CAFFEIC ACID, CHLOROGENIC ACID, AND DIHYDROCAFFEIC ACID METABOLISM: GLUTATHIONE CONJUGATE FORMATION

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ABSTRACT:

The antioxidant properties of the dietary dihydroxycinnamic acids [caffeic (CA), dihydrocaffeic (DHCA), and chlorogenic (CGA) acids] have been well studied but little is known about their metabolism. In this article, evidence is presented showing that CA, DHCA, and CGA form quinoids and hydroxylated products when oxidized by peroxidase/H₂O₂ or tyrosinase/O₂. Mass spectrometry analyses of the metabolites formed with peroxidase/H₂O₂/glutathione (GSH) revealed that mono- and bi-glutathione conjugates were formed for all three compounds except CGA, which formed a bi-glutathione conjugate only when GSH was present. In contrast, the metabolism of the dihydroxycinnamic acids by tyrosinase/O₂/GSH resulted in the formation of only mono-glutathione conjugates. In the absence of GSH, hydroxylated products and p-quinones of CA or CGA were formed by peroxidase/H₂O₂. DHCA formed a hydroxylated adduct (even though GSH was present), as well as the corresponding p-quinone and dihydroescoolitin, an intramolecular cyclization product. NADPH also supported rat liver microsomal-catalyzed CA-, CGA-, and DHCA-glutathione conjugate formation, which was prevented by benzylimidazole, a cytochrome P450 inhibitor. Furthermore, the cytotoxicity of CA, CGA, and DHCA toward isolated rat hepatocytes was markedly enhanced by hydrogen peroxide or cumene hydroperoxide-supported cytochrome P450 and was prevented by benzyl-midazole. Cytotoxicity was also markedly enhanced by dicumarol, an NADPH/oxidoreductase inhibitor. These results suggest that dihydroxycinnamic acids were metabolically activated by P450 peroxidase activity to form cytotoxic quinoid metabolites.

Chlorogenic acid (CGA)¹, caffeic acid (CA), and dihydrocaffeic acid (DHCA) are nonflavonoid catecholic compounds, which are present in many plants (Fig. 1). These chemicals are present in the diet as part of fruits, tea, coffee, and wine (Buren et al., 1973; Challis and Bartlett, 1975). There is growing interest in the multiple biological and pharmacological properties of nonflavonoid catecholic compounds, such as CA, CA, and DHCA (dihydroxycinnamic acids) (Laranjinha et al., 1994). It has been reported that these catecholic acids have anti-inflammatory, antimutagenic, and anticarcinogenic activities (Challis and Bartlett, 1975; Koshihara et al., 1984; Tanaka et al., 1993a,b). The focus of much of the current research is on their cancer chemoprevention and antioxidant properties (Challis and Bartlett, 1975; Koshihara et al., 1984; Tanaka et al., 1993a,b; Laranjinha et al., 1995; Nardini et al., 1995). However, there is little information available in the literature on the enzymatic oxidation of CA, CA, and DHCA.

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¹ Abbreviations used are: CGA, chlorogenic acid; CA, caffeic acid; DHCA, dihydrocaffeic acid; P450, cytochrome P450; HRP, horseradish peroxidase type I; HPLC, high-performance liquid chromatography; GSH, glutathione; DETAPAC, diethylenetriaminepentaacetic acid; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); UV-VIS, UV-visible; GSSG, glutathione disulfide (oxidized glutathione); NQO, NADPH/quinone oxidoreductase.

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An example of enzyme-directed antimelanoma therapy includes the bioactivation of a phenolic or a catecholic agent to an o-quinone by tyrosinase-containing tumor cells (Riley et al., 1997). An agent that can undergo tyrosinase-mediated oxidation to generate a cytotoxic reactive intermediate species, such as o-quinone, but is not metabolically activated by P450-rich-containing tissues, such as liver or kidneys, would be an ideal candidate for an antimelanoma therapy trial. In search of such a candidate we have investigated the enzymatic oxidation of dihydroxycinnamic acids by three different metabolizing systems, i.e., horseradish peroxidase type I (HRP)/H₂O₂ and tyrosinase/O₂ or rat liver microsomal P450/NADPH systems.

We previously used tandem mass spectrometry, HPLC, and UV spectroscopy to show that quercetin and other flavonoid compounds...
formed glutathione conjugates by HRP/H₂O₂ and tyrosinase/O₂ (Galati et al., 1999, 2001). In this article, we have used similar techniques to show that CA, DHCA, and CAs underwent an enzymatic oxidation by HRP/H₂O₂ or tyrosinase/O₂ to form a transient o-quinone intermediate that formed a conjugate with glutathione or underwent hydroxylation by H₂O₂. Further oxidation of this hydroxylated adduct by O₂ formed a p-quinone.

Glutathione conjugate formation also occurred when these dihydroxyccinnamic acids were metabolized by an NADPH/rat liver microsomes system. Conjugate formation was prevented by benzylimidazole, a cytochrome P450 inhibitor. Dicumarol (an NADPH/oxidoreductase inhibitor) increased hepatocyte cytotoxicity induced by these dihydroxyccinnamic acids, whereas benzylimidazole prevented cytotoxicity. This suggests that dihydroxyccinnamic acids were metabolically activated by cytochrome P450 to form cytotoxic-reactive quinoid intermediates.

### Materials and Methods

**Chemicals.** CGA, DHCA, CA, mushroom tyrosinase, horseradish peroxidase, hydrogen peroxide, magnesium chloride, potassium borohydride, ethyl acetate, NADP⁺, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, glucose, glucose oxidase, cumene hydroperoxide, sodium azide, sodium periodate, benzylimidazole, glutathione (reduced), and 5,5'-dithio-bis(2-nitrobenzoic acid), dimethyl sulfoxide, trichloroacetic acid (DETAPAC), potassium phosphate monobasic, potassium phosphate dibasic, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, glucose oxidase, ascorbic acid, NADPH, and sodium periodate. All concentrations shown were obtained from Sigma-Aldrich (Oakville, ON, Canada). The stock solutions of dihydroxyccinnamic acids were prepared in dimethyl sulfoxide. Other chemicals were prepared in Millipore filtered water (Millipore Corporation, Bedford, MA) or buffer. The stock solution of DTNB was dissolved in Tris/HCl buffer (0.1 M, pH 8.9, containing 1 mM DETAPAC). All concentrations shown were final concentrations.

**Preparation of 4-Propylcatechol.** 4-Propylcatechol was prepared according to the method described by Bolton et al. (1994) and Iverson et al. (1995).

**Oxidation Studies by UV-VIS Spectroscopy.** The spectra of solutions containing 50 to 100 μM CGA, DHCA, and CA were recorded in the absence and presence of glutathione before or after the addition of HRP (0.1 or 3 mM H₂O₂ (50 μM) or tyrosinase (25 units/ml) in a potassium phosphate buffer (0.1 M, pH 7.4, containing 1 mM DETAPAC). All reactions were performed and used at room temperature.

**α-Quinone Stability Kinetics.** Sodium periodate (250 μM) was added to a solution of CA, DHCA, or CGA (250 μM) in 3 ml of sodium phosphate buffer at various pH (4.0, 5.0, 6.0, or 7.4). The absorbance was monitored at 420 nm over a period of 20 min.

**Dihydroxyccinnamic Acids Oxidation Kinetics.** The rate of oxygen consumption was measured for auto-oxidation in a sealed chamber containing dihydroxyccinnamic acids (500 mM) in phosphate buffer (0.1 M, pH 7.4, containing 1 mM DETAPAC) by a Clark-type O₂ electrode. The initial oxygen concentration was determined as 224 nmol/ml at standard temperature and pressure (Umbricht et al., 1964).

For tyrosinase enzymatic-mediated oxidation rate measurement, tyrosinase (25 units/ml) was added to a solution of dihydroxyccinnamic acids (250 μM) in the presence or absence of GSH (1 mM) in phosphate buffer (0.1 M, pH 7.4, containing 1 mM DETAPAC), and the rate of oxygen consumption was measured by a Clark type O₂ electrode.

The rate of α-quinone formation was followed spectrophotometrically at 420 nm for chemical oxidation (by H₂O₂ or cumene hydroperoxide) or HRP/H₂O₂ enzymatic-mediated oxidation of dihydroxyccinnamic acids. Hydrogen peroxide (2 mM) or HRP (0.01 μM/H₂O₂ (500 μM) was added to a solution of dihydroxyccinnamic acid (250 μM) in phosphate buffer (0.1 M, pH 7.4, containing 1 mM DETAPAC). Cumene hydroperoxide (130 μM) was added to a solution of dihydroxyccinnamic acid (250 μM) in methanol (20%, v/v) phosphate buffer (0.1 M, pH 7.4, containing 1 mM DETAPAC). The absorbance of the aqueous phase before and after the addition of HRP (0.1 or 3 mM H₂O₂ (50 μM) or tyrosinase (25 units/ml)) was added to a solution of dihydroxyccinnamic acids (250 μM) in 1 ml of sodium phosphate buffer (0.1 M, pH 7.4, containing 1 mM DETAPAC). All reactions were carried out at room temperature.

The distribution coefficient value of the neutral dihydroxyccinnamic acid fraction between the two phases was calculated from

$$D_{p} = \frac{A_{1} - A_{2}}{A_{2}}$$

where Dₚ is the distribution coefficient value at pH 4.0. A₁ and A₂ are the absorbance of the aqueous phase before and after the addition of 1-octanol, respectively. The absorbance of the aqueous and 1-octanol phases, respectively, Log P was calculated from

$$P = \frac{D_{p}}{1}$$

where P, A₁, and A₂ are partition coefficient, degree of ionization at pH 4.0, and distribution coefficient at pH 4.0, respectively.

**Glutathione Depletion Assay.** HRP (0.1 μM) and H₂O₂ (50 μM) were added to a mixture containing dihydroxyccinnamic acids (50 μM) and glutathione (200 μM) in 1 ml of sodium phosphate buffer (0.1 M, pH 4.0, containing 1 mM DETAPAC). The mixture was preincubated for 30 min from which 250 μl was added to 25 μl of 30% (w/v) trichloroacetic acid, vortexed, and left for 5 min. An aliquot of 100 μl of the supernatant was added to a solution containing 25 μl of 2 mg/ml DTNB (prepared in Tris/HCl buffer, 0.1 M, pH 8.9) and 875 μl of Tris/HCl buffer (0.1 M, pH 8.9) and vortexed. The absorbances of the solutions were monitored at 412 nm for CA and DHCA and at 460 nm for CGA. The glutathione depletion assay for CA, DHCA, and CA was also carried out with tyrosinase (20 units/ml) at pH 4.0.

**Mass Spectrometry Analyses.** The reaction mixtures contained CA, CGA, or DHCA (1 mM) and GSH (4 mM) in 1 ml of Millipore filtered water which was added HRP (3 μM/H₂O₂ (2 mM) or tyrosinase (25 units/ml). The reactions were incubated for 5 min at room temperature prior to direct injection into a mass spectrometer (PE Sciex III, Biomolecular Mass; PE Sciex, Toronto, ON, Canada). Mass spectrometry analysis was also performed when GSH was added to the reaction mixture 5 min after HRP/H₂O₂ or tyrosinase addition.

**Glutathione Conjugate Formation by Rat Hepatocyte Microsomes.** Adult male Sprague-Dawley rats, 250 to 300 g, were obtained from Charles River Canada Laboratories (Montreal, QC, Canada), fed ad libitum, and allowed to acclimatize for 1 week on clay chip bedding. The animals were anesthetized by sodium pentobarbital (60 mg/kg of body weight). Livers were removed under sterile potassium buffer/KCl solution (1.18%, w/v, 4°C) as previously described (Anari et al., 1997a). Hepatic microsomes were prepared as described by Dullner (1978).

Glucose 6-phosphate (7.5 mM) was added to a mixture of the test compound (0.5 mM), NADP⁺ (0.5 mM), magnesium chloride (5 mM), microsomes (1 mg/ml), and glucose-6-phosphate dehydrogenase (2.5 units/ml) in 1 ml Tris/HCl buffer (0.1 M, pH 7.4, containing 1 mM DETAPAC). GSH concentration was 0.5 mM when it was present. The mixture was preincubated at 37°C from which 250-μl samples were taken at 30 and 60 min and added to 25 μl of 30% (w/v) trichloroacetic acid, vortexed, left for 5 min, and centrifuged to discard the protein pellet. An aliquot of 62.5 μl of 2 mg/ml DTNB (prepared in Tris/HCl buffer, pH 8.94) was added to 100 μl of the supernatant. The absorbance was monitored at 412 nm for CA and DHCA and at 460 nm for CGA. The assay was repeated in the presence of benzylimidazole (300 μM final concentration) as a P450 inhibitor.

**Isolated Rat Hepatocyte Cytotoxicity Studies.** Hepatocytes (10 μl) (10⁵ cells/ml) were preincubated at 37°C under an atmosphere of 95% O₂ and 5% CO₂ in Krebs-Henseleit buffer, pH 7.4, with CA (5 mM), 4-propylcatechol (0.3 mM), dihydrocaffeic acid (5 mM), and chlorogenic acid (5 mM). Where the following inhibitors were used: 100 μM benzylimidazole (a P450 inhibitor; Quan et al., 1992), 20 μM dicumarol (an NADPH/quinone oxidoreductase inhibitor; Preusch et al., 1991), 4 mM sodium azide (a catalase inhibitor), and 200 μM bromoheptane (a GSH-depleting agent; Khan and O’Brien, 1991). Catalytic agents used included hydrogen peroxide (10 mM glucose/1 unit/ml glucose oxidase) or cumene hydroperoxide (130 μM), or tyrosinase (100 units/ml) in the presence or absence of benzylimidazole (100 μM). Cell viability at 60, 120, and 180 min of incubation was determined by trypan blue uptake. None of the inhibitors or catalytic agents affected the viability of control hepatocytes at the concentrations used. Three separate experiments were carried out. Values shown are means ± S.E.
The rate of hydrogen peroxide generated by glucose/glucose oxidase system was determined using a Clark type oxygen electrode.

**HPLC Analysis of GSH and GSSG Contents of Isolated Rat Hepatocytes.** A modified method reported by Reed et al. (1980) was used for the HPLC analysis of GSH and GSSG. An 800-μl aliquot of the isolated rat hepatocytes reaction mixture was added to 200 μl of 25% w/v metaphosphoric acid in a glass tube, vortexed, left for 30 min at room temperature, and centrifuged. A 500-μl aliquot of the supernatant and 50 μl of 15 mg/ml freshly prepared iodoacetic acid in water were co-transferred to a glass tube containing sodium bicarbonate (100–200 mg), vortexed, left up to 1 h or overnight at room temperature in a dark room. To this was then added 500 μl of 5% w/v 2,4-dinitro-fluorobenzene (prepared in ethanol), which was vortexed and left to stand at room temperature in a dark room for a period of 4 to 6 h for immediate HPLC processes.

An autoinjector (WISP 710B; Waters Scientific Ltd., Milford, MA) was used to inject a 50-μl sample of the reaction mixture into an HPLC column (μBondapak NH₂, aminopropylsilyl bonded amorphous silica) 125Å, 10 μM, 3.9 × 300 mm; Waters Scientific Ltd.). The gradient mobile phase used to elute the sample comprised two solvent systems: solvent A, methanol/water 80:20; and solvent B, methanol/acetic buffer 80:20. Acetic buffer was prepared by the addition of sodium acetate trihydrate (270 g) and acetic acid glacial (378 ml) to Millipore water (128 ml). The HPLC pump (model 501; Waters Scientific Ltd.) was programmed at a flow rate of 1 ml/min for the mobile phase A/B with ratio of 90:10 (0 min), 10:90 (25 min), 90:10 (27 min), and 90:10 (30 min). The LC spectrophotometer (Lambada-Max, model 481; Waters Scientific Ltd.) was set at 365 nm to detect GSH and GSSG, with retention times of 17.5 and 20.5 min, respectively. Software (Maxima 820 chromatography workstation version 3.30; Dynamic Solutions, a division of Millipore Corporation) and an IBM compatible computer were used for analysis of the data and integration.

**Results**

**Oxidation Studies by UV-VIS Spectroscopy.** There were varying rates of oxidation for dihydroxycinnamic acids by HRP/H₂O₂ or tyrosinase/O₂-oxidizing systems (Table 1). DHCA was found to possess the lowest rate of oxidation by HRP/H₂O₂ but had the fastest rate of oxidation by tyrosinase and oxygen. CGA and CA, on the other hand, were rapidly oxidized by HRP/H₂O₂ than tyrosinase/O₂.

**UV-VIS spectroscopy of the CGA solution showed a distinct peak at 325 nm with a characteristic shoulder at 295 nm, whereas CA had a double peak at 285 and 310 nm. DHCA, which lacked the double bond in its side chain, demonstrated a single peak at 280 nm (Table 2).** In the presence of HRP/H₂O₂ or tyrosinase/O₂ at pH 4.0, CA, CGA, and DHCA formed a new peak at 420 nm that was extractable with ethyl acetate. In addition, CA and CGA showed a large peak at 250 nm with a shoulder at 260 nm, whereas DHCA displayed an increase in its peak absorbance at 280 nm. CGA, CA, and DHCA oxidation by HRP/H₂O₂ formed a product at 260 nm that was extractable with ethyl acetate and was tentatively identified as p-quinone. The conversion of the o-quinone to the corresponding p-quinone was increased at higher H₂O₂ concentrations (Fig. 2).

Addition of sodium borohydride to a solution mixture containing CA, CGA, or DHCA with HRP/H₂O₂ or tyrosinase/O₂, or sodium periodate, reduced the quinones formed. A slight shift of the absorbance maxima to the bathochromic region (a red shift) after metabolism by enzymatic systems and reduction by excess borohydride in comparison with the absorbance maxima of CA, CGA, or DHCA before metabolism probably resulted from the addition of a third hydroxyl group to the aromatic ring (Rinaldi et al., 1995). This red shift in the spectra increased as the pH was increased from 4.0 to 7.4.

**Addition of glutathione after 5 min to the mixture of CA or CGA and HRP/H₂O₂ resulted in the development of a new peak at 275 nm with significant loss in the absorbance of the 250-, 260-, and 420-nm peaks. The 275-nm product was not extractable with ethyl acetate (Table 2). Addition of glutathione after 5 min to DHCA and HRP/ H₂O₂ decreased the absorbance of the 420-nm peak but instead two new peaks at 255 and 490 nm were formed.** Similar spectral changes occurred if GSH was present at the beginning of the metabolism reaction.

**Summary of UV spectra for the dihydroxycinnamic acids oxidation by HRP/H₂O₂ or tyrosinase/O₂ or sodium periodate and their glutathione conjugates**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Extinction Coefficient</th>
<th>Parent Compound</th>
<th>o-Quinone</th>
<th>p-Quinone</th>
<th>HO-Product</th>
<th>GSH Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>ε₂₅₀ = 19,510</td>
<td>285, 310</td>
<td>250, 420</td>
<td>260sh, 490</td>
<td>295, 325</td>
<td>275</td>
</tr>
<tr>
<td>CGA</td>
<td>ε₂₅₀ = 19,680</td>
<td>295, 325</td>
<td>250, 420</td>
<td>260sh, 490</td>
<td>300, 345</td>
<td>275</td>
</tr>
<tr>
<td>DHCA</td>
<td>ε₂₅₀ = 5,895</td>
<td>280</td>
<td>420</td>
<td>490</td>
<td>285</td>
<td>290</td>
</tr>
</tbody>
</table>

Spectra data are given in nanometers at pH 7.4.
**o-Quinone Stability Kinetics.** The stability of the o-quinones formed was found to be related to the pH and followed first order kinetics. The $t_{1/2}$ stability of the o-quinones formed by each compound decreased with an increase in pH. As shown in Table 1, the $t_{1/2}$ of o-quinone stability in a decreasing order was CA > DHCA > CGA. The CA o-quinone formed was slightly more stable than the DHCA o-quinone and was almost twice as stable as the CGA o-quinone at pH 7.4.

**Dihydroxycinnamic Acids Oxidation Kinetics.** As shown in Table 1, the rate of oxidation of dihydroxycinnamic acids by HRP/H$_2$O$_2$ was CA, CGA >> DHCA. Negligible oxidation (<1 nmol/ml/min) occurred by H$_2$O$_2$ in the absence of HRP. The rate of oxidation of dihydroxycinnamic acids by tyrosinase/O$_2$ in the presence of GSH was DHCA > CGA > CA. Negligible oxidation (<1 nmol/ml/min) occurred in the absence of tyrosinase.

**Distribution Coefficient Value.** The distribution coefficient measurements for CA, CGA, and DHCA were carried out at pH 4.0 (Table 3). The distribution coefficient value for CGA (50 μM) at pH 4.0 in a mutually presaturated phosphate buffer and 1-octanol solvent system was 1.1 ± 0.1, which means that CGA distributes equally between water and 1-octanol at pH 4.0. The distribution coefficient was higher for DHCA at pH 4.0, which was 3.0 ± 0.4. DHCA, therefore, partitions more into the organic phase than the aqueous phase at pH 4.0. CA with a distribution coefficient value of 14.0 ± 0.1 at pH 4.0, demonstrated a higher organic phase solubility than DHCA or CGA. The distribution coefficients of CA and CGA at pH 7.4 were lower than at pH 4.0.

**Graph:**

![Graph](image-url)

**Fig. 2. Dihydrocaffeic acid metabolism by HRP/H$_2$O$_2$ or tyrosinase/O$_2$.**

**TABLE 3**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molar Equivalents of GSH Depleted*</th>
<th>Distribution Coefficient</th>
<th>$pK_a$</th>
<th>Log P</th>
<th>LD$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HRP/H$_2$O$_2$</td>
<td>Tyrosinase/O$_2$</td>
<td>Microsomes</td>
<td>pH 4.0</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>CA</td>
<td>1.8 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>14.0 ± 0.1</td>
<td>0.029</td>
</tr>
<tr>
<td>CGA</td>
<td>1.6 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>0.001</td>
</tr>
<tr>
<td>DHCA</td>
<td>1.9 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>3.0 ± 0.4</td>
<td>0.003</td>
</tr>
</tbody>
</table>

* HRP (0.1 μM)/H$_2$O$_2$ (50 μM) and a mixture of dihydroxycinnamic acids (50 μM) and GSH (200 μM) incubated in Tris/HCl buffer (0.1 M pH 7.4 at 37°C for 30 min. An aliquot of 25 μl DTNB (2 mg/ml) was added to 100 μl of the reaction mixture and the volume made up to 1 ml by Tris/HCl buffer 0.1 M, pH 8.9 containing 1 mM DETAPAC. Absorbance was monitored at 412 nm for both DHCA and CA and at 436 nm for GSH. Tyrosinase (20 units/ml) was used instead of HRP/H$_2$O$_2$. The data are the average of three separate measurements. Negligible GSH depletion (<0.1 molar equivalent) occurred in the absence of the enzyme.

* Log P, the octanol/water partition coefficient of each compound at pH 7.4.

- $^a$ Log P, the octanol/water partition coefficient of each compound at pH 4.0.
- $^d$ Log P, the octanol/water partition coefficient of each compound at pH 7.4.
- $^{e}$ Log P, the octanol/water partition coefficient of each compound at pH 7.4.
- $^{f}$ Log P, the octanol/water partition coefficient of each compound at pH 7.4.

**Notes:**

- All the values are mean of three separate experiments.
- Distribution coefficient value at pH 7.4 was calculated from $P = D_{4.0}/(1 - a)$, where $P$, $a$, and $D_{4.0}$ are partition coefficient, degree of ionization at pH 7.4, and distribution coefficient at pH 4.0, respectively.
- The absorbance of the aqueous solution was monitored at 300 and 325 nm for CA and CGA, and 280 nm for DHCA before and after each 1-octanol addition. The values are mean of three measurements.
- Values shown are means.
Table 4
Mass spectrometry analyses results for dihydroxybenzene:glutathione conjugates

Glutathione (4 mM) was added either before or after HRP (3 μM)/H$_2$O$_2$ (2 mM) addition to a CGA, CA, or DHCA (1 mM) solutions in Millipore filtered water. Tyrosinase (25 unit/ml) was also used instead of HRP/H$_2$O$_2$. Results are reported as m/z [M + 1]$^+$ (relative intensity percentage).

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Compound</th>
<th>Compound + GSH (Mono-Conjugate)</th>
<th>Compound + 2 GSH (Bi-Conjugate)</th>
<th>Hydroxylated Compound (HO-Catechol)</th>
<th>Hydroxylated Compound + GSH (Mono-Conjugate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA$^a$</td>
<td>181 (50%)</td>
<td>486 (100%)</td>
<td>791 (9%)</td>
<td>197 (11%)</td>
<td></td>
</tr>
<tr>
<td>CA$^b$</td>
<td>181 (45%)</td>
<td>486 (100%)</td>
<td>791 (15%)</td>
<td>197 (11%)</td>
<td></td>
</tr>
<tr>
<td>CGA$^a$</td>
<td>355 (6%)</td>
<td>660 (100%)</td>
<td>965 (6%)</td>
<td>371 (20%)</td>
<td></td>
</tr>
<tr>
<td>CGA$^b$</td>
<td>355 (100%)</td>
<td>660 (3%)</td>
<td>965 (6%)</td>
<td>371 (20%)</td>
<td></td>
</tr>
<tr>
<td>DHCA$^a$</td>
<td>183 (100%)</td>
<td>488 (33%)</td>
<td>793 (80%)</td>
<td>199 (13%)</td>
<td>504 (7%)</td>
</tr>
<tr>
<td>DHCA$^b$</td>
<td>183 (100%)</td>
<td>488 (33%)</td>
<td>793 (50%)</td>
<td>199 (33%)</td>
<td>504 (33%)</td>
</tr>
<tr>
<td>CA$^a$ + Tyrosinase/O$_2$</td>
<td>181 (40%)</td>
<td>486 (20%)</td>
<td>197 (100%)</td>
<td>197 (100%)</td>
<td></td>
</tr>
<tr>
<td>CA$^b$ + Tyrosinase/O$_2$</td>
<td>181 (59%)</td>
<td>486 (20%)</td>
<td>197 (100%)</td>
<td>197 (100%)</td>
<td></td>
</tr>
<tr>
<td>CGA$^a$ + Tyrosinase/O$_2$</td>
<td>355 (42%)</td>
<td>660 (42%)</td>
<td>371 (100%)</td>
<td>371 (100%)</td>
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</tr>
<tr>
<td>CGA$^b$ + Tyrosinase/O$_2$</td>
<td>355 (21%)</td>
<td>660 (100%)</td>
<td>371 (29%)</td>
<td>371 (29%)</td>
<td></td>
</tr>
<tr>
<td>DHCA$^a$ + Tyrosinase/O$_2$</td>
<td>183 (57%)</td>
<td>488 (15%)</td>
<td>199 (100%)</td>
<td>199 (100%)</td>
<td></td>
</tr>
<tr>
<td>DHCA$^b$ + Tyrosinase/O$_2$</td>
<td>183 (92%)</td>
<td>488 (18%)</td>
<td>199 (100%)</td>
<td>199 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ GSH was present at the beginning of the reaction.

$^b$ GSH was added 5 min after the reaction started.

Previously published by Choudhury et al. (1999) as 0.04 and 0.02, respectively. These low distribution coefficients were expected to be due to the ionization of the carboxylic acid moieties at a higher pH.

Glutathione Depletion Assays. In the HRP/H$_2$O$_2$ oxidation system, CGA, CA, and DHCA depleted 1.6, 1.8, and 1.9 M equivalents of GSH, respectively, which suggests that 60, 80, and 90% of their mono-glutathione conjugates underwent a second glutathione conjugation, respectively, catalyzed by peroxidase. However, CA, CA, and DHCA depleted only 1.1, 1.0, and 0.8 M equivalents of GSH in the tyrosinase/O$_2$ oxidation system, respectively, indicating that DHCA was the least suitable substrate for tyrosinase in comparison with CA and CGA. Unlike HRP/H$_2$O$_2$ oxidizing system, the mono-glutathione conjugate of CA, CA, and DHCA were not substrates for the oxidative enzyme tyrosinase. Negligible GSH depletion (<0.1 M equivalent) occurred in the absence of the enzymes.

Mass Spectrometry Analyses. The glutathione conjugate metabolites were identified by direct injection of the samples into a mass spectrometer (Table 4). Both mono- and bi-glutathione conjugates were identified for CA, DHCA, and CGA when glutathione was present before HRP/H$_2$O$_2$ addition.

The mass spectrometry analyses of CA adducts in HRP/H$_2$O$_2$/GSH system when glutathione was present at the beginning of the reaction revealed signals at m/z [M + 1]$^+$ of 660 and 965, which corresponded to the mono- and bi-glutathione conjugates of CA, respectively. Similar results were obtained when GSH was added to the reaction mixture after HRP/H$_2$O$_2$, except that the bi-glutathione conjugate was not observed for CA under the conditions used. Instead, a signal at m/z [M + 1]$^+$ of 371 was detected, which corresponded to a hydroxylated CA adduct.

CA was also oxidized by HRP/H$_2$O$_2$ to form both mono- and bi-glutathione conjugates with m/z [M + 1]$^+$ of 486 and 791, respectively. A hydroxylated product of CA was also detected at m/z [M + 1]$^+$ of 197 when GSH was added to the reaction mixture 5 min after HRP/H$_2$O$_2$. Mass spectrometry analyses of DHCA when oxidized by oxidative enzymes HRP/H$_2$O$_2$ when GSH was added either before or after the enzyme led to the formation of similar hydroxylated products and mono- and bi-GSH conjugates to that of CA. A mono-glutathione conjugate of a hydroxylated DHCA adduct with m/z [M + 1] of 504 was also formed (Table 4). In addition DHCA unlike CA and CGA, formed a hydroxylated DHCA adduct with m/z [M + 1]$^+$ of 199 with HRP/H$_2$O$_2$/GSH even when GSH was present before HRP/H$_2$O$_2$ addition.

There was no bi-glutathione conjugate detected for CGA under tyrosinase/O$_2$ oxidation system whether GSH was added first or last but a hydroxylated product and mono-glutathione conjugate of CGA were detected. Similar results were obtained for CA and DHCA. Under all conditions when GSH was present, no CA-, CA-, and DHCA-dimers or glutathione conjugates of these dimers were formed.

Glutathione Conjugate Formation by Rat Hepatocyte Microsomes. As shown in Table 3, the amount of GSH depleted as a result of dihydroxybenzene acid oxidation catalyzed by rat liver microsomes/NADPH was determined to be 0.2, 0.5, and 0.6 equivalents of GSH per mole of CA, CA, and DHCA, respectively. This suggests that only 20, 50, and 60% of the corresponding compounds in the reaction mixture underwent glutathione conjugation. The CA-, CA-, and DHCA-induced GSH depletion by the microsomes/NADPH metabolic system was largely inhibited by benzylimidazole (a cytochrome P450 inhibitor) (data not shown). Negligible GSH depletion (<0.1 M equivalent) occurred in the absence of NADP$^+$. Cytotoxicity in Isolated Rat Hepatocytes. As shown in Table 3, the LD$_{50}$ (2 h) concentrations, determined by trypan blue exclusion technique as a measure of hepatocyte cell membrane intactness and viability, for CA, CGA, and DHCA were 7, 23, and 6 mM, respectively. The cytotoxicity of all three compounds was dose-dependent with a ranking order of DHCA, CA > CGA.

As shown in Table 5, CA was not toxic at 1 mM but became toxic if a nontoxic concentration of hydrogen peroxide (glucose/glucose oxidase system) or cumene hydroperoxide was added. However, this toxicity was prevented by benzylimidazole, a nonspecific P450 inhibitor (Quan et al., 1992), which suggests the involvement of cytochrome P450 in CA metabolism. NADPH/quinone oxidoreductase (NQO) inactivated hepatocytes (using dicumarol as described under Materials and Methods; Preusch et al., 1991) were also markedly susceptible to CA (or 4-propylcatechol), which suggests that CA or 4-propylcatechol were metabolized to toxic quinoid species that could be detoxified by NQO. Furthermore, benzylimidazole abolished CA (5 mM)-induced cytotoxicity. However, catalase-inactivated hepatocytes and GSH-depleted hepatocytes were much more prone to CA. Similar results were also observed for CA, 4-propylcatechol, and DHCA. Tyrosinase also increased the cytotoxicity of 1 mM caffeic acid.
Caffeic acid-, chlorogenic acid-, and dihydroxycinnamic acid-induced cytotoxicity involves metabolic oxidation

TABLE 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytotoxicity (% Trypan Blue Uptake)</th>
<th>% Hepatocyte GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60 min</td>
<td>120 min</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ caffeic acid (5 mM)</td>
<td>19 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>+ benzylicidazole (100 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ dicumarol (20 μM)</td>
<td>22 ± 1</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>+ NaNO₂ (4 mM)</td>
<td>22 ± 2</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>+ GSH-depleted hepatocytes</td>
<td>28 ± 3</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>+ 4-propylcatechol (0.5 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ dicumarol (20 μM)</td>
<td>30 ± 3</td>
<td>49 ± 5</td>
</tr>
<tr>
<td>+ dihydrocaffeic acid (5 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ dicumarol (20 μM)</td>
<td>33 ± 3</td>
<td>50 ± 2</td>
</tr>
<tr>
<td>+ chlorogenic acid (5 mM)</td>
<td>32 ± 2</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>+ dicumarol (20 μM)</td>
<td>22 ± 3</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>+ hydrogen peroxide (10 mM glucose/g l unit/ml g o)⁴⁻⁵</td>
<td>25 ± 4</td>
<td>60 ± 4</td>
</tr>
<tr>
<td>+ H₂O₂ + benzylicidazole (100 μM)</td>
<td>29 ± 2</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>+ cumene hydroperoxide (130 μM)</td>
<td>33 ± 3</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>+ cumene hydroperoxide + benzylicidazole (100 μM)</td>
<td>21 ± 1</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>+ tyrosinase (100 units/ml)⁶</td>
<td>33 ± 4</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>+ 4-propylcatechol (0.5 mM)</td>
<td>24 ± 3</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>+ cumene hydroperoxide (130 μM)</td>
<td>41 ± 5</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>+ tyrosinase (100 units/ml)⁶</td>
<td>26 ± 3</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>+ tyrosinase (100 units/ml)⁶</td>
<td>38 ± 4</td>
<td>62 ± 6</td>
</tr>
</tbody>
</table>

⁴ Data were significantly different from the control (P < 0.05). g.o., glucose oxidase.
⁵ The rate of hydrogen peroxide generated by the glucose/glucose oxidase system was 40 nmol/ml/min, which had no toxicity effect on hepatocytes (determined using a Clark type oxygen electrode).
⁶ None of the inhibitors (benzylicidazole, dicumarol, sodium azide), hydrogen peroxide-generating system (glucose/glucose oxidase system), hydroperoxides, and tyrosinase were toxic at the dose given alone (data not shown).

None of the inhibitors (benzylicidazole, dicumarol, sodium azide), hydrogen peroxide-generating system (glucose/glucose oxidase system), hydroperoxides, or tyrosinase alone caused hepatocyte cytotoxicity at the dose given (data not shown).

Hepatocyte GSH Levels. As shown in Table 5, hepatocyte GSH was depleted by the hydroxycinnamic acids with the following order of effectiveness: 4-propylcatechol ≫ CGA > CA > DHCA. The GSH levels of NOQ-inactivated hepatocytes were also much more readily depleted by DHCA or 4-propylcatechol, which suggests that DHCA or 4-propylcatechol cytotoxicity was caused by a quinoid metabolite that was reductively detoxified by NOQ. The cytochrome P450 inhibitor benzylicidazole prevented CA-induced hepatocyte GSH depletion. Similar results were also obtained for CGA, DHCA, or 4-propylcatechol (results not shown) and suggest that these catechols are metabolically activated by cytochrome P450.

Discussion

Using mass spectrometry analyses, we have shown for the first time that mono- and bi-glutathione conjugates were formed when GSH was present during the peroxidase/H₂O₂-catalyzed oxidation of CA, DHCA, or CGA. Hydroxylated adducts were formed in the absence of GSH or if GSH was added later. This hydroxylation reaction competed with GSH conjugate formation. Metabolism of the dihydroxycinnamic acids by tyrosinase/O₂/GSH, however, resulted in the formation of hydroxylated adducts and mono-glutathione conjugates only. Previously, Ploemen et al. (1993) also suggested a glutathione conjugate of CA was formed with the tyrosinase system but did not provide mass spectrometry evidence.

The bi-glutathione conjugates formed with HRP/H₂O₂ and not with tyrosinase/O₂ may be related to differences in the catalytic activity of the enzymes HRP/H₂O₂ and tyrosinase/O₂ as well as the differences in the oxidation mechanisms of the two enzymes. Peroxidase catalyzes a one-electron oxidation of catechols (Nakamura et al., 1985, 1989; Garcia-Moreno et al., 1999; Espin et al., 2000), whereas tyrosinase oxidizes catechols through a two-electron oxidation mechanism, thus bypassing the short-lived semiquinone intermediate (Passi and Nazzaro-Porro, 1981; Espin et al., 2000).

Although the mass spectrometry analyses do not show the pattern of glutathione conjugation, it is easy to predict the pattern for the formation of a mono-glutathione conjugate of dihydroxycinnamic acids. C-2 and C-5 of the aromatic ring of CA, CGA, or DHCA-o-quinones are almost equally electrophilic reactive centers because they are adjacent to the carbonyl groups of the o-quinone formed, whereas the C-6 is the least electrophilic center (Fig. 3). However, what distinguishes the three electrophilic centers is the steric hindrance in an increasing order of C-5 < C-6 < C-2. We have recently reported ¹H NMR data on the mixture of three possible isomers of mono-glutathione conjugate of catechin even though the isolation and purification of each mono-glutathione conjugate of catechin was not possible (Moridani et al., 2001). Using the integration of the protons, chemical shifts, and coupling constants for ¹H NMR on protons of the B-ring of catechin glutathione conjugate metabolite, we found that the steric hindrance was playing a major role as a distinguishing factor for glutathione to attack C₆ glutathione conjugate center more readily than C-2, despite the fact that C-2 is more electrophilic than the C-6' center in catechin. The C-5' center is the most electrophilic and the least sterically hindered center and therefore it does not come as a surprise that its glutathione conjugate derivative M1 was formed 1.5- and 3-fold more than the other two metabolites M2 and M3 (Fig. 3). Therefore, we have concluded that we could have a similar pattern for the mono-glutathione conjugates of CA, CGA, or DHCA except that C-2 on dihydroxycinnamic acids is less crowded than C-2' on catechin. This might lead to the different amounts of M2 and M3 formation found for dihydroxycinnamic acids.

Spectral evidence of a transient o-quinone intermediate for CA, CGA, and DHCA was obtained at pH 4 by using HRP/H₂O₂-metabolizing system. These intermediate products reacted rapidly with glutathione or underwent addition by H₂O₂. Further oxidation of this hydroxylated adduct by O₂ formed a p-quinone. The immediate product formed during enzymatic oxidation had a peak at 250 nm and...
they can cross the isolated rat hepatocyte membrane and are metabolized by P450 to form a cytotoxic o-quinone. However, there is no obvious immediate correlation between LD50 and log P, whereas there is a correlation between LD50 and distribution coefficient values measured at pH 4 or calculated for pH 7.4; the higher the distribution coefficient the more toxic the compounds (Table 3). We also showed for the first time that CA, CGA, and DHCA were metabolically activated by cytochrome P450 peroxidase activity (Anari et al., 1997a,b). Further evidence that the cytotoxic metabolite is probably an o-quinone is the marked increase in hepatocyte susceptibility to CA, CGA, and DHCA if NQO1 was inhibited. Most of the quinone substrates of NQO1 are p-quinones but two o-quinones, 9,10-phenanthrenequinone and cyclized dopamine o-quinone, have also been shown to be NQO1 substrates (O’Brien, 1991; Segura-Aguilar et al., 1992).

Tyrosinase is often abundant in melanoma and therefore can be considered as a useful enzyme for bioactivating CA, CGA, and DHCA if used for antimelanoma therapy. Enzyme-directed antimelanoma therapy includes the use of phenols or catechols that form o-quinones that are highly toxic to tyrosinase-containing melanoma tumor cells (Riley et al., 1997). Quinones are highly reactive species that can react with a variety of nucleophiles such as water, thiol-containing compounds, amino acids, and protein thiols, which are normally found in the cell. Glutathione is an important mechanism for cellular defense against reactive quinones (O’Brien, 1991). However, it needs to be shown that CA, CGA, and DHCA act as a substrate for melanoma tyrosinase and depletes GSH and protein thiols in the melanoma cells, resulting in cell death.

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References


Anari MR, Khan S, Jutie SD, and O’Brien PJ (1997b) Cytochrome P450-dependent xenobiotic activation by physiological hydroperoxides in intact hepatocytes. Eur J Drug Metab Pharma-


Choudhury R, Rais SK, Debnam E, and Rice-Evans CA (1999) Urinary excretion of hydroxy-


Garcia-Moreno M, Moreno-Ceseda M, Rodriguez-Lopez JN, Rodriguez-Lopez JN, Garcia-


