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Effects of Tea Polyphenols and Flavonoids on Liver Microsomal Glucuronidation of Estradiol and Estrone

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Administration of 0.5 or 1% lyophilized green tea (5 or 10 mg tea solids per ml, respectively) as the sole source of drinking fluid to female Long–Evans rats for 18 days stimulated liver microsomal glucuronidation of estrone, estradiol and 4-nitrophenol by 30–37%, 15–27% and 26–60%, respectively. Oral administration of 0.5% lyophilized green tea to female CD-1 mice for 18 days stimulated liver microsomal glucuronidation of estrone, estradiol and 4-nitrophenol by 33–37%, 12–22% and 172–191%, respectively. The *in vitro* addition of a green tea polyphenol mixture, a black tea polyphenol mixture or (–)-epigallocatechin gallate inhibited rat liver microsomal glucuronidation of estrone and estradiol in a concentration-dependent manner and their IC_{50} values for inhibition of estrogen metabolism were approximately 12.5, 50 and 10 μg/ml, respectively. Enzyme kinetic analysis indicates that the inhibition of estrone glucuronidation by 10 μM (–)-epigallocatechin gallate was competitive while inhibition by 50 μM (–)-epigallocatechin gallate was noncompetitive. Similarly, several flavonoids (naringenin, hesperetin, kaempferol, quercetin, rutin, flavone, α-naphthoflavone and β-naphthoflavone) also inhibited rat liver microsomal glucuronidation of estrone and estradiol to varying degrees. Naringenin and hesperetin displayed the strongest inhibitory effects (IC_{50} value of approximately 25 μM). These two hydroxylated flavonoids had a competitive mechanism of enzyme inhibition for estrone glucuronidation at a 10 μM inhibitor concentration and a predominantly noncompetitive mechanism of inhibition at a 50 μM inhibitor concentration. © **1998 Published by Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Estrone and estradiol (two major endogenous estrogenic hormones) are phenolic steroids that are eliminated from the body by metabolic inactivation (oxidative and conjugative reactions) followed by excretion in the urine and bile. Uridine 5′-diphosphoglucuronic acid (UDPGA)-dependent glucuronidation of estrone and estradiol to form water-soluble glucuronides is a major conjugation reaction catalyzed by microsomal glucuronosyltransferase present in liver and many extrahepatic tissues [1–5]. Studies combining molecular biology and biochemical approaches have shown that the microsomal glucuronosyltransferase is comprised of a large family of isozymes with overlapping but distinguishable substrate specificities [6–8]. The glucuronosyltransferase isozymes with high catalytic activity for the glucuronidation of estrone and estradiol are very different with regard to their biochemical and molecular biology characteristics from the isozyme for the glucuronidation of 4-nitrophenol, although each of the isozymes has some catalytic activity for the other substrates [6–11]. Moreover, studies with different inducers of microsomal enzymes also showed that different glucuronosyltransferase isozymes can be independently regulated [1, 5, 12].

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Abbreviations: UDPGA, uridine 5′-diphosphoglucuronic acid; BTP, black tea polyphenol(s); GTP, green tea polyphenol(s); EGCG, (–)-epigallocatechin gallate; HPLC, high-pressure liquid chromatography.

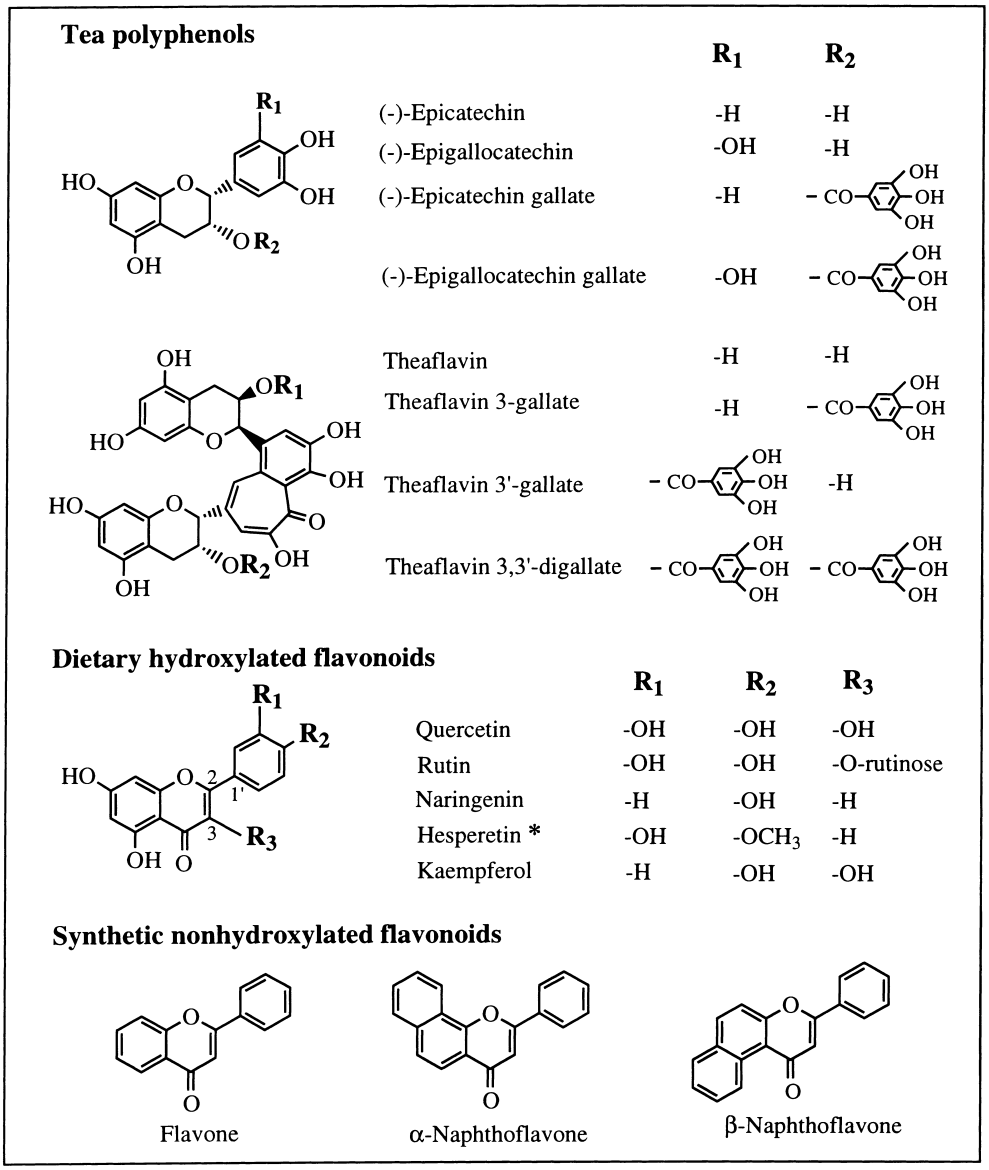

Fig. 1. Structures of several tea polyphenols and several natural or synthetic flavonoids tested in this study. *For the structure of hesperetin (which is a flavanone), there should be a single bond between C2 and C3 and the bond between C1′ and C2 should be represented by a dashed line.

It is known that many naturally occurring hydroxylated dietary chemicals such as catechins in tea and hydroxylated flavonoids in fruit juices are present in significant amounts in our everyday diet (reviewed in Refs. [13–15]. Many of these compounds share polyphenol structures (shown in Fig. 1) and they are extensively glucuronidated in the body by the same UDPGA-dependent conjugation system that participates in estrogen glucuronidation [16, 17]. The possible modulatory effect of tea polyphenols and hydroxylated flavonoids on the metabolic glucuronidation of estrone and estradiol, however, has not been studied and thus is the focus of our present study.

We evaluated the effects of chronic administration of crude extracts of green tea as the sole source of drinking fluid to female Long–Evans rats and female CD-1 mice on hepatic microsomal glucuronosyltransferase activity for estrone and estradiol. The modulatory effect of tea administration on microsomal estrogen glucuronosyltransferase activity was compared with its effect on the enzyme activity for 4-nitrophenol. We also evaluated the *in vitro* effect of tea polyphenols and some flavonoids on the glucuronidation of estrone and estradiol by liver microsomes from untreated female Long–Evans rats. Kinetic mechanisms by which tea polyphenols and flavonoids

inhibit liver microsomal glucuronidation of estrogens were also determined.

MATERIALS AND METHODS

Chemicals and tea components

Estrone, estradiol, UDPGA triammonium salt, quercetin, rutin, naringenin, hesperetin, kaempferol, flavone, α-naphthoflavone and β-naphthoflavone were obtained from Sigma Chemical Co. (St. Louis, MO). (−)-Epigallocatechin gallate (EGCG), a black tea polyphenol (BTP) mixture, a green tea polyphenol (GTP) mixture and theaflavins were gifts from the Thomas J. Lipton Company (Englewood Cliffs, NJ). The composition of the BTP mixture, the GTP mixture and the theaflavins are shown in Table 1 and the purity of EGCG was approximately 90% based on HPLC analysis (data not shown). The HPLC analysis was carried out as described earlier [18]. Samples of lyophilized green tea solids (prepared from the hot-water extracts of green tea leaves) that were used for preparing green tea solution for the *in vivo* animal experiments contained 11% EGCG, 9% (−)-epigallocatechin, 3% (−)-epicatechin gallate, 3% (−)-epicatechin, 1% (+)-catechin, 0.1% gallic acid and 6% caffeine. The lyophilized green tea solids were reconstituted with water (5 or 10 mg tea solids per ml) and administered to rats or mice as the sole source of drinking fluid. [6,7-^{3}H]Estrone and [6,7-^{3}H]estradiol (specific activities of 57 and 55 Ci/mmol, respectively) were obtained from Du Pont New England Nuclear Research Products (Boston, MA). All solvents used in this study were of HPLC grade and were obtained from Fisher Scientific Co. (Springfield, NJ).

Preparation of liver microsomes

Eight-week-old female Long–Evans rats were obtained from Harlan Sprague–Dawley (Indianapolis, IN) and six-week-old female CD-1 mice were from the Jackson Laboratory (Bar Harbor, ME). Animals were allowed free access to water and an AIN-76A diet (a semisynthetic laboratory animal diet prepared by Ralston Purina Co., St. Louis, MO). The female Long–Evans rats were divided into three groups with 3–4 animals in each group: one group of animals was given regular drinking water and the other two groups of animals were given a solution containing 0.5 or 1% lyophilized green tea solids (5 or 10 mg of tea solids per ml, respectively) as the sole source of drinking fluid for 18 days. The female CD-1 mice were divided into two groups: one group of 4–6 animals was given regular drinking water and the other group of 4–6 animals was given a solution containing 0.5% lyophilized green tea as the sole source of drinking fluid for 18 days. Freshly prepared green tea solutions were replaced every two days. After 18 days of treatment with 0.5 or 1% lyophilized green tea solutions, the animals were sacrificed, the liver samples from animals in each treatment group were pooled, and microsomes were prepared by differential centrifugation as previously described [19]. Aliquots of the liver microsomes (approximately 15 mg protein per ml) were stored as a suspension in 0.25 M sucrose at

Table 1. *Composition of tea polyphenol fractions as analyzed by high-pressure liquid chromatography. The HPLC analysis was carried out as described previously [18]*

Compound	Green tea polyphenols (GTP) (% of solids)	Black tea polyphenols (BTP) (% of solids)	Theaflavins (% of solids)
		Catechins	
(+)-Catechin	1.7	1.3	
(+)-Gallocatechin	2.4	0.6	
(−)-Epicatechin	9.8	5.0	
(−)-Epigallocatechin	8.6	1.0	
(−)-Epicatechin gallate	13.2	10.0	
(−)-Epigallocatechin gallate	43.7	16.2	
		Theaflavins	
Theaflavin		4.0	8.5
Theaflavin 3-gallate		3.9	8.5
Theaflavin 3′-gallate		1.7	21.5
Theaflavin 3,3′-digallate		2.9	35.6
Gallic acid		4.0	
Caffeine	0.8		
Total	80.2%	50.8%	99.4%

−80°C. The protein content in liver microsomal preparations was determined with the BioRad protein assay kit according to the procedures of the supplier using bovine serum albumin (BSA) as a standard.

Measurement of microsomal glucuronidation of 4-nitrophenol, estrone and estradiol

The glucuronidation of 4-nitrophenol was carried out as described in a previous study [20]. The glucuronidation of estrone and estradiol was done according to the method described in our previous studies [1, 5]. Briefly, the incubation mixture, unless otherwise indicated, consisted of 1.0 mg of microsomal protein, 2 mM UDPGA, 5 mM $MgCl_2$, estrone or estradiol (containing approximately 0.20 μCi ^{3}H-labeled estrogen) and different concentrations of polyphenol inhibitor in a final volume of 1.0 ml Tris–HCl buffer (50 mM, pH 8.75). Nonradiolabeled estradiol or estrone was dissolved in 5 μl ethanol; tea polyphenols and flavonoids were dissolved either in water or in 5 μl ethanol. The reaction was initiated by addition of microsomal protein. Incubations were carried out at 37°C for 15 min and reactions were arrested by immediately placing the test tube on ice and adding 0.5 ml ice-cold 50 mM Tris–HCl solution (pH 8.75). The reaction mixture was then extracted twice with 7 ml toluene to separate unconjugated estrogen from water-soluble estrogen glucuronides. The toluene-phase, which contained the unconjugated estrogen, was removed after each extraction. Portions (200 μl) of the aqueous phase, which contained glucuronidated estrogen metabolites, were precisely removed and measured for radioactivity content in a Beckman liquid scintillation spectrometer (Model-LS 1701). Blank values, obtained from incubations in the absence of UDPGA, were determined for each individual assay and were subtracted. The average intra-assay variation as determined with pooled liver microsomes was within 8%.

RESULTS

Effect of tea administration on liver microsomal glucuronosyltransferase activity in female Long–Evans rats and CD-1 mice

Administration of 0.5 or 1% lyophilized green tea (5 or 10 mg tea solids per ml) in the drinking fluid to female Long–Evans rats for 18 days increased liver microsomal glucuronidation of estrone, estradiol and 4-nitrophenol by 30–37%, 15–27% and 26–60%, respectively (Table 2).

Earlier studies showed that the extent of induction of hepatic glucuronosyltransferase activity in rats by xenobiotics such as butylated hydroxyanisole and butylated hydroxytoluene was substantially less than the induction of this enzyme activity in CD-1 mice [21, 22]. In this study, therefore, we also evaluated the effect of chronic administration of 0.5% lyophilized green tea to female CD-1 mice on hepatic microsomal glucuronosyltransferase activity for estrone, estradiol and 4-nitrophenol. Administration of 0.5% lyophilized green tea as the sole source of drinking fluid to female CD-1 mice for 18 days increased liver microsomal glucuronidation of estrone and estradiol to a similar extent as seen with female Long–

Table 2. Effect of chronic administration of lyophilized green tea to female Long–Evans rats and CD-1 mice on hepatic glucuronosyltransferase activity

Animals (treatment)	Glucuronosyltransferase activity (pmol/mg protein/min)		
	esterone	estradiol	4-nitrophenol
	Female Long–Evans rats		
Control	876	1107	721
0.5% Lyophilized green tea solids	1139 (130%)	1273 (115%)	908 (126%)
1% Lyophilized green tea solids	1200 (137%)	1406 (127%)	1153 (160%)
	Female CD-1 mice		
	Experiment I		
Control	324	614	880
0.5% Lyophilized green tea solids	431 (133%)	688 (112%)	2561 (291%)
	Experiment II		
Control	356	686	896
0.5% Lyophilized green tea solids	487 (137%)	835 (122%)	2441 (272%)

Female Long–Evans rats (6 weeks old) were treated with a solution containing 0.5 or 1% lyophilized green tea (5 or 10 mg of tea solids per ml, respectively) as the sole source of drinking fluid for 18 days. Female CD-1 mice (6 weeks old) were treated with 0.5% lyophilized green tea solution for 18 days. The experiment with CD-1 mice was repeated twice as shown above (experiment I and II). Livers from rats and mice of the same treatment group were pooled and microsomes were prepared as described earlier [19]. The assays of microsomal glucuronidation of 100 μM estrone, estradiol and 4-nitrophenol are described in Section 2. Each value is the mean of 3 to 4 replicate determinations with pooled liver microsomes. The values in parentheses represent percent of control.

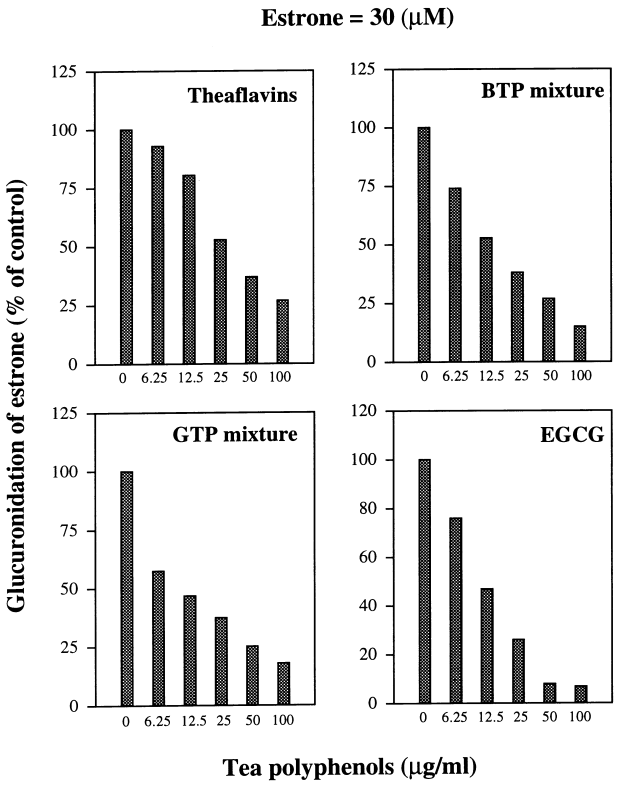

Fig. 2. Inhibition of UDPGA-dependent microsomal glucuronidation of estrone by different concentrations of theaflavins, a BTP mixture, a GTP mixture, or EGCG *in vitro*. The glucuronidation reaction was carried out as described in Materials and Methods. The concentration of estrone was 30 μM and the concentrations of each inhibitor were 6.25, 12.5, 25, 50 and 100 μg/ml as indicated. The rate of microsomal glucuronidation of 30 μM estrone in the absence of inhibitor was 227 pmol/mg protein/min. The relative rate of estrone glucuronidation in the presence of inhibitor was expressed as "% of control", which considers the relative rate of estrone glucuronidation in the absence of tea polyphenols as 100%. Each value is the mean of replicate determinations. This experiment was repeated twice and similar results were obtained.

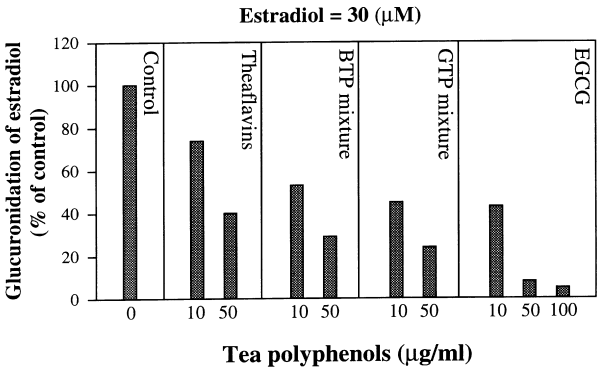

Fig. 3. Inhibitory effect of theaflavins, a BTP mixture, a GTP mixture, or EGCG on UDPGA-dependent microsomal glucuronidation of estradiol *in vitro*. The glucuronidation reaction was carried out as described in Section 2. The concentration of estradiol was 30 μM and the concentrations of EGCG or the tea polyphenol mixtures were 10, 50 or 100 μg/ml as indicated. The rate of microsomal glucuronidation of 30 μM estradiol in the absence of inhibitor was 378 pmol/mg protein/min. The relative rate of estradiol glucuronidation in the presence of each inhibitor was expressed as "% of control", which considers the relative rate of estradiol glucuronidation in the absence of tea polyphenols as 100%. Each value is the mean of replicate determinations.

Evans rats (Table 2), but the liver microsomal glucuronidation of 4-nitrophenol was increased to a much greater extent (172–191%) than was observed with Long–Evans rats (Table 2).

Inhibitory effect of tea polyphenols and flavonoids on microsomal glucuronidation of estrone and estradiol

Using untreated liver microsomes from female Long–Evans rats as a source of glucuronosyltransferase, we examined the direct modulatory effect of tea polyphenols and flavonoids on UDPGA-dependent glucuronidation of estrone and estradiol *in vitro*. Crude extracts of several tea polyphenol fractions (theaflavins, a BTP mixture, a GTP mixture) and highly purified EGCG each inhibited UDPGA-dependent microsomal glucuronidation of estrone in a concentration-dependent manner (Fig. 2). The IC_{50} values for inhibition of microsomal glucuronidation of estrone by a BTP mixture, a GTP mixture, or EGCG were approximately 10 μg/ml (approximately 25 μM for EGCG) and the IC_{50} value for inhibition by a theaflavin mixture was approximately 25 μg/ml (Fig. 2). These tea polyphenolic components also inhibited the UDPGA-dependent microsomal glucuronidation of estradiol in a concentration-dependent manner with similar potencies (Fig. 3).

Several natural and synthetic flavonoids (both hydroxylated and non-hydroxylated flavonoids) all exhibited an inhibitory effect on microsomal glucuronidation of estrone and estradiol (Fig. 4). Among the eight flavonoids tested, naringenin and hesperitin displayed the strongest inhibitory activity (Fig. 4). The IC_{50} values of naringenin and hesperetin for inhibiting estrone glucuronidation were approximately 25 μM (Fig. 5) and similar IC_{50} values were obtained for inhibiting estradiol glucuronidation (data not shown).

We determined the kinetic mechanism of glucuronosyltransferase inhibition by tea polyphenols and flavonoids. In this part of study, estrone was used as a substrate for kinetic analysis because this steroid would only form one glucuronide at the 3-hydroxy group while estradiol would have both the 3- and 17-hydroxy groups available for glucuronidation. Kinetic analysis showed that inhibition of microsomal glucuronidation of estrone by EGCG was essentially competitive at a 10 μg/ml inhibitor concentration but the inhibition was a mixed (competitive plus noncompetitive) mechanism at a 50 μg/ml inhibitor concentration

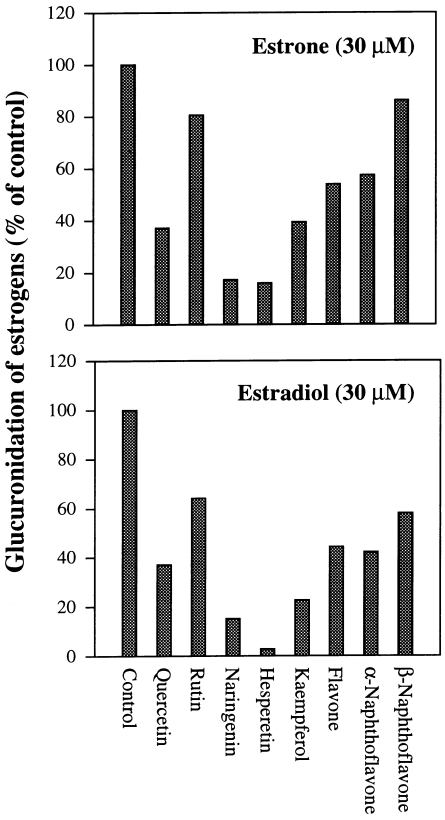

Fig. 4. Inhibitory effect of several natural and synthetic flavonoids on UDPGA-dependent glucuronidation of estrone (*upper panel*) and estradiol (*bottom panel*) *in vitro*. The glucuronidation reaction was carried out as described in Section 2. The concentration of estrone or estradiol was 30 μM and that of the flavonoid inhibitors was 100 μM. The rate of microsomal glucuronidation of 30 μM estrone or estradiol in the absence of inhibitors was 224 or 401 pmol/mg protein/min, respectively. The relative rate of estrogen glucuronidation in the presence of each inhibitor was expressed as "% of control", which considers the relative rate of estrogen glucuronidation in the absence of flavonoids as 100%. Each value is the mean of duplicate determinations. This experiment was repeated twice and similar results were obtained.

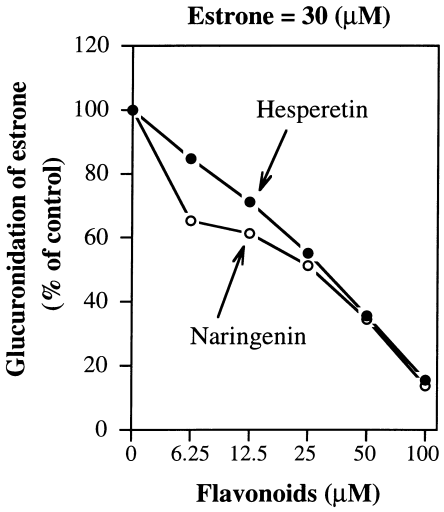

Fig. 5. Inhibition of UDPGA-dependent microsomal glucuronidation of estrone by different concentrations of naringenin and hesperetin *in vitro*. The glucuronidation reaction was carried out as described in Section 2. The concentration of estrone was 30 μM and the concentrations of naringenin and hesperetin were 6.25, 12.5, 25, 50 and 100 μM. The rate of microsomal glucuronidation of 30 μM estrone in the absence of inhibitors was 237 pmol/mg protein/min. The relative rate of estrone glucuronidation in the presence of each inhibitor was expressed as "% of control", which considers the relative rate of estrone glucuronidation in the absence of flavonoids as 100%. Each value is the mean of duplicate determinations.

(Fig. 6). Analysis of the inhibition of microsomal estrone glucuronidation by theaflavins also showed a similar pattern (data not shown).

Kinetic analysis showed that inhibition of microsomal glucuronidation of estrone by naringenin or hesperetin was competitive at a 10 μM inhibitor concentration but the inhibition was essentially noncompetitive at a 50 μM inhibitor concentration (Fig. 7), which is similar to the inhibition pattern seen with EGCG (Fig. 6). In contrast, α-naphthoflavone (a nonhydroxylated flavonoid) inhibited liver microsomal glucuronidation of estrone by a predominantly noncompetitive mechanism at either a 5 or 50 μM concentration (data not shown).

DISCUSSION

The results of our study showed that chronic administration of 0.5 or 1% lyophilized green tea as the sole source of drinking fluid to female Long–Evans rats or female CD-1 mice had only a very small stimulatory effect on liver microsomal glucuronosyltransferase activity for estrone and estradiol (less than 40%; Table 2). In comparison, the liver microsomal glucuronosyltransferase activity for 4-nitrophenol was stimulated in CD-1 mice by ~200% over the control (~60% in female Long–Evans rats). The differential inducibility of liver microsomal glucuronosyltransferase activity for 4-nitrophenol and steroidal estrogens in certain animals has also been observed with other inducers such as 3-methylcholanthrene [1, 5] and butylated hydroxyanisole [22, 23].

Although the assay of the glucuronosyltransferase activities for 4-nitrophenol, estrone and estradiol in this study were done in the absence of a microsomal activating agent (e.g. detergent), our results are in close agreement with a previous report that green tea or green tea polyphenol administration stimulated liver microsomal glucuronosyltransferase activity for 2-aminophenol (assayed in the presence of a detergent) in rats [24]. It should be noted that the use of non-activated microsomes for assay of glucuronosyltransferase activities may give different results

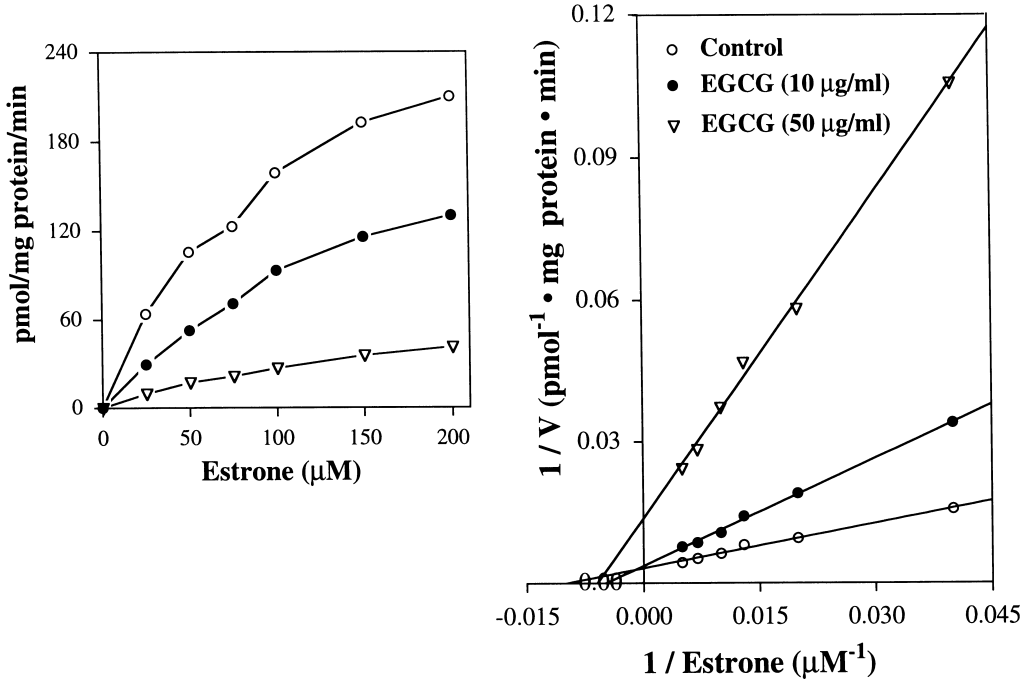

Fig. 6. Double-reciprocal plot of the inhibition of UDPGA-dependent microsomal glucuronidation of estrone by EGCG *in vitro*. The glucuronidation reaction was carried out as described in Section 2. Left inset illustrates the rate of microsomal estrone glucuronidation as a function of substrate concentrations either in the absence or presence of EGCG. Each value is the mean of duplicate determinations.

from the assay of enzyme activities in the presence of a detergent [1, 25, 26]. In an earlier study, we showed that when assays for the glucuronidation of estrone, estradiol, estriol and testosterone were done in the presence or absence of a microsomal activating agent, similar magnitude (fold) of induction of glucuronosyltransferase activity was observed for animals treated with inducers [1]. This was not observed, however, for the glucuronidation of bilirubin [1].

The results of our present study showed that tea polyphenol fractions, EGCG, and some flavonoids are inhibitors of UDPGA-dependent glucuronidation of estrone and estradiol by rat liver microsomes (Figs 2–5). The IC_{50} values for inhibition of estrogen glucuronidation by the tea polyphenol fractions are 10–20 μg/ml (~25 μM for EGCG). The *in vitro* inhibitory effect of tea polyphenols on estrone and estradiol glucuronidation observed in this study may be physiologically relevant because recent studies by Yang and his colleagues showed that the concentrations of tea polyphenols in humans after drinking tea can reach high nM to low μM levels (Ref. [27] and personal communication).

The mechanism of enzyme inhibition by tea polyphenols and hydroxylated flavonoids is competitive at low inhibitor concentrations, and it becomes predominantly noncompetitive at high inhibitor concentrations (Figs 6 and 7). The competitive component of inhibition of microsomal glucuronidation of estrone is expected because tea polyphenols and hydroxylated flavonoids are themselves substrates for glucuronidation by the same UDPGA-dependent enzyme system [16, 17]. However, the mechanism for the noncompetitive component of enzyme inhibition seen at a high inhibitor concentration (50 μM) is not clear. Since nonhydroxylated α-naphthoflavone (not a substrate for the UDPGA-dependent glucuronidation) inhibited microsomal glucuronidation of estrone by a noncompetitive mechanism at both low and high concentrations (data not shown), it is likely that the noncompetitive inhibition is probably due to a nonspecific modification of the microsomal glucuronosyltransferase which alters the enzyme activity and/or alters the interaction of the enzyme with the estrogen substrate.

In summary, chronic administration of green tea as the sole source of drinking fluid to female rats or mice increases, to a small extent, the level of liver glucuronosyltransferase activity for estrone and estradiol. However, the *in vitro* addition of tea polyphenols has a strong inhibitory effect on the liver microsomal glucuronidation of these hormones. It will be of interest to determine the effect of chronic or acute tea administration on the *in vivo* metabolic

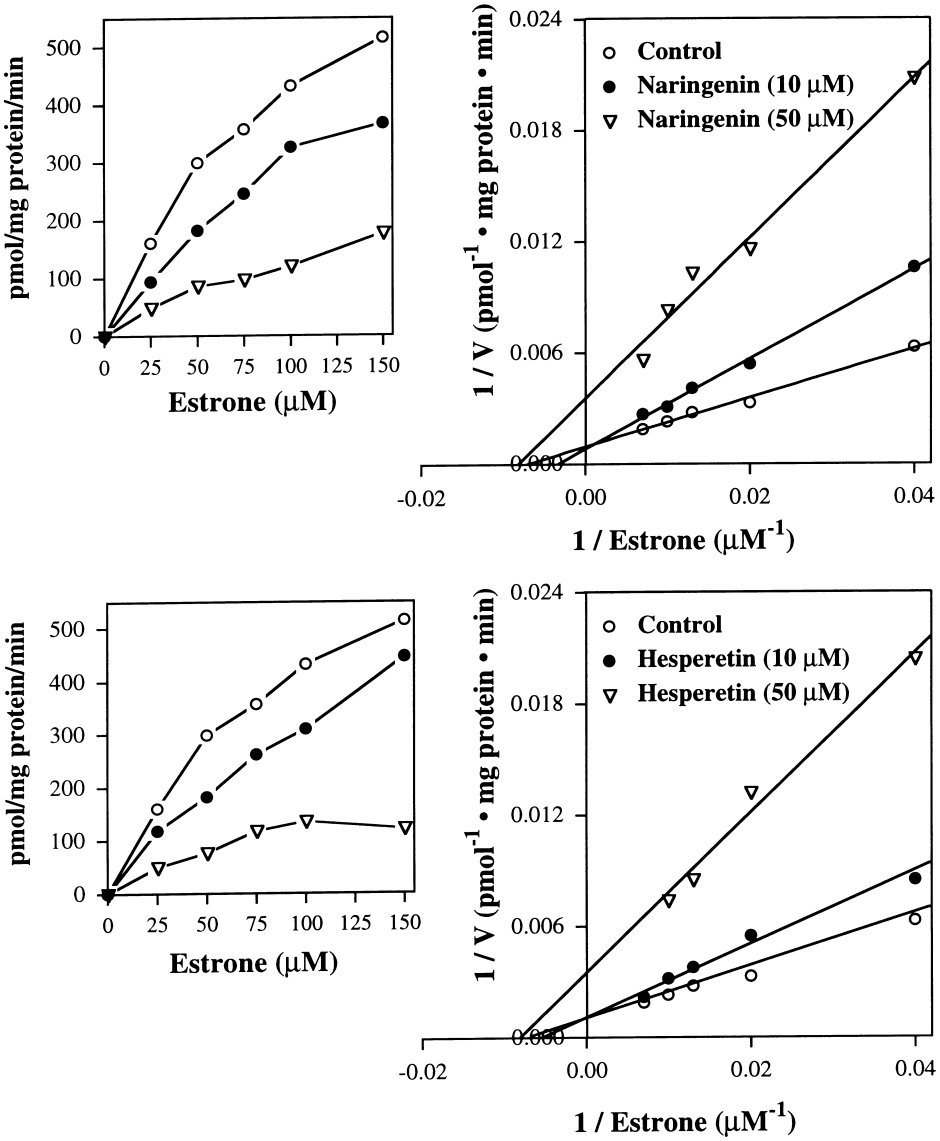

Fig. 7. Double-reciprocal plots of the inhibition of UDPGA-dependent microsomal glucuronidation of estrone by naringenin (upper panel) and hesperetin (lower panel) *in vitro*. The glucuronidation reaction was carried out as described in Section 2. Left insets illustrate the rate of microsomal estrone glucuronidation as a function of substrate concentrations either in the absence or presence of flavonoid inhibitors. Each value is the mean of duplicate determinations. This experiment was repeated twice and similar results were obtained.

glucuronidation of estrone and estradiol in animals and humans.

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