Free Radical Research, October 2006; 40(10): 1047–1053

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healthcare

Effect of a conjugated quercetin metabolite, quercetin 3-glucuronide, on lipid hydroperoxide-dependent formation of reactive oxygen species in differentiated PC-12 cells

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Accepted by Professor H. Sies

(Received 28 March 2006; in revised form 9 May 2006)

Abstract

To assess the efficacy of conjugated quercetin metabolites as attenuators for oxidative stress in the central nervous system, we measured the 13-hydroperoxyoctadecadienoic acid (13-HPODE)-dependent formation of reactive oxygen species (ROS) in pheochromocytoma PC-12 cells in the presence of quercetin 3-*O*-β-glucuronide (Q3GA) and related compounds. A 2′,7′-dichlorofluorescin (DCFH) assay showed that Q3GA significantly suppressed the formation of ROS, when it was coincubated with 13-HPODE (coincubation system). However, it was less effective than quercetin aglycon in the concentration range from 0.5 to 10 μM. In an experiment in which the cells were incubated with the test compounds for 24 h before being exposed to 13-HPODE, Q3GA was also effective in suppressing the formation of ROS in spite that little Q3GA was taken up into the cells. These results suggest that antioxidative metabolites of quercetin are capable of protecting nerve cells from attack of lipid hydroperoxides.

Keywords: *Quercetin metabolites, PC-12 cell, lipid hydroperoxides, 2′,7′-dichlorofluorescin assay*

Introduction

Quercetin, 3,3′,4,5,7-pentahydroxyflavone, is a major flavonol-type flavonid distributed in the plant kingdom and mostly present as a glycoside form in which a sugar moiety substitutes for phenolic hydrogen or hydrogens [1]. The antioxidative activities of quercetin and its glycosides in foods are frequently referred to in relation to the prevention of oxidative stress-related chronic diseases such as cardiovascular heart disease [2,3]. In recent years, central nervous disorders including dementia and Alzheimer's disease, and a decline of cognitive function, have also been suggested to be closely associated with oxidative stress occurring in nerve cells [4–6]. Therefore, quercetin and its glycosides are expected to have a neuroprotective effect resulting in a lower risk of central nervous disorders [7]. Actually, Wang and Joseph [8] have demonstrated that quercetin and its glycosides act as effective antioxidants against exposure to reactive oxygen species (ROS) in cultured rat pheochromocytoma PC-12 cells. However, quercetin glycosides are mostly hydrolyzed and converted into conjugated metabolites during their absorption in the digestive tract [9,10]. Only quercetin conjugates, not aglycon or

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Informa UK Ltd.
DOI: 10.1080/10715760600794287

its glycosides, are detected in human blood [11,12]. Therefore, the question arises whether or not the conjugated quercetin metabolites are able to reach the nerve cells by crossing the blood brain barrier (BBB) to exert their antioxidative effect. Youdim et al. [13,14] demonstrated that the potential of flavonoids and some metabolites to cross the BBB depends on their lipophilicity. Furthermore, quercetin metabolites were detected at low concentrations in the brain tissue of rats and pigs fed high-quercetin diet [15], and rats fed St John's wort extracts [16], a quercetin-rich herbal medicine. These observations prompted us to study the antioxidative activity of conjugated quercetin metabolites in a nerve cell model.

We adopted differentiated PC12 cells and induced oxidative stress by exposing to linoleic acid hydroperoxide (13-hydroperoxyoctadecadienoic acid, (13-HPODE)). We previously found that quercetin 3-*O*-β-glucuronide (Q3GA), a major glucuronide metabolite present in human plasma, retains considerable antioxidative activity for low-density lipoprotein oxidation [17]. Thus, Q3GA was selected as representative of quercetin metabolites and its efficacy as an antioxidant in PC12 cells was compared with that of aglycon, isoquercitrin (quercetin 3-*O*-β-glucoside), and hyperoside, (quercetin 3-*O*-β-galactoside) (Figure 1). The results clearly indicate that this antioxidative metabolite also has the potential to protect nerve cells from attack by lipid hydroperoxides.

Materials and methods

Materials

Quercetin (3,3′,4′,5,7-pentahydroxyflavone), sulfatase type H-5 (from *Helix pomatia*, EC 3.1.6.1), and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were obtained from Sigma Chemical Co (St Louis, MO, USA). Isoquercitrin (Quercetin 3-*O*-β-D-glucoside), hyperoside (Quercetin 3-*O*-β-D-galactoside), luteolin (3′,4′,5,7-tetrahydroxyflavone), isorhamnetin and flavone were obtained from extrasynthese (Genay, France). Quercetin 3-*O*-β-D-glucuronide (quercituron; Q3GA) was chemically synthesized and purified as described previously [17]. 13-HPODE was synthesized using soybean lipoxygenase and purified as reported [18]. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Nalarai Tesque Inc. (Kyoto, Japan). All other reagents were of analytical grade and used without further purification.

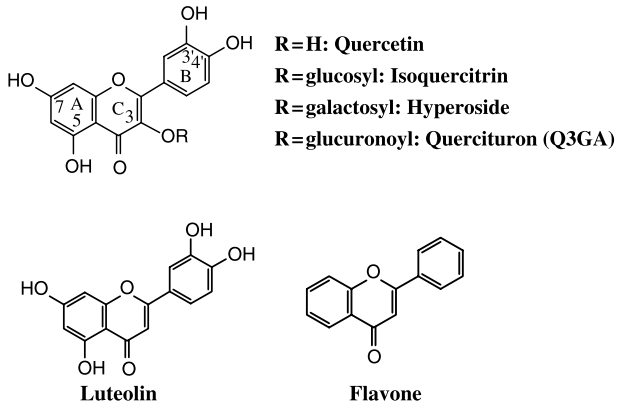

Figure 1. Structures of Q3GA and related compounds used in the current study.

Cell culture

PC12 cells were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma), 5% horse serum (HS; Sigma) and 50 μg/ml gentamicin (Sigma). The medium was changed every 2–3 days. Cells were induced to differentiate by treating them with 50 ng/ml of nerve growth factor-7S (NGF-7S; Sigma) in DMEM containing 1% HS, 0.5% FBS in 60 × 15 mm cell culture dishes (Iwaki Glass, Chiba, Japan) coated with collagen for 7 days the with medium replenished every 2–3 days.

Measurement of cellular generation of reactive oxygen species (ROS)

The generation of cellular ROS was evaluated by examining the ROS-dependent oxidation of 2′7′-dichlorofluorescein (DCFH) to form the fluorescent compound, 2′7′-dichlorofluorescein (DCF) [19]. The fluorescence of each well was measured with a Titertek Fluorskan II (Flow Laboratories, Rockville, MD, USA) fluorescence multiwell plate reader. PC12 cells were seeded at a density of 1×10^4 cells per well in non-fluorescent collagen-coated 96-well plates (Iwaki Glass, Chiba, Japan) one day before the experiments. For the pretreatment experiments, PC12 cells were treated at 37°C for 24 h with the test compounds. After the incubation, the cells were washed twice with phosphate-buffered saline (PBS) containing 2% FBS and then incubated in DMEM without L-glutamine and phenol red containing 1.0% FBS, 0.5% HS and 50 μM DCFH-DA for an additional 45 min at 37°C. The cells were washed and maintained in DMEM without L-glutamine and phenol red containing 1.0% FBS and 0.5% HS (100 μl). Once 13-HPODE (100 μl:final conc., 10 μM was added), the fluorescence was monitored after 1 h at excitation and emission wavelengths of 485 and 538 nm, respectively. In the case of the coincubation experiments, the cells were incubated in DMEM without L-glutamine and phenol red containing 1.0% FBS, 0.5% HS and 13-HPODE (final conc. of 10 μM) and the test compounds (final conc., 0.1–10 μM) at 37°C. Values are expressed as a

percentage of the DCF fluorescence intensity in untreated cells.

Determination of Q3GA and its related compounds in the medium and cellular fraction

The cells were cultured in plates 60 mm in diameter coated with collagen at a density of 2×10^6 cells per plate. When the cells had reached confluence after one day of culture, the medium was replaced with fresh medium containing the test compounds (5 ml), and the cells incubated for 4 h at 37°C. The compounds tested were dissolved in dimethylsulfoxide (DMSO) and diluted with the medium to a final concentration of 10 μM. The final DMSO concentration of the cell culture was less than 0.1% and had no effect on the cells. Following this incubation, the medium was collected, and the cells were washed three times with PBS (pH 7.4). The cells were subsequently dispersed into 5 ml of PBS and centrifuged at 1500 rpm for 5 min at 4°C. The cells were then collected in a solvent of 100 μl of methanol/acetic acid (100:5, v/v) and disrupted by ultrasonic irradiation for 60 s. The disrupted cells were centrifuged at 13,000 rpm for 10 min at 4°C to obtain cellular extracts. To extract quercetin and its metabolites from the medium, a solvent of 100 μl of methanol/acetic acid (100:5, v/v) was added to the same volume of the medium, mixed vigorously, and centrifuged at 13,000 rpm for 5 min, before the resulting supernatant was collected. For enzymatic hydrolysis of conjugated quercetin metabolites, each residue from the medium and cellular extracts was mixed with 25 units of a sulfatase-type H-5 solution in a 100 mM acetate buffer (pH 5.0, 50 μl) and incubated for 50 min at 37°C with shaking. After this incubation, the same volume of methanol/acetic acid (100:5, v/v) was added to the mixture, before it was centrifuged at 13,000 rpm for 5 min. The resulting supernatant solution was used as the sulfatase-type H-5-treated sample for an HPLC analysis.

HPLC analysis of Q3GA and related compounds

Quercetin aglycon, isoquercitrin, hyperoside, luteolin, Q3GA, and flavone were each subjected to reverse-phase HPLC with electrochemical detection at an oxidation potential of +800 mV by an ICA-5212 amperometric detector (Toa Electronics, Tokyo, Japan) with a TSKgel ODS-80Ts column (Tosoh, Tokyo, Japan). The mobile phase consisted of methanol/water/acetic acid (48:50:2, v/v/v, for quercetin aglycon; 38:60:2, v/v/v, for isoquercitrin; 36:62:2, v/v/v, for hyperoside; 48:50:2, v/v/v, for luteolin; and 32:66:2, v/v/v, for Q3GA). All eluents contained 50 mM lithium acetate as the supporting electrolyte and were used at a flow rate of 1.0 ml/min. Flavone was measured by reverse-phase HPLC using a TSK gel ODS-80Ts column (Tosoh). The mobile phase, delivered at a flow rate of 1.0 ml/min, was composed of methanol/water/acetic acid (60:38:2, v/v/v). The eluate was monitored with a Shimadzu SPD-10AV spectrophotometric detector (Shimadzu, Kyoto, Japan) at 350 nm. The compounds were characterized by a chromatographic comparison of the retention times of standards and quantified by using standard curves of the corresponding compounds.

DPPH radical scavenging activity

DPPH radical scavenging activity was measured according to the method of Blois [20]. The procedure was the same as that presented in our previous report [17]. The amount of DPPH trapped by 1.0 mol cysteine was assumed to be 1.0 mol and the activity was expressed as mol DPPH trapped per mol compound.

Calculation of partition coefficient log P of Q3GA and its related compounds

The *n*-octanol/water partition coefficient log *P* is known as a quantitative lipophilicity index of a compound. The log *P* values of Q3GA and related compounds were estimated with the software CLOGP [21]. CLOGP calculates log *P* of a given compound as a summation of the fragment lipophilicity values in the compound [22] and has been widely used for the estimation of log *P* values in quantitative structure–activity relationships [23].

Data analysis

All data were evaluated by a one-way analysis of variance followed by the Bonferroni/Dunn *post hoc* multiple-comparison test to determine the statistical differences between means. A p value less than 0.05 was judged as statistically significant.

Results

Effect of Q3GA and related compounds on 13-HPODE induced cellular ROS production

We examined the effect of Q3GA and related compounds on the 13-HPODE-induced production of ROS in PC12 cells. The cellular concentration of ROS increased with the concentration of 13-HPODE in the range from 5 to 20 μM (Figure 2). The coincubation of either Q3GA or related compounds with 13-HPODE (final conc., 10 μM) showed that all compounds other than flavone significantly inhibited the increase in cellular ROS production at the concentration of 1 μM (Figure 3). The inhibitory effect of Q3GA increased along with its concentration

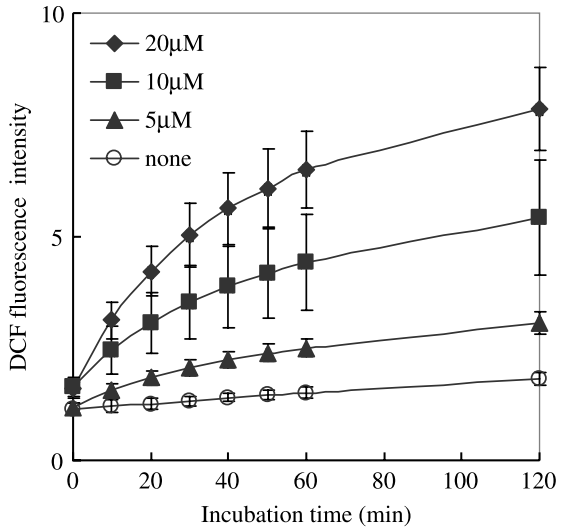

Figure 2. 13-HPODE-induced ROS production in PC-12 cells. PC-12 cells were seeded at a density of 1×10^4 cells per well in non-fluorescent collagen-coated 96-well plates one day before the experiments. The cells were loaded with DCFH-DA (50 μM) for 45 min and then incubated with 13-HPODE (5–20 μM) at 37°C. The fluorescence of the cells from each well was monitored 1 h after the incubation. The *Y*-axis expresses an increase in fluorescence intensity.

(Figure 4). Quercetin aglycon was more inhibitory than Q3GA, as it was effective even at 0.5 μM. We also examined the effect of pretreatment with Q3GA or related compounds on the 13-HPODE-induced production of ROS in PC-12 cells. After PC12 cells had been pretreated with each compound (10 μM) for 24 h and the compound washed out, Q3GA, isoquercitrin, hyperoside, and luteolin significantly inhibited the increase in cellular ROS production (Figure 5). However, quercetin, in addition to flavone, had no effect on the production of ROS.

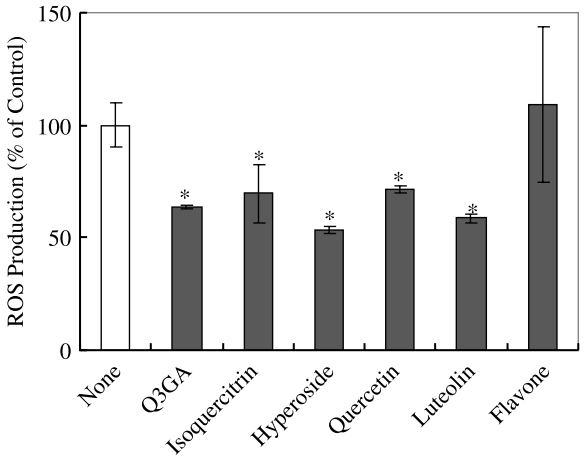

Figure 3. Effect of coincubation of Q3GA and related compounds at 1 μM on 13-HPODE-induced ROS production in PC-12 cells. PC-12 cells were seeded at a density of 1×10^4 cells per well in non-fluorescent collagen-coated 96-well plates one day before the experiments. The cells were treated at 37°C for 24 h with the test compounds (10 μM)m, Then cells were washed with PBS buffer and loaded with DCFH-DA (50 μM) for 45 min and then incubated with 13-HPODE (10 μM). The fluorescence of the cells from each well was monitored 1 h after the incubation. Each value is expressed as a percentage of the DCF fluorescence intensity of the control and is calculated as the mean ± SD from three independent experiments. *Significantly different from the control ($p < 0.05$).

Incorporation of Q3GA and related compounds into PC12 cells

PC12 cells were exposed to Q3GA and related compounds at 10 μM for 4 h. Then the cell extract was separated from the medium to quantify the concentration of each compound. Table I shows the quantity of each compound that was detected in the cellular extracts and the medium after the incubation with PC12 cells. The log *P* value of each compound is also listed as an index of lipophilicity. Compounds with higher log *P* values tended to be detected at higher levels in the cellular extracts. After the incubation of quercetin with PC12 cells, both quercetin and its *O*-methylated metabolite, isorhamnetin, were found in the cellular extracts and the medium (data were not shown here). Hyperoside and isoquercitrin were not detected in the cellular extracts after the incubation. Interestingly, a trace amount of Q3GA was detected in the extracts, although Q3GA had the lowest lipophilicity of all the compounds tested. It should be noted that the recovery of quercetin from the medium was quite low as compared with that of the other compounds tested.

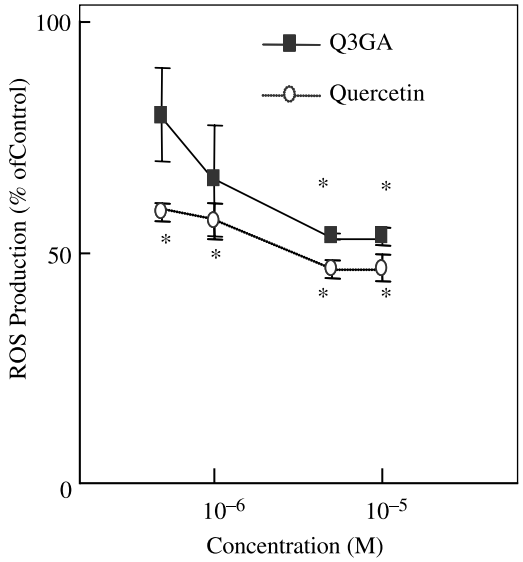

Figure 4. Effect of Q3GA and quercetin aglycon at various concentrations (up to 10 μM) on 13-HPODE-induced ROS production in PC-12 cells. Experimental procedure was the same as that described in the legend of Fig. 3. The cells were incubated after addition of 13-HPODE (10 μM) and each concentration of Q3GA or quercetin aglycon at 37°C. The fluorescence of the cells from each well was monitored 1 h after the incubation. Each value is expressed as a percentage of the DCF fluorescence intensity of the control and is calculated as the mean ± SD from three independent experiments. *Significantly different from the control ($p < 0.05$).

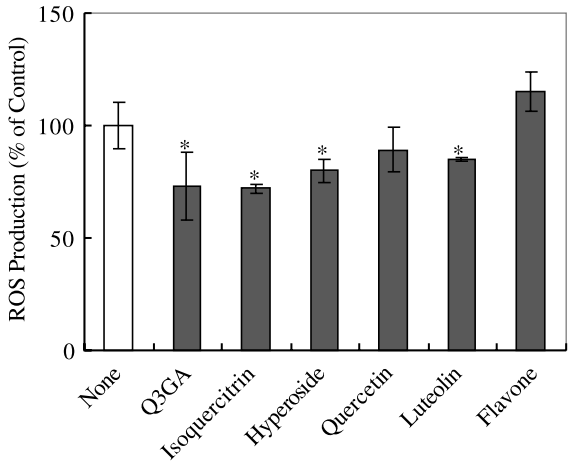

Figure 5. Effect of pretreatment with Q3GA and its related compounds at 10 μM on the 13-HPODE -induced production of ROS in PC-12 cells. PC-12 cells were seeded at a density of 1×10^4 cells per well in non-fluorescent collagen-coated 96-well plates one day before the experiments. The cells were treated at 37°C for 24 h with the test compounds (10 μM)m, Then cells were washed with PBS buffer and loaded with DCFH-DA (50 μM) for 45 min and then incubated with 13-HPODE (10 μM). The fluorescence of the cells from each well was monitored 1 h after the incubation. Each value is expressed as a percentage of the DCF fluorescence intensity of the control and is calculated as the mean ± SD from three independent experiments. *Significantly different from the control ($p < 0.05$).

DPPH radical scavenging activity

Table II shows that the number of DPPH radicals trapped by one molecule of Q3GA was around four. A similar number of radicals could be trapped by one molecule of isoquercitrin, hyperoside and luteolin. On the other hand, one molecule of quercetin aglycon trapped six or seven radicals. Flavone did not show any radical-trapping activity.

Discussion

We used 13-HPODE as an inducer of cellular oxidative stress in PC12 cells. This free fatty acid hydroperoxide is a product of the peroxidation of linoleic acid or released from peroxidized phospholipids via a reaction with phospholipase A_2 in the membranous phospholipid bilayers [24]. Lipid hydroperoxides are known to generate highly reactive lipid alkoxyl radicals and peroxyl radicals by the homolytic cleavage of a hydroperoxide group or a one-electron transfer reaction with metal ions [25]. Cell viability measured by MTT assay was not changed significantly by the incubation with 13HPODE at 10 ~ 20 μM for 1 h (data are not shown here). Thus, it was found that the exposure of the low level 13-HPODE for a short period did not show any severe oxidative stress such as cell death. On the other hand, DCFH is frequently used as a probe of intracellular ROS formation. It was recently clarified that fluorescent DCFH is generated from DCFH in reaction with several reactive intermediates including alkoxyl and peroxyl radicals [26,27]. Yoshida et al. [28] pointed out that DCFH is rather lipophilic ($\log P = 2.62$) and located in both the cellular membranes and the cytoplasm. It is, therefore, likely that ROS formation estimated by the DCFH method reflects lipophilic radicals in the phospholipid bilayer membranes as well as aqueous radicals in intracellular cytosolic fraction.

Sasaki et al. [29] already demonstrated that quercetin and other flavonol-type flavonoids in the aglycon form were effective in the prevention of linoleic acid hydroperoxide-dependent cytotoxicity in PC-12 cells. However, recent studies have demonstrated that quercetin is mostly present as conjugated metabolites such as sulfate, glucuronide and sulfoglucuronide in the human blood stream [11,12]. Quercetin aglycon seems to traverse cellular membranes via passive transport and exert free radical-scavenging activity in the cytoplasm, because it is rather lipophilic as indicated by its $\log P$ value ($= 1.8$). On the other hand, Q3GA and other conjugated metabolites lose lipophilicity and acquire hydrophilic properties as indicated by the lower $\log P$ value of Q3GA. It is unlikely that Q3GA enters the cytoplasm by crossing the lipophilic cellular membranes. On the other hand, the weaker DPPH radical scavenging activity of Q3GA reflects a decrease in the free radical-scavenging activity of quercetin caused by the substitution of the 3-OH group in the C ring.

Table I. Distribution of Q3GA and related compounds in the cellular extracts and the medium.

Compound	Cell (pmol/2 × 10^6 cells)	Medium (% of added contents)	$\log P$[d]
Q3GA	3 ± 2	94 ± 3	−0.82
Isoquercitrin	n.d[a]	90 ± 1	−0.34
Hyperoside	n.d[a]	98 ± 7	−0.39
Quercetin	27 ± 3[b]	6[c]	1.8
Luteolin	45 ± 8	94 ± 3	2.3
Flavone	33 ± 0.4	79 ± 1	3.5

The cells were incubated for 4 h at 37°C after addition of the test compounds (final conc., 5 μM).
[a] Not detected (less than 1 pmol/2 × 10^6 cells).
[b] Isorhamnetin; 19 ± 4 pmol/2 × 10^6cells.
[c] Isorhamnetin 4%.
[d] Calculated by CLOGP.

Table II. DPPH Radical-scavenging activity of Q3GA and related compounds.

Compound	Mol DPPH trapped/mol compound
Q3GA	4.15[a]
Isoquercitrin	4.13
Hyperoside	3.78
Quercetin	6.59
Luteolin	3.67
Flavone	None

DPPH radical scavenging activity of the test compounds was measured in the mixture of ethanol and 0.1M Tris-HCl buffer (pH 7.4) (1:2, v/v). The reaction was carried out for 20 min at room temperature.
[a] Moon et al. [17]

Nevertheless, Table II shows that considerable activity still retains in the 3-OH-substituted quercetin conjugates (Q3GA, isoquercitrin, and hyperoside) and luteolin which lacks a 3-OH group. This phenomenon is based on the fact that the *o*-dihydroxyl group in the B ring is mostly responsible for the free radical-scavenging by quercetin and other flavonol-type flavonoids [30].

Actually, Q3GA significant inhibited the formation of ROS in the coincubation system, although it was less effective than quercetin aglycon at 0.5–10 μM (Figures 3 and 4). In the preincubation system, Q3GA was also active in suppressing the formation of ROS (Figure 5). In contrast, quercetin aglycon did not have any significant effect in the preincubation. This unexpected result can be explained by the disappearance of quercetin aglycon during the preincubation period. Quercetin aglycon was found to be unstable during the preincubation period in the cell culture system as shown by its poor recovery from the medium after 4 h (Table I). We already revealed, using mouse fibroblast cells exposed to H_2O_2, that a poor recovery of quercetin in the medium is associated with little efficacy of quercetin aglycon in the prevention of ROS formation in preincubation conditions [31]. The present study confirms that Q3GA maintains its original structure during the preincubation and possesses the ability to attenuate cellular oxidative stress. Substitution of the 3-OH group seems to be helpful in improving the stability of quercetin in cultured cell system.

We previously found that Q3GA possesses low but significant affinity for phospholipid bilayer membranes [32]. Thus, Q3GA may interact with cellular membranes and scavenge lipid hydroperoxide-derived radicals within membranes, although it is little incorporated into the cytoplasm. Spencer et al. [33] indicated that glucuronidation dramatically decreases the efficacy of cellular uptake of epicatechin and abrogates the antioxidative activity in mouse cortical nerve cells exposed to H_2O_2. Therefore, further studies are required to elucidate the general mechanism for the protection of nerve cells by glucuronide conjugates of flavonoids.

Hydrophilic quercetin metabolites should be transferred to brain tissue by crossing the BBB to protect nerve cells from oxidative stress. It is unlikely that hydrophilic metabolites pass through the BBB without a specific transporter system. However, it is reported that small amounts of such metabolites can be transferred across the BBB [34]. Recent reports indicate that quercetin metabolities accumulate in the brain tissues of experimental animals at the level of 0.02 ~ 0.2 nmol/g tissue after dietary intake of quercetin or a quercetin-containing herbal extracts [15,16]. It is therefore likely that the metabolites have the potential to exert a physiological role in the central nervous system on reaching the target site.

In conclusion, Q3GA, an antioxidative conjugated metabolite of quercetin found in human blood, can act as an attenuator for lipid hydroperoxide-dependent oxidative stress in nerve cells. It has been assumed that antioxidant efficacy of quercetin in nerve cell system disappears because of metabolic conversion to hydrophilic glucuronides, but it is not necessarily the case. The results of the present study indicate that further research is warranted on the effect of quercetin-rich foods on the protection of the central nervous system from oxidative stress.

Acknowledgements

This work was supported by a grant-in-aid for the 21st Century COE program Human Nutritional Science on Stress Control, The University of Tokushima, Japan.

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