JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Covalent Binding of the Flavonoid Quercetin to Human Serum Albumin

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Quercetin is an abundant flavonoid in the human diet with numerous biological activities, which may contribute to the prevention of human disease but also may be potentially harmful. Quercetin is oxidized in cells to products capable of covalently binding to cellular proteins, a process that may be important for its biological activities. In the present study, using radiolabeled drug and quantifying the products after electrophoretic separation, proteins to which oxidized quercetin is binding irreversibly were identified. The binding of quercetin to human serum albumin (HSA) in human blood and the effect of stimulation of neutrophilic myeloperoxidase on this binding were also measured. The in vitro binding of quercetin to eight proteins in the presence of catalytic amounts of horseradish peroxidase and hydrogen peroxide was highly selective for HSA. For all proteins the binding was dramatically decreased by reduced L-glutathione. In the blood samples, the release of neutrophilic myeloperoxidase by phorbol ester caused a 3-fold increase in the binding of quercetin to HSA. This study shows that quercetin in the presence of peroxidase/hydrogen peroxide covalently links to proteins with a particularly high affinity for HSA and that this also may occur in vivo after exposure to quercetin. This provides further insights into the complex behavior of this major dietary flavonoid.

KEYWORDS: Quercetin; covalent binding; albumin, human serum; flavonoids; plasma proteins; peroxidation

INTRODUCTION

Quercetin is one of the most abundant flavonoids in the human diet and has become associated with a myriad of biological activities, many of which may contribute to the prevention of human disease. The mechanisms by which quercetin produces these effects are poorly understood, although many signaling pathways have been indicated (*1*, *2*). A major part of this problem is its very low bioavailability in vivo (*3*) as well as in cultured cells (*4*). As quercetin is widely sold as a food supplement, it is important to improve our understanding of the behavior and actions of this food component.

Quercetin is well-known to be a potent antioxidant (*5*, *6*), contributing to its health benefits. However, it has also been recognized as a prooxidant (*7*, *8*), potentially being harmful (*9*, *10*). Quercetin is oxidized under a variety of conditions (*11*−*15*), a fact that contributes greatly to our lack of understanding of how this major food component is affecting cellular functions.

A recent study demonstrated that quercetin is oxidized in cells, presumably by hydrogen peroxide/peroxidases, to products capable of covalent binding to DNA and, in particular, to cellular proteins (*16*). The latter was shown by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) of the cell lysate to involve only a limited number of proteins. We speculated that quercetin's ability to bind to protein might be important for some of its numerous biological activities. However, our approaches to the identification of these cellular proteins have so far failed, as they appear in the cell in very low amounts.

To identify the types of protein to which oxidized quercetin may bind, we have in the present study examined a group of proteins in a strictly in vitro approach. Human serum albumin (HSA) has previously been indicated to covalently bind quercetin in vivo (*16*). In this study, the covalent protein binding of quercetin was shown to be highly selective for HSA, both in vitro and in human blood.

MATERIALS AND METHODS

Materials. [4-^{14}C]Quercetin dihydrate (52.9 mCi/mmol) was purchased from NCI Radiochemical Repository at ChemSyn Laboratories. HSA (fraction V, high purity) was purchased from Calbiochem. All other proteins and other chemicals were purchased from Sigma-Aldrich.

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10.1021/jf050061m CCC: $30.25
Published on Web 04/09/2005

[^{14}C]Quercetin Binding to Proteins in Vitro. All incubation mixtures contained 25 μM [^{14}C]quercetin and 13.8 μM protein. Incubation mixtures also contained 150 μM hydrogen peroxide, 0.1 μM horseradish peroxidase, or both as indicated. A separate mixture contained 5 mM reduced L-glutathione (GSH) in addition to all components listed above. Incubations were carried out for 30 min or 2 h at 37 °C unless otherwise indicated.

The 25 μM quercetin used can easily be achieved in the intestinal lumen from dietary sources (*17*), although this concentration normally will not be seen in the systemic circulation. The 13.8 μM concentration of the proteins used is considerably lower than the normal HSA concentration in plasma, which is ~600 μM, but probably higher than those of several of the other proteins examined. These proteins were selected for study simply on the basis of commercial availability.

[^{14}C]Quercetin Binding to HSA in Human Plasma. Blood was drawn from human volunteers and placed in heparin-coated tubes to prevent coagulation. To obtain plasma, the blood was centrifuged at 13000*g* for 2 min. Incubation mixtures contained 1 mL of human plasma or whole blood and 25 μM [^{14}C]quercetin with or without 20 nM phorbol 12-myristate 13-acetate (PMA) (*18*). Mixtures were then incubated for 2 h at 37 °C with gentle shaking. After incubation, samples containing whole blood were centrifuged to obtain plasma. Plasma protein content was analyzed according to the method of Lowry et al. (*19*). Five-microliter plasma samples (200 μg of protein) were then prepared for gel electrophoresis as described below.

Separation and Quantitation of Labeled Proteins. The in vitro incubation and plasma samples were treated with sample buffer containing β-mercaptoethanol and boiled for 5 min to denature proteins. Samples (10 or 200 μg of protein for in vitro or plasma, respectively) were then loaded on 12.5% SDS−polyacrylamide gels (*20*). After electrophoresis, the proteins were transferred to nitrocellulose membranes and stained using Ponceau red to identify the locations of proteins. Membranes were then destained using 5% acetic acid, and before destaining was complete, identified protein bands were excised from the membrane. Bands were then destained completely using Tris-buffered saline with 0.2% Tween-20 (TBST) to increase the efficiency of scintillation counting. The destained nitrocellulose membrane protein bands were dissolved in Filter-Count scintillation fluid from Perkin-Elmer, and the amount of [^{14}C]quercetin bound to protein was obtained by liquid scintillation counting.

Calculations and Statistics. Differences among incubation conditions were evaluated using an ANOVA followed by a Tukey post-test (InStat 2.0). A significance level of $p < 0.05$ was used.

RESULTS AND DISCUSSION

As previously shown (*16*), quercetin is efficiently oxidized by 150 μM hydrogen peroxide in the presence of catalytic amounts of horseradish peroxidase (0.1 μM). A large number of products were detected by HPLC-UV, as previously reported (*13*), but could not be identified. Intermediate reactive semiquinones/quinone methides have been implicated after such oxidation (*13*, *14*).

To examine the covalent binding of oxidized quercetin to individual proteins, a selected number of proteins were incubated with [^{14}C]quercetin in the presence of hydrogen peroxide and horseradish peroxidase. The amount of radioactivity irreversibly bound to each protein was determined after electrophoresis of the reaction products (see Materials and Methods). When the oxidative conditions were applied to HSA (**Figure 1**), the extent of covalent binding of quercetin to HSA increased greatly compared to incubations with HSA and quercetin alone. Although hydrogen peroxide by itself induced significant covalent binding, the addition of horseradish peroxidase (HRP) further enhanced this binding by ~4-fold. In a most dramatic fashion, GSH completely abolished this binding at a concentration that normally occurs in many cells (5 mM).

Within the concentration ranges of 1−20 μM for HSA and 0.001−1 μM for HRP, there was no significant difference in covalent binding (**Figure 2A**,**B**). The latter observation emphasizes the need for only catalytic amounts of HRP. Also, the covalent binding increased linearly with time up to 120 min (**Figure 2C**), but did not increase with longer incubation time (data not shown). It would be of interest to know the stoichiometry of oxidized quercetin binding to HSA. Assuming that all of the quercetin is oxidized to a single reactive species that could combine with HSA, which is highly unlikely (*13*), the stoichiometry would be that 1 mol of quercetin binds to ~4−8 mol of HSA.

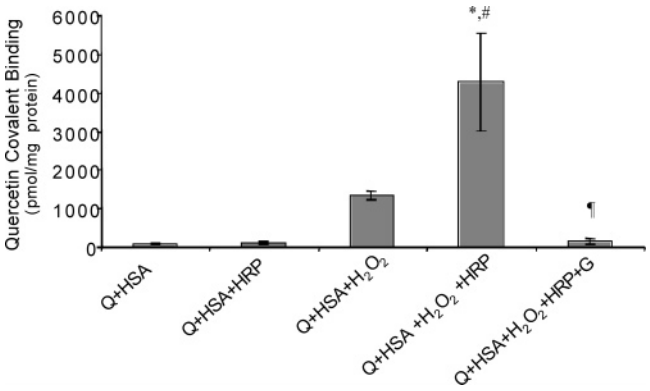

Figure 1. Covalent binding of 25 μM [^{14}C]quercetin (Q) to 13.8 μM human serum albumin (HSA) after 30 min of incubation with 150 μM hydrogen peroxide (H_2O_2) in the absence or presence of 0.1 μM horseradish peroxidase (HRP) and 5 mM GSH (G) as indicated. The samples were processed by SDS-PAGE, as described under Materials and Methods. Mean values ± SEM of four independent experiments are shown: *, higher than Q + HSA or Q + HSA + HRP, $P < 0.001$. #, higher than Q + HSA + H_2O_2, $P < 0.05$. ¶, lower than Q + HSA + H_2O_2 + HRP, $P < 0.001$.

To determine the specificity of the covalent binding of oxidized quercetin to proteins, we compared its binding to HSA with that of seven other distinct proteins, including HRP. As shown in **Table 1**, the covalent binding induced by hydrogen peroxide plus HRP was highly selective for HSA. GSH effectively inhibited the hydrogen peroxide/HRP-induced binding of quercetin to all proteins (data not shown). The higher binding of oxidized quercetin to transferrin than to apotransferrin may be due to the presence of Fe^{3+} ions capable of further oxidizing quercetin (*9*). The higher binding to cytochrome *c* may also be explained similarly.

In a previous clinical study of [^{14}C]quercetin pharmacokinetics we found evidence of covalent binding of quercetin to plasma proteins, potentially contributing to its very long half-life (*16*, *21*). Our previous finding of very high non-covalent association between quercetin and HSA (*22*) and in particular the present study demonstrating covalent binding suggest that this binding is to HSA. The question then becomes one of how and where the bioactivation of quercetin may occur in vivo. One possibility could be via oxidation of quercetin by hepatocytes and binding to HSA as it is synthesized and secreted from these cells. This was tested in Hep G2 cells, which secrete HSA (*23*) and in which effective oxidation of quercetin and binding to other proteins previously had been shown to occur (*16*). Evidence of binding to HSA could, however, not be found, presumably because HSA is protected in vesicles during its processing (*24*).

Previous findings with multiple drugs, such as carbamazepine (*18*), clozapine, and olanzapine (*25*), however, provided another mechanism. These drugs induce agranulocytosis, which is thought to be due to reactive metabolites of these drugs generated by the myeloperoxidase system of the neutrophils. It

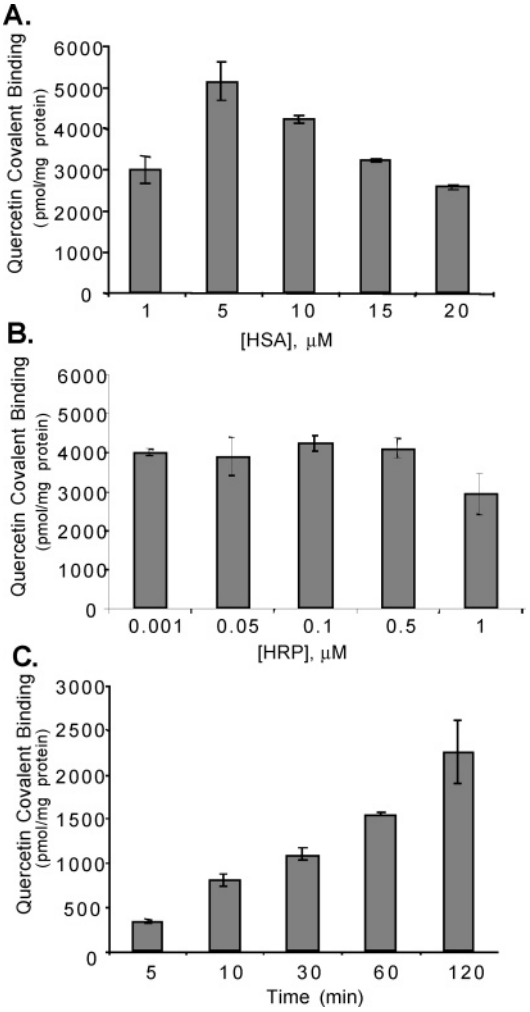

Figure 2. Effects of (**A**) HSA concentration, (**B**) HRP concentration, and (**C**) incubation time on the covalent binding of 25 μM [^{14}C]quercetin to HSA. $N = 3$ for all experiments. The HRP concentration in (**A**) was 0.1 μM, and the HSA concentration in (**B**) was 13.8 μM. The experiment in (**C**) was conducted as in **Figure 1**. The hydrogen peroxide concentration was 150 μM in all cases.

Table 1. Covalent Binding of [^{14}C]Quercetin to Various Proteins[a]

protein	covalent binding (pmol/mg of protein)
horseradish peroxidase	27 ± 28
apo-transferrin	129 ± 17
transferrin	370 ± 38
immunoglobulin G	30 ± 12
trypsin	67 ± 3
tyrosinase	107 ± 29
cytochrome *c*	201 ± 33
HSA	3230 ± 277[b]

[a] The covalent binding of 25 μM [^{14}C]quercetin to 10 μg of various proteins, as induced by 150 μM hydrogen peroxide and 0.1 μM HRP (except when the binding to this protein was determined) was performed as described under Materials and Methods. The experimental conditions were as in **Figure 1**. Data are presented as the mean ± SEM. $N = 10$ for HSA, and $N = 3$ for the other proteins. [b] Significantly different from all other proteins, $P < 0.001$.

is thought that myeloperoxidase produced from neutrophils results in the same oxidation of quercetin as hydrogen peroxidase/H_2O_2 (*26*). However, for this to occur, stimulation by the phorbol ester PMA may be necessary (*18*). Thus, in our experiments we incubated whole blood from six male volunteers with 25 μM [^{14}C]quercetin with and without 20 nM PMA. Incubation with plasma obtained from the same volunteers was used as myeloperoxidase-free controls. After centrifugation of the blood incubates, the samples were analyzed for binding of radioactivity to HSA by SDS-PAGE as above. The basal level of covalent binding of quercetin to HSA in the plasma incubations (negative controls) was 3.5 ± 0.6 pmol/mg of protein (mean ± SEM). This binding may be due to autoxidation of quercetin. As expected, the addition of PMA had no effect (3.3 ± 0.6 pmol/mg of protein). When the incubations were done with whole blood, there was a small increase in covalent binding to HSA (5.1 ± 0.7 pmol/mg of protein), compared to plasma, although not statistically significant. However, the binding increased ~3-fold to 13.2 ± 2.2 pmol/mg of protein ($P < 0.001$) when the blood samples were pretreated with PMA.

Attempts to determine the nature of the covalent binding of quercetin to HSA after tryptic digestion and MALDI-TOF-MS were unsuccessful. This was presumably due to the loss of the covalently linked quercetin under the conditions used. As shown, GSH was highly effective in preventing the covalent binding of quercetin. In recent mechanistic studies, the reaction between oxidized quercetin and GSH has been critically important in the determination of likely reactive sites in the quercetin molecule (*27*). Thus, the quercetin *o*-quinone monoanion in equilibrium with its corresponding quinone methide isomers results in GSH adduct formation at the 6- and 8-positions of the A-ring. These might also be the sites where HSA is linked to quercetin. On the basis of this knowledge, Cys34, which is the only HSA cysteine with a free sulfhydryl group that does not participate in a disulfide linkage (*28*), could be the reactive site in HSA. Cys34 has previously been shown to be susceptible to oxidation by hydrogen peroxide (*29*). However, following a molecular dynamics study of the IIA binding site in HSA, another potential site for quercetin covalent binding may be lysine (*30*). It is well-known that the IIA binding site in HSA provides non-covalent association between many drugs and HSA. This appears to involve quercetin as well (*22*). Interestingly, the study by Diaz et al. (*30*) also showed potential covalent linkage of HSA substrates to a highly reactive residue, Lys 199, which is strategically located in the IIA site.

The biological consequence of quercetin's binding to HSA may be a protective effect. Thus, quercetin is easily oxidized by cells (*4*, *16*) and, as determined in the present study, also in the circulating blood. HSA then promptly binds the resulting *o*-quinones/quinone methides (*13*, *14*, *27*), which, because of their high affinity for HSA and the very high concentration of HSA, might be a detoxification mechanism.

Continued studies should attempt to characterize the nature of the bond between the reactive species of quercetin and HSA and potentially other proteins. From this and a previous study (*16*), the covalent binding of quercetin to proteins appears to be rather selective, in contrast to the situation with many reactive drug molecules (*31*). Whether such oxidation and covalent binding occurs with other flavonoids is not known but may be expected, but only for certain catechol-like flavonoids (*14*, *27*). In this respect, quercetin appears to have a very distinct behavior (*27*).

In summary, this study shows that quercetin oxidized by peroxidase/hydrogen peroxide covalently links to proteins, with particularly high affinity for HSA. Studies in freshly isolated blood from human volunteers indicate that this also occurs in vivo after exposure to quercetin. This provides further insights into the complex behavior of one of the major flavonoids present in the human diet.

ABBREVIATIONS USED

HSA, human serum albumin; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GSH, reduced L-glutathione; PMA, phorbol 12-myristate 13-acetate; TBST, Tris-buffered saline with Tween-20; HRP, horseradish peroxidase.

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Received for review January 11, 2005. Revised manuscript received March 10, 2005. Accepted March 15, 2005. This study was supported by National Institutes of Health Grants GM55561 and RR01070.

JF050061M