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Quercetin metabolites and protection against peroxynitrite-induced oxidative hepatic injury in rats

AKIYUKI YOKOYAMA[1]*, HIROYUKI SAKAKIBARA[1,2]*, ALAN CROZIER[3], YOSHICHIKA KAWAI[4], ASAKO MATSUI[1], JUNJI TERAO[4], SHIGENORI KUMAZAWA[1], & KAYOKO SHIMOI[1,2,5]

[1]*Graduate School of Nutritional and Environmental Sciences,* [2]*Institute for Environmental Sciences, University of Shizuoka, Shizuoka, Japan,* [3]*Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK,* [4]*Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan, and* [5]*Global COE Program, University of Shizuoka, Shizuoka, Japan*

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Abstract
Quercetin has strong antioxidant potency. Quercetin-3′-*O*-sulphate (Q3′S) and quercetin-3-*O*-glucuronide (Q3GA) are the main circulating metabolites after consumption of quercetin-*O*-glucoside-rich diets by humans. However, information about how these quercetin metabolites function *in vivo* is limited. Hence, this study evaluated the efficacy of Q3′S and Q3GA for the protection of oxidative injury using *in vitro* and *in vivo* experiments. Peroxynitrite-mediated hepatic injury in rats was induced by administration of galactosamine/lipopolysaccharide (GalN/LPS). Twenty-four hours after GalN/LPS treatment, plasma ALT and AST levels δ increased significantly. However, pretreatment with 4^{G}-α-D-glucopyranosyl rutin, a quercetin glycoside (30 mg/kg body weight), prevented these increases and reduced nitrotyrosine formation, indicating that consumption of quercetin glycosides prevent oxidative hepatotoxicity. Moreover, physiological levels of Q3′S and Q3GA (1 μM) effectively prevented peroxynitrite-induced nitrotyrosine formation in human serum albumin in *in vitro* experiments. These findings indicate peroxynitrite-induced oxidative hepatotoxicity is protected by the *in vivo* metabolites of quercetin, Q3′S and Q3GA.

Keywords: *Quercetin metabolites, quercetin glycosides, quercetin-3′-sulphate, quercetin-3-glucuronide, peroxynitrite-induced oxidative hepatotoxicity, nitrotyrosine*

Introduction

Quercetin (see Figure 1) is a major dietary flavonol [1], possessing strong antioxidant activity [2]. Several research groups have shown that consumption of quercetin has diverse effects on the prevention of oxidative stress-related chronic diseases such as liver injury, ischemic heart disease and diabetes [3,4]. Moreover, following ingestion, quercetin enters the circulatory system and appears to be readily bioavailable [5]. Quercetin, therefore, has a potential role in protection against oxidative stress-related diseases. However, the degree to which dietary quercetin participates in counteracting oxidative stress *in vivo* has been a matter of some debate.

Quercetin is found in plant foods almost exclusively as *O*-glycosides [1]. Following ingestion by humans, quercetin-*O*-glucosides are hydrolysed by mucosal lactase phloridizin hydrolase and soluble β-glucosidases and the released aglycone converted in the enterocyte to a number of metabolites, principally quercetin-3′-*O*-sulphate (Q3′S) and quercetin-3-glucuronide (Q3GA) and it is these metabolites which accumulate in the bloodstream and during

*These two authors contributed equally to this work.

Correspondence: Kayoko Shimoi, PhD, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1 Yada, Suruga, Shizuoka 422-8526, Japan. Fax: +81 54 264 5787. Email: shimoi@u-shizuoka-ken.ac.jp

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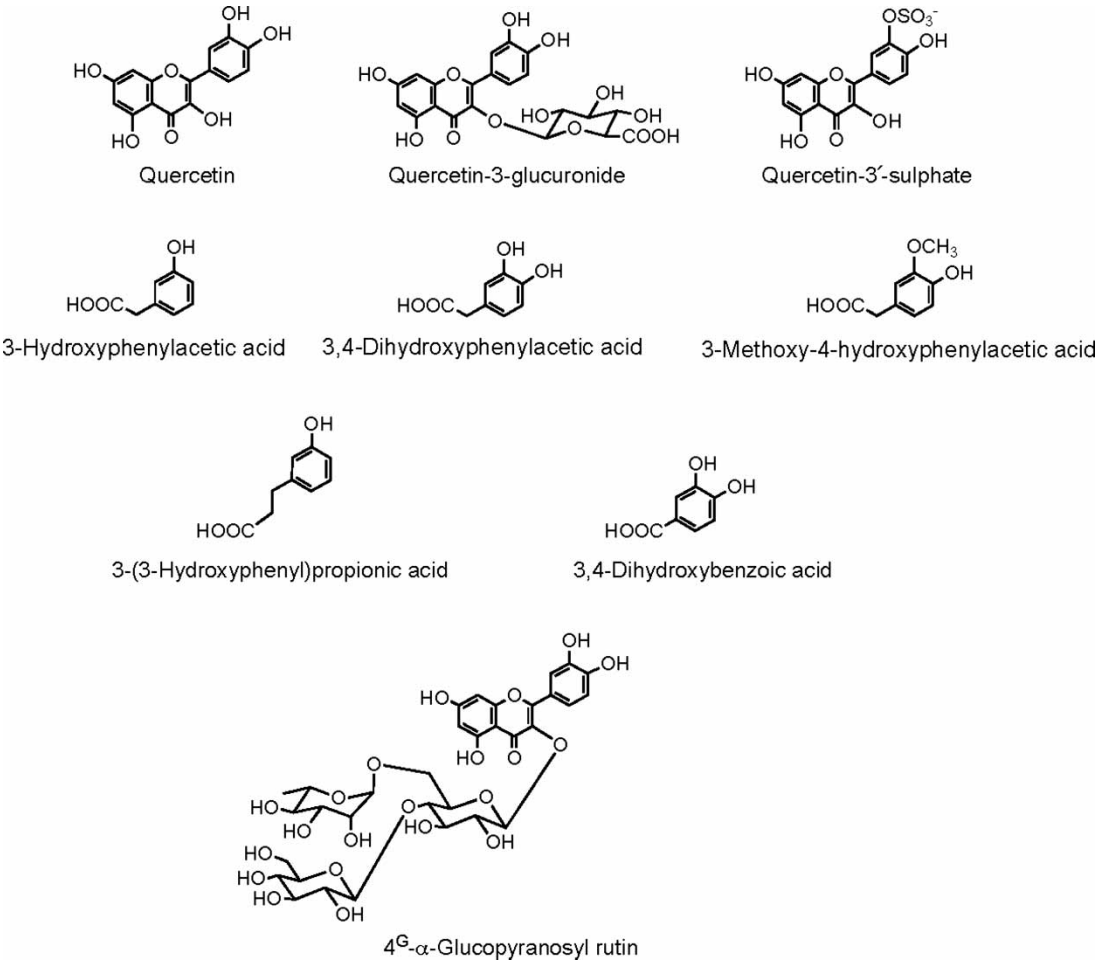

Figure 1. Quercetin and its metabolites used in this study.

passage through the body undergo substantial phase II metabolism, probably in the liver, being excreted in urine [5–9]. The antioxidant activity of flavonoids, including quercetin, is related to the number and position of hydroxyl groups. *Ortho*-dihydroxyl substitution at the 3′ and 4′ position of the B-ring is particularly important for high potency [10,11]. Metabolites such as Q3'S will, therefore, have reduced antioxidative potency compared with the parent aglycone quercetin. Quercetin glycosides not absorbed in the small intestine pass to the large intestine where they are subjected to the action of the colonic microflora which degrade quercetin resulting in the formation of catabolites such as 3,4-dihydroxyphenylacetic acid (DHPAA), 3-hydroxyphenylacetic acid (HPAA) and 3-methoxy-4-hydroxyphenylacetic acid (MHPAA) [12,13]. Thus, in order to ascertain the potential beneficial effects of quercetin *in vivo*, it is necessary to evaluate, at physiological concentrations, the *in vitro* and *ex vivo* bioactivity of not only of the aglycone and its glycoside conjugates but also metabolites, such as Q3′S, Q3GA and catabolites including DHPAA. Information of this type is, however, very limited, with only one report on the impact of various quercetin metabolites, including Q3′S, on physiological activities associated with oxidative stress [14].

In this study, we evaluated the physiological bioactivities of quercetin metabolites and catabolites using *in vitro* and *in vivo* peroxynitrite-induced oxidative injury models. Initially, we investigated the effect of orally-administered a quercetin glycoside on galactosamine/lipopolysaccharide (GalN/LPS)-induced hepatic oxidative injury in rats. GalN/LPS-induced hepatotoxicity is induced via peroxynitrite, which causes nitration of tyrosine [15,16]. Hence, we next studied the effects of *in vivo* quercetin metabolites and catabolites on peroxynitrite-induced oxidation with an *in vitro* model using human serum albumin.

Materials and methods

Materials

The quercetin glycoside used in this study was 4^{G}-α-D-glucopyranosyl rutin (MGR, Figure 1), which was kindly donated from Toyo Sugar Refining Co., Ltd. (Tokyo, Japan). The quercetin trisaccharide was dissolved in deionized water at a concentration of 25 and 50 mg/mL. Q3'S was synthesized according to

the method described by Day et al. [7], while Q3GA extracted from developing seeds of French bean (*Phaseolus vulgaris*) with methanol was purified by liquid–liquid partitioning and preparative HPLC. Quercetin was obtained from Extrasynthèse (Genay, France). Lipopolysaccharide (LPS), HPAA, 3,4-dihydroxybenzoic acid (DHBA), 3(3-hydroxyphenyl)propionic acid (HPPA), DHPAA and MHPAA were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). The structures of quercetin, MGR and the metabolites and catabolites used in this study are illustrated in Figure 1. D-galactosamine-hydrochloride (GalN), diethylenetriaminepentaacetic acid (DTPA) and human serum albumin (HSA) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Anti-nitrotyrosine antibody was obtained from Cayman Chemical (Ann Arbor, MI). Peroxynitrite solution was supplied by Dojindo Laboratories (Kumamoto, Japan). Water was used as ultra pure grade and all other reagents were of the highest grade available.

Animals

Male SD rats (7 weeks; Japan SLC, Shizuoka, Japan) were housed in an air-conditioned room ($23 \pm 1^{\circ}C$) under 12 h dark/12 h light cycles (light on 8:00–20:00 h) with free access to laboratory chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. The animal experiments were killed after 10 days acclimatization. The study was conducted according to the Guidelines for the care and use of laboratory animals of the University of Shizuoka. All efforts were made to minimize animal suffering and to reduce the number of animals used.

GalN/LPS treatment

Rats were randomly divided into six groups ($n = 6$) and treated as follows: control, 15 or 30 mg MGR/kg body weight, GalN/LPS, 15 or 30 mg MGR/kg plus GalN/LPS. Briefly, MGR were administered orally by gavage (15 or 30 mg/12 mL/kg). Animals not receiving MGR received an equivalent volume of deionized water. Two hours after feeding, animals in the GalN/LPS groups were given intraperitoneally GalN (300 mg/kg) and LPS (30 μg/kg) while control animals were injected with an equivalent volume of 0.9% saline. The animals were dissected under ether anaesthesia 24 h after GalN/LPS treatment. Blood was collected from the abdominal vein into heparinized tubes and plasma obtained by centrifuging at 670 *g* for 20 min. The plasma samples were stored at $-80^{\circ}C$ prior to analysis. The liver was removed and frozen immediately with liquid nitrogen and stored at $-80^{\circ}C$ before analysis of nitrotyrosine.

Analysis of markers of hepatic injury

All plasma samples were confirmed haemolysis-free before assay of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are markers of hepatic injury, using Transaminase CII-test Wako (Wako Pure Chemicals Industries) according to the manufacturer's protocol.

Dot blot analysis

Dot blot analysis of nitrotyrosine was used according to the methods reported by Mesenge et al. [17] and Neumann et al. [18] with minor modifications. Briefly, the proteins were extracted from the liver samples using a Mammalian Cell Extraction Kit (BioVision Inc., Mountain View, CA) and the protein content of the extracts were measured with a BCA™ Protein Assay Kit (Pierce, Rockford, IL). After hydration of PVDF membranes (Hybond-P, Amersham Pharmacia Biotech, Buckinghamshire, UK) in PBS, the sample (3 μg protein each) was loaded on the membrane and blocked overnight at $4^{\circ}C$ in Block Ace (DS Pharma Biomedical Co., Osaka, Japan). The membranes were then incubated for 1 h at room temperature with anti-nitrotyrosine diluted 1:1000 in dilution buffer (1/10 of Block Ace), followed by a secondary antibody (anti-mouse horseradish peroxidase conjugated). After incubation for 1 h at room temperature, immunoreactive dots were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and were photographed using a Polaroid camera (Amersham Pharmacia Biotech).

Tyrosine nitration

Nitration of tyrosine in HSA was carried out as reported by Ippoushi et al. [19] with some modifications. The reaction mixture consisted HSA (final concentration of 0.5 mg/mL) and quercetin or its metabolites/catabolites (final concentration of 1 or 10 μM) in PBS containing 100 μM DTPA. For a control, 0.1% of dimethyl sulphoxide in the reaction mixture was used instead of quercetin and its metabolites. After incubation at $37^{\circ}C$ for 5 min, peroxynitrite was added into the reaction mixture at a final concentration of 45 μM and then incubated at $37^{\circ}C$ for 30 min. Tyrosine nitration was measured by Western blotting. The reaction solution (7.5 μL) was treated by 10% SDS-polyacrylamide minigel electrophoresis and proteins were transferred to PVDF membranes. The membranes were blocked for 12 h at $4^{\circ}C$ with a commercial blocking buffer, Block Ace. The blots were then incubated for 1 h at room temperature with anti-nitrotyrosine diluted 1:1000 in dilution buffer (1/10 of Block Ace), followed by a secondary antibodies (anti-mouse horseradish peroxidase conjugated). After incubation for 1 h at room

temperature, immunoreactive dots were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and were photographed using a Polaroid camera (Amersham Pharmacia Biotech).

Analysis for redox state of HSA

The redox state of HSA in the reaction solution was evaluated by using HPLC [20]. The reaction solution was filtrated through a 0.45 μm membrane filter (Nacalai Tesque, Inc., Kyoto, Japan) and a 25 μL aliquot analysed using an HPLC system consisted of a pump (JASCO 880-PV, Tokyo, Japan), column oven (JASCO CO-2065) and fluorescent detector (excitation wavelength, 280 nm; emission wavelength, 340 nm) (JASCO 821-FP). DEAE ion-exchange chromatography was carried out at 37°C using a 100 mm × 7.6 mm (i.d.) Shodex Asahipak ES-502N HPLC column (Showa Denko Co., Tokyo, Japan). The elution was performed by 0.05 M sodium acetate–0.4 M sodium sulphate (pH 4.85) at a flow rate of 1.0 mL/min.

The redox state of HSA was calculated by the following equations:

$$\text{Redox state of HSA (\%)} = [F_{RF}/(F_{RF} + F_{OX})] \times 100$$

Here, F_{RF} and F_{OX} means individual peak areas on the HPLC chromatogram of redox form and oxidized forms of HSA, respectively. The data was indicated as percentage compared with controls that treated with vehicle solvent but without peroxynitrite.

Statistical analyses

Statistical analyses were performed using the Dunnett test with Pharmaco Analyst II (Hakuhousha Co., Ltd., Tokyo, Japan). A p-value of less than 0.05 was considered to indicate a statistically significant difference.

Results

Effects of quercetin glycosides consumption on acute liver injury

The plasma ALT and AST levels in rats were elevated significantly compared with the control group 24 h after injection of GalN and LPS (Figure 2), indicating severe hepatocellular damage. Oral pre-treatment with MGR at a dose of 15 and 30 mg/kg decreased ALT and AST levels in a dose-dependent manner, with the impact of the 30 mg/kg treatment being significantly different in both instances.

Detection of nitrotyrosine in liver of rats administered with GalN/LPS

Nitric oxide (NO) has been reported to play a role in GalN/LPS-induced hepatocellular damage [21]. While moderate levels of NO formation are in principle beneficial [22], excess generation of NO can exert toxic effects via peroxynitrite which induces

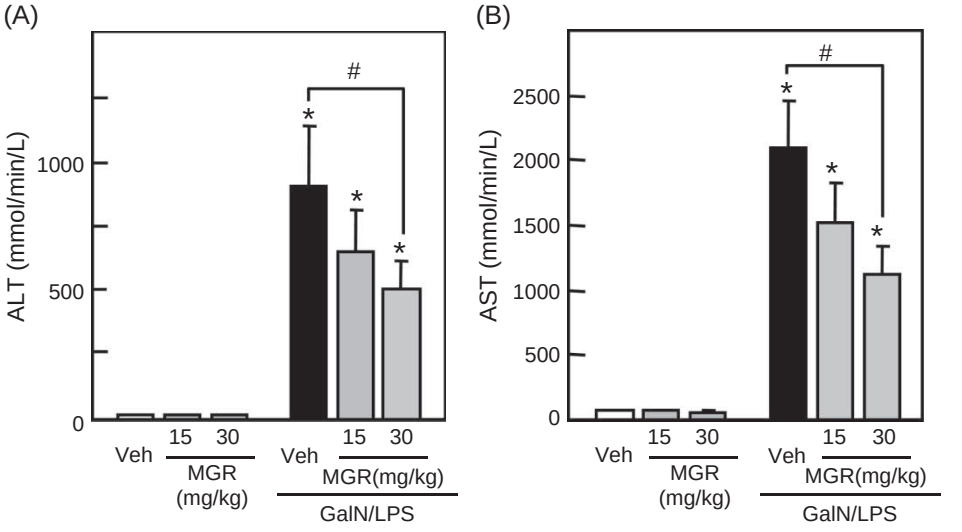

Figure 2. Effect of oral administration of 4^G-α-D-glucopyranosylrutin (MGR) on liver injury in rats treated with GalN/LPS. MGR was administered orally to rats at doses of 15 or 30 mg/kg body weight. After 2 h, galactosamine/lipopolysaccharide (GalN/LPS) was injected intraperitoneally into the rats. The plasma was collected after 24 h, the levels of (A) alanine aminotransferase (ALT) and (B) aspartate aminotransferases (AST) measured. Data presented as mean values ± standard error ($n = 6$–7). *Significant difference vs vehicle control ($p < 0.05$). # Significant difference between the groups ($p < 0.05$). Veh, vehicle (water).

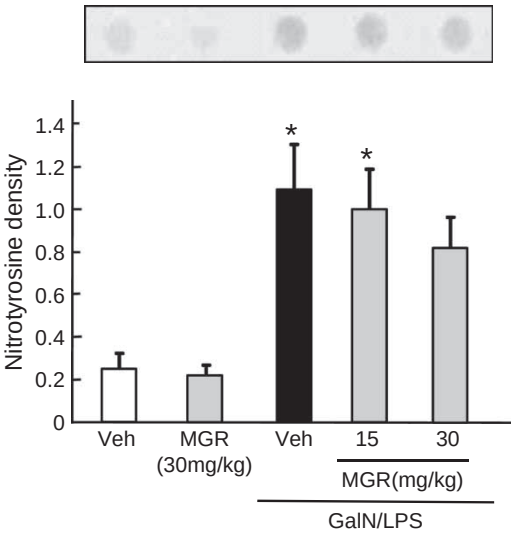

Figure 3. Inhibitory effect of oral administration of MGR on nitrotyrosine formation in the liver of rats treated with GalN/LPS. MGR was orally administered to rats at doses of 15 or 30 mg/kg body weight. After 2 h, galactosamine/lipopolysaccharide (GalN/LPS) was injected intraperitoneally into the rats. Nitrotyrosine formation in the liver was analysed after 24 h using dot blotting. Data presented as mean values ± standard error ($n = 6$–7). *Significant difference vs vehicle control ($p < 0.05$). Veh, vehicle (water).

nitration of tyrosine, forming 3-nitrotyrosine [15,16,23]. We, therefore, used dot blots analysis for measurements of nitrotyrosine levels in the rat livers. The administration of GalN/LPS stimulated nitrotyrosine density and this was reduced in a dose dependent manner by pre-treatment with MGR (Figure 3). These nitrotyrosine levels were strongly correlated with the amounts of ALT and AST in plasma, as shown in Figure 4.

Protective effects of quercetin metabolites against protein damage

We also evaluated the protective effects of quercetin metabolites against peroxynitrite-induce nitration of tyrosine using an *in vitro* test system using human serum albumin (HSA) as a model target. Peroxynitrite-induced nitration of tyrosine residues in HSA was examined by Western blots using anti-nitrotyrosine antibody (Figure 5). Both quercetin metabolites, Q3′S and Q3GA, like their parent aglycone, quercetin, reduced nitrotyrosine formation, with Q3GA being slightly more active than Q3′S. In contrast, the quercetin catabolites and phenolic acids, HPAA, DHPAA, HPPA, DHBA and HMPAA, were either without effect or, in the case of HPAA, DHBA and HMPAA, exhibited only weak activity at the higher 10 μM dose. Figure 6 shows typical HPLC profiles of HSA for control and groups treated peroxynitrite and inhibitors. Reduced form of HSA was eluted faster than oxidized form (Figure 6A), which agreed with the results reported by Hayashi et al. [20]. The incubation of HSA with peroxynitrite reduced dramatically the reduced form of HSA (Figure 6B). On the other hand, coexistence of Q3′S protected the reduction of reduced form of HSA (Figure 6C), but DHPAA was not prevented (Figure 6D). We calculated the effects of every quercetin metabolites as redox state of HSA (%) and these are summarized in Figure 7. While redox state of HSA was decreased substantially when treated with peroxynitrite, this was in part counteracted by quercetin, Q3′S and Q3GA, but not the phenolic acid catabolites.

Discussion

Administration of GalN and LPS to rats induces acute hepatic injury that closely resembles human viral hepatitis in its morphological and functional features. This procedure is, therefore, used as an experimental hepatic injury model for evaluating the efficacy of hepatoprotective agents [21,24,25]. In the current investigation, in keeping with previous reports [26,27], plasma ALT and AST levels in rats were elevated significantly compared with control animals 24 h after i.p. injection of GalN/LPS. Pre-treatment with MGR reduced the effect of

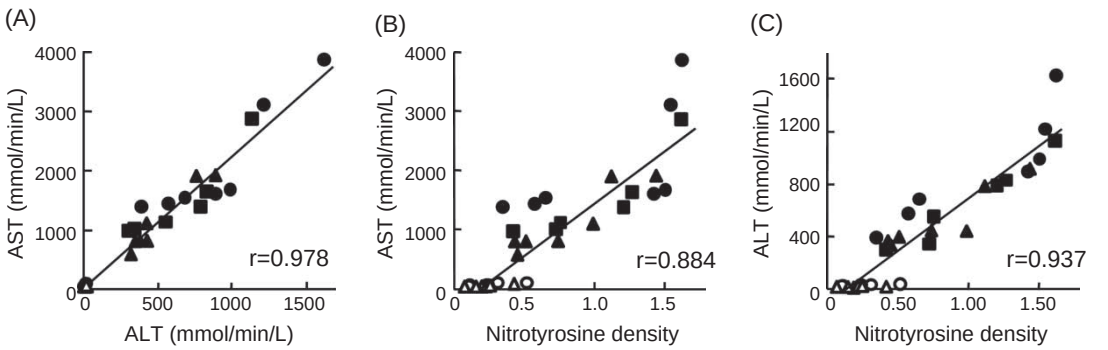

Figure 4. Correlation between nitrotyrosine formation in the liver and plasma levels of ALT or AST. Correlation factors were calculated using the amount of nitrotyrosine in the liver and ALT or AST levels in the plasma of GalN/LPS treated rats. Each symbol indicates: ○, vehicle control; Δ, MGR 30 mg/kg; ●, GalN/LPS; ■, GalN/LPS + MGR at 10 mg/kg; ▲, GalN/LPS + MGR at 30 mg/kg.

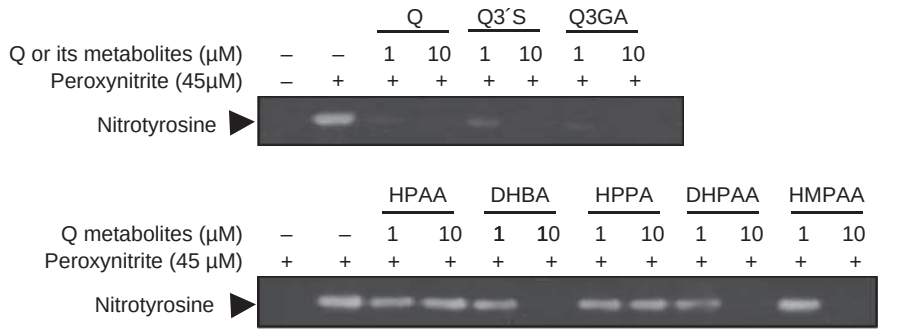

Figure 5. Effect of quercetin and its metabolites on peroxynitrite-induced nitrotyrosine formation on human serum albumin (HSA). The reaction mixtures comprising HSA (0.5 mg/mL) and quercetin or its metabolites (1 or 10 μM) in PBS containing 100 μM DTPA were incubated with peroxynitrite (45 μM) at 37°C for 30 min. Tyrosine nitration was measured by Western blotting. Q, quercetin; Q3′S, quercetin-3′-sulphate; Q3GA, quercetin-3-glucuronide; HPAA; 3-hydroxyphenylacetic acid; DHBA, 3,4-dihydroxybenzoic acid; HPPA, 3(3-hydroxyphenyl)propionic acid; DHPAA, 3,4-dihydroxyphenyl acetic acid; MHPAA, 3-methoxy-4-hydroxyphenylacetic acid.

GalN/LPS on ALT and AST (Figure 2), indicating that consumption of the quercetin glycoside provides protection against the induced acute hepatic injury.

GalN/LPS-mediated hepatic injury is thought to be caused primarily via oxidative stress [28,29], with excess production of NO leading to elevated peroxynitrite which, in turn, results in nitration of tyrosine and the formation of 3-nitrotyrosine [15,16]. In keeping with this proposal, we found that the livers of GalN/LPS-treated rats contained elevated levels of nitrotyrosine (Figure 3), which correlated with the amounts of ALT or AST in plasma shown in Figure 4. These findings confirm that GalN/LPS-induced hepatic injury is related to peroxynitrite-mediated nitrotyrosine production.

MGR, which is the main component in αG-rutin, an approved safety food additive in Japan, inhibits lipid peroxidation induced by Fe-NTA/H_2O_2 and Fe^{2+}/H_2O_2 [30]. In the present study, it was shown that the high nitrotyrosine content of the liver of GalN/LPS-treated rats is partially counteracted by ingestion of MGR (Figure 3), indicating the quercetin glycoside can also provide some degree of protection against hepatic damage.

MGR is rapidly absorbed [31] and, as discussed in the Introduction, it will appear in the circulatory system as phenolic acids and metabolites such as Q3′S and Q3GA. It is, therefore, important to evaluate the ability of these compounds to prevent the oxidative injury. Peroxynitrite, formed in biological systems from the reaction of NO with superoxide anion, is a highly reactive molecule that can lead to cell injury or cell death [32]. Interaction of proteins such as HSA with peroxynitrite results in nitration of tyrosine and the synthesis of nitrotyrosine [23]. In the current study, elevated nitrotyrosine levels were obtained when HSA was incubated with peroxynitrite *in vitro* (Figure 5), in keeping with the data of Ippoushi et al. [19]. Physiological concentrations of Q3GA and quercetin (1 and 10 μM) inhibited the synthesis of nitrotyrosine. Q3′S was also inhibitory but to a lesser degree than Q3GA. The phenolic acid catabolites, HPAA, DHBA, HPPA, DHPAA and HMPAA, exhibited only low activity or were without effect. We also evaluated the effects of quercetin metabolites using the other *in vitro* method, peroxynitrite-induced oxidation of HSA (Figures 6 and 7). Peroxynitrite-induced oxidation of HSA was also partially inhibited by Q3′S and Q3GA. Sadeghipour et al. [11] evaluated the inhibitory ability of flavonoid aglycones against peroxynitrite-induced nitration of tyrosine and found that strong inhibition was

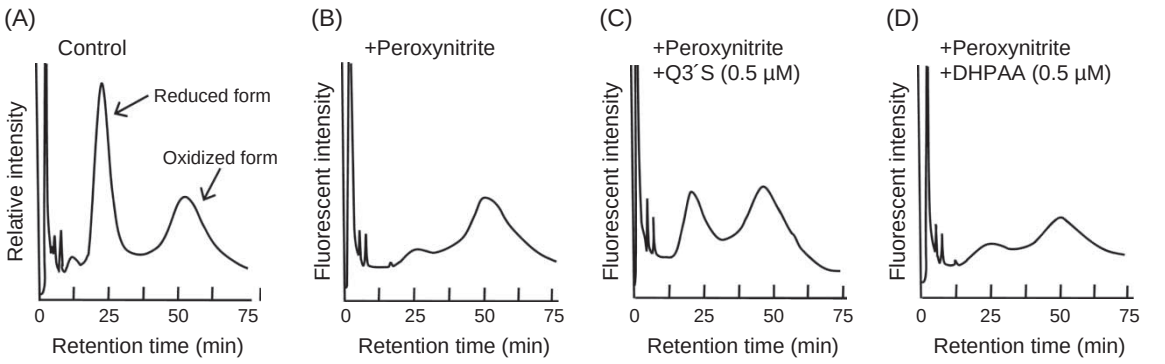

Figure 6. Typical HPLC profiles of HSA for control and groups treated with peroxynitrite and inhibitors. Q3′S, quercetin-3′-sulphate; Q3GA, DHPAA, 3,4-dihydroxyphenyl acetic acid. The detail information was described in the Materials and methods section.

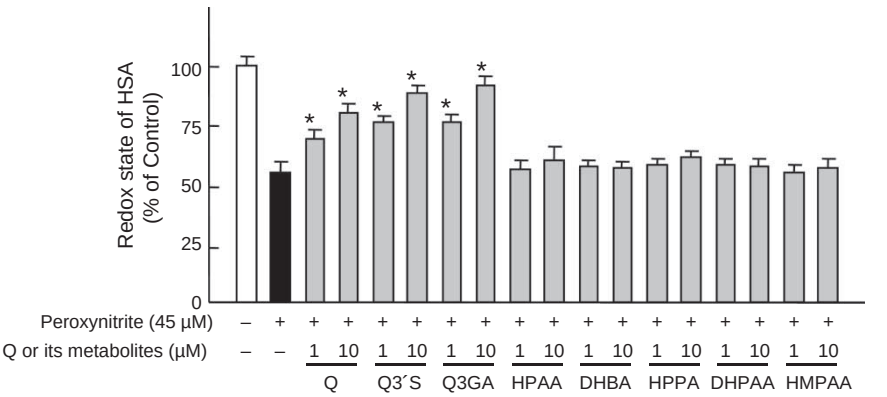

Figure 7. Effects of quercetin and its metabolites on the redox state of HSA. The reaction mixtures, comprising HSA (0.5 mg/mL) and quercetin or its metabolites (1 or 10 μM) in PBS containing 100 μM DTPA, were incubated with peroxynitrite (45 μM) at 37°C for 30 min. The redox state of HSA in the reaction solution was determined using HPLC with fluorescence detection (excitation, 280 nm; emission, 340 nm). Data expressed as mean values ± SE ($n = 6$–7). * Significant difference vs the group with peroxynitrite ($p < 0.05$). Q, quercetin; Q3′S, quercetin-3′-sulphate; Q3GA, quercetin-3-glucuronide; HPAA; 3-hydroxyphenylacetic acid; DHBA, 3,4-dihydroxybenzoic acid; HPPA, 3(3-hydroxyphenyl)propionic acid; DHPAA, 3,4-dihydroxyphenyl acetic acid; MHPAA, 3-methoxy-4-hydroxyphenylacetic acid. HMPAA, 4-hydroxy-3-methoxyphenyl acetic acid.

obtained with flavonoids such as quercetin and catechin with an *ortho*-dihydroxyl structure (3′,4′-OH substitution on ring B), whereas kaempferol and naringenin, which have a single hydroxyl moiety at the 4′, were 50% less effective. Therefore, the strong inhibitory effects of Q3GA on nitrotyrosine formation compared to the impact of Q3′S in our study (Figure 5) agree with this because Q3GA has a 3′,4′-OH substitution whereas Q3′S does not. A similar differential inhibition of lipid peroxidation [33] and oxidation of LDL [34] has also been reported. In the current study, however, there was no significant difference between the inhibitory effects of Q3′S and Q3GA against the reaction of peroxynitrite with HSA (Figure 7), which leads to nitration of tyrosine. Peroxynitrite reacts preferentially with the HSA tyrosine residues at positions 138 and 411 [32] located in sub-domains IB and IIIA, respectively, both of which are known as binding regions for more hydrophobic ligands such as small organic molecules [35], for example polycyclic aromatic hydrocarbon epoxides, with a molecular weight are ~300. Q3′S, which has a molecular weight of *m/z* 381 $(M+H)^+$, is reported to bind to HSA with a higher efficacy than Q3GA, which has molecular weight of *m/z* 477 $(M+H)^+$ [34]. Janisch et al. [34] discussed the relationship between molecular weight and the ability of Q3′S and Q3GA to protect against cupper-induced LDL oxidation of HSA. It is, therefore, plausible that the inhibitory properties of Q3′S against peroxynitrite-induced tyrosine nitration is due to the prevention nitration of the tyrosine residues at positions 138 and 411 via binding to the sub-domains IB and IIIA in HSA. The other possibility is enzymatic deconjugation of not only Q3GA, but also Q3′S, releasing quercetin which, because of its antioxidant activity, is able to counteract LPS-induced inflammation [36]. Basically, the main structural features of flavonoids required for efficient radical scavenging could be summarized as follows: an *ortho*-dihydroxy structure in B ring, C_2–C_3-double bond and hydroxyl groups at positions 3 and 5 [37]. On the other hand, the influence on activity of flavonoids via hydroxyl group at position 3 and C_2–C_3-double bond do not contribute to the inhibiting peroxynitrite-induced nitration of tyrosine [11], indicating that the *ortho*-dihydroxy structure is prior to its inhibition. In this study, our results suggested that the phenolic acid catabolites, DHBA and DHPAA, have weak or no antioxidant efficacy, although they remain *ortho*-dihydroxyl substitutions. Therefore, we guess that the flavonoid structure itself will also have an important rule for inhibition of peroxynitrite-induced nitration of tyrosine on HSA. Further study will be necessary for elucidation of this point. Moreover, recent reports have suggested that the myeloperoxidase pathway, which uses nitrite and hydrogen peroxide or hypochlorous acid as substrates, has a role in tyrosine nitration in lipopolysaccharide-treated rats [38,39]. Interestingly, quercetin metabolites, such as Q3GA, have been reported to be able to act as myeloperoxidase inhibitors [40]. Hence, quercetin metabolites may exhibit antioxidant effects via inhibition of not only the peroxynitrite pathway but also the myeloperoxidase pathway.

In conclusion, consumption of MGR, quercetin trisaccharide conjugate, could prevent the peroxynitrite-induced oxidative hepatic injury. Moreover, physiological concentrations of Q3′S and Q3GA, which are the major quercetin metabolites in the circulatory system after the ingestion of a flavonol-rich meal, effectively prevented the peroxynitrite-induced nitrotyrosine formation and oxidation of HSA in an *in vitro* test system. These findings are in keeping with the quercetin metabolites acting as bioactive components *in vivo*.

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