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Evidence of covalent binding of the dietary flavonoid quercetin to DNA and protein in human intestinal and hepatic cells

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Abstract

Quercetin-rich foods have the potential to prevent human disease. However, knowledge of its biological fate and mechanism of action is limited. This study extends previous observations of the oxidation of quercetin by peroxidases to quinone/quinone methide intermediates and, for the first time, demonstrates covalent binding of [^{14}C]quercetin to macromolecules. This was first demonstrated using horseradish peroxidase and hydrogen peroxide with human liver microsomal protein to trap the intermediates. To extend this observation to the cellular level, human intestinal Caco-2 cells and hepatic Hep G2 cells were incubated for up to 2 hr with [^{14}C]quercetin, and cellular DNA and protein were isolated. The cellular uptake of [^{14}C]quercetin was rapid, and the covalent binding of [^{14}C]quercetin to DNA and protein was determined by liquid scintillation spectrometry after extensive purification. Both cell types demonstrated DNA binding with a maximum level of 5–15 pmol/mg DNA. The level of covalent binding to protein was considerably higher in both cell types, 75–125 pmol/mg protein. To determine potential specificity in the protein binding, Hep G2 cells were treated with [^{14}C]quercetin, and the cell lysate was subjected to SDS–PAGE followed by staining and autoradiography. Several distinct radiolabeled protein bands did not correspond to the major Coomassie blue stained cellular proteins. We propose that this specific binding may mediate part of the antiproliferative and other cellular actions of quercetin.

Keywords: Quercetin; Flavonoids; Peroxidative metabolism; Covalent binding; DNA and protein; Caco-2 cells; Hep G2 cells

1. Introduction

Dietary flavonoids are receiving increasing attention as potential protectors against a variety of human diseases, in particular cardiovascular disease [1–3] and cancer [4–7]. A large number of mechanisms of action have been investigated, including antioxidant properties [8] and effects on enzymes and signal transduction pathways [9–12]. However, evidence of potential toxic actions of flavonoids has also surfaced, at least at high concentrations [13–17].

A main problem concerning flavonoids is our limited knowledge of their bioavailability and metabolism, which will importantly contribute to their magnitude of biological actions. The best example of this may be the most studied flavonoid, quercetin. This flavonoid is present in the diet as a number of glycosides [18,19]. Although it was thought that these glycosides could undergo intestinal absorption [20], more recent studies demonstrate that they are not absorbed as such [21–24]. This is due, at least in part, to an enterocyte efflux mechanism involving MRP2 [25,26]. Instead, quercetin glycosides appear to be efficiently hydrolyzed, not only in the large intestine by bacteria [27,28], but also along the small intestine [29] by enzymes such as β-glucosidases [30] and lactase hydrolase [31]. Once the quercetin aglycone is formed from its glycoside, it is absorbed fairly efficiently in humans. However, although the oral absorption of quercetin is as high as 36–53% [32], the quercetin aglycone is detectable in the circulation only in trace amounts [23,32–34]. The very low oral bioavailability of quercetin in humans is due to extensive presystemic intestinal/hepatic metabolism. This

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Abbreviations: MRP2, multidrug resistance-associated protein 2; HBSS, Hanks' buffered saline solution, pH 7.4.

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includes conjugative pathways, such as glucuronidation [22–24], *O*-methylation [23,33,35] and maybe sulfation [33], but a quantitatively even more important pathway is oxidative degradation, presumably by intestinal bacteria, all the way to carbon dioxide, accounting for as much as 23–81% of a dose [32].

The fraction of the quercetin aglycone that enters the circulation unmetabolized is likely to be taken up rapidly by cells. This has been shown in cell culture [26,36]. However, its lifespan in the cells appears short, which could depend on chemical as well as enzymatic degradation by reactive oxygen species [36]. Previous studies have demonstrated that quercetin may be metabolized this way via quinone/quinone methide intermediates [37–39].

The objective of the present study was to determine the consequence of peroxidase-induced oxidative metabolism of quercetin and its subsequent covalent binding to macromolecules. Most important was the determination of covalent binding to DNA and protein in intact, cultured human cell lines such as the intestinal Caco-2 and hepatic Hep G2 cells. Finally, covalent adducts between proteins and quercetin were sought, using SDS–PAGE and autoradiography.

2. Materials and methods

2.1. Chemicals

[^{14}C]Quercetin (specific activity 53 mCi/mmol, labeled in the 4-position of the C-ring, Fig. 1) was obtained from the NCI Radiochemical Carcinogen Repository at Chemsyn Science Laboratories. Other chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

2.2. Oxidation and binding of quercetin in vitro

Quercetin (50 μM) was incubated with horseradish peroxidase (0.1–2 μM) and/or hydrogen peroxide (5–200 μM) in phosphate buffer (pH 7) for up to 30 min. In some experiments, glutathione (5 mM) was added to the mixture. The reactions were terminated by adding acetic acid and cold methanol. After vortex mixing and centrifugation at 16,000 *g* for 2 min, the supernatant was analyzed by HPLC, using a Symmetry C18 column (Waters), a mobile phase of 35% methanol and 5% acetic acid, and photodiode array detection at 370 nm.

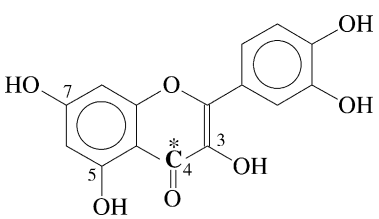

Fig. 1. Chemical structure of quercetin. The asterisk (*) designates the site of ^{14}C-labeling.

In similar experiments with [^{14}C]quercetin (50 μM, 0.5 μCi/sample), pooled human liver microsomes (0.5 mg/mL) (Gentest) were used as acceptor protein to measure covalent protein binding of the oxidized [^{14}C]quercetin in the presence or absence of horseradish peroxidase/hydrogen peroxide (30 min at 37°). To determine if cytochrome P450 contributes to the oxidation of quercetin, some samples were incubated in the presence of 2 mM NADPH. Samples with boiled microsomes or incubated on ice served as controls. Reactions were terminated by the addition of ice-cold trichloroacetic acid and extracted repeatedly (see below, Section 2.5). CYP1A activity in the microsomes was ascertained by measuring the ethoxyresorufin deethylation activity, using a fluorometric assay [40].

2.3. Cell culture

Human intestinal epithelial Caco-2 cells (ATCC), passage number 53–90, were cultured in Earle's Minimum Essential Medium with 10% fetal bovine serum, nonessential amino acids, and penicillin/streptomycin as previously described [21]. Human hepatoma Hep G2 cells (ATCC) were cultured in Williams' Medium E with 10% fetal bovine serum, L-glutamine, and antibiotic/antimycotic solution [41]. Experiments were done in HBSS with 25 mM HEPES (pH 7.4) without phenol red (HBSS) or in cell culture medium.

2.4. Cellular uptake of quercetin

Caco-2 cells in 6-well plates were incubated with 1 mL of 5 μM [^{14}C]quercetin in HBSS for 1–30 min at 37°. The cells were rapidly rinsed three times with ice-cold saline and then digested with sodium hydroxide. The lysate was analyzed for radioactivity using liquid scintillation spectrometry and for protein content according to Lowry *et al.* [42].

2.5. Quercetin binding to DNA

Confluent Caco-2 and Hep G2 cells in 6-well plates were incubated with 5 μM [^{14}C]quercetin in HBSS or complete cell culture medium. The cell monolayers were then rinsed three times with ice-cold saline before the addition of lift buffer (1 mM EDTA in Tris-buffered saline, pH 8) and incubation at 37° until detached. The cells from two wells were combined, pelleted, and resuspended in swell buffer (with spermidine, spermine, EDTA, EGTA) and Triton X-100. After 10 min on ice, the lysed cells were spun down. The pellets were resuspended in swell buffer, and the nuclei were centrifuged at 16,000 *g* for 2 min at 4° through a 30% sucrose cushion. The nuclear pellets were resuspended in Tris/EDTA buffer with RNase, proteinase K, and SDS to digest RNA and protein overnight. The samples were extracted four times with phenol/chloroform (Amresco). To the final aqueous phase was added sodium acetate to 1 M and ice-cold ethanol to precipitate the DNA. The DNA pellets were washed twice with cold ethanol and

dissolved in 1 mL of water. The purity of the DNA was evaluated as the 260/280 nm UV absorbance ratio. In all cases, this ratio was 1.7–1.9, i.e. the DNA was quite pure. The amount of [^{14}C]quercetin bound to DNA was obtained by liquid scintillation spectrometry and the amount of DNA by its 260 nm absorbance.

2.6. Quercetin binding to protein

Confluent Caco-2 and Hep G2 cells in 6-well plates were incubated with 5 μM [^{14}C]quercetin in HBSS or complete cell culture medium. The cell monolayers were then rinsed three times with ice-cold saline. The cells were scraped into PBS and homogenized with a Polytron homogenizer. An aliquot of the homogenate was analyzed by liquid scintillation spectrometry before the addition of 25% trichloroacetic acid to precipitate proteins. After centrifugation (1000 *g* for 5 min at room temperature), the pellet was sequentially extracted with 5% trichloroacetic acid and twice each with 80% methanol, hot 80% methanol, methanol/diethyl ether (50/50), and 80% methanol [43]. The radioactivity in the remaining pellet after this exhaustive extraction procedure represents irreversible binding of [^{14}C]quercetin to cellular protein. The protein content was determined according to Lowry *et al.* [42].

Plasma samples from volunteers given [^{14}C]quercetin orally or intravenously [32] were treated identically, starting with duplicate samples of 1 mL of plasma.

2.7. Statistics

The statistical significance of differences between different incubation conditions or times was evaluated using Student's two-tailed unpaired *t*-test or the Mann–Whitney nonparametric test when appropriate (unequal standard deviations).

2.8. Gel electrophoresis

Confluent monolayers of Hep G2 or Caco-2 cells were washed and lysed in lysis buffer with or without β-mercaptoethanol. The lysates were centrifuged at 12,000 *g* for 2 min at room temperature, and aliquots (200 μg protein) loaded on 8–20% gradient polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie blue, dried on filter paper, and subjected to autoradiography. The films were developed after 1 month at −70°.

3. Results

3.1. Irreversible binding of quercetin to human plasma proteins in vivo

In a recently completed clinical study in which [^{14}C] quercetin was administered both orally and intravenously to normal volunteers, we demonstrated a very long plasma half-life for total radioactivity, i.e. 20–72 hr [32]. As covalent binding of quercetin to plasma proteins may have contributed to this, we tested this hypothesis by precipitating the proteins and extracting them exhaustively to remove any loosely bound quercetin [43]. In the early samples, collected at 2–8 hr after the dose, the irreversible binding of quercetin radioactivity to the proteins was 31 ± 16% (mean ± SEM; N = 6) of the total radioactivity in these samples. In the late samples, collected at 72 hr after the dose, the irreversible binding of radioactivity to proteins was as high as 61 ± 14% (N = 6; $P < 0.05$ compared to the early samples). This would be consistent with the notion that irreversibly bound quercetin is eliminated more slowly than any unbound or reversibly bound quercetin. Furthermore, in control plasma spiked with [^{14}C]quercetin, there was only 12 ± 2% apparent irreversible binding (N = 6), significantly lower than the early patient samples ($P < 0.05$).

3.2. Activation mechanisms and irreversible binding of quercetin in vitro

Previous studies have suggested that quercetin may be metabolized by reactive oxygen species via a quinoid intermediate [37–39]. When quercetin (50 μM) was incubated with horseradish peroxidase (0.1 μM gave the same results as 2 μM) in the presence of various concentrations of hydrogen peroxide (5–200 μM), a concentration-dependent disappearance of the quercetin HPLC peak was observed (Fig. 2). At 50 μM hydrogen peroxide, a concentration shown to be produced *in vivo* during inflammation and ischemia [44], there was a more than 60% reduction in quercetin concentration. Concomitantly, there was increased formation of a new product with a relative

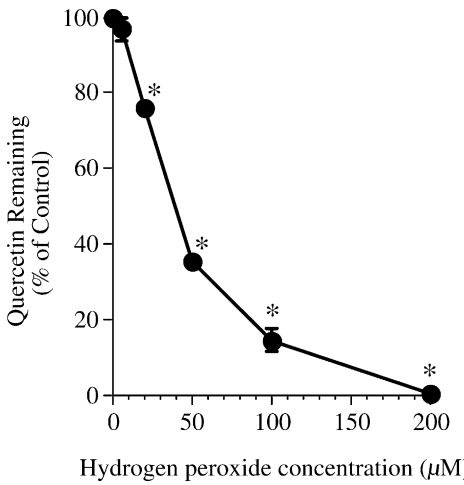

Fig. 2. Degradation of 50 μM quercetin by hydrogen peroxide in the presence of 0.1 μM horseradish peroxidase. The reaction was carried out for 30 min. Mean values ± SEM (N = 3) are shown. Without hydrogen peroxide the remaining quercetin concentration was 44 μM (control). Key: (*) significantly lower than the control, $P < 0.001$.

retention time of 0.30 compared to quercetin and a UV maxima at 264.5, 311.9, and 363.1 nm as compared to 255.1 and 372.5 for quercetin. This is likely due to the quinone/quinone methide previously identified [37–39]. The addition of glutathione did not affect the rate of disappearance of quercetin but led to the formation of two glutathione adducts also described previously [37–39].

To test whether this oxidation would result in irreversible binding of quercetin to protein, the experiment in Fig. 3 with human liver microsomal protein was conducted, using this protein source to trap any species that might bind to macromolecules. Incubates of control as well as boiled microsomes with [^{14}C]quercetin both gave rise to a surprisingly high level of irreversible binding, indicating the presence of oxidative species in the microsomes. In the presence of NADPH, the microsomal incubates demonstrated a clear CYP-type of activity (EROD assay), however, with decreased rather than increased irreversible binding of quercetin to protein. In the presence of 1 μM horseradish peroxidase and 1 mM hydrogen peroxide, the irreversible binding of [^{14}C]quercetin showed a dramatic increase (Fig. 3). As much as 40% of the added radioactivity was irreversibly bound to human liver microsomal proteins in this experiment.

3.3. Caco-2 cell uptake of quercetin

In a previous study we showed accumulation of quercetin by Caco-2 cells using fluorescence microscopy, demonstrating cytoplasmic as well as nuclear localization of this fluorescent flavonoid [26]. The time-course of accumulation of [^{14}C]quercetin by the Caco-2 cells is shown in Fig. 4. After incubation with 5 μM [^{14}C]quercetin, the cells were washed three times with ice-cold saline, and the quercetin content was determined by liquid scintillation spectrometry. The accumulation was rapid

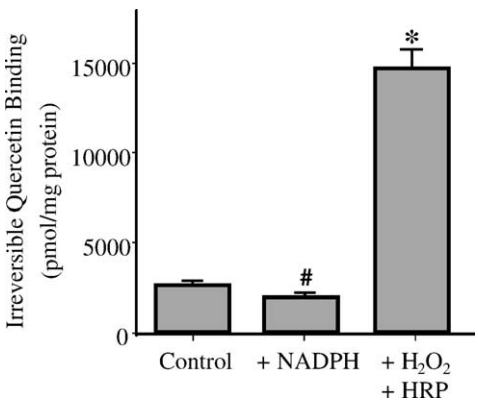

Fig. 3. Irreversible binding of [^{14}C]quercetin (25 μM) to human liver microsomal protein (0.5 mg) under different conditions. NADPH indicates CYP-active microsomes; HRP, horseradish peroxidase (1 μM); H_2O_2, hydrogen peroxide (1 mM). The reactions were carried out for 30 min. Mean values ± SEM (N = 5) are shown. Key: (#) significantly lower than the control, $P < 0.05$; and (*) significantly higher than the control, $P < 0.0001$.

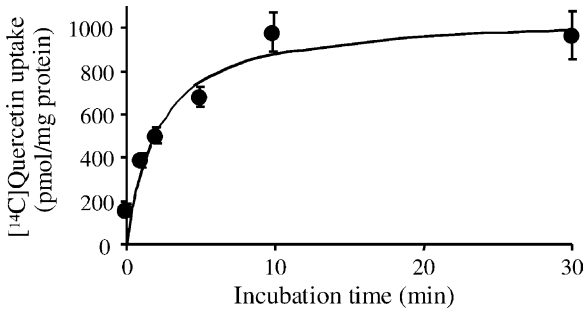

Fig. 4. Time-course of Caco-2 cell uptake of [^{14}C]quercetin. After incubation with 5 μM [^{14}C]quercetin in buffer, the cell monolayers were rapidly washed and lysed with NaOH, and total radioactivity was measured by liquid scintillation spectrometry. Mean values ± SEM are shown (N = 4).

with the maximum concentration reached at 10 min with a cell content of about 1000 pmol/mg protein, i.e. 26% of the added [^{14}C]quercetin.

3.4. Irreversible binding of quercetin in intact cells

These experiments were an attempt to establish whether covalent binding of quercetin to macromolecules could occur in a cell, i.e. under physiologically relevant conditions. A recent study, however, demonstrated that the addition of flavonoids such as quercetin to a cell culture medium may itself lead to the production of hydrogen peroxide [45]. This in turn, as shown above, could lead to increased oxidation and covalent binding of quercetin. To avoid this possibility, all cell culture experiments with quercetin were done both in buffer, where this would pose much less of a problem, and in cell culture medium. In addition, we also performed experiments in the presence of 1 mM ascorbate, which effectively inhibits quercetin autoxidation [39].

3.4.1. Caco-2 cells

Caco-2 cells grown in 6-well plates were incubated for up to 2 hr with 5 μM [^{14}C]quercetin, either in buffer or in cell culture medium. The cells were washed three times with ice-cold buffer, DNA was isolated and purified, and the amount of radioactivity bound to DNA was determined as described in Section 2. Binding to DNA clearly occurred when using buffer and was time-dependent (Fig. 5A) with a maximum binding at 2 hr of 15 pmol/mg DNA. When using cell culture medium, there was a slight binding at only the 2-hr exposure (Fig. 5A). The irreversible binding of [^{14}C]quercetin to protein was determined by exhaustive extraction in identically treated Caco-2 cells. The irreversible binding to protein was substantially higher than to DNA, at both 10 min and 2 hr, when using buffer (Fig. 5B). The binding to protein was about ten times higher than the binding to DNA in the same experiments and corresponded to as much as 10% of the total cellular content of [^{14}C]quercetin (cf. Fig. 4). When using cell culture

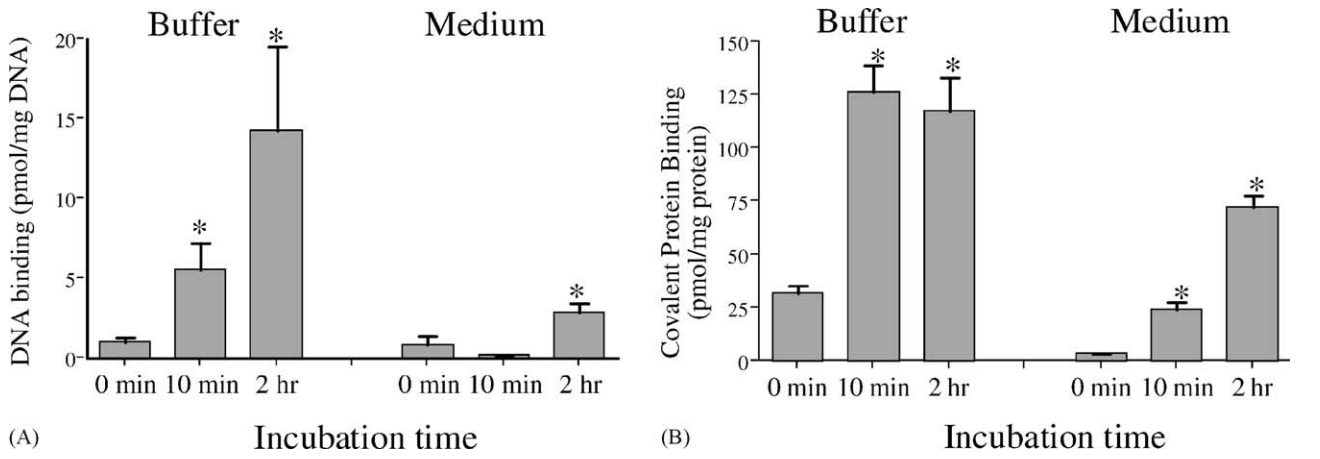

Fig. 5. Irreversible binding of [^{14}C]quercetin in Caco-2 cells. After incubation of the cells with 5 μM [^{14}C]quercetin in HBSS or medium, DNA and protein were isolated as described in Section 2, and the amount of radioactivity bound to DNA (A) and protein (B) was determined by liquid scintillation spectrometry. Mean values ± SEM (N = 3–6) are shown. Key: (*) significantly higher than 0 min, $P < 0.05$.

medium, the irreversible binding to protein was less, but did occur at both the 10-min and 2-hr time points (Fig. 5B). In the presence of ascorbate, the covalent protein binding after incubation of the cells with [^{14}C]quercetin for 10 min in buffer was about 85% of control. This difference was not statistically significant (N = 6).

3.4.2. Hep G2 cells

Hep G2 cells grown in 6-well plates were incubated with 5 μM [^{14}C]quercetin. In analogy with the Caco-2 cell experiment, irreversible binding to DNA and protein was determined (Fig. 6A and B). The binding to DNA of 4–6 pmol/mg DNA was not much different from that of the Caco-2 cells, and was not different when using buffer or cell culture medium (Fig. 6A). The binding to protein of 70–100 pmol/mg protein was somewhat higher when using buffer as compared to cell culture medium and was similar to the Caco-2 cells, although the time course was quite different (Fig. 6B).

3.5. Gel electrophoresis of Hep G2 cell proteins

To be able to determine if there was specificity in the protein binding, total Hep G2 cell lysate, after incubation with [^{14}C]quercetin and washing the cells, was subjected to SDS–PAGE followed by autoradiography (Fig. 7). As expected, Coomassie blue staining showed a large number of protein bands over the whole 10–220 kDa molecular weight range of the gel (Fig. 7A). The corresponding autoradiogram of mercaptoethanol-reduced cell lysate showed about ten bands, three of which were more intense at approximately 55, 58, and 80 kDa (Fig. 7B). Of great significance was the fact that none of these radioactive bands corresponded to bands visible by Coomassie blue staining, indicating that the radiolabeling of proteins by quercetin occurs specifically. When examining identical samples prepared without mercaptoethanol, several additional radiolabeled proteins were visible at about 12–17 kDa (Fig. 7C).

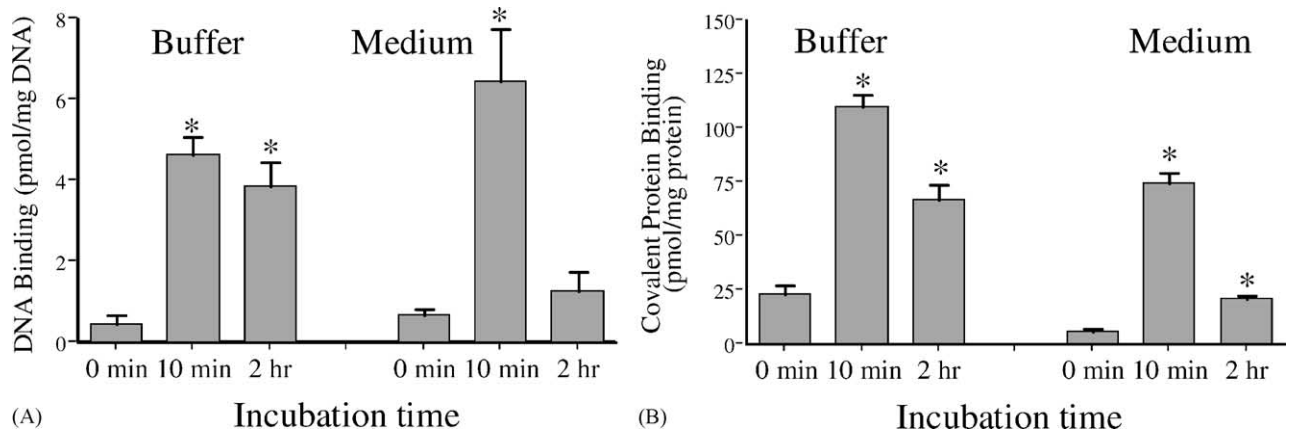

Fig. 6. Irreversible binding of [^{14}C]quercetin in Hep G2 cells. After incubation of the cells with 5 μM [^{14}C]quercetin in HBSS or medium, DNA and protein were isolated as described in Section 2, and the amount of radioactivity bound to DNA (A) and protein (B) was determined by liquid scintillation spectrometry. Mean values ± SEM (N = 3–6) are shown. Key: (*) significantly higher than 0 min, $P < 0.01$.

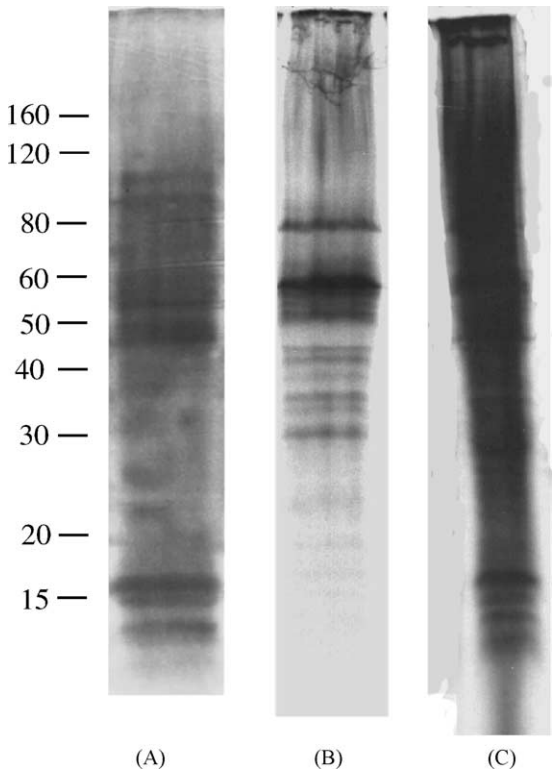

Fig. 7. SDS–PAGE and autoradiography of Hep G2 cell lysate after incubation of the cells with 20 μM [^{14}C]quercetin for 2 hr and washing of the cells. (A) Coomassie blue staining. (B) Autoradiogram of lysate containing β-mercaptoethanol (reducing conditions). (C) Autoradiogram of lysate without β-mercaptoethanol (non-reducing conditions).

It should be pointed out that, due to the low specific activity of the [^{14}C]quercetin used, the time to produce the autoradiogram in Fig. 7 was 1 month.

4. Discussion

This is the first study demonstrating covalent binding of the most ubiquitous of the dietary flavonoids, i.e. quercetin, to cellular DNA and protein. The findings indicate a reactive oxygen-mediated bioactivation of this and potentially other flavonoids within the cell. The binding to proteins appears to be highly selective, suggesting such binding as a potential mechanism of action of quercetin.

Although it is well established that hydrogen peroxide and horseradish peroxidase, or other peroxidases, oxidize quercetin and other flavonoids to quinone/quinone methide intermediates, with subsequent reactions with glutathione [37–39,46], oxidation in intact cells with subsequent covalent binding to macromolecules has not been addressed previously. Although our observations suggest that quercetin is oxidized by reactive oxygen species in both Caco-2 and Hep G2 cells, it may be difficult to demonstrate directly, mainly because of the instability of the oxidized species. Preliminary attempts at this with the Hep G2 cells did produce novel UV-absorbing species, however, at a low level that could not produce complete spectra (data not shown). On the other hand, the covalent binding to DNA and in particular to protein provided strong but indirect evidence for this contention. In a very recent study, conducted with a mouse melanoma cancer cell, quercetin was demonstrated to form two isomeric glutathione adducts, also consistent with cellular oxidation of quercetin [39].

In vivo, based on this study, the extent of covalent binding of quercetin would be expected to be particularly high in enterocytes due to the direct delivery of quercetin at this site from either dietary sources through hydrolysis of quercetin glucosides [29–31] or from dietary supplements of the quercetin aglycone. However, reactions competing with reactive oxygen species in the enterocytes would in particular be glucuronidation and sulfation, metabolic reactions well expressed in the Caco-2 cell enterocyte model system [47]. The extent of covalent binding of quercetin in hepatocytes and other more peripheral tissues would be expected to be lower, mainly due to rather efficient presystemic intestinal/hepatic metabolism of quercetin through multiple pathways [22–24,33,35].

The covalent binding of quercetin to DNA that was observed occurred to a similar extent in Caco-2 and Hep G2 cells. As the extent of binding to DNA was only about one-tenth of that to protein, it might be argued that it could have been due to contaminating protein. However, the DNA isolated from the nuclei appeared very pure, judging from the 260/280 nm UV absorption ratio of about 1.8. The magnitude of maximum DNA binding was about 5–15 pmol/mg DNA after exposure to 5 μM quercetin for 2 hr. As a comparison, this is about one-third of the DNA binding that we have found previously for the well known cooked-food mutagen *N*-hydroxy-PhIP in human mammary epithelial cells under very similar conditions [48]. These observations do add fuel to the debate about potential damage by quercetin and other dietary flavonoids to DNA, a debate that has appeared in cycles in the past [13,14] and more recently [15,17]. Not until more definitive answers have been generated can this issue be settled. The availability of radiolabeled quercetin may be helpful in these efforts.

If bioactivation of quercetin to quinone/quinone methide metabolites [37,38] occurs within the cells, as hypothesized in our study and in the very recent study by Awad *et al.* [39], then covalent binding to protein is not surprising. When examining the labeled proteins from the Hep G2 cells by SDS–PAGE, we expected to see albumin as one of the target proteins. First, albumin is a major protein produced by the Hep G2 cells. Second, our previous studies have determined that the plasma binding of quercetin is more than 99% and that this binding is mainly due to albumin [49]. Third, in our clinical study above, we have evidence of covalent binding of quercetin to plasma proteins. Our conclusion from the gel electrophoresis data in Fig. 7 is that this expectation was not true. It is entirely

possible that this secreted protein was effectively removed from the cells during the extensive washing procedure. Instead, the major radiolabeled proteins appeared at molecular weights of approximately 55–80 kDa. These proteins did not appear to correlate with major cellular proteins, as detected by Coomassie blue staining, indicating them to be proteins to which quercetin binds with high affinity. Although covalent binding of small molecules to proteins, in general, has been considered undesirable, this may not necessarily always be the case. Quercetin is one of the most potent antiproliferative flavonoids with the major mechanism of action believed to involve phosphoinositide 3-kinase [50–52]. The major radioactively labeled bands in the SDS–PAGE autoradiogram of 55–80 kDa could potentially be the regulatory subunits of this important kinase [53]. Thus, the covalent binding may, in part, contribute to the biological actions of quercetin. Multiple other protein bands are labeled by quercetin in the 30–45 kDa range as well as in the 12–17 kDa range, the latter when the gel is run under non-reducing conditions. These observations suggest that further in-depth studies of covalent interactions between quercetin and target proteins may lead to greatly expanded knowledge of the biological actions of this flavonoid.

Based on the study by Long *et al.* [45], oxidation of quercetin and associated covalent binding might have been higher when using cell culture medium rather than buffer in the intact cell experiments. In fact, the opposite was true. The fact that there was not an increased covalent binding of quercetin in the experiments with cell culture medium may be due to the much lower concentrations of quercetin used in our study (5 μM) as compared to that by Long *et al.* (100 μM) [45]. The much lower covalent binding in the experiments with cell culture medium, in particular with the Caco-2 cells, may be due to the extraordinarily high serum protein binding of quercetin [49], limiting the distribution of quercetin into the cell when cell culture medium, containing 10% fetal bovine serum, was present. The Hep G2 cells seemed less sensitive to this effect of the medium, suggesting differences in cellular extraction efficiency of quercetin. Chemical auto-oxidation of antioxidants like quercetin is a potential artifact in this type of studies. However, including a high concentration of ascorbate, similarly to a previous study [39], in some of the experiments without significant reduction of the degree of covalent binding rules out an effect of auto-oxidation.

Thus, although it is well known that quercetin as an antioxidant flavonoid will affect the status of the reactive oxygen species in cells, our current observations suggest that the opposite occurs as well, i.e. reactive oxygen species will metabolize the flavonoids to species that covalently interact with key target proteins. This may, after much further study, help us to understand the many diverse biological actions produced by these dietary components.

Acknowledgments

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