Characterization of CYP1B1 and CYP1A1 Expression in Human Mammary Epithelial Cells: Role of the Aryl Hydrocarbon Receptor in Polycyclic Aromatic Hydrocarbon Metabolism

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ABSTRACT

CYP1B1 and CYP1A1 expression and metabolism of 7,12-dimethylbenz(a)anthracene (DMBA) have been characterized in early-passage human mammary epithelial cells (HMECs) isolated from reduction mammosplasty tissue of seven individual donors. The level of constitutive microsomal CYP1B1 protein expression was donor dependent (<0.01–1.4 pmol/mg microsomal protein). CYP1B1 expression was substantially induced by exposure of the cells to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to levels ranging from 2.3 to 16.6 pmol/mg among the seven donors. Extremely low, reproducible levels of constitutive CYP1A1 expression were detectable in three donors (0.03–0.16 pmol/mg microsomal protein). TCDD inductions were larger for CYP1A1, as compared to CYP1B1, demonstrating substantial variability in the induced levels among the donors (0.8–16.5 pmol/mg). Northern and reverse transcriptase PCR analyses corroborate the donor-dependent differences in protein expression, whereby CYP1B1 mRNA (5.2 kb) was constitutively expressed and was highly induced by TCDD (33-fold). The contributions of CYP1B1 and CYP1A1 to the metabolism of DMBA were analyzed using recombinant human CYP1B1 and CYP1A1, as references, in conjunction with antibody-specific inhibition analyses (anti-CYP1B1 and anti-CYP1A1). Constitutive microsomal activity exhibited a profile of regioselective DMBA metabolism that was characteristic of human CYP1B1 (increased proportions of 5,6- and 10,11-DMBA-dihydropyrrols), which was inhibited by anti-CYP1B1 (84%) but not by anti-CYP1A1. TCDD-induced HMEC microsomal DMBA metabolism generated the 8,9-dihydriodiol of DMBA as the predominant metabolite, with a regioselectivity similar to that of recombinant human CYP1A1, which was subsequently inhibited by anti-CYP1A1 (79%). A CYP1B1 contribution was indicated by the regioselectivity of residual metabolism and by anti-CYP1B1 inhibition (25%). DMBA metabolism analyses of one of three donors expressing measurable basal expression of CYP1A1 confirmed DMBA metabolism levels equivalent to that from CYP1B1. The HMECs of all donors expressed similar, very high levels of the aryl hydrocarbon receptor and the aryl hydrocarbon nuclear translocator protein, suggesting that aryl hydrocarbon receptor and aryl hydrocarbon nuclear translocator protein expression are not responsible for differences in cytochrome P450 expression. This study indicates that CYP1B1 is an important activator of polycyclic aromatic hydrocarbons in the mammary gland when environmental chemical exposures minimally induce CYP1A1. Additionally, certain individuals express low levels of basal CYP1B1 in HMECs, representing a potential risk factor of mammary carcinogenesis through enhanced polycyclic aromatic hydrocarbon bioactivation.

INTRODUCTION

In the United States, breast cancer represents the second leading cause of cancer-related death in women (1). The etiology of breast cancer remains unknown, although developmental, genetic, endocrine, dietary, and environmental factors have been implicated as risk factors for mammary tumorigenesis. Environmental contaminants, including PAHs, have been associated with chemical-mediated carcinogenesis in rodent models. In humans, mammary tissue is an important target site for PAH-mediated carcinogenesis, particularly because PAHs partition into mammary adipose tissue (2–8).

The cytochrome P450 superfamily of drug-metabolizing enzymes mediates the detoxification of environmental contaminants, including PAHs (9–11). However, PAHs often undergo bioactivation, thereby acquiring carcinogenic and mutagenic potential as a consequence of the oxidative metabolic process (12–14). Mutagenesis analyses have shown that rat mammary epithelial cell lines preferentially activate DMBA and benzo(a)pyrene, respectively, to mutagenic dihydriodiol epoxides, which are the most potent cancer initiators produced by these compounds (15). The bioactivated PAHs have been implicated in the development of a variety of rodent tumors, including mammary cancer, by inducing mutations in genes that control cell proliferation (H-ras) or regulate cellular adaptation to chemical-mediated damage (p53; Refs. 16–19). In addition, H-ras gene amplification has been demonstrated in primary HMECs following treatment with activated chemical carcinogens, and the immortalization and transformation of HMECs has been observed following treatment with PAHs (20–23).

This study focuses on the CYP1 superfamily of cytochrome P450 isozymes, which are largely responsible for both PAH activation and detoxification in many cells (24, 25). The CYP1A family of P450 cytochromes is composed of CYP1A1, which is broadly expressed after PAH induction but rarely observed constitutively, and CYP1A2, which is constitutively expressed and TCDD inducible almost exclusively in the liver (26). PAH-mediated induction of CYP1A1 and CYP1A2 is stimulated by ligand binding to AhR (24). TCDD, the most potent agonist of the AhR, mediates the transcriptional activation of CYP1A1 via a cellular cascade initiated by ligand binding, followed by dissociation of the M, 90,000 heat shock protein hsp 90, nuclear translocation, and association with the Arnt protein and nuclear dioxin-responsive elements. The transcriptional activation of human CYP1A1 is highly cell line specific in mammary carcinoma-derived cell lines (27, 28).

This laboratory has recently purified and cloned an additional PAH-responsive cytochrome P450 of the CYP1 family gene, CYP1B1, from C3H10T1/2 mouse embryo fibroblasts and rat adrenal
cells (29, 30). The human orthologue has been cloned from a human squamous carcinoma cell line, SCC-13 (31, 32). CYP1B1 appears to be preferentially localized in tissues of mesodermal origin, particularly those producing steroids (adrenal, testis, and ovary) or those responding to steroids (prostate, uterus, and mammary tissue; Refs. 29, 30, 33). Interestingly, a deficiency in human CYP1B1 protein expression has recently been shown to be responsible for the development of congenital glaucoma (34). CYP1B1 is expressed constitutively and is TCDD inducible in the human mammary carcinoma MCF-7 cell line (35). Human CYP1B1 has been associated with the hydroxylation of 17β-estradiol to form the 4-catecholestrogen in analyses using recombinantly expressed human CYP1B1, as well as in the MCF-7 cell line (36, 37). This activity has been shown to be enhanced in human mammary and uterine tumors relative to the surrounding tissue (38–40). Collectively, these findings clearly establish a developmental, as well as an endogenous, role for this P450 cytochrome in estrogen metabolism, in addition to a role in PAH bioactivation.

Mutagenesis analyses have recently demonstrated that human CYP1B1 activates numerous structurally diverse environmental contaminants, including PAHs and their dihydrodiol derivatives, heterocyclic and aryl amines, and nitroaromatic hydrocarbons, to genotoxic agents (33). The selectivity of these CYP1B1-specific responses differs substantially from those mediated by CYP1A1. Thus, the CYP1B1-mediated activation of many environmentally persistent xenobiotics likely plays a significant role in chemical-induced mammary carcinogenesis. Although previous studies have demonstrated species-related differences in PAH metabolism and bioactivation with respect to rat and human mammary epithelial cells, these studies subjected the cells to prolonged exposures of PAH (24–42 h) and, thus, are representative of inductive as well as activation processes (15, 41, 42). Subsequent analyses have shown that PAH metabolism in cultured rat mammary cells is mediated by both CYP1A1 and CYP1B1, in a cell-selective manner (43). In rat mammary fibroblasts, CYP1B1 was shown to be exclusively expressed both constitutively and following TCDD induction. However, in rat mammary epithelial cells, CYP1A1, absent constitutively, was highly inducible by TCDD, whereas CYP1B1 was scarcely detectable, even under conditions of TCDD exposure. Despite these differences in cell culture, immunohistochemical analyses have recently shown that CYP1B1 is extensively expressed in rat mammary ductal epithelia, demonstrating that CYP1B1 is, indeed, expressed in mammary epithelial cells in vivo (44). Although CYP1A1 and CYP1B1 are each expressed in human mammary tumor cell lines, little is known regarding CYP1A1 and CYP1A1 cell-type-specific expression in cultured human mammary cells. This is of particular importance because many studies have emphasized the multiplicity of cell types existent in HMEC cultures and the dependence of culture conditions on cell-specific expression (45).

This study characterizes the expression of CYP1A1 and CYP1B1, the major PAH-metabolizing P450 cytochromes, in early-passage HMECs. The cells have been cultured under conditions used in previous PAH induction studies (15, 41, 42) and, thus, are composed of a heterogeneous population of basal and luminal epithelial cells. This study represents the first analysis of functional cytochrome P450 expression in cultured HMECs using conditions that measure basal levels of expression and activity, distinct from the induction response. HMECs isolated from seven women following reduction mammoplasty surgeries were used in this study. We have quantitated constitutive and TCDD-inducible expression of CYP1B1 and CYP1A1 with respect to the level of AhR and Arnt expression, two proteins that may influence the extent of PAH-inducible cytochrome P450 expression. DMBA metabolism was used as a biomarker for functional CYP1B1 and CYP1A1 expression. The regioselective profiles of recombinant human CYP1B1 and CYP1A1-mediated DMBA metabolism, in combination with antibody inhibition analyses, have been used to resolve the respective contributions of these P450 cytochromes to both cellular and microsomal metabolism. This study provides evidence that the relative contributions of CYP1B1 and CYP1A1 to cellular bioactivation are highly dependent upon the magnitude of the cellular exposure to environmental chemicals (e.g., PAHs). We provide evidence that CYP1B1 is an important contributor to PAH bioactivation under conditions of low induction from environmental exposure. Additionally, we show that there are substantial differences in CYP1B1 and CYP1A1 expression that may represent a risk factor for chemical-mediated breast cancer.

**MATERIALS AND METHODS**

**Materials.** The MEGM-bullet kit was purchased from Clonetics (San Diego, CA). TC102 reagent was obtained from Life Technologies, Inc. (Grand Island, NY). Random 9-mers and the random-primer DNA labeling kit were obtained from Stratagene (La Jolla, CA). The horseradish peroxidase-conjugated goat antirabbit IgG, Ts DNA polymerase, reverse transcriptase, dNTPs, and RNasin were purchased from Promega (Madison, WI). PCR primers were obtained from Integrated DNA Technologies (Iowa City, IA). The ECL detection system was purchased from Amersham (Arlington Heights, IL). The BCA protein assay reagent was obtained from Pierce (Rockford, IL). Nitrocellulose was supplied by Schleicher and Schuell (Kenne, NH), whereas all other materials used for SDS-PAGE were obtained from Bio-Rad (Richmond CA). DMBA was purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC-grade methanol was purchased from VWR Scientific (Detroit, MI). Human recombinant CYP1B1 and CYP1A1 microsomes and human epoxide hydratase were obtained from Gentest Corporation (Woburn, MA). All other chemicals and reagents were purchased from Sigma Chemical Co. and were of the highest quality available.

**Primary Antibodies and Epoxide Hydratase.** Polyclonal antiserum (IgG fraction) specifically recognizing CYP1A1 was generated in this laboratory, as described previously (46). Antipeptide antiserum specifically recognizing human CYP1B1 was provided by Dr. Craig Marcus (University of New Mexico, Albuquerque, NM) and Dr. William Greenlee (University of Massachusetts, Worcester, MA; Ref. 47). Monoclonal antibodies specific for AhR was provided by Dr. Gary Perdew (Penn State University, State College, PA), whereas polyclonal antiserum specifically recognizing Arnt was a gift from Dr. Richard Pollenz (University of South Carolina, Columbia, SC; Ref. 48). Polyclonal antibodies to mouse CYP1B1 (IgY) were prepared in this laboratory in female Leghorn chickens and purified from the eggs, as described previously (49).

**Cells and Cell Culture.** Human mammary organoid preparations isolated from seven individual donors (Table 1) were provided by Dr. Michael Gould and the University of Wisconsin Comprehensive Cancer Center Cell Bank. The mammary tissue was processed, and organoids were isolated in the Gould laboratory, according to previously published procedures (4, 15). Primary organoids were plated (2 × 10^6 cells/cm²) on plastic and cultured in MEGM supplemented with penicillin-streptomycin (50 units/ml penicillin-0.05 mg/ml streptomycin). Cells were grown at 37°C in a humidified environment of 5% CO₂ and 95% air. Confluent cells (7–10 days postplating) were passaged by differential trypsinization, plated at 1.7 × 10⁶ cells/cm², and subsequently analyzed at day 4, 6, or 13.

Cells either remained untreated or were treated with TCDD (10 nM) for a period of 24 h prior to analysis. Cells used for microsomal preparations were harvested by trypsinization and washed three times with ice-cold 1× PBS. Microsomal fractions were isolated by differential centrifugation, as described previously (46). Protein concentrations were determined according to the BCA procedure using the Pierce BCA protein assay kit, according to manufacturer’s protocol, using BSA as the standard.

**DMBA Metabolism Assay.** Microsomal DMBA metabolism was analyzed as described previously (44). Briefly, microsomal incubations were composed of 0.2 and 1.0 mg/ml microsomal protein for induced and basal metabolism, respectively, 14.2 mM glucose-6-phosphate, 0.06 units glucose-6-phosphate dehydrogenase, 1.2 mM NADP, 6 mM MgCl₂, and 0.1 mM potassium phosphate.
Table 1 Antibody inhibition of microsomal DMBA dihydrodiol formation in primary HMECs

<table>
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<tr>
<th>Treatment/IgG addition</th>
<th>Dihydriodil</th>
<th>Total dihydrodiol formation (pmol/mg/h)</th>
<th>% inhibition</th>
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<tr>
<td></td>
<td>5.6</td>
<td>8.9 + 10.11</td>
<td>3.4</td>
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<td>Primary HMECs</td>
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<td>Constitutive</td>
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<td>Donor D</td>
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<tr>
<td>+ preimmune</td>
<td>0.29</td>
<td>0.34&quot;</td>
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<tr>
<td>+ anti-CYP1A1(b)</td>
<td>0.29</td>
<td>0.35&quot;</td>
<td>0.12</td>
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<tr>
<td>+ anti-CYP1B1(f)</td>
<td>0.07</td>
<td>0.05&quot;</td>
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<td>Donor E</td>
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<td>+ preimmune</td>
<td>0.21</td>
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<td>+ anti-CYP1A1(b)</td>
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<td>+ anti-CYP1B1(f)</td>
<td>0.13</td>
<td>1.01&quot;</td>
<td>0.19</td>
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<td>Recombinant human cytochromes P450</td>
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<tr>
<td>CYP1B1(f)</td>
<td>50</td>
<td>33</td>
<td>27</td>
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<tr>
<td>CYP1A1(f)</td>
<td>189</td>
<td>870</td>
<td>73</td>
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8.9- + 10.11-DMBA-dihydriodils; peaks were poorly resolved at this low level of activity.

a 10 mg IgG/mg microsomal protein.

b 5 mg IgG/mg microsomal protein.

* Metabolism demonstrated by donor D.

Recombinant human CYP1B1 expressed in lymphoblast microsomes (Gentest Corp.), 74 pmol/mg.

Recombinant human CYP1A1 expressed in lymphoblast microsomes (Gentest Corp.), 104 pmol/mg.

(pH 7.6) to a total reaction volume of 1 ml. The reaction was initiated by the addition of 1.5 μM DMBA (HPLC purified) and allowed to incubate for 15 min at 37°C, under conditions of subdued lighting. The analysis of the human recombinant CYP1B1 and CYP1A1 microsomal standards (0.075 and 0.050 mg microsomal protein/ml, respectively) were assayed, as above, with the addition of 0.5 mg/ml human epoxide hydratase. Metabolites were extracted, and the microsomal DMBA metabolite profiles were examined by reverse-phase HPLC analysis. All data were normalized relative to DMBA recovery following extraction procedures.

Cellular DMBA metabolism was examined in 12-well culture plates (3.8 cm² well) on actively growing cells. Each incubation was composed of 0.5 ml of culture medium supplemented with 10 μM DMBA. Basal and TCDD-induced cellular incubations were completed at 37°C for 60 and 30 min, respectively. The incubation medium was removed from the culture vessel and incubated with β-glucuronidase (1000 units in 0.5 M NaOAc, pH 5.2) for 4 h at 37°C to effect the release of all H₂O-soluble metabolites. DMBA metabolites were extracted, and HPLC metabolic profiles were examined, as described above.

Antibody Inhibition Analysis. Antibody inhibition of microsomal DMBA metabolism was analyzed by the addition of anti-CYP1B1 (chicken; IgY, 5 mg antibody/mg microsomal protein) and anti-CYP1A1 (rabbit; IgG, 10 mg antibody/mg microsomal protein), concentrations previously determined to optimally inhibit CYP1B1- and CYP1A1-specific activities in constitutive and TCDD-induced MCF-7 cells. The corresponding preimmune IgY or IgG was added at equivalent concentrations to serve as a control. All reaction mixtures were incubated for 45 min at room temperature prior to assaying DMBA metabolism (35).

Immunoblot Analysis. Microsomal proteins were subjected to SDS-PAGE (3% acrylamide stacking gel-7.5% acrylamide separating gel) followed by immunoblot analysis, as described previously (50, 51). Immuno-reactive proteins were visualized using the ECL method of detection, according to the manufacturer’s protocol. Cytochrome P450 expression was quantitated relative to human recombinant CYP1B1 (75 pmol P450/mg microsomal protein) and CYP1A1 (104 pmol P450/mg microsomal protein) microsomal standards. Quantitation was completed by soft laser scanning densitometry employing a Zeineh Model SL-504-XL soft laser scanning densitometer (Fullerton, CA).

Measurement of mRNA by rt-PCR. Total RNA was prepared from day 6 HMEC cultures that were either untreated or treated with 10 nm TCDD for 6 h prior to isolation. Cells were harvested with TRizol reagent, and the RNA was isolated according to the manufacturer’s protocol. cDNA templates for PCR were synthesized as follows: total RNA (2 μg) and the random 9-mer primer mix (1 μg), in a total volume of 31 μl, were heated to 60°C for 5 min. The samples were allowed to cool slowly to room temperature, and then the reverse transcription master mix, composed of 1.0 mm each dNTP, 40 units of RNasin, 10.0 mm dTT, and 200 units of Moloney murine leukemia virus reverse transcriptase, was added to total of 50 μl. The samples were incubated at 40°C for 1 h, followed by denaturation for 5 min at 100°C.

Primers specific to CYP1B1 (forward, 5'-CGTACCCGCCCCATCATCGT-3'; reverse, 5'-CGAGGCTCATTTGGGTTGGC-3') and CYP1A1 (forward, 5'-AAAGCAGTTGACGAGCTGATG-3'; reverse, 5'-ACATTGGGGTTCTCATTCCACGTGCT-3') were synthesized using an Applied Biosystems 380A synthesizer. All PCR amplifications were completed in a Perkin Elmer/Cetus model 480 DNA thermocycler. CYP1B1 amplifications were composed of 2 μg of cDNA template, 0.22 mm each dNTP, 0.625 units of Taq DNA polymerase, 0.56 μM each primer, and H₂O to a final volume of 22.5 μl. The reaction was initiated under “hot start” conditions, whereby the samples were heated to 94°C for 3 min, followed by the addition of 2.78 mm MgCl₂. The samples were cycled for 1 min at 94°C, 1.5 min at 60°C, and 2.0 min at 72°C, for a total of 30 cycles. CYP1A1 amplifications were composed of 2 μg of cDNA template, 0.6 mm each dNTP, 2.5 mm MgCl₂, 0.625 units of Taq DNA polymerase, 0.5 μM each PCR primer, and H₂O to a total volume of 25 μl. Cycle parameters were 1 min at 94°C, 1.5 min at 57°C, and 2.0 min at 72°C, for a total of 30 cycles. PCR products were analyzed by agarose gel (1.6% agarose) chromatography and visualized by staining in ethidium bromide.

Northern Analysis. cDNA probes specific for CYP1B1 and β-actin were labeled with [α-35S]dCTP (400 Ci/mmol) using the random-primer DNA labeling kit, per manufacturer’s protocol. The poly(A)⁺ mRNA was isolated from day 6 HMEC cultures according to the method of Badley et al. (52). mRNA was fractionated in 1.2% agarose-37% formaldehyde gels, transferred to nitrocellulose, and probed as described previously (53).

RESULTS

HMECs Were Cultured from Seven Individual Donors. Organoid preparations, isolated from reduction mammoplasty procedures and cultured under serum-free conditions, generally demonstrated full attachment within 3–4 days followed by a period of rapid proliferative growth, reaching confluence within 7–10 days of initial plating. The proliferative efficiency varied among cultures of the
seven individual donors, with donors A and C demonstrating the slowest rate of growth.

Cellular DMBA Metabolism Analysis Demonstrated Optimal Enzymatic Activity at Day 6 of Culture. DMBA metabolism was examined in secondary HMEC cultures to evaluate the time dependence of functional cytochrome P450 expression. Cellular DMBA metabolism of three donors was analyzed at days 4, 6, and 13 of culture. Fig. 1 depicts the cellular metabolism of one donor, donor D, but the data are representative of all three individuals examined. HMECs were exposed to 10 nM TCDD for a period of 24 h prior to analysis. The level of TCDD-induced cellular metabolism increased until day 6 of culture, followed by a >60% decline in activity between days 6 and 13. Thus, all subsequent experiments were completed on cells harvested at day 6 of culture, representing optimal expression of functional P450 cytochromes.

TCDD-induced DMBA metabolism was examined in the intact cell (cellular assay), as well as in the isolated microsomal fraction. The rate of TCDD-induced metabolism in the intact cells was of a similar magnitude to that obtained in the microsomal assay (calculating 1 mg microsomal protein/10^7 cells). However, basal cellular metabolism was not measurable at these cell densities (10^5 cells/well) in assays that were of short enough duration to exclude induction by DMBA, an effective AhR agonist (Ref. 54; data not shown). Therefore, we focused all further analyses of DMBA metabolism and antibody inhibition in the isolated microsomal fraction of basal and TCDD-induced cells.

CYP1B1 Is Expressed Constitutively, whereas CYP1B1 and CYP1A1 Are TCDD inducible in Normal HMECs. Microsomal CYP1B1 and CYP1A1 expression was quantitated by immunoblot analysis in seven HMEC preparations at day 6 of culture, relative to recombinant human microsomal standards, as shown in Figs. 2 and 3. These values represent extremely low levels of expression (<0.3–16 pmol/mg) but were reproducibly quantifiable.

Each individual exhibited constitutive CYP1B1 expression that was substantially induced by TCDD (Fig. 2). The variability in TCDD-induced levels among the donors was similar to that observed for basal CYP1B1. The average levels of basal and induced CYP1B1 expression among six of the individuals (donors B–G) were 0.9 ± 0.5 and 10.4 ± 4.7 pmol/mg, respectively. Donor A demonstrated a 3–7-fold lower level of induced CYP1B1 expression, relative to the remaining six individuals. The level of constitutive expression of CYP1A1 was substantially lower than that of CYP1B1. Constitutive microsomal CYP1A1 expression was at the lower limit of detection (<0.02 pmol/mg) in four of the donors (A, B, C, and D), whereas an extremely low level of expression (0.03–1.6 pmol CYP1A1/mg) was detectable in cultures of the donors E, F, and G (using exposure conditions that were 10 times longer than required for CYP1B1 detection; Fig. 3). However, the magnitude of TCDD-mediated induction was substantially greater for CYP1A1 (>100-fold) than for CYP1B1 (5–25-fold; excluding donor A), resulting in comparable TCDD-induced microsomal levels of the two P450 cytochromes. TCDD-induced CYP1A1 expression was more variable between the individual donors than was induced CYP1B1 expression (varying by 21- and 5-fold, respectively, between donors A and B, the donors demonstrating the highest and lowest levels of CYP1A1 expression).

Microsomal DMBA Metabolism Parallels Immunodetectable Cytochrome P450 Expression. The analysis of DMBA metabolism mediated by recombinant CYP1B1 and CYP1A1 expressed in lymphocyte microsomes demonstrated isoform-specific differences with respect to the pattern of regioselective product distribution (Table 1). However, the profiles were not as distinct as demonstrated previously with the rodent orthologues (44). Human CYP1A1-mediated DMBA metabolism demonstrated relatively low levels of 5,6- and 10,11-
The proportion of the 5,6- and 10,11-dihydrodiol metabolites, relative to the 8,9-dihydrodiols, generated by the TCDD-induced microsomes of donor D were consistent with the predominant involvement of CYP1A1. These profiles are typical of trends demonstrated by all donors following TCDD induction.

Antibody inhibition of DMBA metabolism further defined the contributions of CYP1B1 and CYP1A1 to DMBA metabolism in the HMECs (Table 1). The regioselective distribution of metabolism that is blocked by the specific antibody provides a characteristic profile of the inhibited enzyme. The addition of anti-CYP1A1 did not substantially effect basal activity (7% inhibition), whereas a parallel addition of anti-CYP1B1 inhibited DMBA-dihydrodiol formation by 84%. The addition of anti-CYP1A1 to TCDD-induced microsomal fractions reduced dihydrodiol formation by 79%, and left residual activity with a regioselective metabolite distribution consistent with CYP1B1-mediated metabolism. The initial TCDD-induced activity was inhibited by 25% by anti-CYP1B1, whereas the simultaneous addition of anti-CYP1A1 and anti-CYP1B1 to induced microsomes increased the extent of inhibition from 78 to 91%. This suggests a contribution of ~15–25% by CYP1B1 to these TCDD-induced activities. The observed equal expression of the proteins combined with a 6-fold higher specific activity for CYP1A1 predicts a 14% contribution from CYP1B1. This is fully consistent with the antibody inhibition data within the variability of these assays. Thus, when TCDD stimulates DMBA metabolism by 30–40-fold, the 5-fold increase in CYP1B1-mediated activity is overwhelmed by the contribution from the more than 100-fold induction of the more active CYP1A1.

As discussed above, HMECs from donor E consistently exhibited a DMBA product profile characteristic of a mixture of CYP1B1- and CYP1A1-mediated metabolism. This was confirmed by antibody inhibition, whereby CYP1B1 and CYP1A1 each inhibited this metabolism by ~35%, again consistent with equal contributions from each form (Table 1).

The analysis of DMBA metabolism with respect to the measurement of cytochrome P450 protein expression showed that CYP1B1 turnover in HMECs was 6 h⁻¹ (constitutive microsomes; donors D and E), whereas CYP1A1 turnover was 90 h⁻¹ in constitutive microsomes and 21 h⁻¹ in TCDD-induced microsomes. The values for recombinant CYP1B1 and CYP1A1 in lymphoblast microsomes were 4 and 17 h⁻¹, respectively.

rt-PCR and Northern Blot analyses of RNA Expression of CYP1B1 and CYP1A1 in HMECs Support Immunoblot analyses. Constitutive and TCDD-inducible CYP1B1 expression was analyzed using total RNA isolated from cultured HMECs of donor D by rt-PCR methodologies (Table 2). Constitutive and TCDD-inducible CYP1B1

| Method of analysis | Northern hybridization | r-PCR (relative expression level) | Constitutive | 1.0 | 0.6 | 1.01–1.7
| Fold TCDD induction | 12 | 33 | 18 |

*Expression was quantitated by soft laser scanning densitometry of ethidium bromide-stained PCR products. cDNA was synthesized from TCDD-induced MCF-7 cells and used for serial dilution in the analysis of the ethidium bromide detection response.

Poly(A)* RNA (10 µg/lane) was probed for CYP1B1 using a rat CYP1B1 cDNA probe. Expression was quantitated by soft laser scanning densitometry following autoradiography. The relative expression level has been normalized relative to the level of β-actin expression.

Recombinant human CYP1B1 (74 pmol/mg; Gentest Corp.) was used in constructing a standard curve for the quantitation of CYP1B1 protein expression. Immunoreactive proteins were visualized by ECL methodologies, and quantitation was completed by soft laser scanning densitometry.

Metabolism demonstrated by donor D.
mRNA expression was detectable, demonstrating a >10-fold increase in TCDD-induced CYP1B1 mRNA, which was fully consistent with the induction of immunodetectable CYP1B1 protein following TCDD exposure. Similar results were observed in RNA isolated from donors A and B (data not shown). rt-PCR analysis of CYP1A1 expression has been completed for three donors (donors A, B, and D). Constitutive CYP1A1 expression was weakly yet reproducibly detectable in donor B, in parallel with the low magnitude of observed protein expression (data not shown).

Northern blot analysis of mRNA isolated from donor D confirmed the expression of constitutive CYP1B1 mRNA, which was highly inducible by TCDD (>30-fold; Table 2). These results corroborate the results obtained in the immunoblot and rt-PCR analyses.

Ah Receptor and Arnt Expression Are Elevated in Cultured HMECs. Cytosolic AhR expression was analyzed in the cultured HMECs of four donors (donors B, D, E, and F). Immunoblot analysis identified the expression of the Mr 104,000 protein in all individuals examined, the level of which varied by 3-fold (Fig. 4A). TCDD induction resulted in the down-regulation of the cytosolic receptor (~3-fold), which is mediated by nuclear translocation followed by proteolytic turnover (55). The level of receptor expression was many times higher in the cultured HMECs, as compared to MDA-MB-231 cell line, the human mammary carcinoma cell line shown to express the highest level of AhR expression among several mammary cell lines examined.4

The expression of Arnt was also much higher in the cultured HMECs, relative to the MDA-MB-231 human mammary carcinoma cell line (Fig. 4B). The level of Arnt expression varied by only 1.5-fold among the four donors.

Fig. 4. Immunoblot analysis of constitutive and TCDD-induced AhR (A) and constitutive Arnt (B) expression in day 6 secondary HMEC cultures of four individual donors. Total proteins were isolated from constitutive (Lanes C) and TCDD-induced (Lanes T; 10 nm for 24 h) cells by TRIzol procedures and were analyzed by SDS-PAGE. Immunoreactive proteins were visualized by the ECL method, as described in "Materials and Methods." MDA-MB-231 and MCF-7 protein fractions were used as reference standards for comparison with expression levels in immortalized human mammary cell lines. Protein loadings were 20 µg/lane, and membranes were exposed to film for 5 min. Relative expression levels were analyzed by soft laser scanning densitometry using a Zeineh Soft Laser Scanning Densitometer (model SL-504-XL).

DISCUSSION

This study has characterized functional CYP1B1 and CYP1A1 expression in early-passage HMECs isolated from tissues procured from seven women undergoing reduction mammoplasty surgeries. The roles of CYP1B1 and CYP1A1 in PAH metabolism have been quantitatively characterized under conditions that reflect the basal condition of the cultured HMECs. Thus, the potential for PAH-mediated induction of CYP1B1 and CYP1A1 has been eliminated by maintaining short PAH reaction periods in the intact cellular assay and by directly assaying microsomal metabolism. Cytochrome P450 induction has been studied using a nonmetabolizable AhR agonist, TCDD, which substantially induced both isoforms following a 24-h exposure. Through the immunooquantitation of each form, we can account for PAH metabolism in terms of the expressed level of these two P450 cytochromes under both constitutive and TCDD-induced conditions. Previous studies of PAH metabolism in cultured HMECs have used prolonged exposures of the hydrocarbons to the cells (24–42 h), thereby combining P450 induction with P450-mediated bioactivation events (15, 41, 42). In addition, these studies have not addressed which specific P450 isozyme(s) are responsible for PAH metabolism and bioactivation.

This study demonstrates that CYP1B1 is expressed constitutively, at quantifiable levels, in cultured HMECs. In contrast, constitutive CYP1A1 is expressed at extremely low levels (approaching the lower limit of detectability), although both P450 cytochromes are expressed at comparable levels (pmol P450/mg microsomal protein) in the HMECs following TCDD exposure. Northern hybridization and rt-PCR analyses demonstrate that mRNA expression parallels levels of the respective microsomal protein in these cells. The levels of immunodetectable constitutive and TCDD-inducible CYP1B1 expression

4 W. G. R. Angus and C. R. Jefcoate, unpublished observations.
each varied by ~2.5-fold (excluding donor A), whereas induced levels of CYP1A1 varied by as much as 21-fold among the seven individuals.

The HMECs used in this study have been cultured under conditions similar to those applied in the previous studies of HMEC-mediated PAH metabolism, yielding a mixture of luminal and basal epithelial cell populations. Under these culture conditions, the proportion of basal HMECs progressively increased with increased passage and time in culture relative to the luminal subtype (45). In parallel with a predicted increase in the proportion of basal cells (45), we observed a marked decrease in TCDD-induced DMBA metabolism following day 6 of secondary culture. Recent preliminary studies have demonstrated the immunodetectable expression of CYP1A1 in an isolated luminal cell population, whereas CYP1B1 expression was observed in both the isolated basal and luminal epithelia. Thus, the observed increased interdonor variability in CYP1A1 expression, relative to CYP1B1, may potentially arise from differing proportions of the luminal cells among the individual donors or may reflect donor-dependent differences in the magnitude of CYP1A1 expression. These issues are being addressed in ongoing studies.

Although DMBA is a highly discriminating substrate for the functional measurement of rodent CYP1A1 and CYP1B1 (44), elucidating the individual contributions of these forms to DMBA metabolism has been difficult in human cells. Although rodent CYP1B1 and CYP1A1 demonstrate highly distinct regioselective profiles of DMBA metabolite production, the corresponding profiles are less distinct for the human P450 cytochromes. For example, in rodents, CYP1A1-mediated metabolism is characterized by the preferential hydroxylation at the 7-methyl substituent of DMBA and the formation of the 8,9-DMBA-dihydrodiol, whereas CYP1B1 selectively metabolizes DMBA to produce high levels of the 10,11 and 3,4-dihydrodiol metabolites. However, by using recombinant human CYP1B1 and CYP1A1, we have shown that human CYP1A1-mediated metabolism is characterized by the preferential formation of the 8,9-DMBA-dihydrodiol with proportionally less 5,6-dihydrodiol and 7-hydroxy-DMBA than rodent CYP1A1-mediated metabolism. There is also clear production of the 3,4-dihydrodiol, which is absent with the rodent orthologue. Human CYP1B1-directed metabolism yielded relatively high proportions of the 5,6 and 10,11-DMBA-dihydrodiol metabolites, relative to human CYP1A1, but, unlike rodent CYP1B1, showed a similar proportion of the 3,4-dihydrodiol (5%). Human CYP1A1 is 6-fold more efficient in the metabolism of DMBA, as compared to CYP1B1, a much larger difference than observed in the rodent forms.

Secondary HMEC cultures demonstrated low levels of constitutive microsomal DMBA metabolism that was mediated by CYP1B1, consistent with the immunodetectable basal expression of this isoform. The regioselective profile of metabolite distribution generated from these HMECs was consistent with that produced by human recombinant CYP1B1, and this has been confirmed by the selective inhibitory effect of anti-CYP1B1 on DMBA metabolism, as compared to the lack of inhibition with anti-CYP1A1 antibodies. Small but significant differences in the regioselective profile of metabolite formation were observed between the HMECs and the recombinantly expressed protein (HMECs demonstrate a lower 5,6:10,11-dihydrodiol ratio). This may be due to differences in the configuration of CYP1B1 in HMECs and in lymphoblast endoplasmic reticulum. We have recently measured product distribution for DMBA metabolism by recombinant human CYP1B1 expressed in V79 cells, which more closely matches the HMEC product ratios (data not shown). Certainly, for each of these sources, the mobility of the CYP1B1 proteins in SDS-PAGE is indistinguishable. Donor E, one of a group of three individuals demonstrating low levels of basal CYP1A1 immunodetectable expression, yielded a profile of regioselective basal metabolism reflecting approximately equal contributions from both CYP1A1 and CYP1B1. Although the level of basal CYP1A1 expression in donor E is ~10 times lower than basal CYP1B1, the contribution of CYP1A1 to DMBA metabolism is consistent with the 6-fold higher turnover of the isoform. This provides a critical confirmation of the immunodetection of constitutive CYP1A1 in these cultures. The consistent finding of CYP1A1 in cells from these donors and not others cultured in the same medium indicates that this is not due to an inducer in the medium but, rather, results from a selective endogenous activation of CYP1A1 within these donors.

Exposure of the cultured HMEC to TCDD increased the overall rate of DMBA metabolism by 30-40-fold. Although TCDD-induced CYP1B1 and CYP1A1 were expressed at equivalent levels, the induced metabolism was predominantly mediated by CYP1A1. The profile of metabolite regiodistribution paralleled that of recombinant human CYP1A1. Because CYP1B1 is ~6-fold more efficient than CYP1B1 in metabolizing DMBA, we would predict, at equimolar levels of expression, CYP1A1 and CYP1B1 to mediate 86 and 14% of induced DMBA metabolism, respectively. Indeed, antibody inhibition analysis confirmed this activity distribution, within the sensitivity of the assay. We have shown that anti-CYP1A1 substantially inhibited metabolic activity, while yielding a regioselective profile of residual metabolism indicative of predominantly CYP1B1-mediated activity. Furthermore, much of the residual activity was removed by a further addition of anti-CYP1B1 antibodies.

We have estimated the turnover number for DMBA metabolism with the exceptionally low levels of the individual forms of cytochrome P450 calculated in this study. This involves comparison of expression levels with the proportion of the metabolism attributable to the individual form based on antibody inhibition. Constitutive activities from donors D and E indicate CYP1B1 turnover of 6 h⁻¹, in excellent agreement with the turnover of recombinant human CYP1B1 in lymphoblast microsomes (4 h⁻¹). CYP1A1 turnovers were estimated at 90 h⁻¹ in constitutive microsomes from donor E and at 21 h⁻¹ in TCDD-induced microsomes. Again, these rates compare remarkably well with the activity of recombinant human CYP1A1 in lymphoblast microsomes (17 h⁻¹).

Cellular DMBA metabolism was fully consistent with the microsomal activity. Both assays demonstrate TCDD-inducible metabolic activity, and the regioselective distribution of metabolites was consistent between the two methods of analysis. In cells, ~60% of dihydrodiols were released by β-glucuronidase, implicating their conjugation as glucuronides. This agrees with previous measurements of these conjugation ratios (42). The cellular and microsomal assays yielded an equivalent overall rate of metabolic activity, based upon a recovery of 1 mg of microsomal protein/10⁵ cells. The cellular analysis of basal metabolism did not demonstrate measurable activity above nonenzymatic background of DMBA oxidation. However, based on the microsomal assays we would not expect to detect DMBA metabolism from these cellular assays, which typically contained only 10⁵ cells.

Murray et al. (56) have recently demonstrated the immunohistochemical detection of CYP1B1 in tumor tissue but failed to detect CYP1B1 expression in the corresponding donor-matched normal breast tissue, raising questions regarding the magnitude of CYP1B1 expression in normal human breast tissue. We have completed rt-PCR analysis of seven normal and nine tumor-derived tissues in our laboratory and have observed comparable levels of CYP1B1 expression in...
all of the samples examined. Similar results have been reported by Huang et al. (57), who detected CYP1B1 mRNA in 22 of 23 samples examined, while demonstrating no discernible pattern in CYP1B1 expression between tumor and normal tissue from the same individuals (57). Although these studies have been limited to the analysis of CYP1B1 mRNA expression, Dr. Judith Weisz has shown that in situ hybridization analysis of CYP1B1 mRNA expression in breast tissue parallels immunohistochemical protein detection when using the same antibody preparation. The rt-PCR determination of the magnitude of CYP1B1 mRNA expression in the cultured early-passage HMECs presented here fully corroborates our quantitation of low levels of functional microsomal protein expression. Although we have not completed a direct comparative analysis of CYP1B1 mRNA and protein expression in freshly isolated versus cultured HMECs, this study clearly demonstrates the expression of quantifiable levels of functionally active CYP1B1 in early-passage normal human breast epithelia.

AhR levels in the cultured HMECs were up to 5-fold higher than the highest level observed in an established human mammary cell line (MDA-MB-231 cells). TCDD-mediated down-regulation of the cytosolic receptor level clearly showed that the cells in culture were expressing a functional receptor, most of which translocated to the nucleus upon binding the ligand and was degraded, concomitant with nuclear activation (i.e., enhanced transcription of CYP1A1 and CYP1B1). Because the levels are as much as 5 times higher than those in tumor cell lines, such as the MDA-MB-231 cells, in which TCDD is similarly effective in inducing these genes, apparently, AhR is not a limiting factor mediating cytochrome P450 expression in HMECs. This raises major questions regarding the functional significance of such high levels of AhR expression in these cells. Similarly, Arnt, the nuclear partner of the AhR, was elevated in the cultured HMECs, as compared to the human mammary cell lines. It appears that, when the AhR translocates to the nucleus, there is sufficient Arnt for heterodimerization of these proteins. Because an increased requirement for binding to gene enhancer binding elements is unlikely, this high level may function to complex and regulate additional nuclear factors.

Human CYP1B1 and CYP1A1 have previously been shown to discriminate, mediate the bioactivation of numerous procarcinogenic chemicals to genotoxic agents (33). Although the bioactivation of carcinogenic PAHs has previously been attributed to CYP1A1, recent studies by Shimada et al. (33) demonstrate that CYP1B1 mediates the bioactivation of a wide range of procarcinogenic PAH dihydrodiols. However, they report that CYP1B1 was ineffective in activating the associated parent PAHs, which may be attributable to the omission of epoxide hydratase from the assay system. Interestingly, CYP1B1 and CYP1A1 were shown to be highly selective with respect to PAH dihydrodiol activation. CYP1B1 efficiently activated the bay- and fjord-region PAH dihydrodiols, DMBA-3,4-diol, and dibenzo-(a,j)pyrene-11,12-diol, respectively, whereas CYP1A1 was ineffective in the bioactivation of these potent mammary carcinogens. CYP1B1 and CYP1A1 have also been shown to exhibit tissue- and species-specific expression following exposure to B(c)P, a chemical with a fjord-region diol epoxide (B(c)P-3,4-diol, 1,2-epoxide) that is among the most mutagenic and carcinogenic diol epoxides examined to date (58). For example, the treatment of MCF-7 cells with B(c)P resulted in increased CYP1B1 expression in the absence of immunodetectable CYP1A1, whereas exposure of murine epidermal cells to B(c)P resulted in elevated CYP1A1 levels and undetectable CYP1B1 expression. Our present studies indicate that human CYP1B1 should be active in generating dihydrodiol epoxides from DMBA and other PAHs, although the effectiveness, relative to CYP1A1, will certainly be dependent on additional metabolic factors. Clearly, interindividual and cellular specificity of cytochrome P450 expression, coupled with selectivity of CYP1B1- and CYP1A1-mediated bioactivation of procarcinogens, such as environmental PAHs, will largely determine a chemicals carcinogenic potency and an individuals associated risk of tumorigenesis.

This study has confirmed that human mammary epithelia express significant levels of basal CYP1B1 and that certain individuals constitutively express levels of CYP1A1 that are extremely low but, nevertheless, are functionally significant in PAH activation. Although the level of immunodetectable constitutive CYP1A1 expression is 7–70-fold lower than the level of CYP1B1 in these individuals and is only barely detectable by rt-PCR methodologies, the CYP1A1 expression has been demonstrated by the correlative expression of protein with metabolic activity analyses. This activity suggests that any individual expressing CYP1A1 in the mammary gland in this way is at an increased risk for PAH bioactivation, bearing in mind that the level of PAH exposure/bioaccumulation is maintained well below inducing levels due to constitutively active P450s, such as CYP1B1.

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