Enhanced Antioxidant Activity After Chlorination of Quercetin by Hypochlorous Acid


Background: Several epidemiological studies indicate that moderate consumption of red wine decreases both the incidence and mortality associated with cardiovascular disease. Quercetin and rutin (quercetin-3-rutinoside) are polyphenols present in relatively large concentrations in red wine and may play a role in this cardioprotective phenomenon. The precise mechanisms of cardioprotection remain unclear but may involve the action of these polyphenols as antioxidants, which attenuate the tissue injury that results from the production of proinflammatory oxidants such as hypochlorous acid (HOCI).

Methods: To study the interaction of these polyphenols with proinflammatory oxidants, we mixed quercetin or rutin with HOCI (0–150 μM) and analyzed the reaction products by high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance.

Results: Stable mono- and dichlorinated derivates were detected for both quercetin and the glycoside derivative, rutin, which suggests that both the conjugated and unconjugated forms of quercetin reacted with HOCI similarly. Chlorination of quercetin occurred only at two sites, and the derivates (6-chloroquercetin, 6,8-dichloroquercetin) were more potent antioxidants toward oxidative modification of low-density lipoproteins and ABTS radical formation than the unmodified form.

Conclusions: These data suggest that under certain pathological conditions in vivo (e.g., inflammation), flavonols may be converted to chlorinated derivates, which exhibit an enhanced antioxidant potential and thereby play a role in cardioprotection.

Key Words: Quercetin, Rutin, Hypochlorous Acid, Chlorination, High-Performance Liquid Chromatography, Mass Spectrometry, Nuclear Magnetic Resonance.

The flavonols quercetin (3,3',4',5,7-pentahydroxyflavone) and rutin (quercetin-3-O-β-D-glucosyl-O-α-L-rhamnose; vitamin P) are present in relatively high levels in red wine, although they are not present in significant quantities in white wine (Goldberg et al., 1996; Soleas et al., 1997). Several epidemiological studies indicate that the moderate consumption of red wine lowers the incidence and mortality associated with cardiovascular disease (Fuhrman et al., 1995), due largely to a reduction in overall risk for coronary heart disease (Camargo et al., 1997; Doll, 1997; Klatsky et al., 1997; Thun et al., 1997). The 20% to 60% decrease in mortality has been attributed to the ability of moderate levels of alcoholic beverages to exhibit (a) antiatherogenic properties through an increase in high-density lipoprotein (HDL; McConnell et al., 1997), (b) antithrombotic properties by a decrease in platelet aggregation (Renaud and Ruf, 1996) and an increase in fibrinolytic activity (Aikens et al., 1998), and (c) antioxidant properties by minimizing oxidation of biomolecules such as lipids and proteins (Akkus et al., 1997; Croft, 1998; Keli et al., 1996; Svegliati-Baroni et al., 1999). It has been postulated that the red wine polyphenols quercetin and rutin also play an important role in this phenomenon (Nigdikar et al., 1998), although the precise mechanisms by which these flavonols result in cardioprotection remains unclear (Das et al., 1999; Miyagi et al., 1997).

The antioxidant properties of flavonols are well recognized (Bravo, 1998; Croft, 1998), and it generally has been assumed that the cardioprotective potential of the flavonols is due, at least in part, to the scavenging of reactive oxygen species (Hertog et al., 1997; Serafini et al., 1998). In addition, quercetin has been demonstrated to decrease superoxide (O$_2$•⁻) and hydrogen peroxide (H$_2$O$_2$) production by stimulated polymorphonuclear neutrophils (PMNs; Schneider et al., 1979), inhibit neutrophil degranulation (Blackburn et al., 1987; Pagonis et al., 1986), inhibit platelet aggregation stimulated by reactive oxygen species (Xie et al., 1996), reduce the phosphorylation of specific neutrophil...
proteins (Blackburn et al., 1987), and inhibit oxidation of low density lipoproteins (LDL) and attenuate the associated formation of atherosclerotic lesions (Hayek et al., 1997). Rutin also has been demonstrated to inhibit oxidation of LDL (Brown et al., 1998; Day et al., 1999), minimize oxidative renal damage (Shimoi et al., 1997), and limit oxidative DNA damage to human lymphocytes (Noroozi et al., 1998).

In addition, quercetin and rutin were shown to impact the production of hypochlorous acid (HOCl) by PMNs (Pincemail et al., 1988). HOCl is generated by neutrophils through the enzyme myeloperoxidase (MPO), which catalyzes the oxidation of chloride anion (Cl\(^{-}\)) by \(H_2O_2\) to yield HOCl (Winterbourn et al., 2000). The production of HOCl is an integral part of the nonspecific host defense mechanism triggered by opsonized bacteria or activated complement components (Winterbourn et al., 2000) but under certain conditions can also destroy healthy tissues (Pincemail et al., 1988). In this latter context, evidence for HOCl-dependent oxidative damage in chronic inflammatory diseases such as atherosclerosis has been reported (Hazen and Heinecke, 1997; Heinecke, 1997). The ability of quercetin to effectively inhibit MPO activity [Median inhibitory concentration (IC)\(_{50}\) = 3.5 \(\mu\)M; Pincemail et al., 1988] as well as directly scavenge HOCl may limit the vascular injury associated with inflammatory reactions.

Recent data indicate that the reaction between flavonols and HOCl may be more complicated than a simple oxidant-antioxidant interaction, and that phenolic compounds can react with HOCl to form stable chlorinated components, with each product potentially having a unique reactivity (Boersma et al., 1999; Eiserich et al., 1998). These findings are of special relevance to the isoflavones (i.e., genistein) and the flavonols (i.e., quercetin), which share structural similarities with 17\(\beta\)-estradiol (see Fig. 1) and often are referred to as phytoestrogens (Boersma et al., 1999; Davis et al., 1999). Quercetin has been demonstrated to bind to the human estrogen receptor, although with low affinity (Kuiper et al., 1998); however, chlorinated forms of quercetin may have a higher binding affinity to this receptor and thus may have a greater impact. It is anticipated that the binding to the receptor will depend mainly on the extent of chlorination (Garner et al., 1999), as observed for the polychlorinated biphenyls. Estrogens have been reported to exhibit certain cardioprotective properties (Kuroski de Bold, 1999), and chlorinated flavonols may mimic these effects; however, the existence of such molecules remains to be documented.

It has not been demonstrated whether rutin or quercetin can form chlorinated products. This is significant because the metabolites of the flavonols may have biological and chemical properties distinct from the parent compounds. Furthermore, the effects of halogenation on the biological and chemical characteristics of flavonols have not been examined. This is of particular interest with respect to the hydrogen and electron-donating properties critical to the antioxidant function of these molecules, which we hypothesize will be altered as a consequence of modification of the aromatic structure of these compounds. In this series of studies, we demonstrated that chlorinated forms of quercetin and rutin were generated during reaction with HOCl. Various concentrations of each polyphenol (0–150 \(\mu\)M) were incubated with HOCl, and the reaction products were analyzed by high-performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR). Both quercetin and rutin formed stable mono- and dichlorinated derivates with increased activity in inhibiting the oxidation of LDL. The chlorinated derivatives also exhibited significantly greater antioxidant capacity than the unmodified quercetin as determined by the ability of these derivatives to delay formation of a chromogenic radical cation. We hypothesized that similar products may be generated under physiological conditions and may play a role in cardioprotection.

**MATERIALS AND METHODS**

**Materials**

Quercetin (3,3',4',5,7-pentahydroxyflavone), rutin (quercetin-3-O-rutinoside), HOCl, diethylenetriamine pentaacetic acid (DTPA), taurine (2-aminoethansulfonic acid), and naringinase (from P. decumbens) were purchased from Sigma Chemical Co. (St. Louis, MO). 5-Thio-2-nitrobenzoic acid (TNB) was a gift from J. Eiserich (University of Alabama at Birmingham).

**Reactions of Flavonols With HOCl**

Each reaction was carried out in a borosilicate glass tube (Fischer Scientific, Pittsburgh, PA) with a final volume of 2 ml. Stock solutions of quercetin and rutin (10 mM, in methanol) were freshly prepared and then diluted with phosphate-buffered saline (PBS; 200 mM Na-P\(_2\)O\(_4\); 150 mM NaCl, pH 7.3) that contained 1 mM DTPA. While continuously mixing, an
aliquot of a freshly prepared HOCl stock solution (in PBS/DTPA) was added to the quercetin or rutin solution, which resulted in final concentrations for each component in the range of 0 to 150 μM. The HOCl-flavonol reactions were rapid, and the samples were processed immediately after the mixing.

Measurement of HOCl (Taurine Assay)

The HOCl concentrations in the assays were determined by conversion of exogenously added taurine into stable N-chlorotaurine as previously described (Eiserich et al., 1998). Briefly, after reaction of quercetin or rutin with HOCl the assays were adjusted to a taurine concentration of 3.85 mM and then were incubated for 30 min at room temperature to allow a complete reaction of any free HOCl with taurine. Then, TNB was added (40 μM) and incubated for 5 min, and the absorbance of the oxidized TNB was measured at 405 nm. For quantification, fresh prepared HOCl standards (0–40 μM) were assayed in parallel.

Extraction of Quercetin

Quercetin and its derivatives were acidified with phosphoric acid (0.85%) and then extracted by adding ethyl acetate to the sample (2:1). After mixing, the samples were centrifuged for 5 min at 4000 rpm, and the upper organic layer was transferred to a glass tube. The sample then was dried in a rotary vacuum apparatus (Speed-vac; Savant Instruments, New York), and the resulting pellet was resuspended in 80% methanol for subsequent analysis by HPLC.

Naringinase Assay

The rutin samples first were adjusted to a pH of 4.0 with o-phosphoric acid, and then naringinase (2 mg/ml; 10-fold stock in distilled water) was added to cleave the quercetin from the rutinoside. The reaction mixtures were incubated at 37°C for 90 min, followed by an extraction of the quercetin as described previously.

HPLC Analysis of Reaction Products

The reaction products were analyzed by reverse-phase HPLC, by using a C18 MICROSOORB-MV™ column (15 cm, 5 μm; Ranin Instrument Co., Woburn, MA) for separation and a diode array detector (235-C; Perkin Elmer, Norwalk, CT) for monitoring absorbance at 365 nm. Before the sample (100 μl) was injected, the column was equilibrated first at a flow rate of 1.5 ml/min for 5 min with running buffer (20% acetonitrile, 0.05% phosphoric acid, pH 2.0). The samples were eluted with a linear gradient with acetonitrile (20–50%) for 10 min, followed by 50% to 90% acetonitrile from 10 to 12 min. The column was washed with 90% acetonitrile (in 0.05% phosphoric acid, pH 2.0), with a flow rate constant of 1.5 ml/min. To quantify the quercetin or rutin peaks, standards of each flavonol were run in parallel. The quantities of the reaction products were expressed as quercetin or rutin equivalents.

Isolation of the Reaction Products and Storage

Based on the HPLC analysis, we isolated each peak of interest by collecting fractions at the relevant time points by using the HPLC method described previously. After several runs, the fractions that contained each component were pooled and then extracted with ethyl acetate as described previously. The samples were dried and then resuspended in a smaller volume of acidified ethyl acetate. These concentrated sample solutions then were divided in aliquots and the samples were dried for subsequent analysis. For each component, one sample aliquot was resuspended in 80% methanol and separated by HPLC to verify the identity of the compound as well as to quantify the isolated component. The remaining dried samples were sealed with parafilm and kept in the dark at 4°C.

MS Analysis of Reaction Products

The isolated, dried material of a single peak (described previously) was resuspended in 80% methanol and analyzed by MS as described previously (Boersma et al., 1999). Briefly, the samples were subjected to reverse-phase HPLC by using 10 mM ammonium acetate buffer and then eluted with an acetonitrile gradient (0–90%). The column eluent was passed into the electrospray (IonSpray™) interface of a PE-Sciex API III triple quadrupole mass spectrometer (Concord, Ontario, Canada) with the needle voltage set at ~4900 V. Negative ion spectra were recorded over an m/z range of 280 to 380 for quercetin samples and of 535 to 695 for rutin samples. Data were analyzed by using software provided by the manufacturer.

NMR Analysis of Reaction Products

Approximately 125 μg of quercetin and its reaction products were isolated by preparative HPLC as described previously. A one-dimensional proton NMR spectrum of the dried material was provided by Reading Scientific Services Limited (Lord Zuckerman Research Center, Reading, UK).

LDL Isolation, Purification, and Lipid Oxidation Assay

Human LDL was isolated from plasma of healthy donors by differential centrifugation by using the method previously described (Chung et al., 1980). The isolated LDL was dialyzed against Ca2+/Mg2+-free PBS that contained 10 μM DTPA and then was sterilized by filtration through a 0.2 μm pore size filter. LDL was stored at 4°C and used within 1 week of preparation. The concentration of LDL protein was determined by using BioRad protein assay reagent (BioRad, San Francisco, CA). For the LDL oxidation assay, stock solutions of Cu2+ were prepared in distilled water and mixed with LDL to final concentrations of 5 μM Cu2+ and 75 μg/ml LDL in PBS. Oxidative modification of LDL was assessed by measuring the formation of conjugated dienes at 37°C as previously described (Esberbauer et al., 1989). The antioxidant effects of quercetin and its derivatives were assessed by adding different concentrations (0–1 μM) of each isolated component to LDL before the addition of Cu2+.

Total Radical Antioxidant Potential

The total antioxidant capacity of quercetin and its chlorinated derivatives was analyzed with a modification of the inhibition assay of Rice-Evans and Miller (1994) by using a centrifugal analyzer as previously described in detail (Tan et al., 2001). The ABTS™ radical was formed from the interaction of 1600 μM ABTS with ferrylmyoglobin radical species, generated by the activation of 27 μM metmyoglobin with 480 μM H2O2. Absorbance readings at 734 nm were taken in a volume of 360 μl every 15 sec for 12.5 min at 25°C. The end-point absorbance at 12.5 min and the area under the curve for all the absorbance values over time were determined. Samples (100 μM) of quercetin and its mono- and dichloro-derivatives were compared to a standard curve generated from known concentrations of Trolox in isotonic 5 mM PBS, pH 7.4 and 25°C.

Statistical Analysis

All values are expressed as mean ± SEM. Comparisons between groups were made by ANOVA with post hoc comparison of means by Student-Newman-Keuls test. All analysis was conducted with SAS System for Microsoft Windows, Release 6.08 (SAS Institute, Cary, NC).

RESULTS

The Flavonols React With HOCl to Form New Products

In the first series of experiments, quercetin or rutin (both 50 μM) were reacted with concentrations of HOCl that ranged from 0 to 150 μM. The samples then were subjected
had longer retention times than the original unmodified polyphenol; for the quercetin products, the retention times were 6.6 and 7.9 min, compared with 5.6 min for quercetin. The retention time for rutin was 2.4 min, whereas the two new products had retention times of 3.6 and 4.4 min, respectively. Interestingly, the reaction of rutin with HOCl was quite similar to quercetin (Fig. 2A and 2B); most notable, both flavonoids resulted in the formation of only two additional peaks. This phenomenon was confirmed by additional experiments in which the HOCl concentration was kept constant at 50 \( \mu \text{M} \), while quercetin or rutin was added at concentrations that ranged from 0 to 15 \( \mu \text{M} \). As seen in Fig. 2C, both rutin and quercetin consumed HOCl in a similar manner, which resulted in nondetectable levels of free HOCl at a flavonol concentration of 15 \( \mu \text{M} \).

At concentrations of HOCl above 50 \( \mu \text{M} \), there appeared to be a greater amount of new rutin products than was observed at a similar concentration of quercetin (Fig. 2B). The amount of new products formed was about two times higher for rutin than for quercetin at 50 \( \mu \text{M} \) (Fig. 2A and 2B). HOCl concentrations of 50 and 150 \( \mu \text{M} \) markedly reduced the amounts of detectable products, which suggests that the difference in extent in product formation between quercetin and rutin may be due to diminished susceptibility of the rutin products to the effects of HOCl.

The destructive character of HOCl was more obvious in a related set of experiments, in which the concentration of HOCl was kept constant at 50 \( \mu \text{M} \) and quercetin or rutin was added at concentrations of 0 to 150 \( \mu \text{M} \). At polyphenol concentrations below 50 \( \mu \text{M} \), neither the parent components nor new products were detected (data not shown), whereas when the polyphenol and HOCl were at identical concentrations (e.g., 50 \( \mu \text{M} \) HOCl and 50 \( \mu \text{M} \) quercetin or rutin), a ratio of products similar to those in Fig. 2A and 2B was observed. When the polyphenols were present at a higher concentration (e.g., 150 \( \mu \text{M} \)), most of the quercetin or rutin remained in the unmodified form, with two new products generated for each polyphenol (Fig. 3A, and 3B).

Interestingly, although modified products were detected for both quercetin and rutin under these experimental conditions, the ratios of the products formed differed between the polyphenols. With regard to the reaction of quercetin (150 \( \mu \text{M} \)) with HOCl (50 \( \mu \text{M} \)), the peak with a retention time of 5.6 min (unmodified quercetin) contained ~60 \( \mu \text{M} \) of unmodified quercetin, whereas the peaks at 6.6 and 7.9 min (new products) resulted in concentrations of ~20 and ~6 \( \mu \text{M} \) (relative to quercetin standards), respectively (Fig. 3A). In contrast, only about 45 \( \mu \text{M} \) rutin remained of the initial 150 \( \mu \text{M} \) after reaction with HOCl (50 \( \mu \text{M} \)), whereas the peaks of the two new products with retention times of 3.6 and 4.5 min resulted in concentrations of ~45 and ~17 \( \mu \text{M} \) (relative to rutin standards), respectively (Fig. 3B).

To confirm that the peaks detected in the rutin assay were due to the quercetin and not the associated disaccharide, the mixture was treated with the enzyme naringinase to convert rutin to the aglycone, quercetin. As shown in Fig.
3C, the enzyme clearly shifted the three peaks to the right, which resulted in retention times of 5.5, 6.6, and 7.9 min, which corresponded to the retention times for quercetin and its two related new products. When we used unmodified quercetin as a standard, the areas of the three peaks corresponded to concentrations of about 46, 46, and 13 μM, which are very similar to the concentrations predicted for the original rutin forms (45, 45, and 17 μM, see previous results). Thus, the new products observed for rutin seemed to be exclusively due to reactions of HOCl with the quercetin component of the molecule.

Monochlorination of Rutin and Quercetin

In an additional series of experiments, the resulting polyphenol-HOCl reaction products were further characterized by MS. The eluent of the peaks that resulted from the reaction mixtures of 150 μM of quercetin or rutin with 50 μM HOCl was collected and subjected to analysis by HPLC-MS. The analysis by MS showed an [M-H]\(^{-}\) molecular ion of mass to charge ratio (\(m/z\)) of 301 for unmodified quercetin (Fig. 4A, Table 1) and 609 for unmodified rutin (Fig. 5A, Table 1).

Reaction of quercetin and rutin resulted in the formation of new products with slightly longer retention times of 6.6 min and of 3.6 min, which were determined to have an \(m/z\) value of 335 and 643, respectively (Figs. 4B and 5B, Table 1). The addition of an \(m/z\) value of 34 units indicates the addition of chlorine to each of the two flavonols, which corresponded to a monochlorinated quercetin (quer-Cl\(_1\)) and a monochlorinated rutin (rutin-Cl\(_1\)). The 335/337 and 643/645 ratios are characteristic of the isotopes of chlorine and help confirm the presence of a single chlorine. The second series of resultant peaks had even longer retention times of 7.9 and 4.4 min and \(m/z\) values of 369 and 677, respectively. These \(m/z\) values and increased retention times are consistent with the formation of dichlorinated forms of quercetin (quer-Cl\(_2\)) and rutin (rutin-Cl\(_2\); Figs. 4C and 5C, Table 1). The 369/371 and 677/679 ratios also confirm the presence of two chlorines.

In additional studies, the reaction mixture of 150 μM rutin with 50 μM HOCl was treated with naringinase, and the resulting aglycones were subjected to HPLC-MS. As anticipated, the \(m/z\) values for the three components matched those of quercetin, quer-Cl\(_1\), and quer-Cl\(_2\) (data not shown), which supported the contention that the new products for reaction of rutin with HOCl are exclusively based on reactions of HOCl with the quercetin part of the molecule.

A modification of the aromatic structure of the flavonoids should influence the absorption pattern as well on the extinction coefficient. Indeed, the mono- and dichlorination of quercetin and rutin slightly altered the ultraviolet-absorption spectra under the acidic conditions of the HPLC solvent. As shown in Table 1, the single chlorination in-
increased the absorption maximum by about 5 nm, whereas the dichlorination caused an additional 3 to 4 nm shift.

To define the sites of chlorination on the quercetin molecule, about 125 \( \mu \)g of quercetin as well as its mono- and dichlorinated forms was fractionated by HPLC and then analyzed by \(^1\)H NMR. As shown in Fig. 6A, the unmodified quercetin gave clear signals for its five protons at the \( \text{C}_2', \text{C}_5', \) and \( \text{C}_6' \) site of the B-ring as well as at the \( \text{C}_6 \) and \( \text{C}_8 \) sites of the A-ring (see Fig. 1A and 1B). The monochlorinated quercetin showed only a singlet at 6.35\( \delta \) instead of the meta-coupled pair of doublet signals at 6.17\( \delta \) and 6.37\( \delta \) for quercetin, which indicated that this quercetin form was chlorinated at the \( \text{C}_6 \) site of ring A (Fig. 6B). The aromatic region of the spectrum of the dichlorinated quercetin showed only signals assigned to hydrogens at the B-ring, which indicated that chlorination took place at the \( \text{C}_6 \) and \( \text{C}_8 \) sites of ring A exclusively (Fig. 6C). Thus, the reactions of quercetin with hypochlorous acid resulted in the formation of the two new molecules 6-chloroquercetin and 6,8-dichloroquercetin.

Fig. 4. Mass spectra of the reaction products formed between quercetin and HOCl. Quercetin (150 \( \mu \)M) was reacted with HOCl (50 \( \mu \)M), and the reaction products were subjected to reverse-phase HPLC-MS analysis by using electrospray ionization MS. During each HPLC run, single peaks (see Fig. 3A) were isolated by collecting the eluent and analyzed by electrospray ionization mass spectrometry. A, B, and C are the negative ion spectra for the three components of the quercetin assay (A, quercetin; B, new product 1; C, new product 2). The increase in the m/z value of 34 indicates that the new product 1 is a monochlorinated derivate of the native molecule, whereas the increase in the m/z value of 68 indicates that the new product 2 is a dichlorinated derivative.

The Chlorination of Quercetin Enhances the Inhibition of LDL Oxidation

Because chlorination of quercetin could alter its capacity as an antioxidant, the antioxidant effects of quercetin and its chlorinated derivatives were determined in a copper-mediated LDL oxidation system. Figure 7A shows representative time courses for copper-dependent LDL oxidation in the presence of increasing amounts of quercetin. Similar time courses were observed with either the monoor dichlorinated quercetin derivative. A typical time course for oxidation consists of an initial lag phase followed by a propagation phase and finally termination (Patel et al., 1997). As shown in Fig. 7, quercetin inhibited copper-dependent LDL oxidation in a dose-dependent manner, as indicated by an increase in lag time of oxidation (indicated by the right shift in the oxidation curve), which is consistent with previous studies (Brown et al., 1998). The antioxidant potency of quercetin and its chlorinated derivatives was compared by measuring the increase in lag times that resulted from the unmodified and HPLC purified mono- and dichlorinated products (0.5 \( \mu \)M; Fig. 7B). Both the mono- and dichlorinated derivatives were markedly more effective (~2- to 3-fold) at inhibiting copper-mediated LDL oxidation than unmodified quercetin.

Chlorination of Quercetin Increases the Total Radical Antioxidant Potential

Additional studies were conducted by using a modified total radical antioxidant potential assay to determine antioxidant capacity, because copper-induced LDL oxidation potentially could be effected by the ability of quercetin to act as a metal chelator (Ferrali et al., 1997; Fremont et al., 1999). A 100 \( \mu \)M quercetin solution exhibited a Trolox equivalent antioxidant capacity of 330 \( \pm \) 10 \( \mu \)M, which suggested that unmodified quercetin was ~3.3 times more potent an antioxidant than Trolox, on a molar basis (see Fig. 8). The monochlorinated quercetin derivative was significantly (\( p < 0.05 \)) more effective at inhibiting formation of ABTS*\(^+\) radical than unmodified quercetin, with a Trolox equivalency of 580 \( \pm \) 2 \( \mu \)M. The dichloroquercetin was significantly more effective than either unmodified or monochlorinated quercetin with a Trolox equivalency of 607 \( \pm \) 5 \( \mu \)M. The enhanced antioxidant potentials of the chlorinated derivatives (1.8- to 1.9-fold) are similar to those
observed with copper-induced lipid peroxidation, which suggests that chlorination results in derivatives with enhanced antioxidant activity.

**DISCUSSION**

The present studies demonstrated that the polyphenols, quercetin and its glycoside derivative rutin, could react with HOCl to form modified chlorinated products. Mass spectrometric analysis of the HPLC purified peaks indicated that the products represented mono- and dichloro- derivatives of quercetin and rutin. Analysis of the HPLC purified products by $^1$H NMR indicated that monochlorinated quercetin derivative was modified at the C$_6$ site of the A-ring (6-chloroquercetin), whereas chlorination occurred at the C$_6$ and C$_8$ sites in the dichlorinated derivative (6,8-dichloroquercetin). Chlorination of quercetin also increased the antioxidant capacity of the molecule with formation, the mono- and dichloro- products being two to three times more effective at inhibiting LDL oxidation and ABTS$^{+\cdot}$ radical than quercetin. In summary, these studies used a combination of analytical techniques to demonstrate that stable mono- and dichlorinated derivatives of quercetin and rutin were formed on reaction with HOCl and that the chlorination may have physiological consequences with regard to enhanced antioxidant capacity.

The observed chlorination of the red wine polyphenols, quercetin and rutin, are consistent with previous studies in which it was demonstrated that the phenolic compound 4-hydroxyphenylacetic acid (Eiserich et al., 1998), as well as the soy bean isoflavones genistein and daidzein, can be chlorinated in reactions with HOCl (Boersma et al., 1999). These in vitro data suggest that chlorination of other phenolic and polyphenolic components of red wine may occur in vivo. It has been estimated that polyphenols are present in plasma at concentrations of 0.2 to 2 μM (Croft, 1998; Manach et al., 1998; Paganga and Rice-Evans, 1997), whereas feeding rats a quercetin-augmented diet could increase the plasma levels of quercetin and its metabolites up to 10 to 100 μM (Morand et al., 1998). The concentrations of quercetin and rutin used in this study were from 1.5 to 150 μM and may be in the high end of the physiological range, although there are limited data available. The present study suggests that the extent of chlorination of the flavonols depends more on the ratio of flavonols and HOCl rather than the actual concentration of the reactants. There are insufficient data to allow estimation of the HOCl production rates in tissues (e.g., MPO-containing atherosclerotic plaques; Daugherty et al., 1994), but PMNs are normally present in the human blood at a titer of ~$1 \times 10^6$ cells/ml and on stimulation show an MPO activity that can generate about 1 μM HOCl per min (Eiserich et al., 1998). Thus, it can be speculated that at physiological concentration of flavonols in the plasma (e.g., nM), the increased production of HOCl (e.g., at local sites of inflammation) could lead to the formation of chlorinated derivatives.

It was important to analyze rutin in parallel with quercetin—not only because rutin is present in red wine at levels similar to quercetin (Goldberg et al., 1996), but because it is generally assumed that quercetin circulates in human plasma in a conjugated form (Manach et al., 1998; Paganga and Rice-Evans, 1997). Definitive data to confirm that quercetin derivatives are present in human plasma are not currently available. However, experiments with rats have shown that quercetin is present in the plasma as a conjugate linked to gluconic acid (Morand et al., 1998), although the extent and sites of the gluconidation have not been elucidated. Glycosylation at site 3 of the C-ring (Fig. 1) is common to many natural quercetin derivatives, so rutin serves as a good model of the chlorination events for glucuronidated and/or glycosylated quercetin. More-

**Table 1. Formation of Chlorinated Polyphenols by HOCl**

<table>
<thead>
<tr>
<th>Flavonol</th>
<th>Retention time (min)</th>
<th>Maximum absorption (nm)</th>
<th>Mass-to-Charge ratio (m/z)</th>
</tr>
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<tbody>
<tr>
<td>Quercetin</td>
<td>5.6</td>
<td>256/372</td>
<td>301</td>
</tr>
<tr>
<td>Quercetin-Cl$_1$</td>
<td>6.6</td>
<td>259/377</td>
<td>335</td>
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<td>Quercetin-Cl$_2$</td>
<td>7.9</td>
<td>263/380</td>
<td>369</td>
</tr>
<tr>
<td>Rutin</td>
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<td>257/356</td>
<td>609</td>
</tr>
<tr>
<td>Rutin-Cl$_1$</td>
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<td>263/361</td>
<td>643</td>
</tr>
<tr>
<td>Rutin-Cl$_2$</td>
<td>4.5</td>
<td>266/362</td>
<td>677</td>
</tr>
</tbody>
</table>

**Fig. 5.** Mass spectra of the reaction products formed between rutin and HOCl. Rutin (150 μM) was reacted with HOCl (50 μM), and the reaction products were subjected to reverse-phase HPLC-MS analysis by using electrospray ionization MS. Single peaks were isolated by collecting the eluent of each HPLC run and were analyzed by electrospray ionization MS. A, B, and C are the negative ion spectra for the three components of the rutin assay (A, rutin; B, new product 1; C, new product 2). The increase in the m/z value of 34 indicates that the new product 1 is a monochlorinated derivate of the native molecule, whereas the increase in the m/z value of 68 indicates that the new product 2 is a dichlorinated derivative.
over, there is evidence that rutin exists in human plasma (Paganga and Rice-Evans, 1997).

It is interesting that rutin reacted with HOCl in a manner similar to quercetin, which suggests that conjugation, at least at the C₃ site of the C-ring (Fig. 1 A and 1C), does not interfere significantly with the chlorination reaction. This allows speculation that all quercetin derivatives with a similar structure (e.g., isoquercitrin [quercetin-3-glucoside], hyperoside [quercetin-3-galactoside]) also can undergo chlorination to form mono- and dichlorinated derivatives exclusively at the aglycone. Although both quercetin and rutin form similar mono- and dichlorinated derivatives (Figs. 2 and 3), the reaction of rutin with HOCl typically resulted in the formation of more chlorinated products, even twice as much under certain conditions. The increased formation of chlorinated products may be attributed to the ability of the disaccharide to enhance the turnover rate during the chlorination reaction or a protective influence of the disaccharide against secondary reactions in which the primary chlorination products are destroyed.

Chlorination of quercetin by HOCl conceivably could occur at five different sites at the molecule: at the C₂', C₅', and C₆' site of the B-ring as well as at the C₆ and C₈ sites of the A-ring (Fig. 1). However, the observation that only one mono- and one dichlorinated form of quercetin and rutin were detected by HPLC and MS made it reasonable to assume that only two sites of the quercetin molecule were chlorinated. This contention was supported by the NMR studies, which clearly showed that chlorination occurred only at the C₆ and C₈ site of the A-ring (Fig. 1A). These findings are consistent with the observations of Divi and Doerge (1996) on the iodination of the isoflavone, biochanin A, which is identical to quercetin except for the
then is limited to the C8 site. Based on these findings, we can first before a second chlorination event can take place, which genistein derivatives. Interestingly, although both the C6 and quercetin (Q-Cl2). Quercetin was approximately 3.3 times more potent that Trolox, whereas the mono- and dichlorinated derivatives were 5.8 and 6.1 times more potent, respectively, than Trolox on a molar basis. Control vehicle experiments demonstrated that methanol exhibited no demonstrable effect on antioxidant capacity. Values are expressed as mean ± SEM. *p < 0.05 vs. quercetin.

C3' site (no hydroxyl group) and the C4' site (–OCH3 instead of –OH) of the B-ring. In a thyroid peroxidase-catalyzed reaction with H2O2 and I−, iodination occurred at only three sites: C6, C8, and C3'. Our data are also consistent with previous data (Boersma et al., 1999) which suggest that the isoflavone, genistein, was chlorinated by HOCl at C6 and C8 to form mono- and dichlorinated genistein derivatives. Interestingly, although both the C6 and C8 sites of quercetin can be chlorinated, monochlorinated quercetin showed chlorination only at the C6 site of the A-ring. This suggests that 6-chloroquercetin must be formed first before a second chlorination event can take place, which is limited to the C8 site. Based on these findings, we can postulate that the chlorinated rutins are formed in a similar manner, which result in 6-chloroquercetin-rutinoside and 6,8-dichloroquercetin-rutinoside.

The antioxidant behavior of flavonoids and flavonols generally is assumed to be related to the structure of the compound, and it also is assumed that the antioxidant capacity increases as a function of the number of hydroxyl groups and available H atoms (Cao et al., 1997). Several studies have shown that the o-dihydroxyl structure in the B-ring of quercetin (Fig. 1B) is required to exert maximum free radical scavenging activity and is also important for Cu2+ chelation (Brown et al., 1998; Yamamoto et al., 1999). By using copper oxidation of LDL as a model system, we investigated the effects that chlorination of quercetin has on the antioxidant properties of this flavonol. On a molar basis, the mono- and dichlorinated quercetin derivatives were more effective (approximately 2- to 3-fold) at inhibiting copper-mediated LDL oxidation than the unmodified form. Although the precise mechanism for the enhanced antioxidant effect is unknown, it could involve both copper chelation and hydrogen atom donation. The chlorinated derivatives of quercetin also exhibited an enhanced antioxidant capacity when we used a method designed to assess total radical antioxidant potential. Thus, chlorination of the polyphenols, and perhaps the conjugated derivatives, may markedly enhance their antioxidant capacity in vivo and increase the likelihood that nanomolar levels of the polyphenolics would be of physiological relevance.

Additional cytoprotective mechanisms are possible in vivo. Quercetin is recognized as a phytoestrogen, based on its structural similarity to estradiol (Fig. 1B and 1D). It has been reported that quercetin can bind by both estrogen receptors (ER-α, ER-β) and induce estrogen-dependent processes; however, quercetin is not very potent in this regard (Kuiper et al., 1998). These results may be due, at least in part, to the experimental conditions and models selected (Davis et al., 1999). It is also possible that unmodified quercetin will not bind the estrogen receptor with high affinity, whereas the chlorinated derivatives will. This concept is supported by the observation that the catechol metabolites of selected polychlorinated biphenyls exhibited in vitro estrogenicity, which was dependent on the extent as well as on the position of the chlorination (Garner et al., 1999). Thus, if chlorinated derivatives of quercetin, as well as other red wine polyphenols, can occur in vivo and can mimic the effects of estrogens, these components might play a pivotal role in reducing the risk of cardiovascular disease that is associated with consumption of moderate levels of red wine.

**REFERENCES**


