Carcinogenesis vol.17 no.4 pp.821–828, 1996

The effects of dietary ellagic acid on rat hepatic and esophageal mucosal cytochromes P450 and phase II enzymes

Dean Ahn[1], David Putt, Laura Kresty[2], Gary D.Stoner[2], David Fromm[1] and Paul F.Hollenberg[3]

Departments of Pharmacology and [1]Surgery, Wayne State University, Detroit, MI 48201 and [2]Laboratory of Cancer Chemoprevention and Etiology, Department of Preventive Medicine, The Ohio State University, Columbus, OH 43210, USA

[3]To whom correspondence should be addressed at: Department of Pharmacology, University of Michigan, 2301 MSRB III, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0632, USA

Ellagic acid (EA), a naturally occurring plant polyphenol possesses broad chemoprotective properties. Dietary EA has been shown to reduce the incidence of *N*-2-fluorenylacetamide-induced hepatocarcinogenesis in rats and *N*-nitrosomethylbenzylamine (NMBA)-induced rat esophageal tumors. In this study changes in the expression and activities of specific rat hepatic and esophageal mucosal cytochromes P450 (P450) and phase II enzymes following dietary EA treatment were investigated. Liver and esophageal mucosal microsomes and cytosol were prepared from three groups of Fisher 344 rats which were fed an AIN-76 diet containing no EA or 0.4 or 4.0 g/kg EA for 23 days. In the liver total P450 content decreased by up to 25% and P450 2E1-catalyzed *p*-nitrophenol hydroxylation decreased by 15%. No changes were observed in P450 1A1, 2B1 or 3A1/2 expression or activities or cytochrome b_5 activity. P450 reductase activity decreased by up to 28%. Microsomal epoxide hydrolase (mEH) expression decreased by up to 85% after EA treatment, but mEH activities did not change. The hepatic phase II enzymes glutathione S-transferase (GST), NAD(P)H:quinone reductase [NAD(P)H:QR] and UDP glucuronosyltransferase (UDPGT) activities increased by up to 26, 17 and 75% respectively. Assays for specific forms of GST indicated marked increases in the activities of isozymes 2-2 (190%), 4-4 (150%) and 5-5 (82%). In the rat esophageal mucosa only P450 1A1 could be detected by Western blot analysis and androstendione was the only P450 metabolite of testosterone detectable. However, there were no differences in the expression of P450 1A1, the formation of androstendione or NAD(P)H:QR activities between control and EA-fed rats in the esophagus. Although there was no significant decrease in overall GST activity, as measured with 1-chloro-2,4-dinitrobenzene (CDNB), there was a significant decrease in the activity of the 2-2 isozyme (66% of control). *In vitro* incubations showed that EA at a concentration of 100 μM inhibited P450 2E1, 1A1 and 2B1 activities by 87, 55 and 18% respectively, but did not affect 3A1/2 activity. Using standard steady-state kinetic analyses, EA was shown to be a potent non-competitive inhibitor of both liver microsomal ethoxyresorufin *O*-deethylase and *p*-nitrophenol hydroxylase activities, with apparent K_i values of ~55 and 14 μM respectively. In conclusion, these results demonstrate that EA causes a decrease in total hepatic P450 with a significant effect on hepatic P450 2E1, increases some hepatic phase II enzyme activities [GST, NAD(P)H:QR and UDPGT] and decreases hepatic mEH expression. It also inhibits the catalytic activity of some P450 isozymes *in vitro*. Thus the chemoprotective effect of EA against various chemically induced cancers may involve decreases in the rates of metabolism of these carcinogens by phase I enzymes, due to both direct inhibition of catalytic activity and modulation of gene expression, in addition to effects on the expression of phase II enzymes, thereby enhancing the ability of the target tissues to detoxify the reactive intermediates.

Introduction

Ellagic acid (EA*) is a naturally occurring plant polyphenol, present in high concentrations in various fruits and nuts regularly consumed by humans (1). EA has been shown to possess numerous anticarcinogenic and antimutagenic properties towards a variety of different carcinogens, including nitrosamines, azoxymethane, mycotoxins and polycyclic aromatic hydrocarbons (2–7). EA also reduces the incidence of *N*-2-fluorenylacetamide-induced carcinogenesis in rats (8) and *N*-nitrosomethylbenzylamine (NMBA)-induced tumors in the rat esophagus (9,10). Dietary administration of 400 p.p.m. ellagic acid for 16 weeks inhibited the incidence of hepatocellular neoplasms induced in rats by *N*-2-fluorenylacetamide by 70% (from 100 to 30%) (8). When administered orally to rats in a AIN-76 diet at concentrations of 0.4 and 4.0 g/kg EA produced a significant decrease (21–60%) in the average number of NMBA-induced esophageal tumors after 20–27 weeks (9,10). EA also decreased the number of esophageal preneoplastic and neoplastic lesions induced by NMBA (9,10).

Several mechanisms have been proposed to explain the broad antimutagenic and anticarcinogenic effects of EA (4,11–16). One of the mechanisms proposed involves inhibition of cytochrome P450 enzymes (P450) (11). EA has been shown to lower total P450 content in rat hepatic (11,17,18), lung (11) and esophageal microsomes (18). It has also been shown to inhibit the bioactivation of procarcinogens which are metabolized by the P450 1A family, including benzo[*a*]pyrene, 3-methylcholanthrene (3-MC) and the food mutagen 2-amino-3-methylimidazole[4,5-*f*]quinoline (IQ) (2,15,17,19–22). Barch and Fox (18) showed that dietary EA significantly reduced total esophageal and hepatic P450 levels and significantly reduced the esophageal microsomal metabolism of NMBA. Subsequent studies *in vivo* by Barch and Fox (14) demonstrated

*Abbreviations: EA, ellagic acid; NMBA, *N*-nitrosomethylbenzylamine; P450, cytochrome P450; 3-MC, 3-methylcholanthrene; IQ, 2-amino-3-methylimidazole[4,5-*f*]quinoline; DMSO, dimethylsulfoxide; GST, glutathione S-transferase; NAD(P)H:QR, NAD(P)H:quinone reductase; PNP, *p*-nitrophenol; CDNB, 1-chloro-2,4-dinitrobenzene; BNF, β-napthoflavone; PB, phenobarbital; BCA, bicinchoninic acid; KPi, potassium phosphate; ECL, enhanced chemiluminescence; mEH, microsomal epoxide hydrolase; EROD, ethoxyresorufin dealkylase; PROD, pentoxyresorufin dealkylase; UDPGT, UDP glucuronosyltransferase; MI, metabolic intermediate.

that dietary EA significantly reduced NMBA metabolic activation and DNA alkylation in the esophagus. Wilson *et al.* showed that EA [dissolved in dimethylsulfoxide (DMSO)] inhibited P450 2E1-catalyzed *p*-nitrophenol hydroxylation (23).

Another mechanism proposed for the broad chemoprotective effects of EA involves induction of glutathione S-transferase (GST) activity in the liver (2,11). Majid *et al.* also demonstrated that dietary administration of EA to mice increased the levels of reduced glutathione and glutathione reductase in liver (13).

The purpose of this study was to identify any changes in specific rat hepatic and esophageal mucosal P450s and phase II enzyme activities and/or expression following dietary treatment with EA. First, it has not yet been determined whether EA is specific in its inhibitory effects on P450 enzymes. Das *et al.* suggested that EA treatment may alter the composition of cytochrome P450 isozymes in mice (11). However, Aryton *et al.* suggested that EA is a non-selective inactivator of P450s (17). Second, NMBA is bioactivated by P450 enzyme(s) in rat esophageal mucosa (24). Barch and Fox (18) found that dietary EA decreased the total amount of NMBA metabolism in the rat esophagus. However, it was not determined if the reduction in esophageal microsomal metabolism of NMBA following dietary administration of EA was due to a decrease in the concentration of a specific P450 form or to inhibition of esophageal P450 activity. Third, although earlier studies had reported an inhibitory effect of EA on P450 2E1 activity (23), the EA was dissolved in DMSO, which has been shown to be a competitive inhibitor of P450 2E1 activity (25) and appropriate control studies had not been performed. Fourth, the mechanism of inhibition of P450 enzymes by EA has not been determined. Finally, the effect of EA on the phase II enzymes GST and NAD(P)H:quinone reductase [NAD(P)H:QR] in the rat esophageal mucosa had not been investigated.

In this study the effects of dietary EA on the *in vivo* expression of specific forms of P450 and selected phase II enzymes in rat liver and esophageal mucosa and its *in vitro* effects as an inhibitor of the activities of several P450 forms were examined.

Materials and methods

Chemicals

EA, *p*-nitrophenol (PNP), erythromycin, 1-chloro-2,4-dinitrobenzene (CDNB), uridine diphosphoglucuronic acid triammonium salt, nicotinamide adenine dinucleotide, flavin adenine dinucleotide, β-napthoflavone (BNF), phenobarbital (PB), testosterone, 14α-OH testosterone, 11β-OH testosterone, 2,6-dichloroindophenol, dicumarol [3,3′-methylene-bis(4-hydroxycoumarin)] and cytochrome c were purchased from Sigma Inc. (St Louis, MO). Ethoxyresorufin and pentoxyresorufin were purchased from Molecular Probes Inc. (Eugene, OR). Acetone was purchased from Baxter Inc. (McGaw Park, IL). Bicinchoninic (BCA) reagents were purchased from Pierce Inc. (Rockford, IL).

Animal care and study design

Male Fischer 344 rats (5–6 weeks old) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). The animals were quarantined for 2 weeks and then divided into three groups. Group 1 consisted of 54 rats fed an AIN-76 modified diet (no. 110107; Dyets Inc.) for 23 days; group 2 consisted of 51 rats fed the AIN-76 diet containing 0.4 g/kg EA for 23 days; group 3 consisted of 51 rats fed the AIN-76 diet containing 4.0 g/kg EA for 23 days.

Preparation of microsomes

Harvesting of tissues. Fasted rats were sacrificed by CO_2 asphyxiation and the livers and esophagi were immediately excised. Esophagi were harvested from each rat and livers were harvested from six randomly selected rats per treatment group. A total of 153 esophagi and 18 livers were collected. The tissues were rinsed in ice-cold KCl solution and frozen in dry ice after harvesting.

Livers. Rat livers from each group were pooled and homogenized in a Waring blender in 100 mM potassium phosphate (KPi) buffer, pH 7.4, containing 150 mM KCl and 1 mM EDTA. The homogenate was centrifuged in a Sorvall RC2-B centrifuge at 16 270 *g* for 35 min. The supernatant was filtered through four layers of cheesecloth and the pellet was discarded. The supernatant was ultracentrifuged at 100 000 *g* for 75 min and the pellet was resuspended in 100 mM sodium pyrophosphate, 1 mM EDTA, pH 7.4, using a Potter–Elvehjem homogenizer and recentrifuged at 100 000 *g* for 75 min. The supernatant (cytosol) was saved and stored at −70°C. The pellet was then suspended in 100 mM KPi, 1 mM EDTA, 20% sucrose, pH 7.4 buffer, and stored at −70°C.

Esophagi. The esophagi were rinsed in ice-cold KCl solution after harvesting. The esophageal mucosae were then mechanically stripped off the submucosae and any muscular tissues. The mucosae were then slit longitudinally and frozen in dry ice. After the esophageal mucosae were harvested from all rats they were transferred from dry ice into ice-cold 0.15 M KCl w/EDTA. The mucosae were blotted and weighed, and to each gram of mucosa was added 4.5 ml homogenizing buffer (150 mM KCl, 10 mM KPi, 0.5 mM EDTA). The tissues were then homogenized with a teflon–glass homogenizer and the homogenate centrifuged at 9000 *g* for 30 min at 0°C. The supernatant was then carefully removed and saved, the pellet discarded and the supernatant centrifuged at 105 000 *g* for 90 min at 0°C. The resulting supernatant (cytosol) was removed and stored at −70°C. The pellet was resuspended in 0.25 M sucrose buffer (0.5 ml/g tissue) and the microsomes stored at −70°C.

Quantitation of total cytochrome P450

The total P450 content of the rat hepatic microsomes was determined using the spectral method of Omura and Sato (26). The total P450 content of the esophageal microsomes could not be determined since the P450 level in the esophageal microsomes was below the detection limits of the spectral assay.

The P450 specific contents of the hepatic microsomes were 0.56, 0.42 and 0.47 nmol P450/mg protein respectively for control and 0.4 and 4.0 g/kg EA-treated animals (Table I).

Electrophoresis and Western blot analysis

The effect of EA on expression of specific rat hepatic and esophageal mucosal cytochrome P450 forms was determined by immunoblot analysis. SDS gel electrophoresis of rat hepatic and esophageal mucosal microsomes was performed using 7.5% acrylamide gels according to the method of Laemmli (27). Following electrophoresis the proteins were electroblotted onto a nitrocellulose membrane and blocked for 1 h with a 5% solution of dried milk powder. The membrane was immersed in the antibody solution either for 4 h or overnight (depending on the antibody) and then for 1 h in horseradish peroxidase–protein A conjugate solution (BioRad). The antibody–antigen complex was visualized using the enhanced chemiluminescence (ECL) method (Amersham). Western blots were performed using polyclonal antibodies raised against rabbit anti-rat cytochrome P450 1A1 (a gift from Dr Henry Strobel, University of Texas Medical School, Houston, TX), 2B1 (prepared as described previously; 28), 3A1/2 (a gift from Dr Fred Guengerich, Vanderbilt University), sheep anti-rabbit P450 2E1 (a gift from Dr Dennis Koop, University of Oregon Health Science Center), P450 reductase (prepared as described previously) (29) and rabbit anti-rat hepatic microsomal epoxide hydrolase (mEH) (a gift from Drs Sang G.Kim and Raymond Novak, Wayne State University). Silver staining was performed as described by Wray *et al.* (30). Densitometric analyses of the immunoblots were performed using the AMBIS™ Radioanalytic Imaging System and the AMBIS QuantProbe™ Software version 3.0 from AMBIS Inc. (San Diego, CA).

Protein determination

Protein concentrations of the rat hepatic samples were determined by the method of Lowry *et al.* (31). The protein concentrations of the esophageal microsomes and cytosols were determined by the BCA assay (32), since reliable estimates of protein concentration of these microsomes were unobtainable using the Lowry method.

P450 enzyme activity assays

The effect of ellagic acid on enzyme activities associated with specific rat hepatic P450s was studied by assaying ethoxyresorufin dealkylation (EROD) (33), pentoxyresorufin dealkylation (PROD) (34), PNP hydroxylation (35), erythromycin *N*-demethylation (36) and testosterone hydroxylation (using 14α-OH testosterone as an internal standard for the hepatic microsomes) (37). The testosterone hydroxylation assay was also performed on the esophageal mucosal microsomes, using 11β-OH testosterone as an internal standard.

Phase II enzyme assays

Total GST and NAD(P)H:QR activities in rat liver and esophageal mucosal cytosol were assayed as described by Habig *et al.* (38) and Benson *et al.* (39) respectively. The effect of EA on enzyme activities associated with specific

Table I. Effects of dietary EA on the catalytic activities of rat hepatic P450s and other microsomal enzymes

Enzyme activity	Control[a]	EA (0.4 g/kg)	EA (4.0 g/kg)
P450[b]	0.56 ± 0.03	0.42 ± 0.01 (75)[c,j]	0.47 ± 0.01 (84)[k]
EROD-1A1[d]	21.11 ± 1.16	18.26 ± 1.10 (86)	22.64 ± 0.96 (107)
PROD-2B1[e]	2.97 ± 1.46	2.23 ± 0.56 (75)	3.68 ± 0.98 (124)
Erythromycin *N*-demethylase-3A1/2[f]	14.41 ± 0.54	12.15 ± 0.94 (84)	12.33 ± 0.90 (86)
PNP 2E1[g]	0.44 ± 0.01	0.37 ± 0.02 (84)[k]	0.38 ± 0.02 (86)[k]
P450 reductase[h]	124.96 ± 4.03	89.85 ± 10.06 (71)[k]	98.50 ± 2.15 (79)[j]
Cytochrome b_5[i]	0.331 ± 0.004	0.302 ± 0.008 (91)	0.331 ± 0.003 (100)

Rats were fed EA in the diet and microsomes were prepared as described in Materials and methods. P450 levels and enzyme activities were determined as described in Materials and methods.
[a]Data are expressed as mean ± SD (n = 3 for all assays).
[b]Specific content nmol P450/mg protein.
[c]Percent of control.
[d]EROD, pmol resorufin formed/min/mg.
[e]PROD, pmol resorufin formed/min/mg.
[f]nmol HCHO formed/min/mg.
[g]nmol *p*-nitrocatechol formed/min/mg.
[h]nmol/min/mg.
[i]nmol/mg.
[j]Significantly different from control by one-tailed Student's *t*-test, $P < 0.01$.
[k]Significantly different from control by one-tailed Student's *t*-test, $P < 0.05$.

GST isozymes was studied by using substrates and inhibitors specific for the six basic GST activities in rat liver (40–42). UDP glucuronosyltransferase (UDPGT) activity was assayed only in rat liver microsomes, since sufficient quantities of esophageal microsomes were not available for these assays. UDPGT activity was assayed as described by Burchell and Weatherill (43). Microsomal mEH activity was assayed as described by Guenthner *et al.*, with slight modifications (44).

Determination of cytochrome P450 reductase and cytochrome b_5

Cytochrome b_5 concentrations in microsomes from control and EA-fed rats were determined as described by Levin *et al.* (45). Cytochrome P450 reductase activity was determined as described by Phillips and Langdon (46).

EA inhibition studies of specific P450 isozyme activities in vitro

EA was dissolved in buffer just prior to the incubations. The EA solution (1 mM) in 50 mM KPi buffer (pH either 6.8 or 7.4, depending on the assay performed) was filtered through a 0.5 μm Millipore filter, yielding a clear filtrate. The pH of the buffer did not change with addition of EA. The absorbance spectrum of the filtrate was determined from 200 to 600 nm using a Uvikon 930 spectrophotometer, and the EA concentration calculated using an extinction coefficient of 8.51×10^3/M/cm for the absorbance observed at 360 nm (47). The appropriate amount of the EA solution was then added to the incubation mixture to give the desired final concentration.

The effect of EA *in vitro* on rat hepatic P450 activities was examined by incubating EA (100 μM) with the microsomes indicated in enzyme assays thought to be specific for the different forms of P450. The assays used and the forms were: EROD (1A1) activity, using hepatic microsomes from BNF-treated rats (80 mg/kg i.p. for 4 days); PROD (2B1) and erythromycin *N*-demethylation (3A1/2) activities, using hepatic microsomes from PB-treated rats (0.1% in drinking water for 12 days); PNP hydroxylation (2E1) activity, using hepatic microsomes from acetone-treated rats (1.0% in drinking water for 7 days). The effect of EA *in vitro* on rat hepatic P450 reductase activity was examined by incubating EA (100 μM), dissolved in DMSO, with PB-induced microsomes in the assay described by Phillips and Langdon (46), DMSO added to PB-induced microsomes without EA served as controls. The specific P450 contents of the microsomes were 1.44, 2.54 and 0.97 nmol/mg protein for BNF-, PB- and acetone-treated rats respectively.

Determination of inhibition constants (K_i) for EA

The K_i of EA for the inhibition of EROD activity (1A1) was determined using hepatic microsomes from BNF-treated rats (0.1 nmol P450/reaction) and the standard EROD assay procedure (33). Five concentrations of ethoxyresorufin were used, 0.1, 0.3, 0.5, 0.7 and 0.9 μM, and the concentrations of EA used were 0, 50 and 100 μM. The K_i value was determined from double reciprocal plots of enzyme activity versus substrate concentration (48). The K_i of EA for inhibition of PNP hydroxylation activity (2E1) was determined using hepatic microsomes from acetone-treated rats (0.1 nmol P450/reaction). Five concentrations of PNP were used, 20, 60, 100, 140 and 180 μM, and the concentrations of EA used were 0, 30 and 70 μM. The K_i value was determined from double reciprocal plots of enzyme activity versus substrate concentration (48).

Metabolic intermediate (MI)–heme complex formation studies

These experiments were performed to determine if EA inhibited P450 via the metabolism-dependent formation of an inactive metabolic intermediate (MI)–heme complex intermediate with P450 (49). The procedure was similar to that used to dissociate troleandomycin from rat liver microsomes (49).

Binding spectra

Hepatic microsomes from BNF- or acetone-treated rats were diluted to 1 mg/ml in 50 mM KPi buffer, pH 7.4 (containing 20% glycerol), the solutions divided into tandem cuvettes and the baselines recorded. EA in concentrations ranging from 0 to 300 μM was added to the microsomal and buffer compartments of sample and reference cuvettes respectively and incubated at 25°C for 5 min. The samples were then scanned repeatedly from 360 to 660 nm and the difference spectra were recorded using a Uvikon 930 spectrophotometer.

Metabolism of EA by liver microsomal P450

To determine if EA was a substrate for P450, EA (400 μM) was incubated with hepatic microsomes (2 nmol of P450/reaction) from non-induced or BNF-, acetone- or PB-treated rats in 50 mM KPi buffer, pH 7.4, in a final volume of 1 ml. NADPH (1 mM) was added to initiate the reaction and samples were incubated at 37°C for 10 min. Reactions were terminated by addition of 3 ml ethyl acetate. Test tubes were vortexed, placed on ice for 5 min and centrifuged at 800 *g* for 15 min. The organic phase was removed, evaporated with compressed air and the residue dissolved in 100 μl methanol. Metabolite formation from EA was examined by HPLC analysis, using the HPLC method described by Boukharts *et al.* (6).

Results

As shown in Table I, the P450 specific contents of the hepatic microsomes of the EA-fed rats were decreased by 16–25% from those of control rats. For the P450 catalytic activities of the hepatic microsomes from control and EA-fed rats the only statistically significant change in activities was seen for P450 2E1-catalyzed PNP hydroxylation (Table I). Although the decrease in PNP hydroxylation activity after EA treatment was small (~15%), it was statistically significant ($P < 0.05$). The P450 reductase activities also were decreased by up to 29% (Table I). No changes were observed in the activities of the other P450 forms tested nor in the levels of cytochrome b_5.

Testosterone hydroxylation activity, a general assay which can be used to monitor several forms of P450, showed no

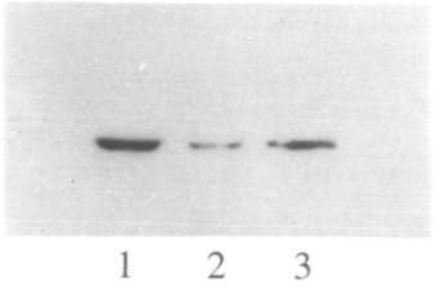

Fig. 1. Effect of EA on the expression of epoxide hydrolase in rat liver microsomes. Rats were fed EA (0.4 or 4.0 g/kg) in the diet and hepatic microsomes were prepared as described in Materials and methods. The microsomal proteins were separated by SDS–PAGE and Western blot analysis was performed as described in Materials and methods. Each lane contained 20 μg microsomal protein. **Lane 1**, control; **lane 2**, microsomes from rats fed 0.4 g/kg EA; **lane 3**, microsomes from rats fed 4.0 g/kg EA.

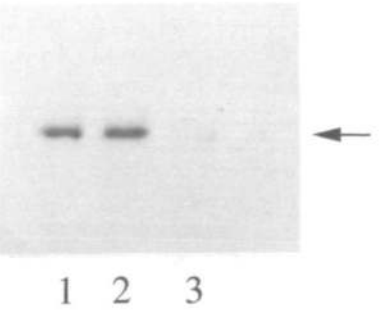

Fig. 2. Effect of EA on the expression of P450 1A1 in rat esophageal microsomes. Rats were fed EA (4.0 g/kg) in the diet and esophageal microsomes prepared as described in Materials and methods. The microsomal proteins were separated by SDS–PAGE and Western blot analysis was performed as described in Materials and methods. Each lane contained 80 μg esophageal microsomal protein. **Lane 1**, control; **lane 2**, microsomes from rats fed 4.0 g/kg EA; **lane 3**, purified P450 1A1 (45 ng).

significant differences between liver microsomes of control and EA-fed rats (data not shown). Androstendione was readily measurable as the only observable metabolite of testosterone formed by esophageal mucosal microsomes, however, there were no statistically significant differences between control and EA-fed rats (data not shown).

SDS–PAGE of the rat hepatic microsomes showed no significant differences in the banding patterns and apparent densities in the P450 region between the control and EA-fed groups when proteins were detected using either Coomasie or silver staining (data not shown). Western blot analysis of rat hepatic microsomes from control and EA-fed rats using antibodies raised against P450 1A1, 2B1, 2E1 and 2A1/2 or P450 reductase showed no significant differences between controls and animals treated with EA (data not shown). In contrast, Western blotting of rat hepatic microsomes using an antibody raised against rat liver mEH showed a significant decrease in the levels of mEH in the EA-treated animals (Figure 1). Densitometry of the bands in Figure 1 indicated an 85% decrease in mEH in animals exposed to 0.4/kg EA (data not shown).

SDS–PAGE of the rat esophageal microsomes showed no significant differences in the banding patterns and apparent densities in the P450 region between control and EA-fed groups when proteins were detected by Coomasie or silver staining. A protein band having the same mobility as P450 1A1 was observed in both control and treated samples. Western blot analysis of the rat esophageal mucosal microsomes confirmed that detectable levels of P450 1A1 were present in esophageal mucosal microsomes from both control and EA-fed (4.0 g/kg) rats, although no significant differences in the levels of P450 1A1 were observed after EA treatment (Figure 2). P450 2B1, 2E1 and 3A1/2 could not be detected in rat esophageal mucosal microsomes by immunoblotting (data not shown).

The effects of EA (100 μM) as an inhibitor of specific rat liver microsomal P450 and P450 reductase activities *in vitro* was investigated. EA caused significant inhibtion of P450 1A1-catalyzed EROD activity (50%), P450 2E1-catalyzed PNP hydroxylation (87%) and P450 2B1-catalyzed PROD activity (18%). However, EA had no effect *in vitro* on P450 3A1/2-catalyzed erythromycin *N*-demethylase activity and only a small inhibitory effect on P450 reductase activity (11%). In order to further characterize the inhibition of P450 catalytic activity by EA studies were performed in which the concentration of the P450 substrate in the assay mixture was systematically varied while the concentration of EA was maintained constant and the initial rates for product formation were determined. This was done using two different concentrations of EA with each of the substrates showing significant inhibition (EROD and PNP). Double reciprocal plots of the data for inhibition of P450 1A1-catalyzed EROD and P450 2E1-catalyzed PNP hydroxylation activities by EA are shown in Figures 3 and 4 respectively. As shown in Figure 3, the inhibition of EROD activity by EA appears to be non-competitive and a K_i of ~55 μM was determined. As shown in Figure 4, the inhibition of PNP hydroxylation by EA also appears to be non-competitive and a K_i of ~14 μM was determined.

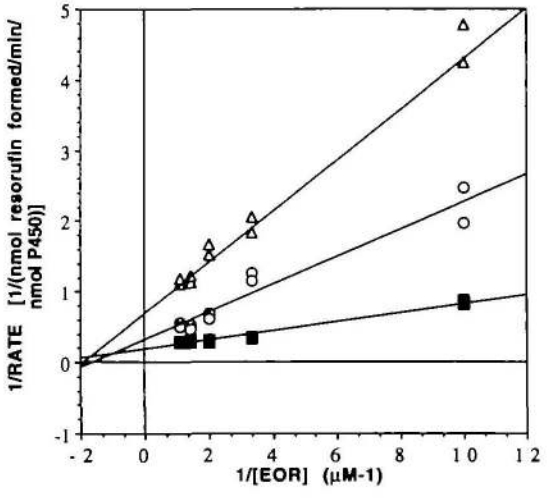

Fig. 3. Double reciprocal plot of the initial rate data for the inhibition of cytochrome P450 ethoxyresorufin *O*-deethylase activity by EA The inhibition studies were performed as described in Materials and methods using liver microsomes from BNF-treated rats. Concentrations of ellagic acid added to reaction incubations were 0 (■), 50 (○) and 100 μM (△).

In order to investigate the possible basis for the significant (up to 25%) decrease in spectrally observable P450 without a corresponding decrease in P450 detectable by Western blotting we investigated the possibility that EA forms an MI–heme complex with P450. However, the lack of spectral change in the Soret region following addition of potassium ferricyanide to microsomes from EA-treated rats (data not shown) ruled out the formation of a P450 MI complex with EA. In addition, EA did not exhibit either type I or II binding spectra with

P450 1A1- or 2E1-enriched microsomes at concentrations of EA as high as 300 μM (data not shown). Finally, EA was not metabolized to any significant extent by microsomes from BNF-pretreated (1A1-enriched) or acetone-pretreated (2E1-enriched) rats (data not shown).

The effects of dietary EA on the catalytic activities of selected phase II enzymes in rat liver and esophageal mucosa were also investigated. As shown in Table II, increases of ~26, 17 and 75% were observed in hepatic GST, NAD(P)H:QR and UDPGT activities respectively. Hepatic mEH activities did not change after dietary EA treatment. As can be seen in Table II, however, EA had no significant effect on the activities of GST or NAD(P)H:QR in rat esophageal mucosa. In order to gain a better understanding of the effect of EA on GST we investigated the changes in the activities of several specific GST isozymes in response to EA treatment. As shown in Table III, significant increases in isozymes 2-2 (190%), 4-4 (150%) and 5-5 (82%) were seen in the liver. In the esophagus there was a slight (66% of control), but significant, decrease in GST 2-2 activity.

Discussion

EA has been shown to possess numerous anticarcinogenic and antimutagenic properties (2–7). It also has been shown to inhibit NMBA-induced tumors in rat liver (8) and esophagus (9,10). Several different mechanisms have been proposed to explain the chemoprotective effects of EA (4,11–16). The purpose of this study was to examine in detail the effects of dietary EA on specific forms of P450 and also on selected phase II enzymes in rat liver and esophageal mucosa.

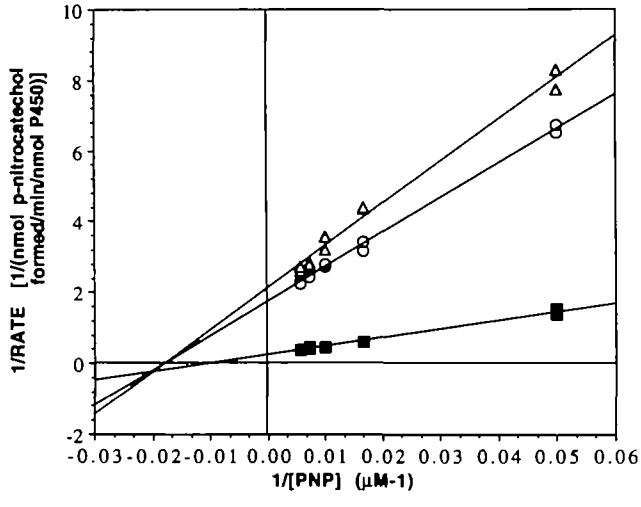

Fig. 4. Double reciprocal plot of the initial rate data for the inhibition of cytochrome P450 PNP activity by EA. The inhibition studies were performed as described in Materials and methods using liver microsomes from acetone-treated rats. Concentrations of ellagic acid added to reaction incubations were 0 (■), 30 (○) and 70 μM (△).

In this study dietary EA resulted in a decrease in total hepatic cytochrome P450 of up to 25%. This is in agreement with earlier reports (11,17,18). However, this decrease did not appear to be dose-dependent for the two concentrations of EA (0.4 and 4.0 g/kg) used, which may be attributable to the fact that both concentrations were in excess of the maximum amount required to suppress P450 or to the variable oral absorption of EA (50). The only statistically significant decrease in any of the individual hepatic P450 enzyme activities assayed was for PNP hydroxylase, presumably due to a decrease in P450 2E1. P450 2E1 in rats and humans is the principle enzyme for the bioactivation of a variety of low molecular weight suspect carcinogens, such as benzene, styrene and vinyl chloride, as well as nitrosamine carcinogens such as *N*-nitrosodimethylamine (51). Das *et al.* demonstrated earlier that hepatic and lung aryl hydrocarbon hydroxylase and 7-ethoxycoumarin *O*-deethylase activities (markers for P450 1A1) decreased in BALB/C mice after chronic administration of EA in drinking water for 16 weeks or acute i.p. administration of EA for 5 days (11). In this study dietary administration of EA over 23 days did not affect rat hepatic ethoxyresorufin dealkylase activity, an assay highly specific for P450 1A1 (33).

The most marked effects of EA on P450 activities were seen *in vitro*. These results suggest several interesting aspects with respect to the effects of EA on various forms of P450. First, EA appears to be a strong inhibitor of P450 2E1 and P450 1A1, two forms which are important in the bioactivation of a variety of suspect and known procarcinogens in rats and humans (51). P450 2E1-catalyzed PNP hydroxylation was inhibited 87% by 100 μM EA. Although inhibition of PNP hydroxylation by EA has previously been reported (23), those studies were done using EA dissolved in DMSO, a potent inhibitor of 2E1 (25). Our results conclusively demonstrate that the inhibition *in vitro* is due to EA rather than DMSO. These studies also show that EA exhibits a differential inhibition of P450 isozymes. While EA was a potent inhibitor of both P450 2E1 and 1A1 activities, P450 3A1/2 activity was not significantly affected by 100 μM EA. Finally, although oral absorption and bioavailability of EA are variable (50), the *in vitro* data presented here suggest that if EA is present

Table II. Effect of dietary EA on rat hepatic and esophageal mucosal phase II enzyme activities

	Liver				Esophagus	
	GST[a,b]	NAD(P)H:QR	UDPGT	mEH	GST	NAD(P)H:OR
Control	0.224 ± 0.009	0.018 ± 0.001	193.37 ± 54.41	6.00 ± 1.71	0.091 ± 0.005	1.144 ± 0.086
EA 0.4 g/kg	0.272 ± 0.004[c]	0.016 ± 0.001	285.45 ± 45.49	6.35 ± 1.44	0.080 ± 0.004	1.092 ± 0.106
EA 4.0 g/kg	0.283 ± 0.018[d]	0.021 ± 0.001[c]	338.86 ± 47.07[c]	6.61 ± 1.34	0.089 ± 0.006	1.108 ± 0.039

Rats were fed EA in the diet and hepatic and esophageal cytosols and microsomes were prepared as described in Materials and methods. Enzyme activities were determined as described in Materials and methods.

[a]GST activity is expressed as μmol 1-chloro-2,4-dinitrobenzene conjugated/min/mg, NAD(P)H:QR activity is expressed as μmol 2,6-dichloroindophenol reduced/min/mg, UDPGT activity is expressed as nmol 4 nitrophenyl-glucuronide formed/min/mg and mEH activity is expressed as nmol diol product formed from [^{14}C]styrene oxide/min/mg.

[b]Values are mean ± SD (n = 3 for all assays).

[c]Significantly different from control by Student's t-test, $P < 0.05$.

[d]Significantly different from control by Student's t-test, $P < 0.01$.

Table III. Effect of dietary EA on rat hepatic and esophageal mucosal GST enzyme activities

Isozyme	Liver		Esophagus	
	Control	EA-treated	Control	EA-treated
CDNB	0.348 ± 0.075[a,b]	0.650 ± 0.087[c]	0.0389 ± 0.0029	0.303 ± 0.002
1-1	0.006 ± 0.001	0.006 ± 0.001	0.00163 ± 0.00024	0.00178 ± 0.00027
2-2	0.067 ± 0.001	0.199 ± 0.014[c]	0.0133 ± 0.0014	0.00889 ± 0.00065[c]
3-3	0.030 ± 0.002	0.031 ± 0.002	0.00202 ± 0.00015	0.00218 ± 0.00018
4-4	0.037 ± 0.002	0.094 ± 0.017[d]	0.01448 ± 0.00164	0.01425 ± 0.00244
5-5	2.757 ± 0.090	5.038 ± 0.457[d]	0.00427 ± 0.00015	0.00461 ± 0.00025
6-6	0.331 ± 0.039	0.334 ± 0.010	0.03073 ± 0.00173	0.02902 ± 0.00216

Rats were fed EA (4.0 g/kg) in the diet and hepatic and esophageal cytosols were prepared as described in Materials and methods. Enzyme activities were determined as described in Materials and methods.
[a]Enzyme activity is expressed as μmol/min/mg protein.
[b]Values are mean ± SD (n = 3 for all assays).
[c]Significantly different from control by Student's t-test, $P < 0.01$.
[d]Significantly different from control by Student's t-test, $P < 0.05$.

in sufficient concentrations in target organs it may inhibit bioactivation of procarcinogens by specific forms of P450. Mandal and Stoner (9) have demonstrated that the concentration of EA in the esophageal tissue of rats fed a 4 g/kg EA diet, as described here, is ~15 μM, suggesting that tissue levels *in vivo* are of the same order of magnitude as the K_i values for the inhibition of 1A1 and 2E1 determined here.

The effect of dietary EA on cytochromes P450 in the rat esophageal mucosa was also examined. Barch and Fox had previously demonstrated that dietary EA decreased total esophageal P450s, but they did not determine changes in specific P450s (18). We were not able to determine total P450 content of the rat esophageal mucosal microsomes, since we did not have sufficient quantities of these microsomes available for this measurement. Of the forms investigated by Western blot analysis only P450 1A1 could be detected in rat esophageal mucosal microsomes, but EA administration did not affect the level of its expression. Although it is conceivable that EA may inhibit NMBA-induced tumors in the rat esophagus by inhibiting rat esophageal mucosal P450 1A1 activity, we were unable to test this hypothesis, since the limited quantities of esophageal microsomes available did not allow us to perform the EROD assay. However, as noted previously, EA is a potent non-competitive inhibitor of liver 1A1.

Western blots demonstrated that EA administration resulted in a marked decrease in the expression of hepatic mEH. mEH is important both in the bioactivation of the procarcinogen benzo[*a*]pyrene to the ultimate carcinogen, benzo[*a*]pyrene 7,8-diol-9,10-epoxide-2 (52), as well as detoxification of other procarcinogens (53). The decrease in the expression of mEH is of interest, since EA has been shown to inhibit polycyclic aromatic hydrocarbon carcinogenesis (2,15,19–22). However, in this study dietary EA treatment had no effect on mEH catalytic activity as measured by the metabolism of styrene oxide. Das *et al.* earlier demonstrated that chronic oral feeding of EA over 16 weeks or acute i.p. administration of EA over 5 days to BALB/c mice did not effect EH activities in liver or lung (11), as we have seen in rat liver. Mukhtar *et al.* demonstrated that chronic oral feeding of EA to BALB/c mice over 120 days did decrease EH activity in skin (2). The explanation for the discrepancy between the decrease in mEH expression and lack of change in mEH activity is unclear.

This study also examined the effects of dietary EA on several phase II enzymes in the rat liver and esophageal mucosa. EA increased hepatic GST activity by up to 26%, which agrees with earlier studies (2,11). However, much more significant increases were seen when the activities of specific GSTs were measured, with increases ranging from ~80% (5-5) up to 190% (2-2). These GST isozymes were selected for further study since they are major hepatic forms, are fairly well characterized with regard to substrate preference and they are thought to play important roles in the detoxication of a variety of carcinogens (54). Form 2-2 is a homodimer thought to act as a non-selenium-dependent GSH peroxidase. Form 4-4 is a homodimeric GST which metabolizes a variety of mutagenic arene oxides. GST 5-5 is also a homodimer and shows very low activity with CDNB. In addition, dietary EA increased hepatic UDPGT and NAD(P)H:QR activities by up to 75 and 17% respectively. The induction of hepatic UDPGT activity was one of the most significant changes seen in any of the drug metabolizing enzymes tested in this study. The UDPGTs are a superfamily of enzymes which catalyze glucuronidation of various xenobiotics and endogenous compounds in most tissues (55). A significant decrease in GST form 2-2 (66%) was observed in rat esophagus. Other than that change, no other significant changes were observed in GST or NAD(P)H:QR activities in the rat esophageal mucosal cytosol. Therefore, dietary EA appears to have a more profound effect on the activities of phase II enzymes than phase I enzymes. Also, its effects on phase II enzymes exhibit some tissue specificity. The significant induction of several phase II enzymes in the liver may play an important role in the ability of EA to protect the liver against hepatotoxins and carcinogens metabolized by these enzymes.

In conclusion, EA is a broad chemopreventive agent which may exert its anticarcinogenic effects by several mechanisms. EA inhibits hepatic cytochrome P450 1A1 and 2E1 in a non-competitive manner. EA also induces some hepatic phase II enzymes. In this study there was no effect of EA on the P450s of the esophageal mucosa, but it did have significant effects on some esophageal GSTs. Since EA appears to only induce phase II enzymes and not P450s, EA may be a monofunctional anticarcinogenic enzyme inducer (56). These results may explain in part the chemoprotective properties of EA towards several carcinogens. Finally, its ability to act as a potent inhibitor of some forms of P450 may also be an important aspect of its chemoprotective activity.

Acknowledgements

We would like to thank the following people for their generous gifts of antibodies: Drs Sang G.Kim and Raymond Novak of Wayne State University (anti-microsomal epoxide hydrolase), Dr Henry Strobel of the University of Texas Medical School at Houston (anti-1A1), Dr Dennis Koop of the University of Oregon Health Science Center (anti-2E1), Dr Tom Primiano of Johns Hopkins University (anti alpha, mu, and pi GST) and Dr Fred Guengerich of Vanderbilt University (anti-3A1/2). We would like to thank Corey Dean for his assistance with the GST assays. This research was supported in part by NCI grants CA 28950 to GDS and CA 16954 to PFH.

References

1. Daniel,E.M., Krupnick,A.S., Heur,Y.H., Blinzler,J.A., Nims,R.W. and Stoner,G.D. (1990) Extraction, stability and quantitation of ellagic acid in various fruits and nuts. *J. Fd Comp. Anal.*, **2**, 338–349.
2. Mukhtar,H., Das,M. and Bickers,D.R. (1986) Inhibition of 3-methylcholanthrene-induced skin tumorigenicity in BALB/C mice by chronic oral feeding of trace amounts of ellagic acid in drinking water. *Cancer Res.*, **46**, 2262–2265.
3. Mandal,S., Ahuja,A., Shivapurkar,N.M., Cheng,S.J., Groopman,J.D. and Stoner,G.D. (1987) Inhibition of aflatoxin B_1 mutagenesis in *Salmonella typhimurium* and DNA damage in cultured rat and human tracheobronchial tissues by ellagic acid. *Carcinogenesis*, **8**, 1651–1656.
4. Dixit,R. and Gold,B. (1986) Inhibition of *N*-methyl-*N*-nitrosourea-induced mutagenicity and DNA methylation by ellagic acid. *Proc. Natl Acad. Sci. USA*, **83**, 8039–8043.
5. Rao,C.V., Tohumo,K., Rigotty,J., Zang,E., Kelloff,G. and Reddy,B.S. (1991) Chemoprevention of colon carcinogenesis by dietary administration of piroxicam, α-difluoromethylornithine, 16α-fluoro-5-androsten-17-one, and ellagic acid individually and in combination. *Cancer Res.*, **51**, 4528–2534.
6. Boukharts,M., Jalbert,G. and Castonguay,A. (1992) Biodistribution of ellagic acid and dose-related inhibition of lung tumorigenesis in A/J mice. *Nutr. Cancer*, **18**, 181–189.
7. Lesca,P. (1983) Protective effects of ellagic acid and other plant phenols on benzo[*a*]pyrene-induced neoplasia in mice. *Carcinogenesis*, **4**, 1651–1653.
8. Tanaka,T., Iwata,H., Niwa,K., Mori,Y. and Mori,H. (1988) Inhibitory effect of ellagic acid on *N*-2-fluorenylacetamide-induced liver carcinogenesis in male ACI/N rats. *Jpn. J. Cancer Res. (Gann)*, **79**, 1297–1303.
9. Mandal,S. and Stoner,G.D. (1990) Inhibition of *N*-nitrosobenzylmethylamine-induced esophageal tumorigenesis in rats by ellagic acid. *Carcinogenesis*, **11**, 55–61.
10. Daniel,E.M. and Stoner,G.D. (1991) The effects of ellagic acid and 13-*cis*-retinoic acid on *N*-nitrosobenzylmethylamine-induced esophageal tumorigenesis in rats. *Cancer Lett.*, **56**, 117–124.
11. Das,M., Bickers,D.R. and Mukhtar,H. (1985) Effect of ellagic acid on hepatic and pulmonary xenobiotic metabolism in mice: studies on the mechanism of its anticarcinogenic action. *Carcinogenesis*, **6**, 1409–1413.
12. Wood,A.W., Huang,M.T., Chang,R.L., Newmark,H.L., Lehr,R.E., Yagi,H., Sayer,J M., Jerina,D.M. and Conney,A.H. (1982) Inhibition of the mutagenicity of bay-region diol epoxides of polycyclic aromatic hydrocarbons by naturally occurring plant phenols: exceptional activity of ellagic acid. *Proc. Natl Acad. Sci. USA*, **79**, 5513–5517.
13. Majid,S.M., Khanduja,K.L., Gandhi,R.K., Kapur,S. and Sharma,R.R. (1991) Influence of ellagic acid on antioxidant defense system and lipid peroxidation in mice. *Biochem. Pharmacol.*, **42**, 1441–1445.
14. Barch,D.H. and Fox,C.C. (1988) Selective inhibition of methylbenzylnitrosamine-induced formation of esophageal O^6-methylguanine by dietary ellagic acid in rats. *Cancer Res.*, **48**, 7088–7092.
15. Mukhtar,H., Del Tito,B.J., Jr, Marcelo,C.L., Das,M. and Bickers,D.R. (1984) Ellagic acid: a potent naturally occurring inhibitor of benzo[*a*]pyrene metabolism and its subsequent glucuronidation, sulfation and covalent binding to DNA in cultured BALB/C mouse keratinocyte. *Carcinogenesis*, **2**, 1565–1571.
16. Gali,H.U., Perchellet,E.M. and Perchellet,J.P. (1991) Inhibition of tumor promoter-induced ornithine decarboxylase activity by tannic acid and other polyphenols in mouse epidermis *in vivo*. *Cancer Res.*, **51**, 2820–2825.
17. Aryton,A.D., Lewis,D.F.V., Walker,R. and Ionnides,C. (1992) Anti-mutagenicity of ellagic acid towards the food mutagen IQ: investigation into possible mechanisms of action. *Fd Chem. Toxicol.*, **30**, 289–295.
18. Barch,D.H. and Fox,C.C. (1989) Dietary ellagic acid reduces the esophageal microsomal metabolism of methylbenzylnitrosamine. *Cancer Lett.*, **44**, 39–44.
19 Deltito,B.J., Jr, Mukhtar,H. and Bickers,D.R. (1983) Inhibition of epidermal metabolism and DNA-binding of benzo[*a*]pyrene by ellagic acid. *Biochem. Biophys. Res. Commun.*, **114**, 388–394.
20. Dixit,R., Teel,R.W., Daniel,R.B. and Stoner,G.D. (1985) Inhibition of benzo(*a*)pyrene and benzo(*a*)pyrene-*trans*-7,8-diol metabolism and DNA binding in mouse lung explants by ellagic acid. *Cancer Res.*, **45**, 2951–2956.
21. Teel,R.W., Dixit,R. and Stoner,G.D. (1985) The effect of ellagic acid on the uptake, persistence, metabolism and DNA-binding of benzo[*a*]pyrene in cultured explants of strain A/J mouse lung. *Carcinogenesis*, **6**, 391–395.
22. Mukhtar,H., Mukul,D., Deltito,B.J., Jr and Bickers,D.R. (1984) Protection against 3-methylcholanthrene-induced skin tumorigenesis in BALB/C mice by ellagic acid. *Biochem. Biophys. Res. Commun.*, **119**, 751–757.
23. Wilson,T., Lewis,M.J., Cha,K.L. and Gold,B. (1992) The effect of ellagic acid on xenobiotic metabolism by cytochrome P450 2E1 and nitrosodimethylamine mutagenicity. *Cancer Lett.*, **61**, 129–134.
24. Labuc,G.E. and Archer,M.C. (1982) Esophageal and hepatic microsomal metabolism of *N*-nitrosomethylbenzylamine and *N*-nitrosodimethylamine in the rat. *Cancer Res.*, **42**, 3181–3186.
25. Yoo,J.S.H., Cheung,R.J., Patten,C.J., Wade,D. and Yang,C.S. (1987) Nature of *N*-nitrosodimethylamine demethylase and its inhibitors. *Cancer Res.*, **47**, 3378–3383.
26. Omura,T. and Sato,R. (1964) The carbon monoxide-binding pigment of liver microsomes. *J Biol. Chem.*, **239**, 2379–2385.
27. Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*, **227**, 680–685.
28. Shen,T., Zhuang,Z., McCauley,R., Putt,D. and Hollenberg,P.F. (1991) Differential effects of proteinase K on the components of the liver microsomal cytochrome P450 mixed function oxidase system. *Drug Metab. Dispos.*, **19**, 1016–1021.
29. Kim,H., Putt,D., Reddy,S., Hollenberg,P.F. and Novak,R.F. (1993) Enhanced expression of rat hepatic CYP2B1/2B2 and 2E1 by pyridine: differential induction kinetics and molecular basis of expression. *J. Pharmacol. Exp. Ther*, **267**, 927–936.
30. Wray,W., Boulikas,T., Wray,V.P. and Hancock,H. (1981) Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.*, **118**, 197–203.
31. Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,A.J. (1951) Protein measurements with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
32. Redinbaugh,M.G. and Turley,R.B. (1986) Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. *Anal. Biochem.*, **153**, 267–271.
33. Burke,M.D. and Mayer,R.T. (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.*, **2**, 583–588.
34. Lubet,R.A., Mayer,R.T., Cameron,J.W., Nims,R.W., Burke,M.D., Wolf,T. and Guengerich,F.P. (1985) Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P450 by phenobarbital and other xenobiotics in the rat. *Archs Biochem. Biophys.*, **238**, 43–48.
35. Koop,D.R., Laethem,C.L. and Tierney,D.J. (1989) The utility of *p*-nitrophenol hydroxylation in P450 2E1 analysis. *Drug Metab. Rev.*, **20**, 541–551.
36. Wrighton,S.A., Schuetz,E.G., Watkins,P.B., Maurel,P., Barwick,J., Bailey,B.S., Hartle,H.T., Young,B. and Guzelian,P.S. (1985) Demonstration in multiple species of inducible hepatic cytochromes P450 and their mRNAs related to the glucocorticoid-inducible cytochrome P450 of the rat. *Mol. Pharmacol.*, **28**, 312–321.
37. Wood,A.W., Ryan,D.E., Thomas,P.E. and Levin,W. (1983) Regio- and stereoselective metabolism of two C_{19} steroids by five highly purified and reconstituted rat hepatic cytochrome P450 isozymes. *J. Biol. Chem.*, **258**, 8839–8847.
38. Habig,W.H., Pabst,M.J. and Jakoby,W.B. (1974) Glutathione-S-transferases: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.*, **249**, 7130–7139.
39. Benson,A.M., Hunkeler,M.J. and Talalay,P. (1980) Increase of NAD(P)H:quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. *Proc. Natl Acad. Sci. USA*, **77**, 5216–5220.
40. Habig,W.H. and Jakoby,W.B. (1981) Assays for differentiation of glutathione S-transferases. *Methods Enzymol.*, **77**, 398–405.
41. Mannervik,B. and Jensson,H. (1982) Binary combinations of four protein subunits with different catalytic specificities explain the relationship between six basic glutathione S-transferases in rat liver cytosol. *J. Biol. Chem.*, **257**, 9909–9912.
42. Valcin,S., Jensson,H. and Mannervik,B. (1983) A set of inhibitors for discrimination between the basic isozymes of glutathione transferase in rat liver. *Biochem. Biophys. Res. Commun.*, **114**, 829–834.
43. Burchell,B. and Weatherill,P. (1981) 4-Nitrophenol UDP glucuronyl-

transferase (rat liver). In Jakoby,W.B. (ed.), *Methods in Enzymology*. Academic Press, New York, NY, Vol. 77, pp. 169–170.
44. Guenthner,T.M., Bentley,P. and Oesch,F. (1981) Microsomal epoxide hydrolase. In Jakoby,W.B. (ed.), *Methods in Enzymology*, Academic Press, New York, NY, Vol. 77, pp. 344–346.
45. Levin,W., Ryan,D., West,S. and Lu,A.Y.H. (1974) Preparation of partially purified, lipid-depleted cytochrome P-450 and reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase from rat liver microsomes. *J. Biol. Chem.*, **249**, 1747–1754.
46. Phillip,A.H. and Langdon,R.G. (1962) Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization, and kinetic studies. *J. Biol. Chem.*, **237**, 2652–2660.
47. Windholz,M., Budavari,S., Stroumtsos,L.Y. and Fertig,M.N (eds) (1976) *The Merck Index, an Encyclopedia of Chemicals and Drugs*, 9th Edn. Merck and Co., Rahway, NJ, p. 466.
48. Lehninger,A.L. (1970) *Biochemistry*. Worth Publishers, New York, NY, pp. 147–167.
49. Wrighton,S.A., Maurel,P., Schuetz,E.G., Watkins,P.B., Young,B. and Guzelian,P.S. (1985) Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450$_p$. *Biochemistry*, **24**, 2171–2178.
50. Smart,R.C., Huang,M.T., Chang,R.L., Sayer,J.M., Jerina,D.M. and Conney,A.H. (1986) Disposition of the naturally occurring antimutagenic plant phenol, ellagic acid, and its synthetic derivatives, 3-*O*-decylellagic acid and 3,3′-di-*O*-methylellagic acid in mice. *Carcinogenesis*, **7**, 1663–1667.
51. Guengerich,F.P. and Shimada,T. (1991) Oxidation of toxic and carcinogenic chemicals by human cytochrome P450 enzymes. *Chem. Res. Toxicol.*, **4**, 391–407.
52. Conney,A.H. (1982) Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons. *Cancer Res.*, **42**, 4975–4917.
53. Oesch,F. (1988) Antimutagenesis by shift in monooxygenase isoenzymes and induction of epoxide hydrolase. *Mutat. Res.*, **202**, 335–342.
54. Mannervik,B. (1985) The isoenzymes of glutathione transferase. *Adv. Biochem. Related Areas Mol. Biol.*, **57**, 357–417.
55. Burchell,B. and Coughtrie,M.W.H. (1989) UDP-glucuronosyltransferases. *Pharmacol. Ther.*, **43**, 261–289.
56. Prochaska,H.J. and Talalay,P. (1988) Regulatory mechanisms of monofunctional and bifunctional anticarcinogenic enzyme inducers in murine liver. *Cancer Res.*, **48**, 4776–4782.

Received on June 23, 1995; revised on December 21, 1995; accepted on December 21, 1995