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Inhibition of CYP3A-mediated oxidation in human hepatic microsomes by the dietary derived complex phenol, gallic acid

Ieva Stupans, Hai-Wei Tan, Amra Kirlich, Kellie Tuck, Peter Hayball and Michael Murray

Abstract

Plant polyphenols, such as gallic acid, have been reported to have a range of biological activities including antimutagenic effects. Previously, we reported that gallic acid (3,4,5-trihydroxybenzoic acid), an agent found in wine and tea, inhibits androstenedione 6β-hydroxylase activity (Ki 70 μM), a cytochrome P450 (CYP3A) marker in human liver microsomes. The preincubation of gallic acid (100 μM) with human liver microsomes in the absence of NADPH, as compared with the presence of NADPH, before assay of androstenedione 6β-hydroxylase activity significantly increased the inhibitory effects of the gallic acid (0.03 ± 0.03 nmol (mg microsomal protein)$^{-1}$ min^{-1} compared with 0.20 ± 0.06 nmol (mg microsomal protein)$^{-1}$ min^{-1} ($P < 0.05$)). The antioxidant ascorbic acid and the radical scavenger glutathione prevented this observed increase in inhibition. Removal of gallic acid-derived products from the incubation completely restored CYP3A activity. In contrast, the activities of CYP1A and CYP2E, and non-CYP mediated reductive microsomal 17β-hydroxysteroid dehydrogenase activity were refractory to inhibition by gallic acid.

Introduction

Hepatic microsomal enzymes, in particular the cytochromes P450 (CYP), are important in the metabolism of xenobiotic substrates, such as drugs, and endogenous substrates. Their inhibition or induction can have profound effects on clearance of drug substrates and therefore pharmacotherapy. CYPs have also been widely documented as having an important role in the activation of carcinogens (reviewed Guengerich & Shimada 1998). Previously, we reported that gallic acid (a complex phenol found in red wine (Blanco et al 1998) and in green and black tea (Constable et al 1996; Arce et al 1998)) inhibited androstenedione 6β-hydroxylase activity (Ki determined in these studies was 70 μM), a CYP3A marker in human liver microsomes (Stupans et al 2000). Other studies (Wang et al 1988) have not determined inhibition of CYP3A by gallic acid but have focused on CYP1A. CYP3A accounts for up to 60% of hepatic CYPs (Guengerich 1995). Importantly, it has been identified in many non-hepatic sites such as the lung (reviewed Raunio et al 1999) and gut (Kolars et al 1994) which are frequently target sites for cancers. Guengerich et al (1998) identified CYP3A as activating carcinogens such as aflatoxin B_1.

The CYPs may be inhibited by a number of different mechanisms, which may be reversible or irreversible (reviewed Murray 1999). This study was undertaken to

Center for Pharmaceutical Research, School of Pharmaceutical, Molecular and Biomedical Sciences, University of South Australia, SA 5000, Australia

Ieva Stupans, Hai-Wei Tan, Amra Kirlich, Kellie Tuck, Peter Hayball

School of Physiology and Pharmacology, University of New South Wales, NSW 2052, Australia

Michael Murray

Correspondence: I. Stupans, Center for Pharmaceutical Research, School of Pharmaceutical, Molecular and Biomedical Sciences, University of South Australia, SA 5000, Australia. E-mail: ieva.stupans@unisa.edu.au

examine in detail the mechanism of inhibition of CYP3A by gallic acid. Our results indicated the formation of a gallic acid derivative, most probably a radical species, which, under some conditions, was able to essentially abolish CYP3A activity. The inhibition observed was found to be reversible. This postulated radical did not alter chlorzoxazone 6-hydroxylase or ethoxyresorufin-*O*-deethylase activity, indicating a degree of CYP isozyme selectivity, or microsomal reductive 17β hydroxysteroid dehydrogenase (17β HSD).

Materials and Methods

Materials

A sample of pooled human liver microsomes was purchased from Human Biologics International (Scottsdale, AZ). The male pool 2.0 microsomes are GLP characterized for several CYP activities and are sourced from seven individuals. NADP, NADPH, isocitrate dehydrogenase, glutathione, ascorbic acid, resorufin, ethoxyresorufin, unlabelled androst-4-ene-3,17-dione (androstenedione) and unlabelled chlorzoxazone were purchased from the Sigma Chemical Co. (St Louis, MO). [4-^{14}C]Androstenedione (sp.act. 2.0 GBq mmol^{-1}) and [2-^{14}C]chlorzoxazone (sp.act. 2.11 GBq mmol^{-1}) were purchased from Amersham (Sydney, Australia). Hydroxylated androstenedione standards were obtained from the MRC Steroid collection or Steraloids, Inc. (Wilton, New Hampshire). Gallic acid was purchased from ICN (Aurora, OH) and 3,4-dihydroxybenzoic acid was purchased from BDH (Poole, UK). 1,4-Benzoquinone and catechol were purchased from Aldrich (Milwaukee, WI).

Assays and spectra

Microsomal androstenedione 6β-hydroxylase was assayed essentially as described by Stupans et al (2000). Microsomal fractions were incubated in a 1-mL reaction mixture containing isocitrate (4 mM), isocitrate dehydrogenase (0.4 IU), $MgCl_2$ (8 mM), 1 mM NADP and androstenedione (43.75 μM, 4.8 MBq mmol^{-1}) for 10 min at 37°C, after which the incubation mixtures were extracted twice with 3 mL ethyl acetate. Application to, and development of, TLC plates was carried out as described by Stupans & Sansom (1991). Zones corresponding to hydroxylated androstenedione standards and to testosterone were visualized under UV light and scraped into vials for scintillation spectrometry (ACS, Amersham, Sydney, Australia).

Chlorzoxazone 6-hydroxylase activity was determined essentially as described by Draper et al (1998). Microsomal fractions were incubated in a 1-mL reaction mixture containing isocitrate (4 mM), isocitrate dehydrogenase (0.4 IU), $MgCl_2$ (8 mM), 1 mM NADP and chlorzoxazone (500 μM, 37 MBq mmol^{-1}) for 20 min at 37°C.

7-Ethoxyresorufin-*O*-deethylase activity was determined essentially as described by Lake (1987). In this assay after incubation of the 7-ethoxyresorufin with the microsomes and generating system, the dealkylated resorufin formed is quantified by fluorescence spectrophotometry. Microsomal fractions were incubated in a 1-mL reaction mixture containing isocitrate (4 mM), isocitrate dehydrogenase (0.4 IU), $MgCl_2$ (8 mM), 1 mM NADP and 10 μM ethoxyresorufin for 15 min at 37°C. After the addition of 0.5 mL 5% $ZnSO_4$, 0.5 mL saturated $Ba(OH)_2$ and centrifugation at 2000 *g* for 10 min, 2 mL 0.1 M glycine–NaOH buffer (0.5 M glycine, pH 8.5) was added to the supernatant and the fluorescence was measured at excitation and emission wavelengths of 535 and 582 nm, respectively, using a Perkin-Elmer MPF-3L Fluorescence spectrophotometer. Product formation was determined using a resorufin standard.

Where used, potential inhibitors were added in water. Where used, antioxidants (ascorbic acid) and scavengers (glutathione) were added in buffer containing 1 mM EDTA. Appropriate control incubations were performed concurrently. Pre-incubation was carried out with test compounds, microsomes and buffer with or without NADPH (1 mM), before the addition of substrate and generating system.

In some assays, pre-incubation was carried out as above except that the buffer contained 1 mM EDTA as chelating agent. After pre-incubation the reduced–carbon monoxide spectra were recorded as described by Omura & Sato (1964).

In some experiments microsomes were pre-incubated in the presence or absence of gallic acid (0.1 mM) and NADPH for 15 min. The microsomal protein was then separated from NADPH/NADP and gallic acid through Centricon 10 microconcentrators. The removal of gallic acid from the microsomes was verified by HPLC (not shown) and protein content was determined before androstenedione 6β-hydroxylase activities were determined as described above.

Spectra of gallic acid and 3,4-dihydroxybenzoic acid in 0.1 M phosphate buffer, pH 7.4 were recorded against water. A concentration of 800 μM of the compounds was used; spectra were recorded between 200 and 700 nm. The solutions were warmed (37°C) during the period over which recordings were made. Recordings were

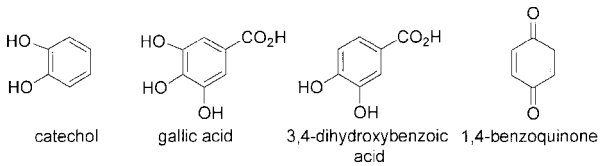

Figure 1 Structures of catechol, gallic acid, 3,4-dihydroxybenzoic acid and 1,4-benzoquinone.

Table 1 Effects of pre-incubation with NADPH in the presence or absence of test compounds (0.1 mM) on androstenedione 6β-hydroxylase activity concentration in human liver microsomes.

Test compounds	Androstenedione 6β-hydroxylase activity (nmol (mg microsomal protein)$^{-1}$ min^{-1})	
	Pre-incubation in the presence of NADPH	Pre-incubation in the absence of NADPH
No compound	0.53±0.09	0.43±0.09
Gallic acid	0.20±0.06**	0.03±0.03**#
Gallic acid+ascorbic acid	0.40±0.04	0.34±0.05
Ascorbic acid	0.39±0.04	0.34±0.05
Gallic acid+glutathione	0.56±0.04	0.49±0.04
Glutathione	0.58±0.04	0.50±0.04
3,4-Dihydroxybenzoic acid	0.60±0.06	0.49±0.06
Benzoquinone	0.07±0.06**	0.07±0.04**
Catechol	0.23±0.06**	0.12±0.04**#

Glutathione concentration used was 5 mM, ascorbic acid concentration was 1 mM. Values are means±s.d. of three independent experiments carried out and conducted in duplicate. **$P < 0.05$, incubations with added compounds as compared with incubations carried out with no added compound. #$P < 0.05$, incubations carried out after pre-incubation without NADPH as compared with NADPH.

made at time intervals to 1 h (0, 10, 20, 30, 45 and 60 min). Some recordings were made in the presence of NADPH (1 mM) or ascorbic acid (1 mM). In some cases NADPH (1 mM) was added after this recording period.

All assay conditions were optimized with respect to time, protein concentration and substrate concentrations to ensure linearity.

Data analysis

For each experiment data are expressed as the means± s.d., a measure of variability of the data, of three independent experiments, conducted in at least duplicate unless stated otherwise. Statistical analysis was carried out using one-way analysis of variance (STATVIEW, Berkeley, USA) followed by a Scheffe F-test to detect differences.

Table 2 Investigation of the nature of gallic acid inhibition of androstenedione 6β-hydroxylase activity by 0.1 mM concentration of gallic acid in human liver microsomes.

Gallic acid	NADPH	Androstenedione 6β-hydroxylase activity (nmol (mg microsomal protein)$^{-1}$ min^{-1})
+	+	0.39±0.02
+	−	0.26±0.04*
−	+	0.41±0.04
−	−	0.21±0.07*

Human liver microsomes were incubated in the presence or absence of NADPH and the presence or absence of gallic acid (0.1 mM) for 15 min. The NADPH/NADP and gallic acid were then removed from the pre-incubation mix by the use of Centricon 10 microconcentrators. Androstenedione 6β-hydroxylase activities were then determined. Values are means±s.d. of two independent experiments carried out in triplicate. *$P < 0.05$ compared with the pre-incubation carried out in the presence of NADPH.

Results

Stupans et al (2000) demonstrated the inhibition of CYP3A activity in human liver microsomes by gallic acid. To determine whether complex formation was occurring for CYP3A, assays to quantify androstenedione 6β-hydroxylase activity and CYP content were carried out using human liver microsomes pre-incubated with and without NADPH and gallic acid.

Structures of gallic acid and other test compounds are shown in Figure 1. Our assay did not distinguish between the several forms of CYP3A.

Results for these experiments are shown in Table 1. In the absence of test compound such as gallic acid, pre-incubation of human liver microsomes in the presence or absence of NADPH did not alter androstenedione 6β-hydroxylase activity significantly ($P < 0.05$). However, in the absence of NADPH, pre-incubation with gallic acid essentially abolished androstenedione 6β-hydroxylase activity (i.e. to 5% of control activity 0.03±0.03 nmol (mg microsomal protein)$^{-1}$ min^{-1} compared with 0.53±0.06 nmol (mg microsomal protein)$^{-1}$ min^{-1}). When ascorbic acid or glutathione were

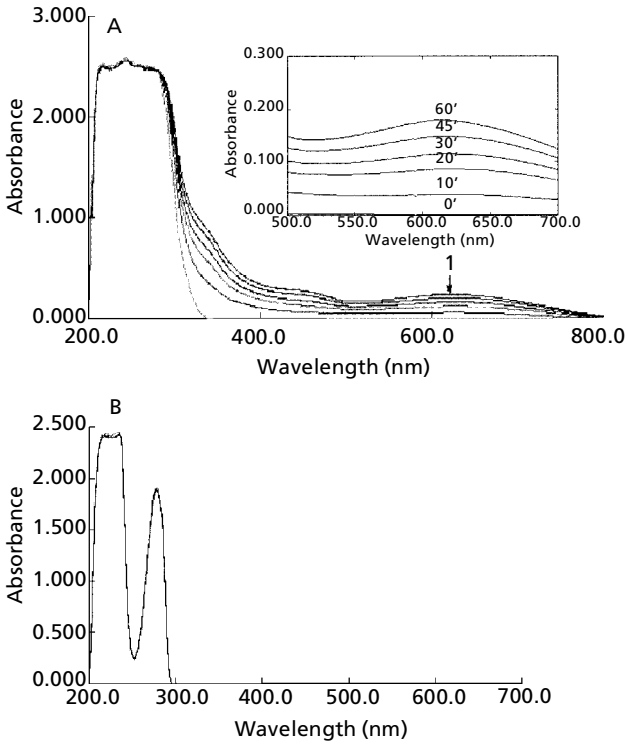

Figure 2 Time dependent change in UV-visible spectrum of gallic acid. (A), Gallic acid. The wavelength at which maximal changes occurred is shown as 1 (620 nm). The inset for (A) shows in detail 500–700 nm. (B), 3,4-Dihydroxybenzoic acid. Scans were taken at 0, 10, 20, 30, 45 and 60 min.

Table 3 Effects of pre-incubation with NADPH and gallic acid on 17β HSD, chlorzoxazone 6-hydroxylase and ethoxyresorufin-*O*-deethylase by 0.1 mM concentration of gallic acid in human liver microsomes.

Activity		NADPH	nmol (mg microsomal protein)$^{-1}$ min^{-1}
17β HSD	No compound	+	0.18±0.04
		−	0.13±0.02
	Gallic acid	+	0.11±0.03
		−	0.08±0.03
Chlorzoxazone 6-hydroxylase	No compound	+	0.61±0.04
		−	1.25±0.01
	Gallic acid	+	0.61±0.04
		−	1.15±0.02
Ethoxyresorufin-*O*-deethylase	No compound	+	0.031±0.001
		−	0.031±0.002
	Gallic acid	+	0.034±0.001
		−	0.026±0.002

Values are means±s.d. of three independent experiments carried out and conducted in duplicate. Human microsomes were incubated first with 0.1 mM gallic acid in the absence or presence of NADPH for 15 min. The pre-incubation mixtures were then diluted, substrate and generating system added and the activities determined.

included in the incubation the effects that were observed with gallic acid, without NADPH, were essentially abolished. We examined inhibition by a structural analogue 3,4-dihydroxybenzoic acid, using human liver microsomes in the presence or absence of NADPH. Studies using human liver microsomes demonstrated a significant decrease in androstenedione 6β-hydroxylase activity with gallic acid pre-incubated without NADPH; this was not observed with 3,4-dihydroxybenzoic acid. The structurally related metabolites catechol and benzoquinone were examined. In the presence of catechol, a significantly lower androstenedione 6β-hydroxylase activity was determined after pre-incubation in the absence of NADPH compared with pre-incubation carried out in the presence of NADPH. Benzoquinone essentially abolished androstenedione 6β-hydroxylase activity after pre-incubation in the presence and in the absence of NADPH.

In further studies, we evaluated the possibility that enhanced inhibition of CYP3A activity might be due to irreversible inactivation. Free polyphenol-derived products were removed by ultrafiltration before the determination of inhibition. After this procedure androstenedione 6β-hydroxylase activity was unchanged from that in control incubations, thus indicating the reversible nature of inhibition (Table 2).

The CO-reduced CYP difference spectra (not shown) were similar irrespective of pre-incubation conditions. The CYP content in the absence of test compound was found to be 0.25±0.05 and 0.28±0.05 nmol (mg microsomal protein)$^{-1}$ for incubations carried out in the presence or absence of NADPH respectively (n = 3, individual incubations). For incubations in the presence of gallic acid the CYP content was found to be 0.27± 0.05 nmol (mg microsomal protein)$^{-1}$ irrespective of whether incubations were carried out in the presence or absence of NADPH (n = 3, individual incubations).

Absorbance spectra were recorded over a 60-min period for gallic acid, gallic acid with NADPH or ascorbic acid and for 3,4-dihydroxybenzoic acid. Results obtained for gallic acid and 3,4-dihydroxybenzoic acid are shown in Figure 2. Concentrations of compounds used were such that changes could be readily demonstrated. The spectra recorded for gallic acid alone indicated the formation of a radical species with a λ_{max} of 620 nm. This was not observed in the presence of the antioxidants ascorbic acid or NADPH (not shown).

Spectra were recorded also for gallic acid alone in 0.1 M K_2HPO_4 or 0.1 M KH_2PO_4. The formation of a radical species was observed in 0.1 M K_2HPO_4 only (not shown), consistent with formation in an alkaline and not an acidic solution. There was no destruction of the radical species by the addition of NADPH to the cuvette containing incubated gallic acid (not shown). Absorbance spectra were recorded under the same conditions with 3,4-dihydroxybenzoic acid. The formation of a radical species could not be demonstrated.

The assay used to determine androstenedione 6β-hydroxylase activity also allowed us to determine reductive 17β HSD activity. Assays to measure chlorzoxazone 6-hydroxylase and ethoxyresorufin-*O*-deethylase activities were carried out (marker activities for CYP2E and CYP1A, respectively). These activities were determined with microsomes that were pre-incubated with or without gallic acid and with or without NADPH. Results are presented in Table 3 and indicated that there was no inhibition of CYP2E or CYP1A activity in the presence of gallic acid. The results indicated that the abolition of CYP3A activity observed when gallic acid was pre-incubated with microsomes in the absence of NADPH was not observed for reductive microsomal 17β HSD activity or chlorzoxazone 6-hydroxylase and ethoxyresorufin-*O*-deethylase activities.

Discussion

The evidence for the chemoprotective effects of green and black tea is not definitive (reviewed Yang & Wang 1993; Kohlmeier et al 1997; Chow et al 1999). However, this study demonstrated the potential of the red wine and tea-derived complex phenol gallic acid to decrease activation of carcinogens such as aflatoxin B_1.

Experiments were carried out to determine whether gallic acid was a mechanism-based inhibitor of androstenedione 6β-hydroxylase activity. In the case of mechanism based inhibition, an increase in inhibitory potency was observed with pre-incubation of microsomes and compound in the presence of NADPH (reviewed Murray 1999). However, our interpretation of the results was complicated by the results observed when gallic acid was pre-incubated without NADPH before androstenedione 6β-hydroxylase activity determination.

When gallic acid was pre-incubated with human liver microsomes without NADPH, androstenedione 6β-hydroxylase activity was significantly decreased (Table 1). This significant decrease in activity could be prevented by the addition of ascorbic acid or glutathione. Absorbance spectra of gallic acid showed the formation of a new species, the formation of which could be prevented by the presence of ascorbic acid or NADPH. We hypothesize that gallic acid was able to form a quinone radical species (Figure 3), which was able to decrease CYP activity. Our hypothesis is consistent with recent studies examining the stability of gallic acid, which have suggested the formation of unstable quinone intermediates in alkaline conditions (Satoh et al 1999; Friedman & Jurgens 2000). Ascorbic acid is well documented as an antioxidant and glutathione is well known as a reactive species scavenger. The role of ascorbic acid in scavenging radicals of gallic acid has been demonstrated using electron spin resonance spectroscopy (Satoh et al 1999). Experiments to determine whether the reactive quinone radical species was destroyed by the addition of NADPH indicated that this did not occur, clearly confirming the relationship between the inhibition of CYP (Table 1) and the formation of this reactive species. The results for 3,4-dihydroxybenzoic acid showing that there was no formation of a quinone type species detectable by spectroscopy and that there was no significant inhibition of androstenedione 6β-hydroxylase activity by pre-incubation of this compound in the absence of NADPH with human liver microsomes support our hypothesis further. Similarly, the extent of inhibition of androstenedione 6β-hydroxylase activity by benzoquinone and catechol substantiates the potential for inhibition by these quinone-type chemicals (Figure 1).

To determine if irreversible inhibition was occurring, experiments were carried out where gallic acid was

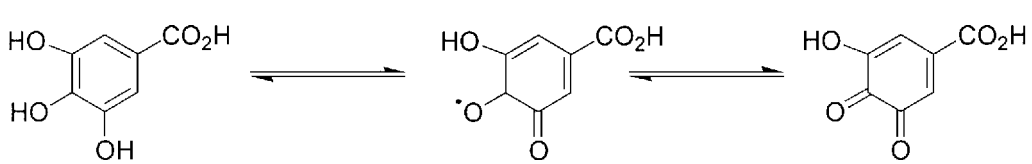

Figure 3 Structure of gallic acid and proposed quinone.

removed from the pre-incubation mix before the addition of androstenedione and the determination of activity (Table 1). These experiments showed that inhibition observed with the reactive intermediate was reversible i.e. no covalent complex was formed between CYP3A and the quinone radical. Thus, when gallic acid/gallic acid radical was removed from the incubation mix, no inhibition of CYP3A activity was observed.

Previously, we reported that gallic acid inhibited reductive microsome mediated 17β HSD activity (50$\pm$4% inhibition at 100 μM gallic acid) (Stupans et al 2000). Pre-incubation with or without NADPH did not increase the inhibition observed i.e. the formation of the postulated quinone did not have any effect on reductive microsome mediated 17β HSD activity. Similarly, chlorzoxazone 6-hydroxylation and ethoxyresorufin-*O*-deethylase activities were not altered by pre-incubation of the gallic acid with the microsomes in the absence of NADPH.

Thus we have provided evidence for the significant inhibition of human liver CYP3A mediated activity by gallic acid after pre-incubation in the absence of NADPH. The inhibition was reversible by removal of the gallic acid/gallic acid radical. Inhibition was not observed for other CYPs or non-CYP activities examined.

The proposed beneficial effects of tea in various neoplasms has led to the examination of gallic acid for its effects on both direct and indirect acting mutagens in the Ames test. However, these studies have only been carried out using induced rodent liver and have not examined pre-incubation effects. Results from a study by Hour et al (1999) have indicated that gallic acid possesses weak antimutagenic activity against a range of indirect acting mutagens such as 2-acetylaminofluorene, benzo(a)pyrene and aflatoxin B_1 at relatively high concentrations (approximately 180 μM). However, Apostolides et al (1997) indicated no apparent antimutagenic activity against 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (inhibitory IC50 >1000 μM).

It is recognized that antimutagenic effects may be the result of reduced formation of the ultimate mutagenic metabolite or by scavenging of reactive mutagenic metabolites to prevent their reaching the critical target sites. Our data, which indicated the formation of quinone radical species of gallic acid which inhibited CYP3A, together with its free radical scavenging capacity (Burns et al 2000; Pulido et al 2000) suggested that under appropriate conditions (such as those where NADPH concentrations are depleted) gallic acid may significantly decrease CYP activation of mutagens.

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