METABOLIC ACTIVATION OF 4-HYDROXYANISOLE BY ISOLATED RAT HEPATOCYTES

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ABSTRACT:

A tyrosinase-directed therapeutic approach for treating malignant melanoma uses depigmenting phenolic prodrugs such as 4-hydroxyanisole (4-HA) for oxidation by melanoma tyrosinase to form cytotoxic o-quinones. However, in a recent clinical trial, both renal and hepatic toxicity were reported as side effects of 4-HA therapy. In the following, 4-HA (200 mg/kg i.p.) administered to mice caused a 7-fold increase in plasma transaminase toxicity, an indication of liver toxicity. Furthermore, 4-HA induced-cytotoxicity toward isolated hepatocytes was preceded by glutathione (GSH) depletion, which was prevented by cytochrome P450 inhibitors that also partly prevented cytotoxicity. The 4-HA metabolite formed by NADPH/microsomes and GSH was identified as a hydroquinone mono-glutathione conjugate. GSH-depleted hepatocytes were much more prone to cytotoxicity induced by 4-HA or its reactive metabolite hydroquinone (HQ). Dicumarol (an NAD(P)H/quinone oxidoreductase inhibitor) also potentiated 4-HA- or HQ-induced toxicity whereas sorbitol, an NADH-generating nutrient, prevented the cytotoxicity. Ethylenediamine (an o-quinone trap) did not prevent 4-HA-induced cytotoxicity, which suggests that the cytotoxicity was not caused by o-quinone as a result of 4-HA ring hydroxylation. Deferoxamine and the antioxidant pyrogallol/4-hydroxy-2,2,6,6-tetramethylpiperidene-1-oxyl (TEMPOL) did not prevent 4-HA-induced cytotoxicity, therefore excluding oxidative stress as a cytotoxic mechanism for 4-HA. A negligible amount of formaldehyde was formed when 4-HA was incubated with rat microsomal/NADPH. These results suggest that the 4-HA cytotoxic mechanism involves alkylation of cellular proteins by 4-HA epoxide or p-quinone rather than involving oxidative stress.

The incidence of malignant melanoma is increasing at an alarming rate among Caucasians (Albino and Fountain, 1993). The lack of effective antimelanoma drugs for treating this form of cancer is partly due to drug resistance (Nathanson and Jilani, 1993). Metastatic melanoma cells are pigmented because active tyrosinase of the melanin-synthesizing pathway is found in melanocytes (Riley, 1991). Thus, the unique ability of melanocytes to produce melanin pigment could be exploited in an enzyme directed melanoma therapy (Jimbow et al., 1993). The selective toxicity of phenolic antimelanoma agents toward iso-

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The incidence of malignant melanoma is increasing at an alarming rate among Caucasians (Albino and Fountain, 1993). The lack of effective antimelanoma drugs for treating this form of cancer is partly due to drug resistance (Nathanson and Jilani, 1993). Metastatic melanoma cells are pigmented because active tyrosinase of the melanin-synthesizing pathway is found in melanocytes (Riley, 1991). Thus, the unique ability of melanocytes to produce melanin pigment could be exploited in an enzyme directed melanoma therapy (Jimbow et al., 1993). The selective toxicity of phenolic antimelanoma agents toward local and metastatic melanoma cells could be achieved if the phenolic agent were bioactivated by melanoma tyrosinase to form reactive o-quinones as long as the agent was not bioactivated by hepatic or renal cytochrome P450. Several phenolic agents have been tested for their antimelanoma effect among which 4-hydroxyanisole (4-HA) had the greatest depigmenting ability.

4-HA was first shown by Riley (1969) to be a melanocytotoxic agent. Depigmentation and tumor shrinkage resulted from both the topical application of 4-HA (Riley, 1969) and intra-arterial infusions of 4-HA into the legs (Morgan, 1984). Unfortunately, 4-HA clinical trials were terminated because serious kidney and liver damage occurred (Rustin et al., 1992). Tyrosinase was shown to catalyze the oxidation of 4-HA to 4-methoxycatechol and its o-quinone, which reacted readily with nucleophiles (Naish et al., 1988a,b). Melanoma toxicity may result from the covalent binding of the o-quinone to protein thiols and/or glutathione (GSH) depletion (Land et al., 1990) and inhibition of mitochondrial electron transport (Passi et al., 1984).

We previously reported a hydroquinone (HQ) mono glutathione conjugate was formed when 4-HA was incubated with rat liver microsomes and hydroprooxide and suggested that O-demethylation was the 4-HA bioactivation route to form benzoquinone (Anari et al., 1995). However, other mechanistic studies showed that incubation of 4-HA with rat liver microsomes (Cheeseman, 1984) and mouse liver microsomes (Schiller et al., 1991) produced little formaldehyde, suggesting that O-demethylation of 4-HA to HQ was not a major metabolic pathway. Furthermore, 3,4-diacetoxanisole, a prodrug of 4-methoxycatechol, was not more toxic than 4-HA toward mouse hepatocytes, indicating that this possible ring hydroxylation metabolite of 4-HA also did not account for its cytotoxicity (Schiller et al., 1991).

In the current work, we have sought to test the validity of these findings by investigating the P450-mediated bioactivation of 4-HA and HQ in isolated rat hepatocytes. It was found that ring epoxidation and/or one electron oxidation rather than O-demethylation/ring hydroxylation/ipso attack was the bioactivation route for 4-HA in rat liver. Furthermore, the cytotoxic mechanism for 4-HA and its metab-
olites HQ, 4-HA epoxide, and HQ-SG conjugate likely resulted from the alkylation of cellular proteins and not oxidative stress.

**Materials and Methods**

**Chemicals.** 4-Hydroxyanisole (4-HA, 98%), reduced GSH, trypan blue, 2,4-dinitro-fluorobenzene, 5,5′-dithiobis-(2-nitrobenzoic acid), iodoacetic acid, catalase (EC 1.11.1.6), superoxide dismutase (EC 1.15.1.1), ammonium acetate, acetic acid, acetyl acetone, trichloroacetic acid, and an AST transaminase diagnostic kit were obtained from Sigma-Aldrich (St. Louis, MO). 1-Bromohexane, oxidized glutathione, and dithiothreitol (DTT) were obtained from Aldrich Chemical Company Inc. (Milwaukee, WI). Collagenase (from Clostridium histolyticum), HEPEs, and bovine serum albumin were obtained from Roche Diagnostics (Laval, QC). Deroxorafine was a gift from Ciba-Geigy Canada Ltd. (Toronto, ON). HPLC-grade solvents were obtained from Caledon Laboratories Ltd. (Georgetown, ON). Hydroquinone (99%) was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). All other chemicals were of the highest grade available commercially.

**In Vivo Hepatotoxicity.** 4-HA (200–400 mg/kg, i.p.) was administered to 30 g male Sprague-Dawley mice (n = 9 per treatment). An increase in plasma transaminase levels (AST) was used as an indicator indicative of liver damage (except deferoxamine, which was co-added with the test substance) and was determined from blood samples taken from the heart 5 h post-treatment. The assay for these enzymes was performed with the respective Sigma diagnostic kit. Aspartate and α-ketoglutaric acid (10 mM) were used as substrates and were incubated with a 20-μl sample of serum for 30 to 60 min at 37°C. The absorbance at 505 nm was measured after the transamination reaction was inhibited by the addition of 20-μl 2,4-dinitrophenol solution and 0.4 M sodium hydroxide solution. Normal saline solution was injected into the control animals.

**Animal Treatment and Hepatocyte Preparation.** Isolated hepatocytes were prepared by collagenase perfusion of the liver as described by Moldeus et al. (1978). Stock solutions of chemicals were made either in incubation buffer or in methanol. A final concentration of 0.1% methanol in the incubation media did not affect hepatocyte viability. All cytotoxicity modulators (except deroxorafine, which was co-added with the test substance) were preincubated with hepatocytes (1 × 10⁶ cells/ml, 10 ml) under an atmosphere of 95% O₂/5% CO₂ or under 1% O₂/5% CO₂/94% N₂ for 20 min prior to the addition of 4-HA or HQ. Where shown, DTT was added 30 min after the addition of other cytotoxicity modulators. GSH-depleted hepatocytes were obtained by preincubating hepatocytes with 1-bromohexane (300 μM) for 20 min prior to the addition of the test compound as described previously (Khan and O’Brien, 1991).

Hepatocytes from rats treated with inducing agents for P450 2E1 and P450 1A2 were prepared by injecting (i.p.) 25 mg/kg/day pyrazole (Krikun and Omin prior to the addition of the test compound as described previously (Khan et al., 1978), for 3 consecutive days before sacrificing the rat.

**Cell Viability.** Hepatocyte viability was assessed by plasma membrane disruption as determined by the trypan blue (0.1% w/v) exclusion test (Moldeus et al., 1978).

**Microsomal Preparation.** Adult male Sprague-Dawley rats, 250–300 g, were anesthetized by sodium pentobarbital (60 mg/kg body) on day 4 after pyrazole or 3-methylcholanthrene i.p. injections. Hepatic microsomes were prepared by differential centrifugation as previously described (Dallner, 1978). The microsomal pellet was suspended in potassium phosphate buffer/KCl solution [50 mM KH₂PO₄, and 0.23% (w/v) KCl, pH 7.4] before storage at −70°C. Microsomal protein was determined by the method of Joly et al. (1975).

**Formaldehyde Formation by 4-Hydroxyanisole in Microsomes/NADPH System.** The incubation mixture contained in a final volume of 2.5 ml of 100 mM potassium phosphate buffer pH 7.4, 4 mg/ml rat liver microsomes, 1 mM NADPH, and 1 mM 4-HA or aminopyrine (Niwa et al., 1999) as a positive control. The mixtures were gently mixed at 37°C from which 1-ml samples were taken at 1- and 2-h intervals points into Eppendorf tubes containing 50-μl trichloroacetic acid (70% w/v). Following protein precipitation and centrifugation for 5 min, Nash’s reagent (750 μl) (Winters and Cederbaum, 1990) was added to a 750-μl aliquot of the assay mixture. The formaldehyde level of the mixtures was determined after 45 min of incubation at 37°C at 412 nm using a Beckman DU-7 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA).

**Hepatocyte Protein Thiols Measurement.** Hepatocyte protein thiols were quantified according to the method of Albano et al. (1985). Tissue protein thiols were detected at 365 nm, with retention times of 17.5 and 20.5 min, respectively. The amount of GSH conjugates formed was determined colorimetrically using Ellman’s reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (Gergel and Cederbaum, 1997). Incubation mixtures (1 ml) contained 100 mM potassium phosphate buffer, pH 7.4, 1 mg/ml rat liver microsomes, 500 μM GSH, 1 mM NADPH, and 1 mM 4-HA. The mixtures were gently mixed at 37°C from which 250-μl samples were removed at 15- and 30-min intervals and mixed with 25 μl of trichloroacetic acid (30% w/v). Following protein precipitation and centrifugation for 5 min, the GSH levels of a 100-μl aliquot of the assay mixture were determined by the addition of 0.4 M Tris/HCl buffer, pH 8.94 (850 μl), and 4 mg/ml DTNB (50 μl). The reduced DTNB formed was determined at 412 nm on a Beckman DU-7 spectrophotometer.

**Hepatocyte Protein Thiol Levels Determination.** Hepatocyte protein thiols were quantified according to the method of Albano et al. (1985). Oxygen Consumption. The rate of HQ auto-oxidation was measured by O₂ uptake using a Clark O₂ electrode (model 5300; Yellow Springs Instrument...
Drugs were administered to 30 g male Sprague-Dawley mice (n = 9 per treatment). Plasma transaminase levels (AST) were determined from blood samples taken 5 h post-treatment. The plasma AST level for control mice was 835 SF units/mL.

<table>
<thead>
<tr>
<th>Condition (Dose i.p.)</th>
<th>Plasma AST (Fold Control)</th>
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<tbody>
<tr>
<td>None</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>4-HA (200 mg/kg)</td>
<td>6.8 ± 0.8</td>
</tr>
<tr>
<td>4-HA (300 mg/kg)</td>
<td>15.9 ± 1.7</td>
</tr>
<tr>
<td>4-HA (400 mg/kg)</td>
<td>18.2 ± 1.6</td>
</tr>
</tbody>
</table>

Co., Inc., Yellow Springs, OH) in a 2.1-ml chamber. HQ (2 mM) was prepared in a Krebs-Henseleit buffer, containing HEPES (12.5 mM, at 37 °C and pH 7.65). The modulating agents GSH (1 mM), catalase (280 units/ml), superoxide dismutase (SOD; 150 units/ml), or deferoxamine (1 mM) were added approximately 1 min after the 4-HA or HQ addition.

Statistical Analysis. Results represent the mean ± standard error of the mean of at least three independent samples. Statistical significance was calculated using the Student’s t test. The minimal level of significance was p < 0.05.

Results

In Vivo Hepatotoxicity. As shown in Table 1, 4-HA showed a dose-dependent increase in the plasma AST levels in mice. The administration of 4-HA (200–400 mg/kg) induced a 7- to 18-fold increase in plasma AST levels 24 h later.

Modulation of 4-Hydroxyanisole-Induced Hepatocyte Cytotoxicity. 4-HA showed a concentration-dependent toxicity toward isolated hepatocytes, and a concentration of 13 mM 4-HA was required to induce 50% cytotoxicity in 2 h at 37 °C as determined by trypan blue uptake (data not shown). As shown in Table 2 and Fig. 1, GSH-depleted hepatocytes were also much more susceptible to 4-HA. Furthermore, hepatocytes isolated from rats treated with pyrazole, a P450 2E1 inducer, were much more susceptible to the toxic effects of 4-HA and a lower concentration of 4-HA (0.6 mM) was sufficient to cause a 65% depletion in hepatocyte GSH after 60 min of incubation (results not shown). Therefore, the reactive oxygen species “ROS” scavengers pyrogallol and 4-hydroxy-2,2,6,6-tetramethylpyperidene-1-oxyl had no effect on 4-HA-induced cytotoxicity or GSH depletion. In the absence of 4-HA, none of the modulators at the doses used affected hepatocyte viability.

Hepatocyte GSH Depletion by 4-Hydroxyanisole. As shown in Table 2, the DT-diaphorase inhibitor, dicumarol (Preusch et al., 1991), markedly increased 4-HA-induced toxicity and GSH depletion. Sorbitol, an agent that elevates cytosolic NADH (Khan and O’Brien, 1995), also prevented 4-HA-induced cytotoxicity. In contrast, the ferric chelator deferoxamine had no or minimal protective effects against 4-HA-mediated cytotoxic insult. Furthermore, the reactive oxygen species “ROS” scavengers pyrogallol and 4-hydroxy-2,2,6,6-tetramethylpyperidene-1-oxyl had no effect on 4-HA-induced cytotoxicity or GSH depletion.

Microsomal Catalyzed 4-Hydroxyanisole Metabolism. Using HPLC coupled with an electrochemical detector, we were able to...
A, prevention of 4-HA-induced GSH depletion in isolated hepatocytes from fasted rats by P450 2E1 inhibitors or dithiothreitol, and B, effect of 4-HA on hepatocyte protein thiol levels in hepatocytes from 3-methylcholanthrene (P450 1A2) treated rats. Hepatocytes (1 × 10⁶ cells/ml) were isolated from rats and maintained under the constant flow of 95% O₂/5% CO₂ except that (A) was under 1% O₂/94% N₂/5% CO₂ atmosphere. GSH levels were determined by the method of Albano et al. (1985). Values represent averages of three experiments with error bars representing standard deviations.

**Table 3**

Modulation of hydroquinone-induced hepatocyte cytotoxicity

Chemicals were incubated with hepatocytes under similar condition to that of Table 2 except that hepatocytes (10⁶ cells/ml) were incubated in Krebs-Henseleit buffer, pH 7.4, at 37 °C under 1% O₂/94% N₂/5% CO₂ atmosphere. Values are expressed as the means of three separate experiments ± S.D. Modulators were not cytotoxic by themselves (data not shown).

<table>
<thead>
<tr>
<th>Addition</th>
<th>% Cytotoxicity at time (min)</th>
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<tbody>
<tr>
<td></td>
<td>30</td>
</tr>
<tr>
<td>None</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>HQ (200 μM)</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>* + GSH depleted hepatocytes</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>* + dicumarol (25 μM)</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>HQ (2 mM)</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>* + catalase (100 units/ml)</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>* + SOD (100 units/ml)</td>
<td>100 ± 100</td>
</tr>
<tr>
<td>* + isoniazid (10 mM)</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>* + ethylenediamine (2 mM)</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>* + dicumarol (25 μM)</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>* + deferoxamine (1 mM)</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>* + pyrogallol (100 μM)</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>* + sorbitol (10 mM)</td>
<td>28 ± 3</td>
</tr>
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*Statistically significant difference from control (p < 0.05).

SELECTIVE DETECTORS OF 4-HYA AND p-QUINONE WITH RETENTION TIME OF 3.1 AND 5.4 MIN, RESPECTIVELY, AS THE 4-HA METABOLITES FORMED BY THE MICROSONAL/NADPH SYSTEM. WHEN GSH (500 μM) AND 4-HA (1 mM) WERE ADDED TO A MICROSONAL/NADPH INCUBATE, THE GSH AMOUNTS DEPLETED BY 4-HA OVER A PERIOD OF 15 AND 30 MIN WERE 10% (50 μM) AND 15% (76 μM), RESPECTIVELY. IN THE ABSENCE OF 4-HA, THE GSH AMOUNTS DEPLETED WERE 3 AND 4%, RESPECTIVELY. LITTLE OXIDIZED GLUTATHIONE FORMATION WAS DETECTED BY HPLC IN THIS STUDY. WHEN A MICROSONAL/HYDROGEN PEROXIDE-GENERATING SYSTEM WAS USED INSTEAD OF MICROSONAL/NADPH, THE AMOUNT OF GSH DEPLETED BY 4-HA OVER A PERIOD OF 15 MIN WAS 89% (445 μM) AND IN THE ABSENCE OF 4-HA WAS 66% (330 μM). HOWEVER, 4-HA DID NOT GENERATE FORMALDEHYDE WHEN INCUBATED WITH NADPH-SUPPORTED RAT LIVER MICROSOMES OVER A PERIOD OF 2 HOURS whereas aminopyrine (Niwa et al., 1999) as a positive control formed formaldehyde as a result of N-demethylation catalyzed by P450.

**GSH Conjugates of 4-Hydroxynisole Metabolites.** LC/MS analysis of the GSH conjugates formed identified the single HPLC peak (tᵢ = 2.8 min) as the hydroquinone mono GSH conjugate and thus was the major metabolite formed by the NADPH-supported liver microsomal system. The hydroquinone-glutathione conjugate was detected by mass spectrometry in positive ion mode at m/z [MH⁺]⁺ 416. Subsequent LC/MS/MS analyses of the HQ-SG parent signal [MH⁺]⁺ = 416 exhibited the following fragmentive ions: m/z 416 [MH⁺]⁺, 341 (MH⁻ – glyoxylate)⁻, 287 (MH⁻ – pyrogallol acid)⁻, 270 (MH⁻ – pyrogallol N-oxide)⁻, 184 (MH⁻ – pyrogallol acid – glyoxylate)⁻, 167 (MH⁻ – pyrogallol acid – glycine – COOH)⁻, 141 (hydroquinone + S – H)⁺, and 130 (pyrogallol acid + H)⁺. A similar fragmentation pattern of mass spectra was obtained for 2-glutathionyl hydroquinone prepared from a reaction between p-benzoquinone and GSH. Both conjugates had similar properties so that the 4-HA metabolite is unlikely to be a rearranged conjugate such as 4-glutathionyl resorcinol.

**Modulation of Hydroquinone-Induced Hepatocyte Cytotoxicity.** HQ showed dose-dependent cytotoxicity toward isolated rat hepatocytes with an LD₅₀ (2 h) of 1.7 mM. As shown in Table 3, the DT-diaphorase inhibitor dicumarol markedly potentiated HQ-induced cytotoxicity. The NADH-generating agent sorbitol also prevented HQ-induced cytotoxicity. HQ-induced cytotoxicity was also markedly increased in GSH-depleted hepatocytes (Table 3). Furthermore, cytotoxicity was delayed by extracellular catalase and prevented by the P450 2E1 inhibitor isoniazid. The extracellular catalase removes H₂O₂ that effluxes the cells and thereby decreases intracellular H₂O₂ levels.

HQ-induced cytotoxicity was markedly exacerbated by the addition of SOD to the incubation mixture and resulted in 100% cell death in 30 min. The ROS scavengers, deferoxamine and pyrogallol, partially...
prevented the HQ-induced cytotoxicity. The o-quinone trap ethylenediamine did not affect HQ-induced cytotoxicity.

Modulation of the Auto-oxidation of Hydroquinone. The addition of HQ to Krebs-Henseleit buffer caused extensive O₂ uptake (42 ± 5 nmol/ml/min) (Table 4) at pH 7.4, which was inhibited by GSH. SOD accelerated 1.5-fold the rate of HQ auto-oxidation. This finding may be explained by a shift in the equilibrium toward p-quinone formation caused as a result of superoxide radical removal by SOD. Furthermore, catalase inhibited auto-oxidation by approximately 60%, suggesting that H₂O₂ may mediate HQ auto-oxidation. Deferoxamine did not affect the rate of HQ auto-oxidation. The addition of HQ or 4-HA to hepatocytes did not induce any cyanide resistant respiration, even at a cytotoxic concentration (results not shown).

Discussion

The hepatotoxic mechanism of 4-HA, a once promising drug used in the treatment of melanoma, has been investigated using isolated rat hepatocytes. 4-HA (200 mg/kg i.p.) administered to mice caused a 7-fold increase in plasma transaminase in 24 h later thereby indicating that 4-HA is hepatotoxic. Furthermore, GSH depletion preceded 4-HA cytotoxicity in isolated rat hepatocytes and GSH-depleted hepatocytes were much more susceptible to toxicity induced by 4-HA or its reactive metabolite HQ. Dicumarol (a NAD(P)H/quinone oxidoreductase inhibitor) potentiated the 4-HA- or HQ-induced toxicity indicating a p-quinone- or semiquinone radical-mediated cytotoxic mechanism. Ethylenediamine (an o-quinone trap) did not prevent 4-HA- and HQ-induced cytotoxicity indicating that an o-quinone did not contribute to cytotoxicity and suggests that ring hydroxylation was not the bioactivation route for 4-HA. Alkylation of intracellular nucleophilic sites or the plasma membrane by the electrophilic metabolites of 4-HA may be responsible for the impaired plasma membrane integrity as hepatocyte protein thiol depletion occurred before cytotoxicity ensued. Prior GSH depletion also markedly increased hepatocyte susceptibility to 4-HA, which suggests that GSH conjugate formation plays a vital role in the detoxification of 4-HA. Using horseradish peroxidase/GSH/H₂O₂-metabolizing system, we have not found any dimers, trimers, or their GSH conjugates for 4-HA whereas we have identified dimers and their GSH conjugates with 2-HA and 3-HA. Therefore it is unlikely that dimers are causing or contributing to the toxicity of 4-HA (results not shown).

Furthermore, liver microsomes/NADPH and/or microsomes/H₂O₂-metabolized 4-HA to form a mono HQ-SG conjugate. The cytoprotection by P450 inhibitors and the increased hepatocyte susceptibility of P450-induced hepatocytes to 4-HA suggests that P450 2E1 and 1A1 play a role in the bioactivation of 4-HA. We only examined the role of P450 2E1 and P450 1A1 in toxicity, and other P450s have not been investigated in the current study and may well subsequently prove to contribute to the bioactivation. The role of P450 2E1 in 4-HA-induced GSH depletion and cytotoxicity may result from its ability to catalyze primarily the ring epoxidation of 4-HA to p-quinone and 4-HA epoxide, which react with GSH to form a HQ-SG conjugate. HQ-SG may then undergo oxidation induced by superoxide radicals generated by P450 2E1 (Dai et al., 1993) to form a semiquinone-SG conjugate and subsequently a Q-SG.

When 4-HA was incubated with a rat liver microsomes/NADPH system, however, negligible amounts of formaldehyde were formed, which suggests that the apparent O-demethylation reaction occurred via epoxidation. Previously, it was reported that ring hydroxylation of 4-HA was not a bioactivation route because 3,4-diacetoxyanisole, a prodrug of the metabolite 4-methoxycatechol, which forms if 4-HA undergoes ring hydroxylation, was not more toxic than 4-HA (Schiller et al., 1991). Further mechanistic studies showed that incubation of 4-HA with rat liver microsomes (Cheeseman, 1984) or mouse liver microsomes (Schiller et al., 1991) produced little formaldehyde, indicating that O-demethylation of 4-HA to HQ was not a major metabolic pathway. However, they speculated that the cytotoxic metabolite of 4-HA was probably an epoxide without elucidating its chemical structure or identifying a GSH conjugate (Schiller et al., 1991).

We hypothesized three mechanisms for 4-HA bioactivation [i.e., ring hydroxylation, O-demethylation, and O-demethoxylation (arene oxide formation)]. The first two mechanisms were ruled out because we were not able to identify 4-methoxycatechol and formaldehyde, respectively. The third pathway is the most likely pathway for the bioactivation of 4-HA because a HQ-SG conjugate was identified. O-demethoxylation can occur as a result of either epoxidation (arene oxide formation) or one electron oxidation or an ipso attack. The epoxidation and one electron oxidation were previously described as a possible mechanism of O-deoxylation for 4-aryloxyphenol but not an ipso attack due to the presence of a hydroxy group at para position to the arylxy group (Ortiz de Montellano, 1995; Testa, 1995; Guengerich, 2001). Using deuterium and tritium substituted phenyl derivatives, Daly et al. (1968) have found that when the main phenyl substituent was not readily ionizable such as methyl, phenyl, and halide groups, 40 to 65% of the deuterium and higher percentages of the tritium migrated via a National Institutes of Health shift mechanism. However, with ionizable substituents such as hydroxy or amino groups, deuterium retention in the molecule ranged from 0 to 30% (Daly et al., 1968). This indicates that when an arene oxide (epoxide) formed as a result of P450-mediated bioactivation, the presence of a hydroxy group at meta or para position to the epoxide leads to the cleavage of the epoxide bond between oxygen and C3 and the formation of a hydroxy group at para position to the phenolic group and subsequent elimination of deuterium. Thus one can conclude that the addition of oxygen to aromatic bonds C1-C2 of 4-HA would yield to an arene oxide that undergoes a rearrangement because of the hydroxy group, a readily ionizable group, at its para position consequently leading to the loss of the methoxy group in 4-HA.

As depicted in Scheme 1, hepatocyte P450 catalyzes the ring epoxidation of 4-HA to form 4-HA epoxide and p-quinone. HQ is also oxidized to form the electrophile p-quinone. Both 4-HA epoxide and p-quinone react with GSH to form a HQ-SG conjugate and alkylate protein thiols, which likely causes a loss of cell function. In addition, HQ-SG undergoes further oxidation and generates an electrophilic p-quinone-SG conjugate. Therefore, it appears that the 4-HA/microsomal-metabolizing system acts as a p-quinone/hydroquinone-generating system. Alternatively, the formation of p-quinone could also proceed through P450-mediated one electron oxidation of phenolic group with subsequent hydroxylation at para position to the phenolic group.

The formation of 4-glutathionyl resorcinol is mechanistically unlikely for two reasons: 1) the C4 is more hindered than C3 when GSH attacks the arene oxide formed, and 2) if GSH attacked the C4 center, 4-methoxyresorcinol should have been also identified by MS analysis. It should also be noted that if 4-HA underwent ring epoxidation on the carbon centers adjacent to 4-hydroxy group, the product of 4-HA bioactivation would have been 4-methoxycatechol- or 4-HA-gluthione conjugate, which clearly is not the case. In the current study, we have provided additional evidence for the formation of hydroquinone and p-quinone when 4-HA was incubated with microsomal/NADPH incubation mixture using a HPLC coupled with an electrochemical detector. LC/MS studies by Anari et al. (1995) also showed that HQ-SG adduct was formed by the t-butylhydroperoxide-supported
microsomal P450 metabolism of 4-HA (known as P450 peroxidase activity).

The thiol-reducing agent DTT did not prevent 4-HA cytotoxicity or affect the depletion of hepatocyte GSH or protein thiols thereby suggesting that mixed protein disulfide formation did not contribute to protein thiol depletion. Deferoxamine, a ferric chelator, which inhibit ROS formation by preventing the participation of iron in the Fenton reaction, and the antioxidant pyrogallol/4-hydroxy-2,2,6,6-tetramethylpiperidene-1-oxyl were not effective in preventing 4-HA-mediated cytotoxicity. GSH-depleted hepatocytes were more susceptible toward 4-HA-induced cytotoxicity. These findings also suggest that protein alkylation by 4-HA epoxide or p-quinone could be a major cause of 4-HA-induced cytotoxicity toward isolated rat hepatocytes rather than oxidative stress. The effects of cytotoxicity modulators on hepatocyte cytotoxicity induced by HQ were in most cases similar to the effects of these modulators on hepatocyte cytotoxicity induced by 4-HA. This strengthens the likelihood that HQ was the reactive metabolite resulting from the 4-HA bioactivation.

The nucleophile GSH likely inhibited HQ auto-oxidation by binding the p-quinone. Catalase delayed HQ-mediated hepatocyte cytotoxicity as well as HQ auto-oxidation in the absence of hepatocytes. The catalytic decomposition of H$_2$O$_2$ by catalase would prevent the oxidation of hydroquinone to semiquinone (Scheme 1), thereby inhibiting superoxide radicals and ROS formation. Catalase may protect against HQ-mediated hepatocyte cytotoxicity by preventing the auto-oxidation of HQ-SG and/or HQ catalyzed by H$_2$O$_2$. The catalase added to incubation medium removes H$_2$O$_2$ that effluxes the cells and thereby decreases intracellular H$_2$O$_2$ levels, which suggests that intracellular H$_2$O$_2$ formation contributes to the oxidation of HQ by P450 2E1. In addition, SOD exacerbated HQ-induced cytotoxicity and HQ auto-oxidation. There is a greater affinity between the semiquinone and molecular oxygen than between p-quinone and O$_2^*$ (Eyer, 1991;
Tayama and Nakagawa, 1994). SOD, by removing superoxide radicals, shifted the equilibrium between semiquinone and quinone towards quinone formation (Scheme 1), thereby accelerating HQ autoxidation. This suggests that increasing extracellular H$_2$O$_2$ by dissmutating O$_2$ increases intracellular H$_2$O$_2$ and P450 2E1 catalyzed HQ oxidation to form p-quinone. This increased quinone and H$_2$O$_2$ formation in turn oxidizes more HQ to semiquinone.

The variety of inhibitors such as isoniazid, phenylimidazole, and dicumarol or modulators such as deferoxamine used in our study have multiple actions, which leads to the problem of drawing definitive conclusions from the use of pharmacologic agents. However, the cumulative evidence drawn from the use of these agents with differing major effects points to our conclusions with respect to the 4-HA mechanism of cytotoxicity discussed in this communication. In summary, the P450-catalyzed bioactivation pathway of 4-HA to form cytotoxic reactive metabolites seems to involve ring epoxidation and/or P450-mediated one electron oxidation as bioactivation routes to convert 4-HA to the reactive intermediate species 4-HA epoxide and/or P450-mediated one electron oxidation as bioactivation routes to convert 4-HA to the reactive intermediate species 4-HA epoxide and p-quinone rather than O-demethylation/ring hydroxylation/ipso attack mechanism. The cytotoxic mechanism for 4-HA is similar to the cytotoxic mechanism of its reactive metabolite HQ and likely results from the alkylation of cellular components by 4-HA epoxide and p-quinone rather than resulting from oxidative stress. Finally, the 4-HA-induced hepatotoxicity found indicates that 4-HA is not suitable for antimelanoma therapy.

References


