ELSEVIER

Biochemical Pharmacology 64 (2002) 393–404

Biochemical Pharmacology

Modulations of P450 mRNA in liver and mammary gland and P450 activities and metabolism of estrogen in liver by treatment of rats with indole-3-carbinol

Thomas L. Horn[a,1], Mark A. Reichert[b], Robin L. Bliss[c], Danuta Malejka-Giganti[a,b,*]

[a]*Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455, USA*
[b]*Veterans Affairs Medical Center, Research Service (151), One Veterans Drive, Minneapolis, MN 55417, USA*
[c]*Biostatistics Core, University of Minnesota Comprehensive Cancer Center, Minneapolis, MN 55455, USA*

Received 24 July 2001; accepted 24 January 2002

Abstract

Indole-3-carbinol (I3C), found in cruciferous vegetables, has been shown to suppress tumorigenesis at estrogen-responsive sites. This effect may be mediated through modification by I3C of the cytochrome P450 (CYP) complement and activities leading to estrogen detoxication. In this study, we examined the effects of 4- and 10-day treatments of female Sprague–Dawley rats with I3C at 5, 25, and 250 mg/kg body weight, administered by oral gavage, on CYP mRNA expression in the liver and mammary gland, CYP-dependent activities, and the metabolism of 17β-estradiol (E2) and estrone (E1) by liver microsomes. The mRNA transcripts for hepatic CYP1A1, 1B1, and 2B1/2 and mammary CYP1A1 were up-regulated after treatment with I3C at 250 mg/kg. However, the level of expression of CYP1B1 in the liver was lower than that of other CYPs. In the mammary gland, CYP1B1 mRNA levels were unaltered by treatment and similar to those of I3C-induced CYP1A1. Hepatic P450 probe activities indicative of induction of CYP1A1, 1A2, and 2B1/2 were increased by I3C in a dose-dependent manner. Treatment with I3C at 250 mg/kg increased the capacity of liver microsomes to metabolize E2 to 2-OH-E2, 2-OH-E1, 6α-OH-E2, 6β-OH-E2, estriol, and 15α-OH-E2, and E1 to 2-OH-E1, 2-OH-E2, 6(α + β)-OH-E1, and 6α-OH-E2. The magnitudes of increases of CYP-dependent activities and rates of estrogen metabolite formation achieved with I3C at 250 mg/kg were smaller after ten than four treatments. The increased rates of formation of 6α-OH-E2, 6β-OH-E2, and 15α-OH-E2 from E2 were also detected after treatment with I3C at 25 mg/kg, and, except for increased 6β-OH-E2 from E2, no other changes in E2 or E1 metabolism occurred after treatment with I3C at 5 mg/kg. The data indicate that alterations in the CYP complement and, thus, metabolite composition from E2 and E1 are I3C dose- and treatment duration-dependent, and suggest that potential biological activity of I3C administered at low doses to rats may not involve changes in estrogen metabolism.

Keywords: Indole-3-carbinol (I3C)/Female rats; Liver/Mammary gland P450 (CYP) mRNA expression; CYP activities; Estrogen metabolism

1. Introduction

I3C, an autolysis product of an abundant glucobrassicin in cruciferous vegetables (e.g. broccoli, cabbage, cauliflower, and Brussels sprouts), has been shown to inhibit chemically induced tumorigenesis of the liver, mammary gland, colon, and other tissues in laboratory rodents [1]. The blocking activity of I3C administered before or concurrently with a carcinogen is thought to be mediated through alterations in the levels and activities of Phase I (e.g. P450 or CYP) and Phase II (e.g. GSH *S*-transferase) isozymes in the rodent liver and/or extrahepatic tissues resulting in their increased capacity for detoxication of carcinogens, and thus decreased levels of DNA adducts, and reduced tumorigenic response.

* Corresponding author. Tel.: +1-612-725-2000, Ext. 4583; fax: +1-612-725-2255.
E-mail address: malej001@umn.edu (D. Malejka-Giganti).
[1] Present address: IIT Research Institute, Toxicology and Carcinogenesis Division, Life Sciences Operation, 10 West 35th Street, Chicago, IL 60616.

Abbreviations: AhR, aryl hydrocarbon receptor; BROD, benzyloxyresorufin *O*-dealkylase; cDNA, complementary DNA; CYP or P450, cytochrome P450; DIM, diindolylmethane; E1, estrone; E2, 17β-estradiol; E3, estriol; EROD, ethoxyresorufin *O*-deethylase; I3C, indole-3-carbinol; ICZ, indolo(3,2-*b*)carbazole; MROD, methoxyresorufin *O*-demethylase; NIFOX, nifedipine oxidation; PCR, polymerase chain reaction; PROD, pentoxyresorufin *O*-dealkylase; RT–PCR, reverse transcription–PCR; SD, Sprague–Dawley; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; t_R, retention time.

0006-2952/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0006-2952(02)01190-5

In addition, I3C has been shown to suppress spontaneous carcinogenesis at estrogen-susceptible sites, e.g. mammary gland and endometrium [2,3]. This was ascribed to significant increases in CYP-catalyzed 2-hydroxylation of E2 and E1 to the less estrogenic 2-catechol estrogens by liver microsomes of the I3C-treated rodents. Ingestion of I3C at ~5–7 mg/kg body weight for a week or 2 months by men or women, respectively, increased the extent of 2-hydroxylation of E2 *in vivo* [4], and also increased urinary excretion of 2-catechol estrogens but decreased that of more active estrogens (E2, E1, E3, and 16α-OH-E1) [5]. These findings led the authors to postulate that treatment with I3C may decrease the concentration of metabolites known to activate the estrogen receptor and, thus, reduce estrogenic stimulation in women leading to prevention of breast cancer.

Orally administered I3C induces CYP1A1, 1A2, 2B1/2, and 3A1 in the liver of female rats [6–8]. The efficacy of the ingested I3C as an inducer of CYP1 family genes depends upon activation of AhR by I3C products formed in stomach acid, e.g. ICZ and DIM [6,9]. Among the CYP1 gene family, induction of CYP1B1 in the liver and its up-regulation in extrahepatic tissues including the mammary gland of female SD rats treated with TCDD have been reported [10–12]. Whereas the increases of the hepatic CYP1B1 RNA levels were detectable after chronic treatment with TCDD at doses as low as 3.5 ng/kg/day, those of CYP1B1 protein were determined only after the higher doses of TCDD (>35.7 ng/kg/day) [11]. In response to TCDD treatment, the level of expression (number of copies/μg RNA) of CYP1A1 or CYP1A2 was much greater than that of CYP1B1, although its mRNA level was most increased. In the rat liver, CYP1A2, 2B1/2, and the 3A family are recognized as catalysts of both 2- and 4-hydroxylations of E2 and E1 [13]. In the tissues of TCDD-treated female rats, induction of CYP1A1 and 1A2 appeared to be associated chiefly with increased 2-hydroxylation of E2 and E1 and to a lesser extent with their 4-hydroxylation, whereas the latter oxidation correlated with the induction of CYP1B1 [12]. These oxidations are the major source of catechol estrogens which via oxidation to semiquinones and quinones, especially those derived from 4-catechols, may be involved in estrogen carcinogenicity [14–16]. Hence, it is important to delineate the CYP complement and metabolism of estrogen in response to treatment with modifiers such as I3C, which is consumed by humans with vegetables of the Cruciferae family or as a dietary supplement.

In our previous study, treatment of female rats with I3C at 250 or 500 mg/kg body weight for 4 days (acute) or at 250 mg/kg body weight three times per week for 12 weeks (chronic) increased CYP1A1, 1A2, 2B, and 3A probe activities of hepatic microsomes and the metabolism of E2 and E1 including the increased rates of formation of 2-, 4-, 16α-, 6α-, 6β-, and 15α-OH metabolites [17]. It is presumed that persistent effects of the increased putative carcinogenic and estrogenic 4- and 16α-OH as well as 6α- and 6β-OH metabolites of E2 and E1 might have counteracted those of the less estrogenic 2-catechols, thus failing to suppress carcinogen-initiated mammary tumorigenesis by chronic post-initiation treatment of rats with I3C [18].

It also has been shown that the ratios of acid-catalyzed condensation products of I3C formed *in vitro* change with the concentration of I3C [19]. Since the I3C products elicit distinct biological activities [9,20], it is relevant to determine the effects of treatment with I3C following a broad range of dose levels including those relatively low doses that have been tested in humans [5]. In the present study, treatments of female SD rats included three dose levels of I3C, i.e. 5, 25, and 250 mg/kg body weight, administered by oral gavage in 20% ethanol in olive oil for 4 and 10 days. The dose- and treatment duration-related effects of I3C on (a) CYP mRNA expression in the liver and mammary gland, (b) CYP probe activities of liver microsomes, and (c) CYP-dependent metabolism of E2 and E1 by liver microsomes were examined.

2. Materials and methods

2.1. Materials

RNA*later* was from Ambion, Inc. TRIzol, oligo $dT_{12–18}$, RNaseOUT RNase inhibitor, and Superscript™ II reverse transcriptase were from Life Technologies. DNase I and dNTPs (dATP, dCTP, dTTP, and dGTP) were from Promega. *Taq*Master and Master*Taq* DNA polymerase were from Eppendorf Scientific. PCR primers for CYP1A1, 1A2, 2B1/2, 3A1, and cyclophilin were from the PanVera Corp. and that for CYP1B1 was synthesized by Sigma Genosys. Materials for assays of hepatic P450 activities and microsomal E2 and E1 metabolism were from sources described previously [17]. Nifedipine was a gift of Pfizer, and its oxidation product, 3,5-dimethoxycarbonyl-2,6-dimethyl-4-[2-nitrophenyl]-pyridine, was provided by Dr. F. Peter Guengerich. HPLC columns were from Phenomenex, Inc., and Oasis SPE cartridges from the Waters Corp.

2.2. Treatment of rats

Six-week-old female SD rats (specific pathogen-free from Harlan Sprague Dawley, Inc.) were housed in a controlled environment on a 12 hr light/dark cycle and maintained on Teklad Certified Rodent Diet (Harlan Teklad) and water *ad lib.* Treatment of rats (a total of 32) started at 7 weeks of age. A stable solution of I3C in nitrogen-saturated absolute ethanol was mixed with olive oil (1:4) just before treatment by oral gavage. I3C (5, 25, or 250 mg/kg body weight) or vehicle (20% ethanol in olive oil) at 2.5 mL/kg body weight was administered once daily for 4 and 10 days. Rats were euthanized 24 hr after the last

dose. Livers were perfused with 10 mL of ice-cold sterile saline. A portion (~200 mg) of the right liver lobe was removed immediately for RNA analyses and frozen in liquid nitrogen, while the remaining liver was immersed in ice-cold saline, cleaned, cut in small pieces, and then frozen in liquid nitrogen. Mammary glands were excised and placed in RNA*later*, cleaned by removing excess fat, blood vessels, and lymph nodes, and then blotted and frozen in liquid nitrogen. All tissues were stored at −80°.

2.3. RNA extraction

Livers (~250 mg) and mammary glands (~400 mg) were quickly thawed and homogenized in 4–5 mL of TRIzol, and total RNA was isolated according to the directions from Life Technologies. RNA was resuspended in RNase-free water, and DNase I was digested to remove contaminating chromosomal DNA, quantitated using UV spectrophotometry, and stored at −80°.

2.4. CYP semiquantitative RT–PCR

Reverse transcription was performed in a 20 μL volume that contained 0.5 μg oligo $dT_{12–18}$, 0.5 mM dNTPs, either 1 μg total liver or 4 μg total mammary gland RNA, 1× buffer [50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$], 5 mM dithiothreitol, 40 U RNase inhibitor, and 200 U reverse transcriptase and was incubated for 1 hr at 42°. In a total volume of 20 μL, the PCR mixture contained 1× *Taq*Master buffer, 1× buffer [50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$], 0.25 mM dNTPs, and 1 U Master*Taq* DNA polymerase. The final concentration of PCR primers and $MgCl_2$, the volume of reverse transcribed cDNA used, and the PCR amplification conditions are shown in Table 1. An aliquot (8 μL) of the RT–PCR reactants was separated on a 2% agarose gel containing ethidium bromide, visualized under UV light, photographed, and analyzed by densitometry using Scion Image software. The housekeeping gene cyclophilin served as an internal loading control for semiquantitative analyses [21].

2.5. P450 content and P450 isozyme specific activities

Liver microsomal fractions were prepared as previously described [22]. Protein was determined with the Coomassie Blue Plus assay (Pierce Chemical Co.). The P450 content was determined from the carbon monoxide-difference spectra using the extinction coefficient of 91 mM^{-1} cm^{-1} [23]. EROD, MROD, BROD, and PROD activities [24] and NIFOX [25] were assayed as described. All rates were linear with time and protein concentration. Controls contained microsomes inactivated by immersion in a boiling water bath for 5 min.

2.6. Microsomal metabolism of [4-^{14}C]-labeled E2 or E1

The substrates [4-^{14}C]E2 and [4-^{14}C]E1 were determined to be >98% pure by HPLC, and used for the assay of their metabolism described previously [17]. Briefly, hepatic microsomes (1 mg protein) in buffer (100 mM Tris–Cl; 50 mM HEPES, pH 7.4; 1 mM ascorbic acid; 3 mM $MgCl_2$), E2 or E1 (40 μM) containing 0.1 μCi of the ^{14}C-labeled compound and an NADPH-generating system (0.25 mM NADP; 4.75 mM glucose-6-phosphate; 1 U of glucose-6-phosphate dehydrogenase) in 1 mL final volume were incubated for 30 min. Controls were devoid of the NADPH-generating system or contained heat-inactivated microsomes. The reaction was terminated by the addition of 1 vol. of methanol. A mixture of 0.25 mM each of E3, 2-OH-E3, 15α-, 6α-, 6β-, 4-, and 2-OH-E2, and 2-, 4-, and 16α-OH-E1, and E1 and E2 in 10 μL methanol:isopropanol (2:1) was added as carrier. After centrifugation at 14,000 *g* for 1 min at room temperature, the supernatant was applied to an Oasis 30 mg SPE column, fractionated, and processed as described previously [17]. The residue containing the ^{14}C-labeled compounds was resuspended in 30–50 μL isopropanol:methanol (2:1), and 10 μL-aliquots

Table 1
PCR conditions for P450 gene expression in the liver and mammary gland (MG)

Gene[a]	Final concentration		RT cDNA (μL)[b]	Number of PCR cycles		PCR product (bp)
	Primers	$MgCl_2$ (mM)		Liver	MG	
CYP1A1	0.25 nM	1.5	1	30	30	332
CYP1A2	0.25 nM	1.5	1	25	35	237
CYP2B1/2	0.25 nM	1.5	1	25	33–35	550
CYP3A1	0.25 nM	1.5	1	25	30–33	581
CYP1B1[c]	2.00 μM	2.5	1–2[d]	45	30	427
Cyclophilin	0.25 nM	1.5	1	25	25	266

[a] Reaction conditions for CYP1A1, 1A2, 2B1/2, 3A1, and cyclophilin: denatured for 2 min at 94°; amplified at 94° 30 sec, 56° 30 sec, 72° 30 sec; final extension step for 5 min at 72°.
[b] Volume of reverse transcribed cDNA in a 20 μL PCR reaction.
[c] Reaction conditions for CYP1B1 (denatured for 5 min at 95°; amplified at 94° 30 sec, 60° 30 sec, 75° 30 sec; final extension step for 5 min at 75° were as previously described [11,12].
[d] Two microliters for liver and 1 μL for mammary gland were used for PCR amplification.

Table 2
Retention times (t_R) of E1, E2, and E3 derivatives separated by HPLC

Compound	t_R (min)	Compound	t_R (min)
6-keto-E3	4.9	6α-OH-E1[a]	11.2
15α-OH-E3	4.9	6β-OH-E1[a]	11.2
2-OH-E3	5.1	16α-OH-E1	12.6
15α-OH-E2	6.2	4-OH-E2	16.2
6α-OH-E2	6.7	2-OH-E2	17.2
E3	8.2	2-OH-E1	20.5
[15β-OH-E2][b]	8.6	4-OH-E1	23.8
6β-OH-E2	9.2	E2	25.2
15α-OH-E1[a]	10.4	E1	27.2

HPLC analysis and the standard compound mixture used are described in Section 2.

[a] Compounds obtained via oxidation of the respective E2 derivatives by liver homogenate and NAD [17].

[b] Standard compound was unavailable; its t_R was calculated based on the relative t_R of E2 in the HPLC system of Suchar *et al.* [27].

were analyzed by HPLC. The HPLC system and equipment used were described previously [17]. Retention times (t_R) of the standard compounds in the above system are shown in Table 2. Metabolites were identified based on coelution with the standards and quantitated by equating the fraction of radiolabel in each peak to a fraction of substrate added. Recovery of the added radiolabel averaged 96%. Detection and quantitation required 1 and 2 pmol of the ^{14}C-labeled metabolite, respectively.

2.7. Statistical analyses

Values for CYP mRNA expression in the liver (Fig. 1) and mammary gland (Fig. 2) were compared among the I3C dose levels separately for each of 4- and 10-day treatment regimens using ANOVA. If the overall F-statistic indicated a statistically significant difference among the I3C dose levels, post *t*-tests were conducted on all pairwise combinations. Values for the relative levels of CYP mRNAs in the tissues of rats treated with I3C (250 mg/kg) for 4 or 10 days (Table 3) were transformed using the natural log derivative in order to meet the assumptions of linearity and constant variance. These transformed values were then compared using repeated measures ANOVA, with a P450 as the 'within-subjects factor.' If the overall F-statistic indicated a statistically significant difference, contrasts were constructed between individual pairs of CYPs and tested using ANOVA. Values for P450 activities (Fig. 3) and rates of formation of estrogen metabolites (Figs. 4 and 5; Table 4) were transformed using the natural log or square root derivative in order to satisfy the assumption of normality. For each P450 activity or substrate (E2 or E1), ANOVA was used to examine the effects of dose levels of I3C, within the 4- or 10-day treatment regimen. Pairwise contrasts between each I3C dose level and vehicle (control), and between individual I3C dose levels were constructed. All *P* values shown were adjusted using the Bonferroni method.

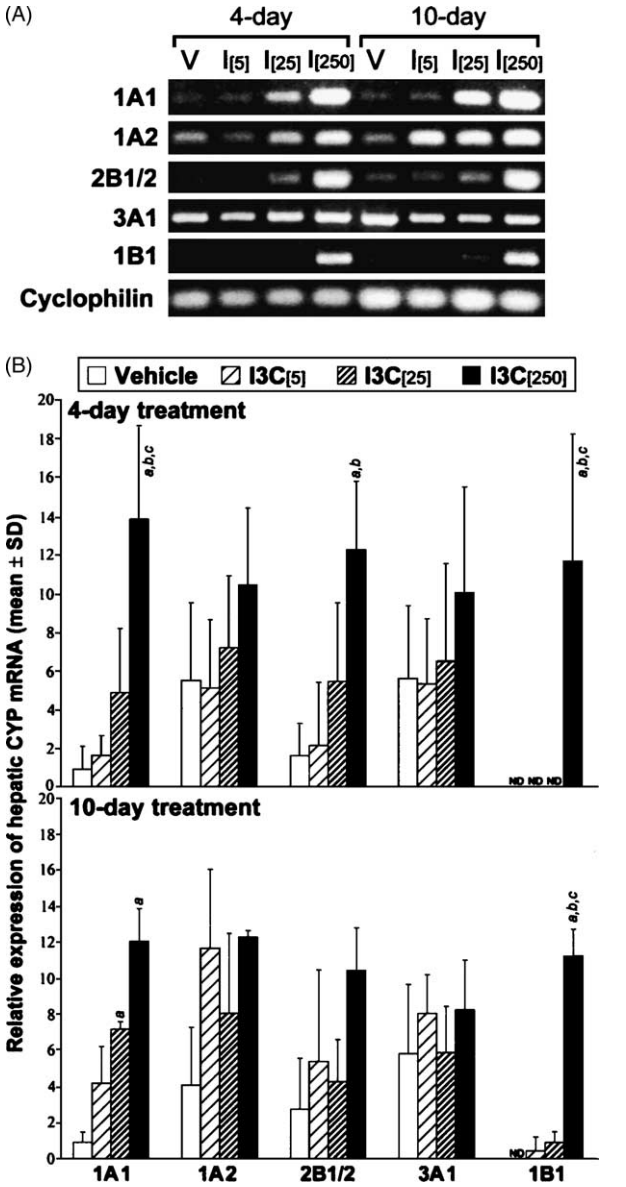

Fig. 1. Effect of treatment of rats with I3C on hepatic CYP mRNA expression. Rats were treated once daily with vehicle (V, 20% ethanol in olive oil) or 5, 25, or 250 mg/kg body weight of I3C ($I_{[5]}$, $I_{[25]}$, or $I_{[250]}$, respectively) by oral gavage for 4 or 10 days and were killed 24 hr after the last treatment. (A) Total liver RNA (1 μg) was isolated for semiquantitative RT–PCR analyses. Hepatic CYP transcripts were separated on 2% agarose gels, stained with ethidium bromide, and visualized under UV light. Data shown are representative for 1 of 3–4 identically treated rats. (B) Relative CYP mRNA expression was normalized to cyclophilin and determined using densitometry. Values (means ± SD; N = 3–4) were obtained by dividing the CYP densitometry value by that of cyclophilin and multiplying by 10. Significantly different ($P < 0.05$): (*a*) $I3C_{[250]}$- or $I3C_{[25]}$- vs vehicle-treated rats; (*b*) $I3C_{[250]}$- vs $I3C_{[5]}$-treated rats; and (*c*) $I3C_{[250]}$- vs $I3C_{[25]}$-treated rats. ND, RT–PCR band not detected, value assumed to be 0 (i.e. ≤ background).

3. Results

3.1. Hepatic and mammary gland CYP mRNA expression

The effects of treatment of rats with I3C on the hepatic and mammary mRNA transcripts for CYP1A1, 1A2, 2B1/2,

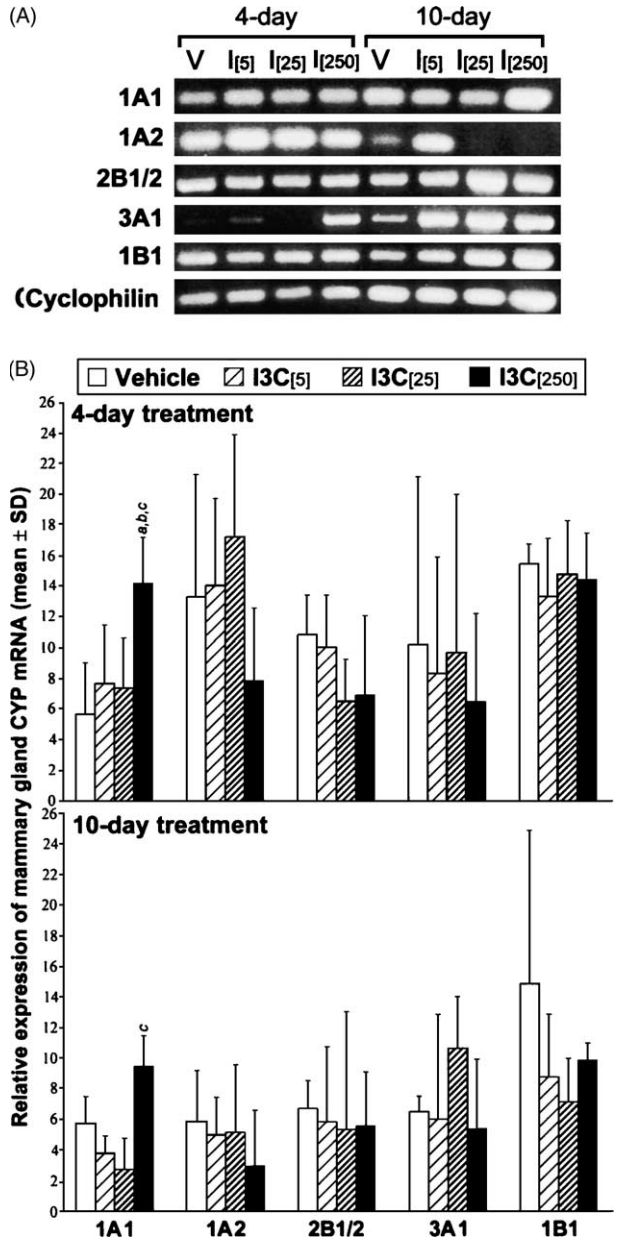

Fig. 2. Effect of treatment of rats with I3C on mammary gland CYP mRNA expression. Rats were treated once daily with vehicle (V, 20% ethanol in olive oil) or 5, 25, or 250 mg/kg body weight of I3C ($I_{[5]}$, $I_{[25]}$, or $I_{[250]}$, respectively) by oral gavage for 4 or 10 days and were killed 24 hr after the last treatment. (A) Total mammary gland RNA (4 μg) was isolated for semiquantitative RT–PCR analyses. Mammary gland CYP transcripts were separated on 2% agarose gels, stained with ethidium bromide, and visualized under UV light. Data shown are representative for 1 of 3–4 identically treated rats. (B) Relative CYP mRNA expression was normalized to cyclophilin and determined using densitometry. Values (means ± SD; N = 3–4) were obtained by dividing the CYP densitometry value by that of cyclophilin and multiplying by 10. Significantly different ($P < 0.05$) for: (*a*) $I3C_{[250]}$- vs vehicle-treated rats; (*b*) $I3C_{[250]}$- vs $I3C_{[5]}$-treated rats; and (*c*) $I3C_{[250]}$- vs $I3C_{[25]}$-treated rats.

3A1, and 1B1 were determined with the RT–PCR parameters shown in Table 1. Since CYP mRNA expression in extrahepatic tissues is lower than in the liver, 4 μg of total mammary gland RNA as compared to 1 μg of total liver RNA was used for RT–PCR, but the number of cycles used to amplify the CYPs in both tissues was similar. The CYP2B1 and 2B2 were co-amplified because their sequences are 97% homologous [26]. RT–PCR products separated by electrophoresis on 2% agarose gels and CYP mRNA expression relative to the housekeeping gene cyclophilin are shown in Figs. 1 (liver) and 2 (mammary gland). After four treatments with I3C at the highest dose level (250 mg/kg body weight), the hepatic mRNA transcripts for CYP1A1 were 15.3-, 8.4-, or 2.9-fold greater than in vehicle-, I3C (5 mg/kg)-, or I3C (25 mg/kg)-treated rats, respectively, and those for CYP2B1/2 were 7.5- or 5.8-fold greater than in vehicle- or I3C (5 mg/kg)-treated rats, respectively (Fig. 1B). After ten treatments with I3C at 25 or 250 mg/kg, CYP1A1 mRNA transcript levels were increased 8- or 13.4-fold, respectively, compared with that of the vehicle group. The CYP1B1 mRNA transcripts were undetectable in the liver of vehicle-treated rats or after four treatments with I3C at 5 or 25 mg/kg, but low levels were detected after ten treatments. With I3C at 250 mg/kg for 4 or 10 days, the hepatic CYP1B1 mRNA expression was induced ~12- to 25-fold.

In the mammary gland, only CYP1A1 transcripts were up-regulated by treatments of rats with I3C at 250 mg/kg. Transcript levels after 4 days were 2.5- or 1.9-fold greater than in vehicle- or I3C (5 or 25 mg/kg)-treated rats, respectively, and after 10 days, 3.4-fold greater than in I3C (25 mg/kg)-treated rats (Fig. 2B). In contrast to the liver, CYP1B1 mRNA was expressed in the mammary gland of vehicle-treated rats, and its level was unaffected by treatment with I3C.

Since significant up-regulation of mRNA transcripts for several hepatic CYPs and mammary CYP1A1 was determined in rats treated with I3C at 250 mg/kg (Figs. 1 and 2), the relative CYP mRNA expression levels in the liver and mammary gland of these rats were directly compared in a separate set of experiments (Table 3). The liver had greater relative CYP mRNA levels than the mammary gland as 4 times more total RNA was required to amplify mammary CYPs. The hepatic mRNA transcripts for CYP1A2 and 2B1/2 after four treatments, and those for CYP1A1, 1A2, 2B1/2, and 3A1 after ten treatments with I3C were significantly greater than for CYP1B1. The latter was not detected in this assay because 1 μL of reverse transcribed liver cDNA and 25 amplification cycles rather than 2 μL cDNA and 45 cycles (Table 1) were used. In the mammary gland, the levels of expression of CYP1A1 and 1B1 were similar (Table 3). The mammary mRNA transcripts for CYP1B1 likely reflect its constitutive level, whereas those of CYP1A1 comprise chiefly the induced species.

3.2. Hepatic P450 content and P450-dependent activities

After four or ten treatments with I3C at 250 mg/kg, the P450 level was increased ~2-fold relative to that of the vehicle- or I3C (5 or 25 mg/kg)-treated rats (Fig. 3). Assays

Table 3
Relative CYP mRNA expression in the liver and mammary gland of rats treated with I3C (250 mg/kg body weight)

Treatment	CYP1A1	CYP1A2	CYP2B1/2	CYP3A1	CYP1B1
Liver					
4-day	1.80 ± 0.46	2.42 ± 0.20[a]	2.53 ± 0.29[a]	2.42 ± 0.43	0 ± 0[b]
10-day	1.76 ± 0.22[a]	2.37 ± 0.14[a]	2.36 ± 0.33[a]	2.17 ± 0.29[a]	0 ± 0
Mammary gland					
4-day	1.82 ± 0.28	0.53 ± 0.30	1.18 ± 0.49	0.16 ± 0.28	1.58 ± 0.19
10-day	2.11 ± 0.05[c]	0.30 ± 0.37	0.94 ± 0.28	0 ± 0	2.11 ± 0.19

Treatment of rats, semiquantitative RT–PCR, and statistical analysis are described in Section 2. Total liver (1 μg) or mammary gland (4 μg) RNA was reverse transcribed into cDNA, and 1 μL of the cDNA was aliquoted for PCR with 25 or 30 cycles, respectively. Values are means ± SD (N = 3) on the natural log scale.

[a] Significantly different ($P \leq 0.05$) from CYP1B1.

[b] RT–PCR band was not detected; value is assumed to be 0 (i.e. ≤ background).

[c] Significantly different ($P < 0.05$) from CYP3A1.

of EROD, MROD, BROD/PROD, and NIFOX activities were used as probes to determine the hepatic activities of CYP1A1, 1A2, 2B1/2, and 3A1/2, respectively [24,25]. After 4 days, EROD activity of rats treated with I3C at 5, 25, and 250 mg/kg was increased ~3-, 13-, and 26-fold, and after 10 days, ~3-, 10-, and 20-fold, respectively, compared with the vehicle-treated group (Fig. 3). After four treatments with I3C at 25 or 250 mg/kg, the activities of MROD, BROD, and PROD were increased 7- or 15-fold, 23- or 230-fold, and 5- or 105-fold, respectively, and after ten

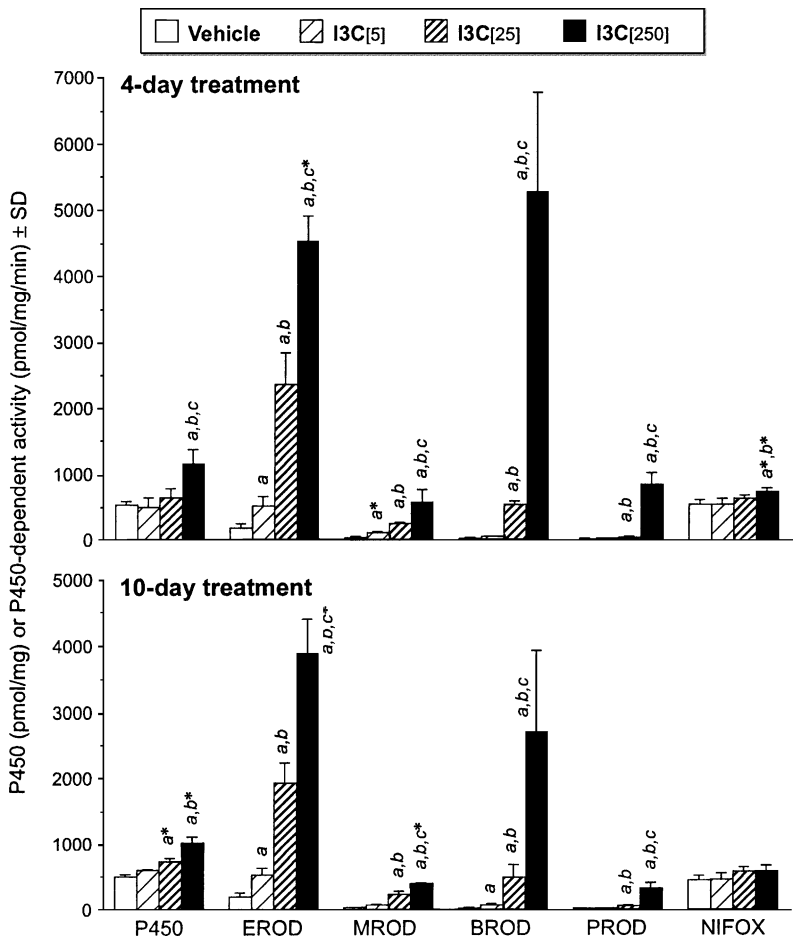

Fig. 3. Effects of treatment of rats with I3C on P450 content and P450-dependent enzyme activities. Treatment of rats, assays of P450 and P450-dependent alkoxyresorufin *O*-dealkylase (EROD, MROD, BROD, PROD) and NIFOX activities of liver microsomes, and statistical analysis of data are described in Section 2. Values in the brackets denote the dose level (mg/kg body weight) of I3C administered by oral gavage. Each bar represents the mean ± SD of duplicate assays with liver microsomes from 4 separate rats. Significant differences are shown for: $I3C_{[250]}$-, $I3C_{[25]}$- or $I3C_{[5]}$- vs vehicle-treated rats at (*a*) ($P < 0.001$) or (a^*) ($P \leq 0.02$); $I3C_{[250]}$- or $I3C_{[25]}$- vs $I3C_{[5]}$-treated rats at (*b*) ($P < 0.001$) or (b^*) ($P < 0.02$); and $I3C_{[250]}$- vs $I3C_{[25]}$-treated rats at (*c*) ($P < 0.001$) or (c^*) ($P < 0.02$).

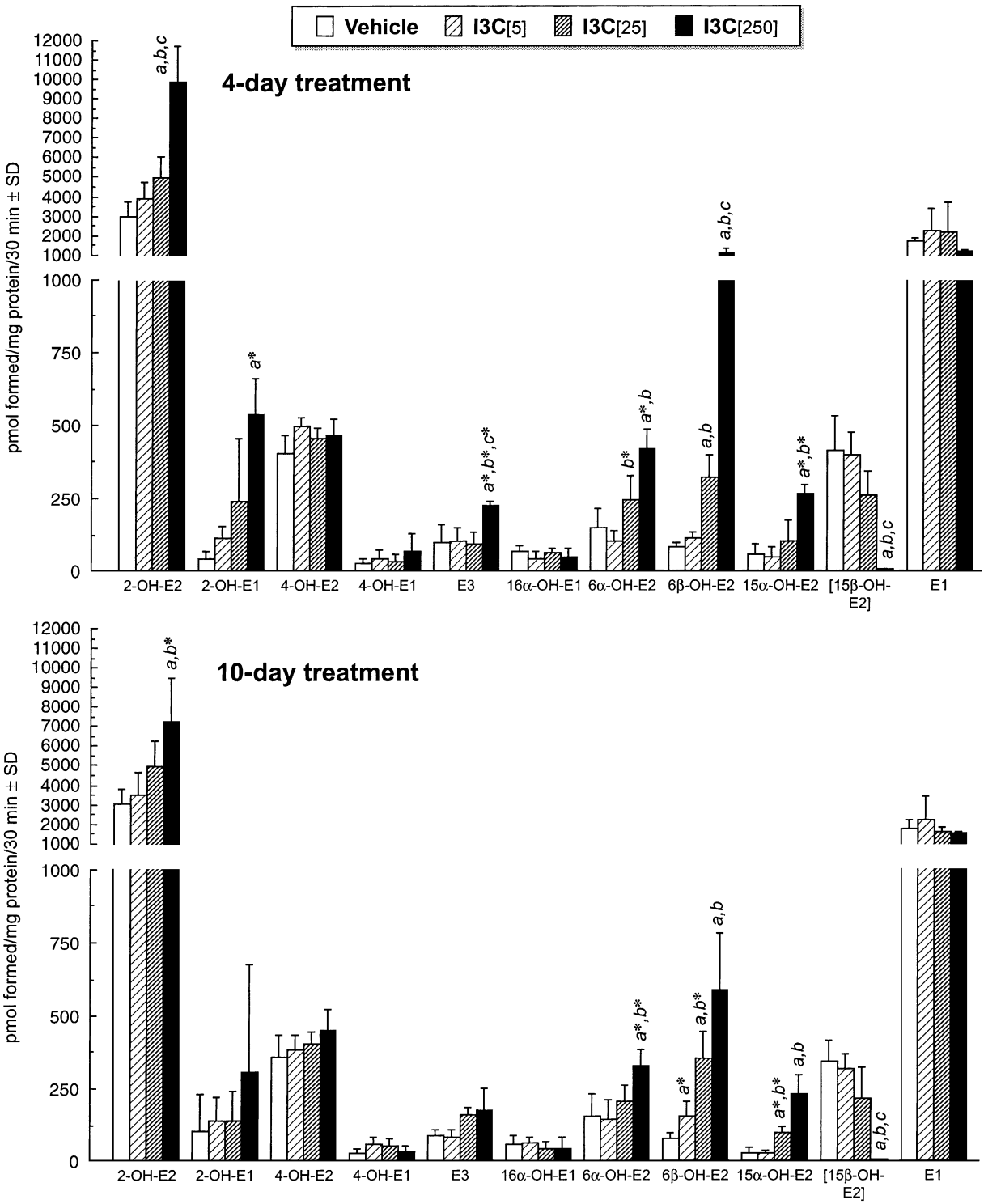

Fig. 4. Effect of treatment of rats with I3C on metabolism of E2 by hepatic microsomes. Treatment of rats, assays of E2 metabolism, and statistical analysis of data are described in Section 2. Values are the means ± SD from duplicate incubations with microsomes of 4 separate livers. Significant differences are shown for: $I3C_{[250]}$-, $I3C_{[25]}$- or $I3C_{[5]}$- vs vehicle-treated rats at (*a*) ($P < 0.01$) or (*a**) ($P < 0.05$); $I3C_{[250]}$- or $I3C_{[25]}$- vs $I3C_{[5]}$-treated rats at (*b*) ($P < 0.001$) or (*b**) ($P < 0.05$); and $I3C_{[250]}$- vs $I3C_{[25]}$-treated rats at (*c*) ($P < 0.02$) or (*c**) ($P < 0.05$).

treatments, these activities were increased 9- or 16-fold, 34- or 193-fold, and 5- or 45-fold. The small (~3- to 4-fold) increases in the activities of MROD after four, and of BROD after ten treatments with I3C at 5 mg/kg versus vehicle, were also significant. In addition, the increases of EROD, MROD, BROD, and PROD activities after treatment with I3C at 250 mg/kg were greater than those determined with lower doses of I3C. NIFOX activity was increased only ~1.4-fold in I3C (250 mg/kg)- versus I3C (5 mg/kg)- or vehicle-treated rats after 4 days.

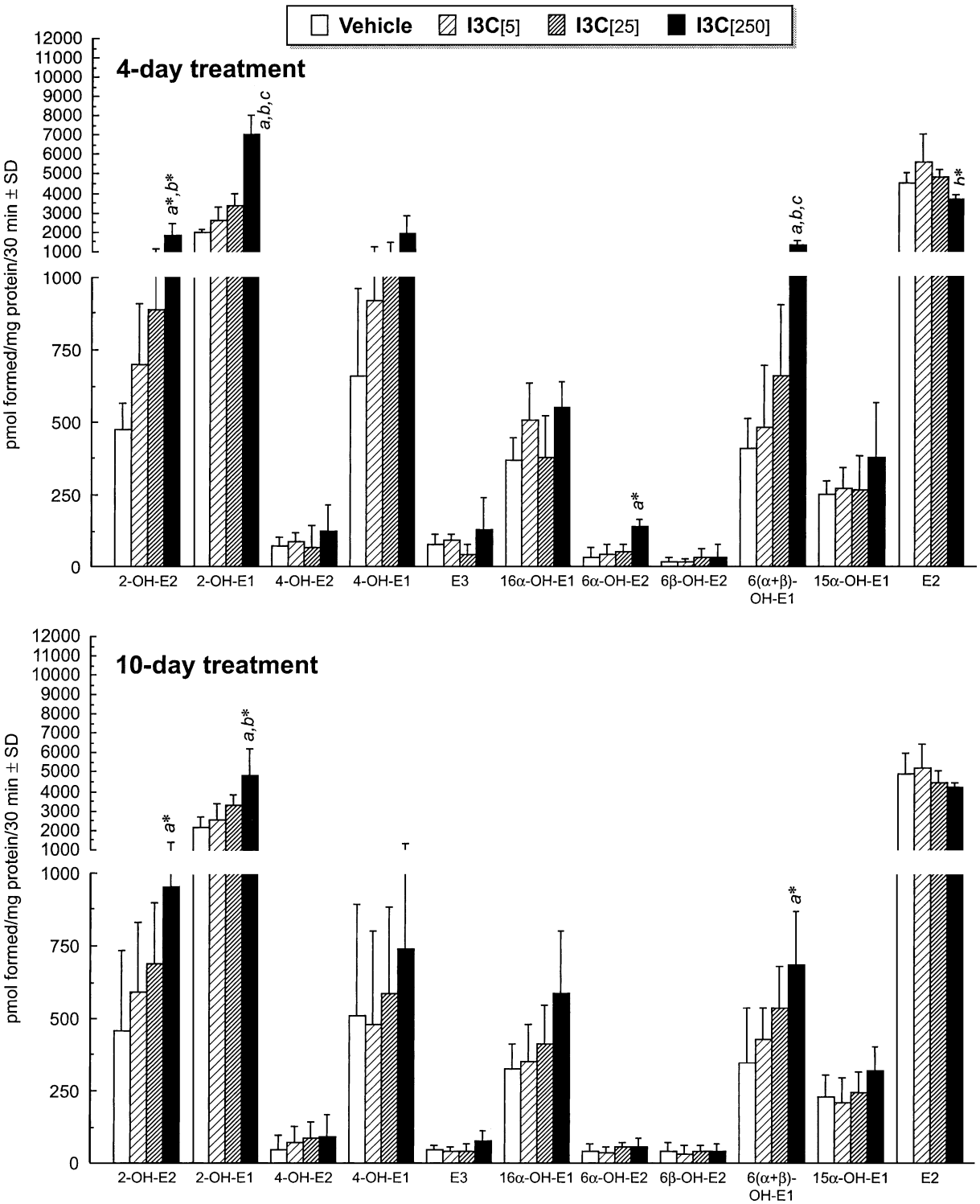

Fig. 5. Effect of treatment of rats with I3C on the metabolism of E1 by hepatic microsomes. Treatment of rats, assays of E1 metabolism, and statistical analysis of data are described in Section 2. Values are the means ± SD from duplicate incubations with microsomes of 4 separate livers. Significant differences are shown for: $I3C_{[250]}$- vs vehicle-treated rats at (*a*) ($P < 0.01$) or (*a**) ($P < 0.05$); $I3C_{[250]}$- vs $I3C_{[5]}$-treated rats at (*b*) ($P < 0.001$) or (*b**) ($P < 0.05$); and $I3C_{[250]}$- vs $I3C_{[25]}$-treated rats at (*c*) ($P < 0.02$).

3.3. Effects of treatment of rats with I3C on the metabolism of E2 and E1 by hepatic microsomes

The capacity of hepatic microsomes to metabolize E2 and E1 was increased ~2-fold after four or ten treatments of rats with I3C at the highest dose level of 250 mg/kg as compared to vehicle treatment (Table 4). In all groups, 2-OH-E2 was the major metabolite formed from E2, accounting for 46–70% of the total metabolites (Fig. 4). After four treatments with I3C at 250 mg/kg, the rates of

Table 4
Effects of treatment of rats with I3C on overall metabolism of E2 and E1 hepatic microsomes

Substrate	Treatment	Total metabolites[a] (nmol/mg protein/30 min)			
		Vehicle	$I3C_{[5]}$[b]	$I3C_{[25]}$	$I3C_{[250]}$
E2	4-day	6.50 ± 0.94	8.00 ± 0.42	9.31 ± 2.32	14.35 ± 2.21[c]
	10-day	6.55 ± 1.53	7.71 ± 1.66	8.69 ± 1.65	11.42 ± 3.02[c]
E1	4-day	8.89 ± 0.80	11.33 ± 1.26	11.62 ± 1.89	15.41 ± 2.09[c]
	10-day	9.18 ± 2.69	9.98 ± 2.11	10.48 ± 1.59	12.56 ± 2.77

Treatment of rats and assays of metabolites are described in Section 2.
[a] Total metabolites include all known and unknown radioactive metabolites eluted by HPLC. Values are the means ± SD from duplicate incubations with microsomes of 4 separate livers.
[b] The number in brackets denotes the dose level (mg/kg body weight) of I3C.
[c] Significantly ($P < 0.05$) different from vehicle-treated rats.

formation of 2-OH-E2 were 3.3-, 2.5-, or 2-fold greater than those of vehicle-, I3C (5 mg/kg)-, or I3C (25 mg/kg)-treated rats, respectively, and after ten treatments, they were 2.4- or 2.2-fold greater than those of vehicle- or I3C (5 mg/kg)-treated rats. After four treatments with I3C at 25 or 250 mg/kg, the rates of formation of 6α-OH-E2 and 6β-OH-E2 were 1.6- and 4-fold or 2.8- and 13.3-fold greater, respectively, than those of vehicle- or I3C (5 mg/kg)-treated rats. After ten treatments with I3C at 250 mg/kg, the rates of formation of 6α-OH-E2 and 6β-OH-E2 were increased 2.1- and 7.5-fold, respectively, and those of 6β-OH-E2 were increased 2- or 4.5-fold with I3C at 5 or 25 mg/kg, respectively, compared with vehicle rats. Hence, the longer treatment with I3C at the lowest dose level (5 mg/kg) yielded the detectable induction of 6β-OH-E2, whereas in rats treated with I3C at the highest dose level (250 mg/kg), the magnitude of induction of this metabolite (comprising ~7% of the total metabolites from E2) decreased by >40%. The rate of formation of E3, i.e. 16α-OH-E2, was increased ~2.3-fold only after four treatments with I3C at the highest dose level.

After four or ten treatments with I3C at 250 mg/kg, the rate of formation of 15α-OH-E2 was increased 4.9- or 8.3-fold, respectively, and after ten treatments with I3C at 25 mg/kg, it was increased 3.4-fold compared with vehicle- or I3C (5 mg/kg)-treated rats. By contrast, formation of the presumed 15β-OH-E2 was inhibited completely after treatment with I3C at 250 mg/kg. The identity of this metabolite was based on calculation of its t_R relative to the t_R of E2 in the HPLC system of Suchar *et al.* [27], who had the standard 15β-OH-E2. Similar calculations of the relative t_Rs for the other standards gave the values close to their actual t_Rs in the HPLC system used herein (Table 2).

The rates of formation of E1 and its metabolites from E2 were unaffected by I3C treatment except for a 12.5-fold increase in the formation of 2-OH-E1 by I3C (250 mg/kg)-versus vehicle-treated rats after 4 days (Fig. 4). After 4 or 10 days, E1 accounted for up to 30% of the total metabolites formed from E2, by vehicle- or I3C (5 or 25 mg/kg)-treated and for ~10% by I3C (250 mg/kg)-treated rats. The smaller fraction of E1 in the latter was likely due to significant increases in the rates of formation of metabolites of E2 described above.

With E1 as the substrate, 2-OH-E1 and E2 accounted for 22–29 and 41–51%, respectively, of the total metabolites after four treatments of rats with vehicle or I3C (5 or 25 mg/kg) (Fig. 5). The respective fractions were 46 and 24% after treatment with I3C (250 mg/kg). A smaller fraction of E2 in the latter group was consistent with the increases of 3.9- and 2.1-fold in the rates of formation of 2-OH-E2 after four and ten treatments, respectively. After four treatments with I3C (250 mg/kg), the rate of formation of 2-OH-E1 was 3.6-, 2.7-, and 2.1-fold greater than that of vehicle-, I3C (5 mg/kg)-, and I3C (25 mg/kg)-treated rats, respectively, and after ten treatments, it was ~2-fold greater than that of vehicle- or I3C (5 mg/kg)-treated rats. After four and ten treatments with I3C (250 mg/kg), the rate of formation of 6(α + β)-OH-E1 was increased 3.3- and 2-fold, respectively, and that of 6α-OH-E2 was increased 4.3-fold after four treatments. The rates of formation of 6(α + β)-OH-E1 from E2 or 15α-OH-E2 from E1 were low (<30 pmol/mg protein/30 min) and unaffected by the treatment (not shown in Figs. 4 and 5).

4. Discussion

The effects of treatment of rats with I3C at 5, 25, and 250 mg/kg body weight versus vehicle for 4 or 10 days on the relative expression of mRNA for CYP1A1, 1A2, 1B1, 2B1/2, and 3A1 in the liver and mammary gland were examined using semiquantitative RT–PCR. Significant increases in mRNA levels for CYP1A1 and 2B1/2 in the liver (Fig. 1) and CYP1A1 in the mammary gland (Fig. 2) were shown only in rats treated with I3C at the highest dose level. In an earlier study, the increases in the hepatic transcript levels for CYP1A1 and 2B1 and also CYP1A2 and 3A1 were shown after a 100-day treatment of female SD rats with I3C (~500 mg/kg body weight), and coincided with the increases of CYP-specific catalytic activities: EROD (CYP1A1), MROD (CYP1A2), and BROD (CYP2B1) [8]. In our study, the increases of EROD and MROD activities were determined at all three dose levels of I3C (5, 25, and 250 mg/kg body weight) and those of

BROD or PROD (CYP2B1/2) at the two higher dose levels (Fig. 3). Although potential overlap in the substrate specificity for assays of CYP-catalyzed *O*-dealkylase activities may exist [24,28], the increases of CYP activities herein (Fig. 3) were consistent with the induction of CYP1A1, 1A2, and 2B1/2 proteins in the liver of female SD rats after one oral dose of I3C (100 mg/kg body weight) [6,7]. A very small increase in the CYP3A1 activity of I3C (250 mg/kg)-treated rats (Fig. 3) reflected lack of induction by I3C of this CYP protein in the mature female rats [7].

The increases in the EROD, MROD, BROD, and PROD activities achieved with the highest dose of I3C after 4 days decreased by 14, 31, 49, and 63%, respectively, after 10 days, whereas those determined with I3C at 5 or 25 mg/kg remained unchanged (Fig. 3). This suggested a saturating effect of the highest dose of I3C for induction of P450 activities and/or partial inhibition by I3C (or its products formed *in vivo*) of the induced activities. Inhibition by DIM of rat and human CYP1A1, human CYP1A2, and rat CYP2B1 *in vitro* using CYP-specific activity assays was reported [28]. Inhibition by I3C and DIM of the TCDD-induced EROD activity in the AhR-responsive T47D human breast cancer cells was ascribed to the AhR antagonist effects [29]. Hence, I3C may act as both AhR agonist and antagonist *in vivo*, which would affect the course of CYP-dependent metabolism of xeno- and endo-biotics with the duration of treatment.

The observed induction of CYP1B1 mRNA in the liver of I3C (250 mg/kg)-treated rats (Fig. 1) was not detected when the amount of reverse transcribed cDNA and the number of PCR cycles for all hepatic CYPs were equalized (Table 3). This indicated that the level of expression of CYP1B1 mRNA in the liver of I3C-treated rats is lower than that of other CYPs, consistent with reports showing lower transcript and protein levels of CYP1B1 relative to those of CYP1A1 and 1A2 inducible in the liver of TCDD- or β-napthoflavone-treated female SD rats [11,12,30,31]. In contrast to the liver, CYP1B1 mRNA was detected in the mammary gland of vehicle-treated rats, and its level was unaltered by I3C treatment (Fig. 2). Likewise, the mRNA transcript levels for CYP1A2, 2B1/2, and 3A1 expressed in the mammary gland of vehicle-treated rats were unaffected by I3C treatment. On the other hand, the increases in mRNA levels for CYP1A2 and 1B1 shown in the mammary gland of TCDD-treated rats [12] are likely due to a higher affinity of TCDD than I3C or its products for AhR [6,9]. A higher level of induction by TCDD than ICZ of mRNAs for CYP1A1 and 1B1 in the MCF-7 human breast cancer cells exposed to equimolar doses of these AhR ligands also has been reported [32]. Treatment with TCDD or polycyclic aromatic hydrocarbons (PAHs) of rat mammary gland epithelial and stromal cells in culture induced CYP1A1 (mRNA, protein, and regioselective PAH metabolism) chiefly in the epithelial cells and CYP1B1 selectively in the stromal fibroblasts [33]. It remains to be established whether or not the cell type-specific distribution of these as well as other CYPs occurs in the mammary gland after treatment of rats with I3C. Furthermore, expression of CYP mRNAs and isozyme proteins in the mammary gland depends on the age and hormonal status of the rat [34].

After four treatments with I3C at 250 mg/kg, the capacities of hepatic microsomes to metabolize E2 to 2-OH-E2, 2-OH-E1, E3, 6α-OH-E2, 6β-OH-E2, and 15α-OH-E2 (Fig. 4), and E1 to 2-OH-E2, 2-OH-E1, 6α-OH-E2, and 6(α + β)-OH-E1 (Fig. 5) were increased significantly, and except for the formation of 2-OH-E1 and E3 from E2 and of 6α-OH-E2 from E1, the increases albeit of smaller magnitudes were retained after ten treatments. The data were consistent with the lower levels of induction of CYP1A1, 1A2, or 2B1/2 and no induction of CYP3A probe activities after ten treatments (Fig. 3). In general, the increases achieved after a 4-day treatment with I3C at 250 mg/kg in the hepatic P450 content, CYP specific activities, and microsomal metabolism of E2 and E1 were similar to those reported by us previously for this I3C regimen [17]. Small differences between our two studies in the extents of induction of the hepatic CYP activities and estrogen metabolism suggest variable modification of the CYP complement in rats of an outbred strain in response to treatment with the vehicle (40% ethanol in corn oil used previously vs 20% ethanol in olive oil used herein) and/or the modifier itself. In view of the induction of the hepatic CYP1B1 in rats treated with I3C at 250 mg/kg (Fig. 1), the lack of increases in the formation of 4-catechols from E2 and E1 (Figs. 4 and 5) was rather unexpected, but it may be explained by a lower level of expression of CYP1B1 relative to other CYPs (Table 3).

CYP-catalyzed estrogen metabolism in the mammary gland, a target tissue for estrogen, may affect mammary carcinogenesis, leading to an enhancement or suppression of the process. In our previous study, formation of 2-OH-E2 by the mammary gland microsomes was induced by acute treatment of rats with I3C at 250 mg/kg and that of 4-OH-E2 was detected in both vehicle- and I3C-treated rats [17]. This pattern was consistent with the induction by I3C (250 mg/kg) of CYP1A1 mRNA and the constitutive expression of a relatively high level of CYP1B1 mRNA in the mammary gland determined herein (Fig. 2). In the tissues of TCDD-treated rats, induction of CYP1A1/1A2 and CYP1B1 was associated chiefly with the formation of 2- and 4-catechol estrogens, respectively [12]. Furthermore, alterations in the CYP1B1/1A1 ratio, such as those induced by E2 or xenoestrogens in the MCF-7 breast cancer cells, are considered of critical importance for the metabolism and, thus, toxicity and carcinogenicity of E2 for mammary cells [35].

Treatment of rats with I3C at 25 mg/kg increased the capacity of hepatic microsomes to metabolize E2 to 6β-OH-E2, 6α-OH-E2, and/or 15α-OH-E2, but that with I3C at 5 mg/kg had no effect, except for an ∼2-fold increase in 6β-OH-E2 after ten treatments (Fig. 4). The data indicate that the low levels of CYP1A1, 1A2, and CYP2B1/2 probe

activities induced by I3C at 5 mg/kg (Fig. 3) are insufficient for increasing the capacity of hepatic microsomes to metabolize E2 (Fig. 4) or E1 (Fig. 5). Inhibition of mammary tumor growth in SD rats has been accomplished with low doses of DIM (10 doses at 5 mg/kg body weight over 20 days), which did not induce EROD activity in liver microsomes [36]. Hence, the above antiestrogen-like effects of DIM may not concern alterations in CYP-dependent estrogen metabolism, but may involve changes in activation and signaling pathways of the estrogen receptor [20,37,38]. Further studies are needed to establish the dose efficacy of I3C and/or its products for suppression of estrogen-dependent tumorigenesis and elucidate mechanisms of antiestrogenic action of these compounds.

Acknowledgments

This study was supported by Biomedical Research Funds, the US Department of Veterans Affairs, a grant from the National Cancer Institute (CA-28000), the US Public Health Service, and a grant from the Minnesota Medical Foundation at the University of Minnesota.

References

[1] Verhoeven DTH, Verhagen H, Goldbohm RA, van den Brandt PA, van Poppel G. A review of mechanisms underlying anticarcinogenicity by brassica vegetables. Chem Biol Interact 1997;103:79–129.
[2] Bradlow HL, Michnovicz JJ, Telang NT, Osborne MP. Effects of dietary indole-3-carbinol on estradiol metabolism and spontaneous mammary tumors in mice. Carcinogenesis 1991;12:1571–4.
[3] Kojima T, Tanaka T, Mori H. Chemoprevention of spontaneous endometrial cancer in female Donryu rats by dietary indole-3-carbinol. Cancer Res 1994;54:1446–9.
[4] Michnovicz JJ, Bradlow HL. Induction of estradiol metabolism by dietary indole-3-carbinol in humans. J Natl Cancer Inst 1990;82:947–9.
[5] Michnovicz JJ, Adlercreutz H, Bradlow HL. Changes in levels of urinary estrogen metabolites after oral indole-3-carbinol treatment in humans. J Natl Cancer Inst 1997;89:718–23.
[6] Jellinck PH, Forkert PG, Riddick DS, Okey AB, Michnovicz JJ, Bradlow HL. Ah receptor binding properties of indole carbinols and induction of hepatic estradiol hydroxylation. Biochem Pharmacol 1993;45:1129–36.
[7] Jellinck PH, Newcombe A-M, Forkert PG, Martucci CP. Distinct forms of hepatic androgen 6β-hydroxylase induced in the rat by indole-3-carbinol and pregnenolone carbonitrile. J Steroid Biochem Mol Biol 1994;51:219–25.
[8] Grubbs CJ, Steele VE, Casebolt T, Juliana MM, Eto I, Whitaker LM, Dragnev KH, Kelloff GJ, Lubet RL. Chemoprevention of chemically-induced mammary carcinogenesis by indole-3-carbinol. Anticancer Res 1995;15:709–16.
[9] Bjeldanes LF, Kim J-Y, Grose KR, Bartholomew JC, Bradfield CA. Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol *in vitro* and *in vivo*: comparisons with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Proc Natl Acad Sci USA 1991;88:9543–7.
[10] Walker NJ, Gastel JA, Costa LT, Clark GC, Lucier GW, Sutter TR. Rat CYP1B1: an adrenal cytochrome P450 that exhibits sex-dependent expression in livers and kidneys of TCDD-treated animals. Carcinogenesis 1995;16:1319–27.
[11] Walker NJ, Portier CJ, Lax SF, Crofts FG, Li Y, Lucier GW, Sutter TR. Characterization of the dose-response of CYP1B1, CYP1A1, and CYP1A2 in the liver of female Sprague–Dawley rats following chronic exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicol Appl Pharmacol 1999;154:279–86.
[12] Badawi AF, Cavalieri EL, Rogan EG. Effect of chlorinated hydrocarbons on expression of cytochrome P450 1A1, 1A2 and 1B1 and 2- and 4-hydroxylation of 17β-estradiol in female Sprague–Dawley rats. Carcinogenesis 2000;21:1593–9.
[13] Zhu BT, Conney AH. Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis 1998;19:1–27.
[14] Dwivedy I, Devanesan P, Cremonesi P, Rogan E, Cavalieri E. Synthesis and characterization of estrogen 2,3- and 3,4-quinones. Comparison of DNA adducts formed by the quinones versus horseradish peroxidase-activated catechol estrogens. Chem Res Toxicol 1992;5:828–33.
[15] Yager JD, Liehr JG. Molecular mechanisms of estrogen carcinogenesis. Annu Rev Pharmacol Toxicol 1996;36:203–32.
[16] Cavalieri EL, Stack DE, Devanesan PD, Todorovic R, Dwivedy I, Higginbotham S, Johansson SL, Patil KD, Gross ML, Gooden JK, Ramanathan R, Cerny RL, Rogan EG. Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. Proc Natl Acad Sci USA 1997;94:10937–42.
[17] Ritter CL, Prigge WF, Reichert MA, Malejka-Giganti D. Oxidations of 17β-estradiol and estrone and their interconversions catalyzed by liver, mammary gland and mammary tumor after acute and chronic treatment of rats with indole-3-carbinol or β-naphthoflavone. Can J Physiol Pharmacol 2001;79:519–32.
[18] Malejka-Giganti D, Niehans GA, Reichert MA, Bliss RL. Post-initiation treatment of rats with indole-3-carbinol or β-naphthoflavone does not suppress 7,12-dimethylbenz[*a*]anthracene-induced mammary gland carcinogenesis. Cancer Lett 2000;160:209–18.
[19] Grose KR, Bjeldanes LF. Oligomerization of indole-3-carbinol in aqueous acid. Chem Res Toxicol 1992;5:188–93.
[20] Riby JE, Chang GHF, Firestone GL, Bjeldanes LF. Ligand-independent activation of estrogen receptor function by 3,3′-diindolylmethane in human breast cancer cells. Biochem Pharmacol 2000;60:167–77.
[21] Cavicchioli L, Flanigan TP, Dickson JG, Vantini G, Dal Toso R, Fusco M, Walsh FS, Leon A. Choline acetyltransferase messenger RNA expression in developing and adult rat brain: regulation by nerve growth factor. Brain Res Mol Brain Res 1991;9:319–25.
[22] Ritter CL, Malejka-Giganti D. Mixed function oxidase in the mammary gland and liver microsomes of lactating rats. I. Effects of 3-methylcholantrene and β-naphthoflavone. Biochem Pharmacol 1982;31:239–47.
[23] Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein structure. J Biol Chem 1964;239:2370–8.
[24] Nerurkar PV, Park SS, Thomas PE, Nims RW, Lubet RA. Methoxyresorufin and benzyloxyresorufin: substrates preferentially metabolized by cytochromes P4501A2 and 2B, respectively, in the rat and mouse. Biochem Pharmacol 1993;46:933–43.
[25] Guengerich FP, Martin MV, Beaune PH, Kremers P, Wolff T, Waxman DJ. Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. J Biol Chem 1986;261:5051–60.
[26] Fujii-Kuriyama Y, Mizukami Y, Kawajiri K, Sogawa K, Muramatsu M. Primary structure of a cytochrome P-450: coding nucleotide sequence of phenobarbital-inducible cytochrome P-450 cDNA from rat liver. Proc Natl Acad Sci USA 1982;79:2793–7.
[27] Suchar LA, Chang RL, Rosen RT, Lech J, Conney AH. High-performance liquid chromatography separation of hydroxylated estradiol metabolites: formation of estradiol metabolites by liver microsomes from male and female rats. J Pharmacol Exp Ther 1995;272:197–206.

[28] Stresser DM, Bjeldanes LF, Bailey GS, Williams DE. The anticarcinogen 3,3′-diindolylmethane is an inhibitor of cytochrome P-450. J Biochem Toxicol 1995;10:191–201.

[29] Chen I, Safe S, Bjeldanes L. Indole-3-carbinol and diindolylmethane as aryl hydrocarbon (Ah) receptor agonists and antagonists in T47D human breast cancer cells. Biochem Pharmacol 1996;51:1069–76.

[30] Bhattacharyya KK, Brake PB, Eltom SE, Otto SA, Jefcoate CR. Identification of a rat adrenal cytochrome P450 active in polycyclic hydrocarbon metabolism as rat CYP1B1. J Biol Chem 1995;270: 11595–602.

[31] Walker NJ, Crofts FG, Li Y, Lax SF, Hayes CL, Strickland PT, Lucier GW, Sutter TR. Induction and localization of cytochrome P450 1B1 (CYP1B1) protein in the livers of TCDD-treated rats: detection using polyclonal antibodies raised to histidine-tagged fusion proteins produced and purified from bacteria. Carcinogenesis 1998;19:395–402.

[32] Hayes CL, Spink DC, Spink BC, Cao JQ, Walker NJ, Sutter TR. 17β-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1. Proc Natl Acad Sci USA 1996;93:9776–81.

[33] Christou M, Savas U, Schroeder S, Shen X, Thompson T, Gould MN, Jefcoate CR. Cytochromes CYP1A1 and CYP1B1 in the rat mammary gland: cell-specific expression and regulation by polycyclic aromatic hydrocarbons and hormones. Mol Cell Endocrinol 1995; 115:41–50.

[34] Hellmold H, Lamb JG, Wyss A, Gustafsson J-Å, Warner M. Developmental and endocrine regulation of P450 isoforms in rat breast. Mol Pharmacol 1995;48:630–8.

[35] Coumoul X, Diry M, Robillot C, Barouki R. Differential regulation of cytochrome P450 1A1 and 1B1 by a combination of dioxin and pesticides in the breast tumor cell line MCF-7. Cancer Res 2001; 61:3942–8.

[36] Chen I, McDougal A, Wang F, Safe S. Aryl hydrocarbon receptor-mediated antiestrogenic and antitumorigenic activity of diindolylmethane. Carcinogenesis 1998;19:1631–9.

[37] Safe SH. Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds. Pharmacol Ther 1995;67:247–81.

[38] Leong H, Firestone GL, Bjeldanes LF. Cytostatic effects of 3,3′-diindolylmethane in human endometrial cancer cells result from an estrogen receptor-mediated increase in transforming growth factor-α expression. Carcinogenesis 2001;22:1809–17.