress and subsequent tissue injury. Recent studies have demonstrated that quercetin metabolites not only scavenge free radicals directly but also regulate the signaling cascade via a non-free radical scavenging mechanism [33]. We have also recently demonstrated that orally administered quercetin affected hypothalamic–pituitary–adrenal axis response via attenuating MAP kinase signaling in hypothalamus, resulting in the decrease in corticosterone [34]. These observations suggest that quercetin metabolites found in brain could attenuate oxidative stress not only through radical scavenging but also through non-radical scavenging activities. In any case, it should be important that the quercetin metabolites interact with the target sites (probably hippocampus and/or striatum) to attenuate oxidative stress induced by forced swimming stress.

Transport of quercetin metabolites into the brain tissues via the BBB and their effect on the central nervous system have been recently argued [35]. In this study, RBEC1 cells were used as a BBB model, because the interaction of plasma quercetin metabolites and brain capillary endothelial cells is an initial step during the incorporation of plasma constituents into BBB. Because nonconjugated quercetin

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derivatives are not present in human and rat plasma, we treated RBEC-1 cells with Q3GA as a model for incorporation of plasma quercetin metabolites into BBB. We have shown the cellular accumulation of Q3GA, a major metabolite conjugate, in RBEC1 cells (Fig. 5); however, it has been generally accepted that hydrophilic glucuronide metabolites (with logP values below zero) cannot cross the blood–brain barrier. There might be the possibility that Q3GA is attached to the cell surface membranes. The BBB cells control the entry of circulating molecules and cells into the brain by the regulation between influx and efflux. In addition to the influx transport of quercetin metabolites into brain, there might be the efflux transport system(s). Although both influx and efflux mechanisms still remain unclear, we preliminarily found that at least a part of Q3GA binds to cell surface proteins and that nonconjugated quercetins passed through cell membranes. Furthermore, we observed the likelihood of β-glucuronidase activity to deconjugate Q3GA into the hydrophobic aglycone in RBEC1 cells (unpublished result), suggesting the possibility of a transient deconjugation reaction (and subsequent reconjugation through transport) taking place around the BBB. Further investigation of the transport system of quercetin conjugates to the BBB is required in the future. In particular, more physiological BBB models using coculture of pericytes and astrocytes should be tested.

In summary, our results showed that orally administered quercetin accumulated as its metabolite forms in the brain tissue of rats and exerted antioxidative activity in the brain tissue. Quercetin may be a potent nutrient that can access the brain and protect it from disorders associated with oxidative stress.

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