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17β-Estradiol hydroxylation catalyzed by human cytochrome P450 1B1

(catechol estrogen/indole carbinol/dioxin/breast cancer/uterine cancer)

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ABSTRACT **The 4-hydroxy metabolite of 17β-estradiol (E_2) has been implicated in the carcinogenicity of this hormone. Previous studies showed that aryl hydrocarbon-receptor agonists induced a cytochrome P450 that catalyzed the 4-hydroxylation of E_2. This activity was associated with human P450 1B1. To determine the relationship of the human P450 1B1 gene product and E_2 4-hydroxylation, the protein was expressed in *Saccharomyces cerevisiae*. Microsomes from the transformed yeast catalyzed the 4- and 2-hydroxylation of E_2 with K_m values of 0.71 and 0.78 μM and turnover numbers of 1.39 and 0.27 nmol product min^{-1}·nmol $P450^{-1}$, respectively. Treatment of MCF-7 human breast cancer cells with the aryl hydrocarbon-receptor ligand indolo[3,2-*b*]carbazole resulted in a concentration-dependent increase in P450 1B1 and P450 1A1 mRNA levels, and caused increased rates of 2-, 4-, 6α-, and 15α-hydroxylation of E_2. At an E_2 concentration of 10 nM, the increased rates of 2- and 4-hydroxylation were approximately equal, emphasizing the significance of the low K_m P450 1B1-component of E_2 metabolism. These studies demonstrate that human P450 1B1 is a catalytically efficient E_2 4-hydroxylase that is likely to participate in endocrine regulation and the toxicity of estrogens.**

The importance of estrogens in the etiology of breast and uterine cancer is widely recognized (1–3). The carcinogenicity of estrogens has been primarily attributed to their action as agonists of the estrogen receptor, through which concerted gene regulation controls cellular growth and differentiation in estrogen responsive tissues. Increasing evidence of another mechanism of carcinogenicity has focused attention on the catechol estrogen metabolites, which are less potent estrogens than 17β-estradiol (E_2). The 2- and 4-hydroxylated metabolites of both E_2 and estrone (E_1) can directly or indirectly damage DNA, proteins, and lipids through the generation of reactive free radicals by the reductive-oxidative cycling of these catechol estrogens between their semiquinone and quinone forms (4–6).

4-Hydroxylated metabolites represent only a small percentage of the total urinary catechol-estrogen content, and 4-hydroxylation was previously thought to be only a minor metabolic route (7). However, tissue-specific 4-hydroxylation of E_2 may be significant in the metabolic control of estrogen homeostasis. In human (8) and mouse uteri (9), rat pituitary (10), and hamster kidney (11) the rate of E_2 4-hydroxylation approaches or exceeds that of 2-hydroxylation. Interestingly, these organs are targets of estrogen-induced tumorigenesis (2, 12–14), and higher E_2 4-hydroxylase activity has been measured in tumors of the human breast (15, 16) and uterus (8), each compared with normal tissue. Furthermore, in the male hamster kidney, the carcinogenic and DNA-damaging activity of 4-hydroxyestradiol (4-OHE_2), and lack of activity of 2-hydroxyestradiol (2-OHE_2), (17–19), implicate the 4-hydroxylated metabolites in estrogen-induced carcinogenesis. Pertinent to elucidating the contribution of 4-OHE_2 to the development of human cancer is the identification of the enzyme(s) that produce this metabolite.

Previous studies demonstrated that treatment of MCF-7 breast cancer cells with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an environmental pollutant and potent agonist of the aryl hydrocarbon (Ah)-receptor, resulted in greater than 10-fold increases in the rates of E_2 4- and 2-hydroxylation (20). Human cytochrome P450 1A1 was shown to catalyze 2-hydroxylation in these cells, but the 4-hydroxylase was not identified (21). The recent cloning of the TCDD-inducible human P450 1B1 cDNA (22, 23) led to the hypothesis that the encoded enzyme catalyzed the observed E_2 4-hydroxylation. In MCF-7 cells, treatment with TCDD increased the amount of the 5.1-kb P450 1B1 mRNA, and antibodies to mouse P450 1B1 significantly inhibited microsomal E_2 4-hydroxylation (24).

To determine whether human P450 1B1 is an E_2 4-hydroxylase and to compare the kinetics of this reaction with other known human E_2 hydroxylases, we expressed the P450 1B1 protein in *Saccharomyces cerevisiae*. We also investigated the effects of treatment with indolo[3,2-*b*]carbazole (ICZ), a dietary-derived Ah-receptor ligand (25), on the expression of P450 1A1 and P450 1B1 mRNAs and on the corresponding metabolism of E_2 in MCF-7 cells.

MATERIALS AND METHODS

Protein Expression Plasmids. Five P450 1B1 expression plasmids were constructed; the preparation of two of these is described. One construct, Δ0, was designed to express the entire deduced amino acid sequence. Since the amino-terminal sequence reported for rat P450 1B1 did not contain the first four amino acids of the deduced amino acid sequence (26), another expression construct, Δ3, was prepared that did not encode three amino acids after the initial methionine. To prepare the Δ0 and Δ3 expression plasmids, the *Hin*dIII (nucleotide −133 relative to ATG at +1)–*Ssp*I (nucleotide +1762) fragment of P450 1B1 cDNA clone p128 (23), containing the complete coding sequence, was inserted into *Hin*dIII–*Xho*I-digested yeast expression vector, pYES2 (Invitrogen). The *Xho*I site was filled-in with Klenow DNA polymerase, and the plasmid was closed by ligation. This plasmid was digested with *Hin*dIII and *Not*I, which removed all of the 5′

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Abbreviations: E_1, estrone; E_2, 17β-estradiol; 16α-OHE_1, 16α-hydroxyestrone; 2-OHE_1, 2-hydroxyestrone; 2-OHE_2, 2-hydroxyestradiol; 2-$MeOE_2$, 2-methoxyestradiol; 4-OHE_2, 4-hydroxyestradiol; 4-$MeOE_2$, 4-methoxyestradiol; Ah, aryl hydrocarbon; ICZ, indolo[3,2-*b*]carbazole; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DMSO, dimethyl sulfoxide.

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noncoding sequence of P450 1B1 and 384 nucleotides of the coding sequence of the corresponding amino-terminal end.

Two DNA products, each to be inserted where the coding sequence of P450 1B1 had been removed, were generated by PCR. Primer 1 for the Δ0 construct, 5′-CCCAAGCTTAACAGATCATGGGCACCAGCCTC-3′, did not alter the coding region of P450 1B1, but did alter the sequence 5′ of the ATG. These alterations included (*i*) a *Hin*dIII site (the nucleotides AAGCTT), (*ii*) a sequence found 5′ of the initiation site of several abundantly expressed yeast genes (the nucleotides AACA) (27), and (*iii*) a P450 1A1-specific sequence previously shown to increase the expression of P450 1A2 in yeast (the nucleotides GATC), (28). Primer 1 for the Δ3 product, 5′-CCCAAGCTTAACAGATCATGCTCAGCCCG-AACGAC-3′, produced the same changes upstream of ATG as the Δ0 product, and also removed nine nucleotides immediately 3′ of the ATG. Both products had the same reverse complement primer 2, 5′-ACCTTCCAGTGCTCCGAG-TAGT-3′ (nucleotides +428 to +407), located 3′ of a *Not*I site.

For PCR, 5 ng of p128 cDNA was denatured at 99°C for 2 min and chilled on ice, then 50 pmol of each primer, 200 μM of each dNTP, 3.75 mM $MgCl_2$, 1× *Taq* Extender buffer (Stratagene), and 5% formamide were added. Samples were heated to 95°C for 2 min before 5 units of *Taq* polymerase and *Taq* Extender were added. After denaturation at 95°C for 2 min, PCR was performed for 25 cycles (94°C for 30 s, 50°C for 30 s, 72°C for 1 min), followed by final extension for 10 min at 72°C. PCR products were digested with *Hin*dIII and *Not*I, and ligated to the remainder of the coding sequence of P450 1B1 in the pYES2 plasmid. PCR-generated sequence was verified by DNA sequence analysis.

Antipeptide Antibodies. A 20-mg aliquot of synthetic peptide, (C)-TRQPRSRQVLE, P450 1B1 amino acids 158–168 (23), was conjugated to keyhole limpet hemocyanin by addition of an amino-terminal cysteine residue (Biosynthesis, Lewisville, TX). Male New Zealand White rabbits were injected subcutaneously with 1 mg of conjugated peptide suspended in 1 ml of phosphate-buffered saline and emulsified in 1 ml of complete Freund's adjuvant. Subsequent injections consisted of conjugated peptide emulsified in 1 ml of incomplete Freund's adjuvant (0.5 mg at 2 and 4 weeks and finally 1 mg at 12 weeks) (Spring Valley Laboratories, Sykesville, MD). Antisera IgG (13 weeks) was purified by affinity chromatography with a bound-protein A-gel matrix (29).

Expression of Human P450 1B1 Constructs in Yeast. The *S. cerevisiae* strain JL20, *MAT***a** *leu2-3,112 his4-519 ade1-100 ura3-52* (kindly provided by J. C. Loper, University of Cincinnati), was transformed with plasmid DNA (30). To induce P450 1B1 expression, yeast was grown in synthetic dextrose medium without uracil (0.4% galactose, 0.02% glucose) to an absorbance (A_{600}) of 1.7–1.9. Microsomes were prepared by a described method (31) with minor modifications. Yeast were washed twice in buffer A (0.65 M sorbitol/0.1 mM EDTA/0.1 mM dithiothreitol/10 mM Tris·HCl, pH 7.5), collected by centrifugation, and resuspended at 20 ml/g wet weight in buffer B (2.0 M sorbitol/0.1 mM EDTA/0.1 mM dithiothreitol/10 mM Tris·HCl, pH 7.5/1.0 mM $MgCl_2$). Cells were incubated with Zymolyase 20T (ICN) at 9.6 mg/g wet weight for 1 h at 30°C. Spheroplasts were washed twice with ice-cold buffer A containing 0.1 mM α-toluenesulfonyl fluoride and then lysed by nine 10-s bursts of sonication on ice. The supernatant fraction from a 10-min centrifugation at 3000 × *g* was centrifuged for 15 min at 12,000 × *g*. To obtain a microsomal pellet, the 12,000 × *g*-supernatant fraction was centrifuged at 105,000 × *g* for 1 h. Microsomes were resuspended in 10 mM Tris·HCl (pH 7.5), 1.0 mM EDTA, and 20% glycerol and either stored at −80°C or analyzed for P450 content.

Protein concentration (24), total P450 content, and yeast NADPH cytochrome P450 reductase activity were measured as described (32). Microsomal proteins were separated by SDS/PAGE, electroblotted, and incubated with anti-P450 1B1 antibody at 10 μg IgG per ml. Detection was by the Enhanced Chemiluminescence system (Amersham) after incubation with horseradish-peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Promega) at 40 ng/ml. DNA and total RNA were isolated and analyzed as previously described (22). Northern and Southern blots were probed with a [α-^{32}P]dCTP-labeled 1.55-kb *Eag*I fragment of P450 1B1 cDNA from p128 (23), and a 1.0-kb *Bam*HI–*Hin*dIII fragment of yeast genomic actin from plasmid Δ10-AHX3 (33) (kindly provided by V. Culotta, Johns Hopkins University). Hybridization signals were normalized to actin and quantitated by laser densitometry (22).

E_2 Metabolism. Metabolites of E_2 were analyzed by GC/MS after preparation of the metabolite trimethylsilyl derivatives, with quantitation by the stable isotope dilution technique as described (20, 34). For the determination of microsomal E_2 hydroxylase activity, incubation mixtures contained 50 or 75 μg of microsomal protein, 0.1 M sodium phosphate (pH 7.4), 1.4 mM NADPH, 5 mM $MgCl_2$, 2 mM ascorbic acid, and varying amounts of E_2 as substrate. After 10 min at 37°C, the reaction was stopped by extraction with ethyl acetate. In some assays, where indicated, rabbit NADPH P450 reductase (kindly provided by F. P. Guengerich, Vanderbilt University, Nashville) was included. Kinetic data were analyzed by the PENNZYME computer program as previously described (21).

To analyze cellular E_2 metabolism, MCF-7 cells were grown as described (20, 35) in 6-well plates (1.5 cm^2 per well) and exposed for 72 h to medium containing 10 μM ICZ or the solvent vehicle only [0.2% (vol/vol) dimethyl sulfoxide (DMSO)]. The medium was then replaced with medium containing 1 μM or 10 nM E_2 for 6 h or 24 h, respectively. The medium was recovered; metabolite conjugates were hydrolyzed by treatment with β-glucuronidase/sulfatase and the metabolites were extracted and analyzed as described (20, 35).

RNA Expression. MCF-7 cells were exposed for 24 h either to medium containing various concentrations of TCDD or ICZ (kindly provided by L. F. Bjeldanes, University of California, Berkeley) or to their corresponding solvent vehicle controls, 0.1% or 0.2% (vol/vol) DMSO, respectively. Total cellular RNA was analyzed as described with successive hybridization with [α-^{32}P]dCTP-labeled human P450 1B1, P450 1A1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs (22, 23). Hybridization signals were quantitated by laser densitometry or direct PhosphorImaging and normalized to GAPDH.

RESULTS

Expression of Human P450 1B1. Levels of P450 1B1 protein present in the microsomal fraction were quantitated spectrophotometrically. The P450 level (pmol·mg^{-1}) of Δ0 was 36 ± 7 (mean ± SE; n = 4) and of Δ3 was 340 ± 65 (mean ± SE; n = 5) (Fig. 1). These results were verified by immunoblot analysis of the P450 1B1 protein (Fig. 1*D*). Although the reason for the differential expression of Δ0 and Δ3 was not determined, it was not due to differences in plasmid copy number, as demonstrated by Southern blot (Fig. 1*B*), or to differences in mRNA levels, as demonstrated by Northern blot (Fig. 1*C*).

Metabolism of E_2. To determine whether P450 1B1 had E_2 hydroxylase activity we analyzed extracts of microsomal incubations by GC/MS, in both the scanning- and selected-ion-monitoring modes, after preparation of trimethylsilyl derivatives of the metabolites. These analyses revealed that microsomes from Δ0 and Δ3 both catalyzed NADPH-dependent 4- and 2-hydroxylation of E_2 with a product ratio of approximately 5:1 (Fig. 2 and data not shown). Hydroxylation at the C-6α, C-15α, and C-16α positions of E_2 was not detected,

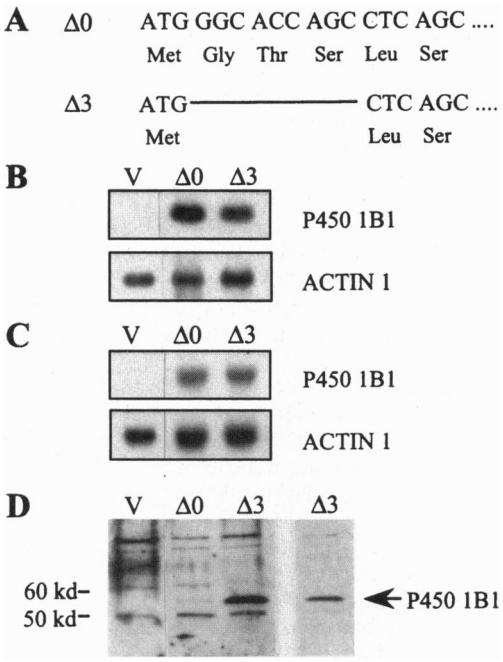

FIG. 1. Expression of human P450 1B1 in *S. cerevisiae*. (*A*) Amino-terminal nucleotide and deduced amino acid sequences of constructs Δ0 and Δ3. (*B*) Southern blot of DNA (5 μg per lane). (*C*) Northern blot of total RNA (20 μg per lane). (*D*) Immunoblot of microsomal protein (4 μg per lane) reacted with human anti-P450 1B1 antibody. The furthest right Δ3 lane is a shorter chemiluminescence exposure, showing the specificity of the antibody. (*B–D*) V, plasmid vector; Δ0 and Δ3, as depicted in *A*. Microsomal P450 levels were 36 ± 7 (n = 4) and 340 ± 65 (n = 5) (pmol·mg^{-1}, mean ± SE) for Δ0 and Δ3, respectively.

(<0.5 pmol·min^{-1}·mg^{-1}). The velocities of the enzymes were determined in triplicate at nine different concentrations of E_2 (Fig. 3) and the resulting K_m and V_{max} values are presented in Table 1.

The turnover numbers [nmol product·min^{-1}·(nmol P450)$^{-1}$] for P450 1B1 Δ0 and Δ3 were determined for three separate microsomal preparations of each expressed protein. The microsomal P450 content of each preparation was measured spectrophotometrically, and E_2 hydroxylase activity was determined in triplicate at saturating E_2 concentrations, with or without the addition of fivefold molar excess of P450 reductase over the amount of P450. The turnover numbers for Δ0 and Δ3 presented in Table 1 are the median determinations for three separate microsomal preparations.

The turnover numbers of three microsomal preparations of Δ0 without added P450 reductase for the formation of 2-OHE_2 ranged from 0.24 to 1.14, and for the formation of 4-OHE_2 ranged from 0.98 to 6.08. The addition of P450 reductase did not increase the turnover numbers in any of the three microsomal preparations of Δ0 (Table 1 and data not shown). The turnover numbers of three microsomal preparations of Δ3 without added P450 reductase for the formation of 2-OHE_2 ranged from 0.03 to 0.08, and for the formation of 4-OHE_2 ranged from 0.14 to 0.39. For each individual preparation of Δ3, the addition of P450 reductase significantly increased (P < 0.05) the turnover numbers approximately two-fold in each of the three preparations (Table 1 and data not shown). Endogenous P450 reductase activities (nmol cytochrome c reduced·min^{-1}·mg^{-1}) of the three preparations of Δ0 and Δ3 microsomes were 73 ± 14 (mean ± SE, n = 3) and 45 ± 6 (mean ± SE, n = 3), respectively. These values were not significantly different from each other.

P450 Induction and E_2 Metabolism in MCF-7 Cells. Treatment of MCF-7 cells with TCDD or ICZ for 24 h resulted in concentration-dependent increases in the amounts of P450 1A1 and P450 1B1 mRNAs (Fig. 4). The EC_{50} for induction by TCDD was 0.1 nM for P450 1B1 and 0.4 nM for P450 1A1. While the increases in the amount of mRNAs did not appear to reach a maximum over the ICZ concentration range tested, ICZ was approximately 5000-fold less potent than TCDD, consistent with the relative potency of these two compounds to induce ethoxyresorufin *O*-deethylase activity in Hepa 1c1c7 cells (25).

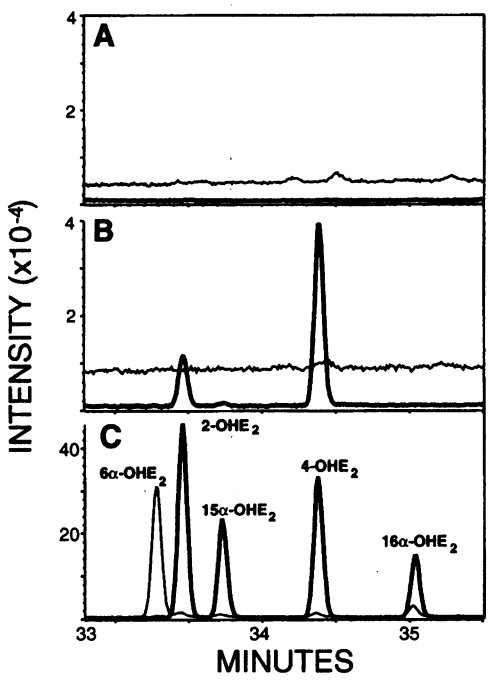

FIG. 2. GC/MS analysis of hydroxylation of E_2 catalyzed by human P450 1B1 expressed in *S. cerevisiae*. Incubation mixtures containing 75 μg microsomal protein from yeast transformed with vector alone (*A*) or Δ3 construct (*B*) and 10 μM E_2 were extracted and derivatized. The trimethylsilyl derivatives were analyzed by capillary GC/MS with selected-ion monitoring at m/z 504.3 (thick line) and m/z 414.2 (thin line). (*C*) Analysis of equimolar mixture of E_2-metabolite standards.

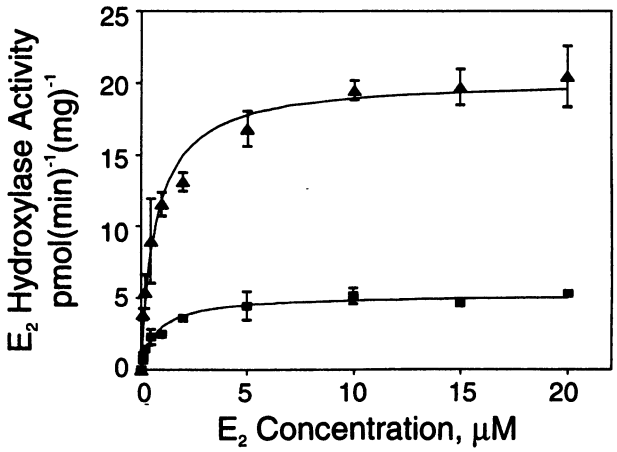

FIG. 3. Dependence of P450 1B1 E_2 hydroxylase activities on E_2 concentration. Incubation mixtures contained 50 μg of microsomal protein from Δ0-expressing yeast and 0.0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, or 20.0 μM E_2 as substrate; metabolites were analyzed as in Fig. 2. The curves were obtained by fitting the data to the Michaelis–Menten equation; values for the derived constants, K_m and V_{max}, are presented in Table 1. Each point represents an average of three determinations and the error bars indicate SE. ▲, 4-Hydroxylation; ■, 2-hydroxylation.

Table 1. Kinetic parameters for E_2 2- and 4-hydroxylation by human P450 1B1 proteins, Δ0 and Δ3

	K_m*, μM		V_{max}*, pmol·min^{-1}·mg^{-1}		Turnover number†, nmol product·min^{-1}·(nmol P450)$^{-1}$			
Yeast	2-OHE_2	4-OHE_2	2-OHE_2	4-OHE_2	2-OHE_2	4-OHE_2	2-OHE_2‡	4-OHE_2‡
Δ0	0.78 ± 0.24	0.71 ± 0.18	5.19 ± 0.35	20.29 ± 1.07	0.27 ± 0.03	1.39 ± 0.18	0.28 ± 0.00	1.33 ± 0.03
Δ3	1.19 ± 0.17	0.89 ± 0.13	14.08 ± 0.50	56.64 ± 1.85	0.05 ± 0.00	0.25 ± 0.02	0.14 ± 0.02§	0.69 ± 0.10§

*Mean ± SE, determined from triplicate assays at nine concentrations of E_2 as described.
†Mean ± SE, determined from triplicate assays with 10 μM E_2. The representative values shown are the median of analyses from three separate preparations of microsomes in which the P450 content had been determined.
‡Corresponding values measured after addition of NADPH P450 reductase [5 mol P450 reductase·(mol P450)$^{-1}$].
§Significantly different from corresponding Δ3 value without added NADPH P450 reductase, $P < 0.02$.

Although the pools of 2- and 4-OHE_2 cannot be accurately measured because they oxidize readily in the culture medium, the methoxyestrogens, 2- and 4-methoxyestradiol (2- and 4-$MeOE_2$), were detected in medium from ICZ-treated cultures after hydrolysis of conjugates by treatment with β-glucuronidase/sulfatase. Treatment of MCF-7 cells with 10 μM ICZ (72 h), followed by replacement of medium containing 1 μM E_2 for 6 h, resulted in a TCDD-like profile of E_2 metabolites (20), with increased hydroxylation at C-2 and C-4, as indicated by the production of 2- and 4-$MeOE_2$, as well as at C-6α and C-15α (Table 2). After similar treatment with 10 μM ICZ and 24-h exposure to 10 nM E_2, 2- and 4-$MeOE_2$ were present in ICZ-treated cultures at 2.33 ± 0.09 and 1.75 ± 0.05 nM (mean ± SE, $n = 3$), respectively, whereas 2- and 4-$MeOE_2$ in control culture could not be detected (<0.2 nM) (Fig. 5).

DISCUSSION

By kinetic analyses of the expressed protein, we demonstrated that human P450 1B1 catalyzes NADPH-dependent hydroxylation of E_2 at C-4 and, to a lesser extent, at C-2. The increase in turnover numbers of Δ3 after the addition of P450 reductase established that endogenous P450 reductase can be limiting in *S. cerevisiae*, as was previously observed in other studies of cDNA-directed P450 expression (36). Turnover numbers of Δ0 were not increased by addition of P450 reductase, indicating that it was not limiting in those yeast that expressed lower levels of P450 1B1. Thus, our results with Δ0 are likely to be good estimates of the turnover numbers for human P450 1B1 E_2 hydroxylation.

The major circulating and excreted catechol estrogens are the 2-hydroxylated metabolites. The liver is the primary site of estrogen metabolism, where the rate of 2-hydroxylation, catalyzed by P450 1A2, P450 3A3, and P450 3A4, greatly exceeds that of 4-hydroxylation. The reported apparent K_m values for E_2 2-hydroxylation catalyzed by these human enzymes ranged from 32 to 156 μM (37), which are considerably higher than the apparent K_m values of 0.71 and 0.78 μM reported here for the E_2 4- and 2-hydroxylase activity of P450 1B1, respectively. The K_m value for 4-hydroxylation of E_2 determined in the present study for P450 1B1 is consistent with a K_m value of 0.88 μM, previously reported from analyses with microsomes from TCDD-treated MCF-7 cells (21).

The turnover number [nmol product·min^{-1}·(nmol P450)$^{-1}$] for the formation of 4-OHE_2 by P450 1B1 (1.39) was similar to the turnover numbers for the formation of 2-OHE_2 by human P450 1A2, P450 3A3, and P450 3A4, which ranged from 0.84 to 3.3 (37, 38), while the turnover number for the formation of 2-OHE_2 by P450 1B1 (0.27) was lower. The catalytic efficiencies (turnover/K_m) of 4- and 2-hydroxylation of E_2 by P450 1B1 were 20- and 4-fold, respectively, higher than the catalytic efficiency of 2-hydroxylation of E_2 by P450 1A2, and 99- and 18-fold, respectively, higher than the catalytic efficiencies of 2-hydroxylation by either P450 3A3 or P450 3A4 (37). In addition to the hepatic enzymes, the E_2 2-hydroxylase activity of human placental aromatase has been determined, reporting a K_m value of 1.58 μM and a turnover number of 0.69 (39). The catalytic efficiency of 4-hydroxylation of E_2 by P450 1B1 was four-fold higher than that of aromatase, while the catalytic efficiency of 2-hydroxylation of E_2 by P450 1B1 was approximately equal. Thus, E_2 4-hydroxylase activity of P450 1B1 has the highest catalytic efficiency of all the E_2 hydroxylases reported, and the K_m values of E_2 4- and 2-hydroxylase activities are the lowest described values for E_2 hydroxylation. These comparisons indicate that the hydroxylase activity of P450 1B1 may be important for the homeostasis of estrogen, especially in extrahepatic organs, which express much lower levels of P450 1A2 and P450 3A4 than does the liver (40).

Strategies for breast cancer intervention that increase the ratio of 2- to 16α-hydroxyestrogens are being investigated. Elevated 16α-hydroxylation of E_2 has been associated with an increased risk of developing breast cancer (41–43). A metabolite of this pathway, 16α-hydroxyestrone (16α-OHE_1), was shown to be uterotrophic in mice and to increase unscheduled DNA synthesis and anchorage-independent growth of mammary cells in culture. In contrast, 2-hydroxyestrone (2-OHE_1) was much less active than 16α-OHE_1 in the uterotrophic assay, did not increase unscheduled DNA synthesis, and suppressed

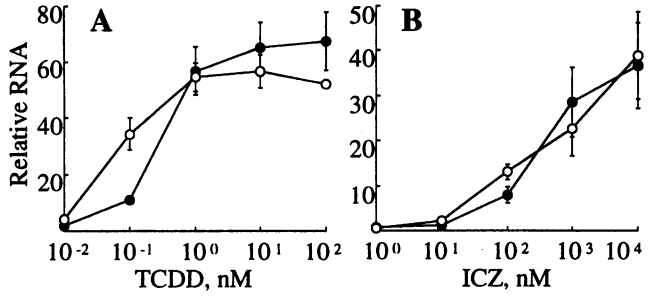

FIG. 4. Concentration-dependent increases in P450 1A1 and P450 1B1 mRNAs in MCF-7 cells treated with TCDD or ICZ. RNA was isolated after 24 h of treatment and analyzed as described. Relative RNA is the value of the hybridization signal at the indicated treatment concentration divided by the value determined for control vehicle alone. Each point represents an average of three determinations and the error bars indicate SE. (*A*) TCDD; control [0.1% (vol/vol) DMSO]. (*B*) ICZ; control [0.2% (vol/vol) DMSO]. ●, P450 1A1; ○, P450 1B1.

Table 2. E_2 metabolism by MCF-7 cells treated with ICZ

	Metabolism, pmol·h^{-1}·(mg cell protein)$^{-1}$*	
Metabolite	Control	ICZ
2-$MeOE_2$	1.48 ± 0.11	23.70 ± 0.89
4-$MeOE_2$	ND†	8.12 ± 0.49
6α-OHE_2	ND†	3.06 ± 0.29
15α-OHE_2	ND†	5.68 ± 0.37
16α-OHE_2	ND†	ND†

MCF-7 cells were treated with 0.2% (vol/vol) DMSO or 10 μM ICZ for 72 h before treatment with 1 μM E_2 for 6 h. Medium was collected and E_2 metabolites were extracted, derivatized, and quantitated by GC/MS.
*Values are the mean ± SE of three plates of cells.
†Not detected [<0.04 pmol·h^{-1}·(mg cell protein)$^{-1}$].

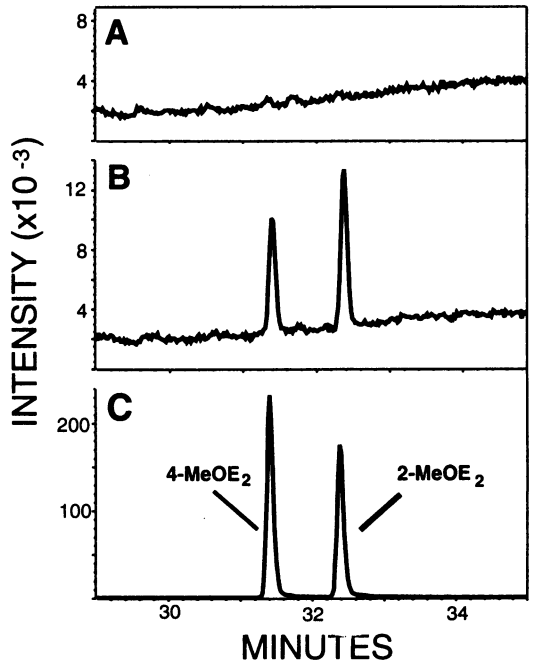

FIG. 5. Methoxyestradiol production by ICZ-treated MCF-7 cells. After treatment for 72 h with 10 μM ICZ, 10 nM E_2 was added for 24 h. The media were then recovered and analyzed for E_2 metabolites. Shown are the GC/MS ion chromatograms at m/z 446.2 for the analysis of the trimethylsilyl ether derivatives of 2- and 4-MeOE_2. (*A*) DMSO-treated [0.2% (vol/vol)]. (*B*) ICZ-treated. (*C*) 4- and 2-MeOE_2 standards.

growth of breast cancer cells in culture (42, 43). It was proposed that because these metabolic pathways compete for estrogen, an increase in the rate of 2-hydroxylation would decrease the formation of 16α-OHE_1 and protect against breast cancer (43).

Because ligand-mediated activation of the Ah-receptor was known to elevate the rate of E_2 2-hydroxylation (44), these chemoprotective strategies were developed using indole-3-carbinol (43, 45), a compound derived from vegetables of the *Brassica* genus, such as broccoli and brussel sprouts, and a precursor of the potent Ah-receptor ligand, ICZ (25). In mice and humans, treatment with indole-3-carbinol resulted in increased rates of E_2 2-hydroxylation; this treatment was protective against spontaneous mammary tumors in mice (43, 45).

The use of indole-3-carbinol as a chemoprotective agent to induce 2-hydroxylation of E_2 and E_1 is controversial because catechol-estrogen production has been associated with breast cancer (15, 16, 46, 47). Furthermore, the potential of indole-3-carbinol to cause increased rates of E_2 4-hydroxylation has not been carefully considered. Results presented here demonstrated parallel concentration-dependent increases in P450 1A1 and P450 1B1 mRNAs in response to treatment of MCF-7 cells with ICZ. Corresponding analysis of E_2 metabolite formation in these treated cells resulted in a TCDD-like profile, as rates of hydroxylation at C-2, C-4, C-6α, and C-15α were highly elevated. Increased rates of metabolism at C-2 and C-4, as evidenced by elevated levels of 2- and 4-MeOE_2, were detected when the concentration of E_2 was lowered to 10 nM, demonstrating that the E_2 4- and 2-hydroxylase activity of P450 1B1 are significant at low, physiologically relevant concentrations of E_2. This indicates that exposure to indole-carbinol compounds may elevate 4-hydroxylation in a number of human tissues where P450 1B1 is expressed (23, 48). Such tissue-specific increases would not necessarily be reflected in the concentrations of estrogens or their metabolites in urine or plasma. In contrast, the increase in excreted 2-hydroxylated estrogen metabolites, measured after administration of indole-3-carbinol, probably reflects the elevation of hepatic P450 1A2.

Except for their importance in the excretion of E_1 and E_2, a physiological role for hydroxyestrogens remains unclear. Catechol estrogens have been proposed to mediate physiological processes such as blastocyst implantation (9) and regulation of ovarian function (49), but biochemical mechanisms of catechol estrogens not involving the estrogen receptor remain elusive. In contrast, the importance of catechol estrogens in toxicity and tumorigenesis has been described in several cell types and tissues including hypothalamic β-endorphin neurons (50) and hamster kidney (17). The mechanism of these toxic effects appears to involve free radicals, generated from the reductive-oxidative cycling of the catechol estrogens with their corresponding semiquinone and quinone forms, which cause cellular damage (4–6).

In the hamster kidney, where 4-OHE_2 is as carcinogenic as E_2, selective chemical inhibition of the E_2 2-hydroxylase by fadrozole hydrochloride indicated that 4-OHE_2 was formed by an enzyme distinct from the enzyme responsible for the majority of the 2-hydroxylation in this organ (11). Similarly, in human uterine tissue, where 4-hydroxylation of E_2 is increased in myomas compared with surrounding myometrium, the 4-hydroxylase activity was suggested to be independent of the 2-hydroxylase activity because of selective inhibition by ethynylated hydrocarbons. Moreover, the 4- and 2-hydroxylase activities of the myoma were inhibited 53% and 17%, respectively, by an antimouse P450 1B1 antibody (8). This inhibition of activity by antimouse P450 1B1 antibody resembled a previous observation with microsomes from TCDD-treated MCF-7 cells (24), which express P450 1B1, and suggests that E_2 4-hydroxylation in human uterine tissue is also catalyzed by P450 1B1. Elevated catechol estrogen production has also been associated with tumors of the breast (15, 16, 47, 51), and in a recent study, P450 1B1 was detected by reverse transcriptase-PCR in 73% of the human breast tumor samples analyzed (52).

In summary, the expression of P450 1B1 and formation of catechol estrogens have been independently associated with estrogen-related tumors in multiple tissues and species. The identification here that human P450 1B1 is a low-K_m E_2 4- and 2-hydroxylase will undoubtedly aid our understanding of the relative importance of specific hydroxylases in estrogen-mediated biological processes and toxic effects, including cancer. Given the specific pathological role that has emerged for 4-OHE_2 and the widespread human tissue expression of P450 1B1, we suggest that breast cancer chemoprotective studies that are based on agents that activate the Ah-receptor and induce estrogen metabolizing P450s, notably P450 1B1, should be approached with caution.

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