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Properties of Quercetin Conjugates: Modulation of LDL Oxidation and Binding to Human Serum Albumin

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Quercetin is an important dietary flavonoid with *in vitro* antioxidant activity. However, it is found in human plasma as conjugates with glucuronic acid, sulfate or methyl groups, with no significant amounts of free quercetin present. The antioxidant properties of the conjugates found *in vivo* and their binding to serum albumin are unknown, but essential for understanding possible actions of quercetin *in vivo*. We, therefore, tested the most abundant human plasma quercetin conjugates, quercetin-3-glucuronide, quercetin-3′-sulfate and isorhamnetin-3-glucuronide, for their ability to inhibit Cu(II)-induced oxidation of human low density lipoprotein and to bind to human albumin, in comparison to free flavonoids and other quercetin conjugates. LDL oxidation lag time was increased by up to four times by low ($<2\,\mu M$) concentrations of quercetin-3-glucuronide, but was unaffected by equivalent concentrations of quercetin-3′-sulfate and isorhamnetin-3-glucuronide. In general, the compounds under study prolonged the lag time of copper-induced LDL oxidation in the order: quercetin-7-glucuronide > quercetin > quercetin-3-glucuronide = quercetin-3-glucoside > catechin > quercetin-4′-glucuronide > isorhamnetin-3-glucuronide > quercetin-3′-sulfate. Thus the proposed products of small intestine metabolism (quercetin-7-glucuronide, quercetin-3-glucuronide) are more efficient antioxidants than subsequent liver metabolites (isorhamnetin-3-glucuronide, quercetin-3′-sulfate). Albumin-bound conjugates retained their property of protecting LDL from oxidation, although the order of efficacy was altered (quercetin-3′-sulfate > quercetin-7-glucuronide > quercetin-3-glucuronide > quercetin-4′-glucuronide = isorahmnetin-3-glucuronide). K_q values (concentration required to achieve 50% quenching) for albumin binding, as assessed by fluorescence quenching of Trp214, were as follows: quercetin-3′-sulfate ($\sim 4\,\mu M$) = quercetin ≥ quercetin-7-glucuronide > quercetin-3-glucuronide = quercetin-3-glucoside > isorhamnetin-3-glucuronide > quercetin-4′-glucuronide ($\sim 20\,\mu M$). The data show that flavonoid intestinal and hepatic metabolism have profound effects on ability to inhibit LDL oxidation and a lesser but significant effect on binding to serum albumin.

Keywords: Quercetin; LDL oxidation; HSA binding; Antioxidant; Flavonoids

INTRODUCTION

Flavonoids are claimed to have anti-atherosclerotic properties, at least in part, by protecting LDL from oxidation *in vitro*.[1–6] These studies used either aglycones or glucosides with human LDL or a mixture of uncharacterised glucuronides (enzymically synthesised from rat liver and intestinal microsomes) tested on rat LDL. However, several studies have now demonstrated that there was no free quercetin detectable in human plasma after consumption of a quercetin rich meal.[7–9] Due to the metabolic activity of human small intestine and liver, it is also unlikely that glucosides are present in human plasma.[9–11] The metabolites present in human plasma 1.5 h after a meal of onions were quercetin-3-glucuronide, isorhamnetin-3-glucuronide and quercetin-3′-sulfate.[11]

Modified LDL plays a major role in atherogenesis, the main cause of heart disease and stroke. The unregulated uptake of modified LDL by macrophages converts them into foam cells, which are the basis for atherosclerotic plaques.[12–14]

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Modification of LDL *in vivo* can be induced in several ways, for example self-aggregation,[14] immune complex formation[14] or oxidation by cells.[12–15] LDL oxidised with Cu(II) ions exhibits the same properties as cell-modified LDL.[15] An extension of the lag time (time until rapid extinction increase occurs) due to the investigated substances correlates with their higher protective capacity.[5,12,16,17]

Serum albumin is the most abundant protein in human plasma. It contributes to the colloid osmotic pressure in the blood vessels, stabilises the concentration of free ligands in the blood and delivers molecules to tissues.[18,19] Serum albumin exhibits a broad specificity for binding,[18,19] the major regions for binding are located in subdomains IIA and IIIA, providing hydrophobic pockets.[19] Tryptophan 214 and Lysine 199 in subdomain IIA and Tyrosine 411 in subdomain IIIA contribute to the binding process,[19,20] which involves hydrophobic interactions.[18] Several studies used the quenching of tryptophan fluorescence of the single Trp 214 to examine the binding of a variety of molecules to the protein in this specific subdomain.[21–26] Quercetin aglycone binds to albumin,[27,28] but it is unknown if the conjugates of quercetin actually present in plasma can either bind to albumin or inhibit LDL oxidation; the aim of this paper was to test this.

MATERIALS AND METHODS

Materials

Quercetin, catechin, Cibachrom Blue 3GA Type 3000-CL and human serum albumin (HSA) fatty acid-free (HSA1) were purchased from Sigma, UK. $Cu(SO_4)$ and Na_2EDTA were from BDH Chemicals Ltd, UK. Isoquercetin was from Apin Chemicals Ltd, UK. The Coomassie Blue reagent was purchased from Pierce Chemicals, UK. The concentration of HSA isolated from blood (HSA2) was determined by a standard protein assay from Pierce Chemicals and by the absorption spectrum: extinction of a 1 mg/ml solution was 0.55 at 280 nm for a molecular weight of 66439 Da.[18]

The glucuronides of quercetin and isorhamnetin, as well as the sulfate of quercetin, were chemically synthesized, stored as stock solutions in either 50% aqueous methanol or 100% methanol and diluted (0.1–3% methanol final concentration) before use.[29,30] The above concentrations of methanol exhibited no effect in the test systems employed (data not shown).

Methods

The results shown are means of at least three individual determinations ($n = 3$) and standard deviations are given as σ_{n-1}. Calculations were performed using Microsoft Excel and GraFit.

Plasma Preparation

The plasma used in these experiments was obtained from the blood of 20 healthy volunteers, aged 25–45 years. The blood was collected in heparinized tubes and centrifuged at 400*g* for 15 min at 10°C immediately after collection. The pooled plasma was stored at −20°C until required.

LDL Preparation

The preparation of LDL was performed by isopycnic ultra centrifugation. Plasma (5 ml), adjusted to a density of 0.3265 g/ml with KBr, was stratified in Beckman Centrifuge tubes with approximately 7.5 ml density solution ($d = 1.006$ g/ml; 11.4 g NaCl, 0.1 g Na_2EDTA, 1 ml 1 M NaOH). After 3.5 h centrifugation at 377855*g* at 7°C, the LDL was removed, filtered (0.22 μm, Millex-GP, Millipore) and EDTA added (final concentration 1 mg/ml) prior to storage at 4°C. Prior to use, the LDL was desalted with an Econo-Paq DG-10 (BioRad) column and the protein content determined using the Coomassie Blue assay.[31]

Copper-induced LDL Oxidation

The assay for continuous monitoring of the copper-induced LDL oxidation is based on a previous method.[32] Samples were added to a phosphate buffered saline (PBS) buffer containing desalted LDL and the reaction was started by the addition of an aqueous $Cu(SO_4)$ solution. The final concentrations for a 1 ml assay were: 0.02 M PBS buffer, pH 7.2 containing 0.15 M NaCl, 1.67 μM $Cu(SO_4)$, 25 μg LDL protein and 0–2 μM quercetin conjugate. The change in the extinction was measured at 234 nm every 10 min for 1000 min at 37°C. The final concentrations were 0.5, 1 and 2 μM for all test substances and each was performed in triplicate.

HSA Isolation

HSA2 for binding studies was purified from human plasma by affinity chromatography. The columns used ($l = 30$ cm, $\varnothing = 1.5$ cm, 10 ml bed volume) contained Cibacron Blue 3GA matrix. Columns were washed and equilibrated with water prior to the application of the plasma (diluted 1:10). After the plasma entered the bed the column was washed with 4–5 bed volumes to remove unbound proteins. Bound HSA was eluted from the column with 0.01 M Tris–HCl buffer, pH 7.5 containing 1.5 M NaCl, and the column regenerated with 0.1 M borate, pH 9.8.[33,34] The resulting HSA was desalted with

Econo-Paq DG-10 (BioRad) columns, concentrated to a stock solution (467 μM) and frozen at −20°C until required.

HSA Binding Study

The fluorescence assay employed was modified from previous methods.[24,26] Samples were diluted in 5 mM sodium phosphate buffer, pH 7.4 and kept in a water bath at 30°C. After the addition of HSA, the mixture was vortexed for 15 s and fluorescence measured immediately. The final concentrations (1 ml assay) were: phosphate buffer 5 mM pH 7.4, 5 μM HSA, 0-50 μM quercetin conjugate. The settings for the fluorescence spectrometer were: excitation 298 nm, emission 290–500 nm, slits width 2.5/5 nm, scan rate 200 nm/min. The quenching can be described by the following equation for single site binding:

$$100(1 - F[C]/F_{\max}) = \frac{[C]Q_{\text{cap}}}{K_q + [C]}$$

where [*C*] is the concentration of the compound used for quenching, Q_{cap} is the maximum quenching capacity for the binding, K_q is the concentration of *C* to reach (1/2) quenching capacity and *F* is the fluorescence.[26,35]

RESULTS

Copper-induced LDL Oxidation

Quercetin-7-glucuronide extended the lag time of LDL oxidation significantly (Fig. 1). The lag time increased in a dose dependent manner, delaying the start of the propagation phase for 110 min even at the lowest concentration of 0.5 μM. Other compounds were assessed in the same way (Fig. 2 and Table I), where quercetin also exhibited inhibition of LDL oxidation as expected.

LDL Oxidation with HSA and Combinations of HSA and Quercetin Metabolites

HSA was added to the copper-induced LDL oxidation at concentrations of 2.5, 5 and 10 μM. Additions of 5 and 10 μM HSA resulted in starting values of the extinction above 1.0 (5 μM: between 0.9 and 1.2; 10 μM: between 1.5 and 1.7) with no determinable lag phase over the measurement period (data not shown). The combination of HSA and quercetin conjugates was, therefore, conducted with concentrations of 2.5 μM HSA and 2 μM of each conjugate. Full binding can be presumed at these concentrations.

The addition of 2.5 μM HSA resulted in a different LDL oxidation curve than usually observed. Over the first 70 min, a short propagation phase with an increase of the extinction of approximately 0.15 points occurred followed by a typical lag phase. Subsequently the onset of a 2nd propagation phase at 337.6 ± 39.6 min was observed (Fig. 3). Two of the five albumin-bound quercetin conjugates gave normal LDL oxidation curves (quercetin-3-glucuronide and quercetin-7-glucuronide). The remaining three conjugates (quercetin-3′-sulfate, isorhamnetin-3-glucuronide and quercetin-4′-glucuronide) exhibited the same curve progression as LDL oxidation with HSA alone.

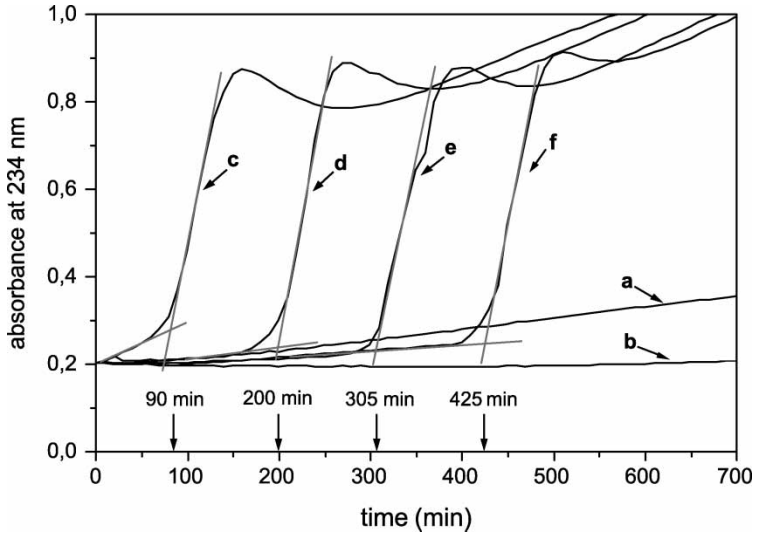

FIGURE 1 Effect of quercetin-7-glucuronide on copper-induced LDL oxidation. Key: **a**—without copper; **b**—without copper/+2 μM quercetin 7-glucuronide; **c**—control; **d**—0.5 μM quercetin-7-glucuronide; **e**—1 μM quercetin-7-glucuronide; **f**—2 μM quercetin-7-glucuronide.

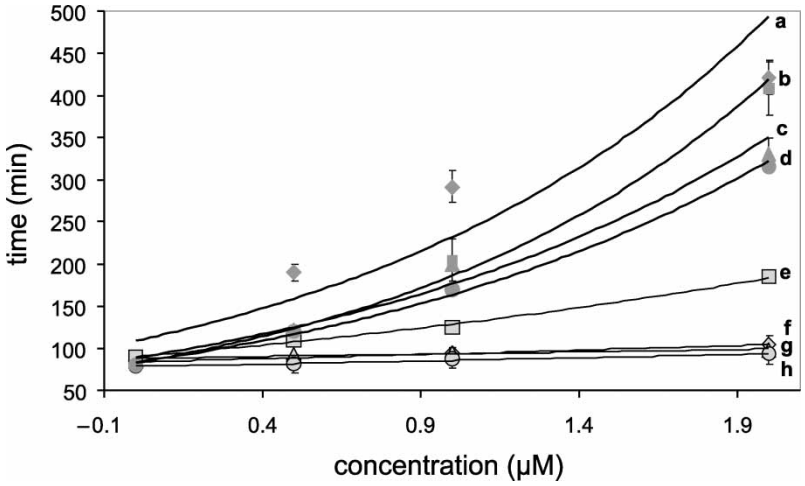

FIGURE 2 Effect of quercetin conjugates and other flavonoids on LDL oxidation. The results shown are means and standard deviations of at least three individual determinations. Calculations were performed using Microsoft Excel and GraFit. Key: ◆ and **a**—quercetin-7-glucuronide; ■ and **b**—quercetin; ▲ and **c**—quercetin-3-glucuronide; ● and **d**—isoquercitrin; □ and **e**—catechin; ◇ and **f**—quercetin-4′-glucuronide; △ and **g**—isorhamnetin-3-glucuronide; ○ and **h**—quercetin-3′-sulfate.

Lag times for LDL oxidation in the presence of HSA and the individual conjugates (albumin bound and free) are shown in Fig. 4. Only quercetin-3′-sulfate demonstrated an elongation of the lag period over and above that of the HSA control. The quercetin-3′-sulfate and HSA combination resulted in a co-operative effect for the lag time prolongation, increasing prolongation for about 115.1 min which corresponds to an extension of about 25% (lag times: quercetin-3′-sulfate = 93.0 min, quercetin-3′-sulfate/HSA = 452.7 min, HSA = 337.6 min; Fig. 4).

The binding of quercetin-3-glucuronide and quercetin-7-glucuronide to HSA resulted in a decrease of the lag time compared to the corresponding free conjugates. Lag times were reduced by 60.5 and 33.6% for quercetin-3-glucuronide and quercetin-7-glucuronide, respectively (lag times: quercetin-3-glucuronide = 332.0 min, quercetin-3-glucuronide/HSA = 131.1 min; quercetin-7-glucuronide = 421.7 min, quercetin-7-glucuronide/HSA = 279.9 min; Fig. 4).

The combination of HSA with quercetin-4′-glucuronide and isorhamnetin-3-glucuronide resulted in an increase in the lag time compared to the free conjugates (lag times: quercetin-4′-glucuronide = 104.8 min, quercetin-4′-glucuronide/HSA = 258.6 min; isorhamnetin-3-glucuronide = 98.3 min, isorhamnetin-3-glucuronide/HSA = 330.9 min; Fig. 4), for quercetin-4′-glucuronide the lag time extension is 147% and for isorhamnetin-3-glucuronide it is 237%. However lag times of these combinations were still significantly shorter than the HSA control.

TABLE I Lag times for quercetin conjugates and flavonoids for copper-induced LDL oxidation

Compound	Concentration (μM)	Lag time for LDL oxidation (min)
Quercetin-7-glucuronide	0.0	85.0 ± 5.0
	0.5	190.0 ± 10.0
	1.0	291.7 ± 18.9
	2.0	421.7 ± 20.2
Quercetin-3-glucuronide	0.0	85.0 ± 5.0
	0.5	121.7 ± 2.9
	1.0	200.0 ± 8.7
	2.0	331.7 ± 17.6
Quercetin-4′-glucuronide	0.0	89.2 ± 1.4
	0.5	90.8 ± 1.4
	1.0	98.3 ± 2.9
	2.0	111.7 ± 5.8
Isorhamnetin-3-glucuronide	0.0	86.7 ± 5.8
	0.5	92.5 ± 10.9
	1.0	94.3 ± 10.4
	2.0	98.3 ± 11.6
Quercetin	0.0	83.8 ± 4.8
	0.5	113.8 ± 7.5
	1.0	205.0 ± 22.7
	2.0	408.3 ± 31.8
Catechin	0.0	90.1 ± 5.2
	0.5	110.2 ± 6.9
	1.0	125.4 ± 7.1
	2.0	185.2 ± 11.1
Isoquercetin	0.0	80.2 ± 4.8
	0.5	121.2 ± 6.5
	1.0	171.6 ± 6.5
	2.0	315.6 ± 15.5

Binding to Human Serum Albumin

We assessed binding to both isolated HSA and commercial fatty acid-free HSA. Both HSA preparations exhibited an emission maximum at 348 nm, only differing in the relative fluorescence intensity. In all experiments, the pure HSA2 preparation demonstrated slightly higher relative fluorescence intensity, with a value about 10% higher than pure HSA1. All substances decreased the fluorescence of the two HSA preparations in a dose-dependent manner without interference of the HSA

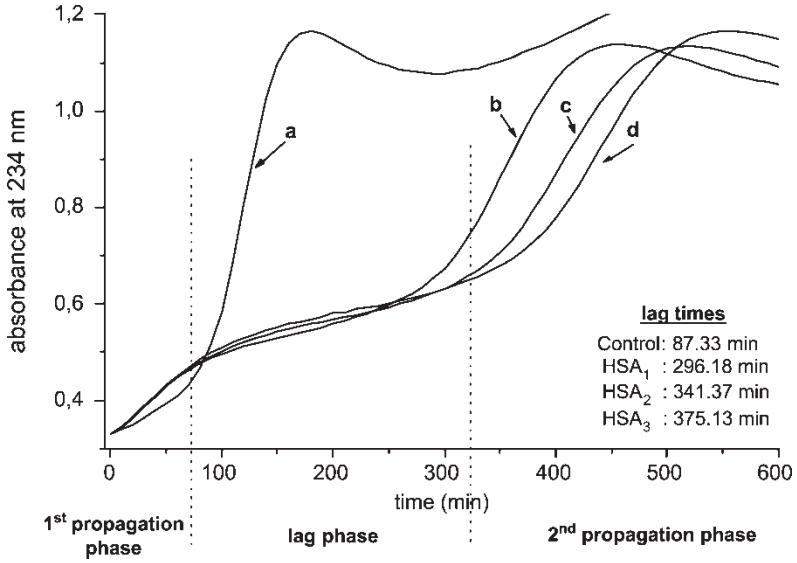

FIGURE 3 Effect of HSA on copper-induced LDL oxidation. Shown are three independent determinations of 2.5 μM HSA with the determined lag times. Key: **a** control; **b**, **c** and **d** replicate analysis of 2.5 μM HSA.

fluorescence maximum (data not shown). The concentration range for the compounds was 1–50 μM, corresponding to molar ratios from 1:0.2 to 1:10 HSA:test compound. A shift to shorter wavelengths of up to 10 nm could be observed with all compounds used (data not shown), indicative of binding. Representative emission spectra for fluorescence quenching with quercetin-7-glucuronide are shown in Fig. 5. There was very little significant difference between the two HSA preparations. In addition, binding with catechin was not reproducible, possibly due to catechin oxidation in the presence of HSA. The quenching of HSA by flavonoids and conjugates is shown in Fig. 6 and Table II. The binding to HSA is dependent on the nature of the conjugate, ranging from quercetin-3′-sulfate and quercetin (~4 μM) to quercetin-4′-glucuronide (~20 μM).

DISCUSSION

Copper-induced LDL Oxidation

Oxidized LDL is involved in the process of atherogenesis leading to heart disease and stroke, and, therefore, it is important to minimise LDL oxidation.[12] It is known that flavonoids inhibit lipid peroxidation

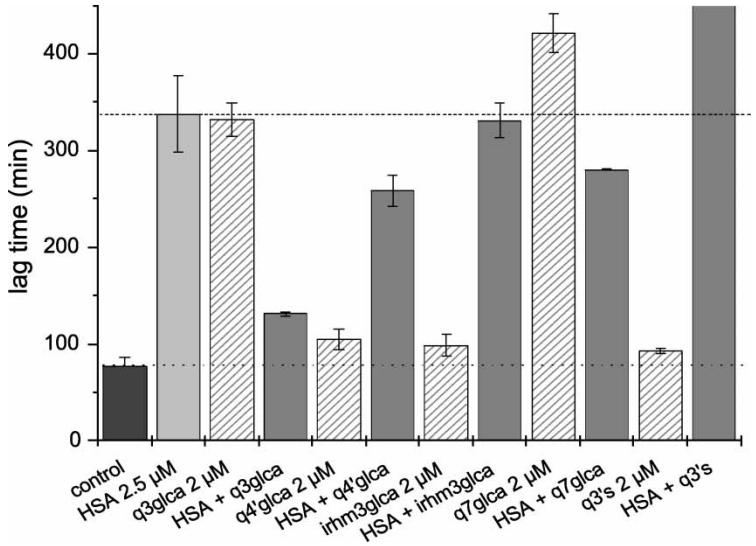

FIGURE 4 Effect of free and albumin-bound quercetin conjugates on copper-induced LDL oxidation. Concentration of HSA used was 5 μM, and quercetin conjugates 2 μM. Values are the mean and standard deviation of three determinations. Key: q3glca—quercetin-3-glucuronide; q4′glca—quercetin-4′-glucuronide; irhm3glca—isorhamnetin-3-glucuronide; q7glca—quercetin-7-glucuronide; q3′glca—quercetin-3′-glucuronide.

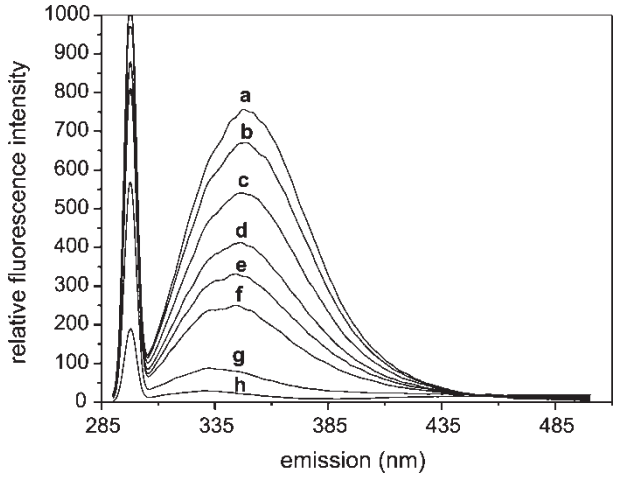

FIGURE 5 Effect of quercetin-7-glucuronide on fluorescence quenching of HSA2. Key: concentrations of quercetin-7-glucuronide: **a**—control; **b**—1 μM; **c**—2.5 μM; **d**—5 μM; **e**—7.5 μM; **f**—10 μM; **g**—25 μM; **h**—50 μM.

and LDL oxidation, and there are studies showing a protective effect for quercetin.[1–3,5] Recently, we and others demonstrated that there was no free quercetin in plasma;[7–9] we positively identified the following conjugates in plasma: quercetin-3-glucuronide, quercetin-3′-sulfate and isorhamnetin-3-glucuronide.[11] We, therefore, tested the real forms of quercetin in the plasma for binding to albumin and inhibition of LDL oxidation. Generally, quercetin conjugates prolonged the lag time of the copper-induced LDL oxidation in the order (quercetin-7-glucuronide > quercetin > quercetin-3-glucuronide = quercetin-3-glucoside > catechin > quercetin-4′-glucuronide > isorhamnetin-3-glucuronide > quercetin-3′-sulfate).

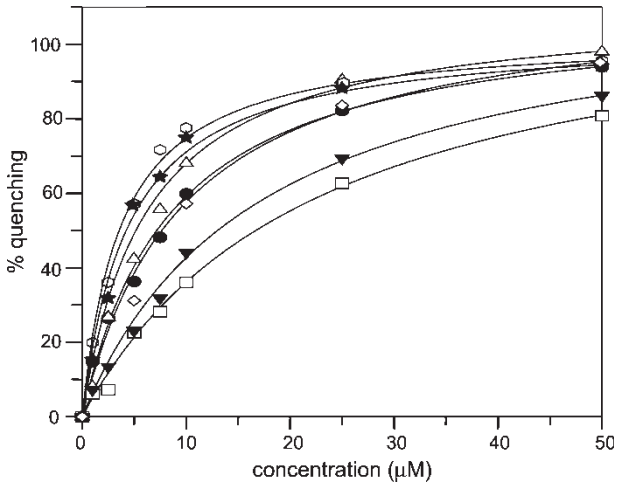

FIGURE 6 Effect of quercetin conjugates and other flavonoids on the quenching of HSA2. The results are the mean and standard deviations of at least three determinations. Calculations were performed using Microsoft Excel and GraFit. The standard deviations were smaller than the symbols shown. Key: △—quercetin-7-glucuronide; •—quercetin-3-glucuronide; □—quercetin-4′-glucuronide; ▼—isorhamnetin-3-glucuronide; ○—quercetin-3′-sulfate; ★—quercetin; ◇—isoquercitrin.

TABLE II K_q-values for quercetin conjugates for quenching HSA binding

Compound	HSA1	HSA2
Quercetin-7-glucuronide	5.26 ± 0.24[a]	6.24 ± 0.51[f]
Quercetin-3-glucuronide	8.54 ± 0.31[b]	8.64 ± 0.33[g]
Quercetin-4′-glucuronide	20.05 ± 1.08[c]	22.62 ± 1.49[h]
Isorhamnetin-3-glucuronide	12.92 ± 0.45[d,*]	17.21 ± 1.01[i,*]
Quercetin-3′-sulfate	4.28 ± 0.65[a]	3.76 ± 0.30[j]
Quercetin	5.73 ± 0.46[a,*]	4.49 ± 0.16[j,*]
Isoquercetin	10.24 ± 0.96[b]	9.91 ± 0.88[g]

[a–j]Different letters indicate significant difference, $P < 0.025$, (paired *t*-test).
*Indicates significant difference between the two HSA preparations, $P < 0.05$, (paired t-test).

Part of this is consistent with the work of Yamamoto *et al.*,[4] who used various quercetin glucosides in an equivalent study.

The protective capacities of flavonoids against LDL oxidation is derived either from (a) binding to the copper receptor of the Apo-B protein of the LDL particle, (b) regeneration of the endogenous antioxidants of the LDL particle,[5] (c) scavenging lipid peroxyl radicals[1,36] or (d) chelating copper(II)ions.[2,4,37] Quercetin has three possible metal-binding sites with a range of binding affinities. The B ring (catechol moiety) is the binding site with the highest affinity compared to the sites between the 3-OH and 4-oxo group or the 4-oxo and 5-OH group.[38] The B ring also exhibits maximal radical-scavenging potential.[1,36,37,39]

The poorest activity against LDL oxidation are thus quercetin conjugates substituted in the B ring (quercetin-3′-sulfate, isorhamnetin-3-glucuronide and quercetin-4′-glucuronide). As a flavan-3-ol, catechin possesses a good radical-scavenging property, which is due to the catechol group in the B ring and the saturated 2,3-bond.[36] Isoquercetin and quercetin-3-glucuronide, possessing a substitution at the 3-position, have similar activities, both having two metal-binding sites between the 3′- and 4′-position and the 4-oxo and 5-OH group[38] and the same predicted radical-scavenging abilities: the catechol group in the B ring, the 2,3-double bond with the linked 4-oxo group and the free 5-OH group.[36] The slightly better protection of quercetin-3-glucuronide could conceivably be due to the glucuronic acid providing another oxo- and hydroxyl-group, which might increase the metal-binding properties of position 4 and 5. The protective ability of quercetin in this study is consistent with the results of Ref. [2]. Quercetin can bind metal cations at three possible sites[38] and possesses the ideal structure for maximum radical scavenging.[1,36,39] The best protective capacity in our study was exhibited by quercetin-7-glucuronide. According to predictions by Yamamoto *et al.*,[4] quercetin-7-glucuronide should prolong the lag time to the same extent as quercetin, possessing the same

possible three metal-binding sites and radical-scavenging features. The observed result could be explained by the substitution of glucuronic acid providing another possible binding site between the carboxyl group of the glucuronic acid and the 5-hydroxyl group of the A ring.

LDL Oxidation with HSA and Combinations of HSA and Quercetin Metabolites

The complete inhibition of LDL oxidation with HSA concentrations of 5 and 10 μM are consistent with the results of Schnitzer *et al.*[40] A total inhibition of the LDL oxidation was reported to be due to the binding of copper to the albumin. Copper-induced LDL oxidation with HSA at 2.5 μM exhibits the same properties as low Cu(II)/LDL ratio conditions.[40,41] As HSA possesses a binding site for copper, it is clear that its addition to the assay results in binding of copper (this is one aspect of the protective properties of HSA[18,19,40,42]). The copper-binding properties are influenced by the binding of quercetin conjugates in addition to the individual protective abilities of the two metabolites quercetin-3-glucuronide and quercetin-7-glucuronide. As the copper/LDL ratio is not changed with a combination of these two conjugates and HSA, it can be assumed that the binding of these two metabolites results in an alteration of the copper-binding ability of HSA which, in turn, leads to a higher copper concentration in the assay. This assumption is consistent with the estimated K_q-values of the two conjugates which indicate good binding to HSA. The results of quercetin-4′-glucuronide, isorhamnetin-3-glcuronide and quercetin-3′-sulfate suggest that, not only the metabolite, but also the copper, is bound to HSA. The lag phase of isorhamnetin-3-glucuronide can be solely contributed to the copper-binding of HSA, the conjugate not contributing to this protective effect. The combination of quercetin-4′-glucuronide and HSA does still eliminate copper from the assay but clearly not in the same range as with isorhamnetin-3-glucuronide and quercetin-3′-sulfate. A higher Cu(II)-concentration in the assay shortens the lag phase of this combination compared to HSA alone. As glucuronic acid is sterically larger than a methyl or sulphate group, the whole quercetin-4′-glucuronide structure is sterically larger than isorhamnetin-3-glucuronide and quercetin-3′-sulfate, and can, therefore, obstruct the copper binding site of HSA. Quercetin-3′-sulfate bound to HSA results in a lag phase prolongation which is above the lag phase of HSA alone. Such cooperative effects between proteins and flavonoids have been observed in this test system previously.[43] According to the K_q-value of quercetin-3′-sulfate it might be expected that the binding of this conjugate would result in a change in the copper-binding properties of HSA as already described for quercetin-3-glucuronide and quercetin-7-glucuronide. Quercetin-3′-sulfate clearly does not interfere with the copper-binding of HSA.

HSA-binding Study

HSA contributes both to the colloid osmotic pressure and the binding of a wide variety of biological compounds.[18,19,26] A common assay for the investigation of binding abilities is the fluorescence quenching of the single tryptophan in the hydrophobic pocket of subdomain IIA.[24,26,28] In all our analyses, defatted HSA was also used since the binding can be influenced by bound fatty acids.[20,44] However, long chain fatty acids bind to subdomain IIIA,[20] whereas subdomain IIA has one crevice that binds small organic compounds and another crevice for binding to medium chain fatty acids situated adjacent. The high affinity binding site corresponding to the subdomain IIIA,[22,24] usually occupied by long chain fatty acids in native HSA, is not a major binding site for quercetin or small molecules in humans.[27] We found no significant difference between isolated native HSA and the commercial fatty acid free HSA.

As quercetin is the most abundant flavonol in human diet, the aglycone has been thoroughly investigated for its antioxidant properties. However, it is conjugated after uptake, and so we have examined the plasma metabolites. All the metabolites tested herein bound to HSA with values that predict there are no free metabolites circulating in the plasma. This result is consistent with the study of Boulton *et al.*,[27] who demonstrated the binding of quercetin to HSA was about 99.4%. Albumin-bound conjugates retained their ability to protect LDL from oxidation, so it can be concluded that the binding of the flavonoid metabolites to HSA occurring in human plasma does not result in the loss of the protective properties of the metabolites. As HSA is mainly a transport protein, it can surely deliver the metabolites to the site of atherogenesis. If an LDL/HSA associated complex exists *in vivo* as postulated by Carbonneau *et al.*,[45] then the binding of the quercetin metabolites provides a protective effect for the LDL particle in atherogenesis.

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