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PRINCIPAL INVESTIGATOR: Colin R. Jefcoate, Ph.D.

CONTRACTING ORGANIZATION: University of Wisconsin
Madison, Wisconsin 53706-4880

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Madison, Wisconsin 53706-4880

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The research characterizes the role of cytochrome P4501B1 (CYP1B1) as a contributor to breast cancer etiology. This enzyme may generate or remove a lipophilic hormone while also converting many environmental polycyclic aromatic hydrocarbons (PAH) to carcinogenic metabolites and estradiol (E_2) to potentially carcinogenic 4-hydroxy E_2. This work now shows that CYP1B1 is the dominant constitutive activator of PAH's and E_2 in human breast cells. Primary epithelial cultures from 7 normal human surgical samples all show constitutive CYP1B1 without the related CYP1A1 form which metabolizes PAH. Addition of the environmental organochlorine compound dioxin elevates CYP1B1 (10x) while introducing CYP1A1. Constitutive expression of CYP1B1 and induction of CYP1A1 varies 5-10 fold between cultures from different individuals apparently in proportion to variable expression of the Ah receptor which mediates dioxin effects. Studies with cultured human breast tumor cell lines indicate that loss of estrogen receptors decreases the ratio of CYP1A1 to CYP1B1. Breast stromal fibroblasts express CYP1B1 stimulated by dioxin but not CYP1A1. Normal and tumor breast tissue consistently express CYP1B1 but not CYP1A1. The regulation of transcription of CYP1B1 has been studied in cultured rat mammary cells using promoter constructs. A gene region that mediates Ah receptor/dioxin regulation has been identified. However, environmental elevation of CYP1B1 is not a contributor relative to high basal expression which may however be subject to genetic variability.

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Introduction

This proposal addresses potential mechanisms for contributions from environmental chemicals to the etiology of breast cancer. In particular, this research examines potential ways in which organochlorine compounds (OC's) and polycyclic aromatic hydrocarbons can synergize in producing biological effects in human breast cells. These ubiquitous environmental pollutants readily concentrate in the breast adipose tissue. PAH's generate DNA adducts that can potentially mediate mutagenesis and this process can be enhanced by the additional presence of OC's. Mutations in Ras and p53 genes that are consistent with PAH mutagenesis have been detected in human breast cancers. Epidemiology studies point to organochlorine compounds (OC's) that accumulate in breast adipose as a risk factor. These mechanisms may be synergistic. PAH's require activation to the ultimately carcinogenic form, the bay-region dihydrodiol epoxide, and this step involves P450 cytochromes. This laboratory has recently cloned a novel cytochrome P450, CYP1B1, that is particularly active in this process (1, 2). This form is related to a second P450 form, CYP1A1 that also metabolizes PAH (3). Each form is induced via the Ah-receptor by dibenzodioxins and planar PCB's, and this laboratory has provided evidence that these forms also metabolize 17β-estradiol to 2- and 4-catecholestrogens (4). CYP1B1 seems to be selectively effective in forming 4-catecholestrogens. This conversion has recently been shown to be elevated in endometrial and breast cancers. Estrogens also exert a potentiating effect on the stimulation of CYP1A1 by Ah-receptor ligands (5). Two additional mechanisms have been identified in our proposal; (a) estrogenic/anti-estrogenic activity of DDE, PCB metabolites (b) Ca^{++}-elevating effects of many OC's An exciting recent report (6) shows that several OC's synergize in their binding and activation of the estrogen receptor. A 100-fold increase in potency may emerge from this synergism. On the other hand, other work points to strong antiestrogenic effects of AhR ligands (7).

The CYP1B1 gene has a very unusual structure (only 2 introns) and produces one of the largest P450 mRNA (5.2 kb), which includes 3 kb of 3'-untranslated sequence (8). CYP1B1 is selectively expressed in hormonally regulated steroidogenic tissues (adrenal, ovary, testis), in the stromal cells of steroid sensitive tissues (mammary, prostate, uterus), and during limited periods in the embryo in tissue undergoing morphogenesis (1, 2). Thus, CYP1B1 may also be a key determinant of the level of an agent that directly regulates tissue development including cancer cells. We have hypothesized, based on the stromal expression pattern, that CYP1B1 may modulate stromal effects on epithelia, a key regulatory mechanism in the breast. Consequently, we are studying the expression of CYP1B1 in mammary stroma from different sources, including normal breast versus breast tumor. Ultimately, we will test whether CYP1B1 activity is related to stromal effects on mammary epithelia.

The initial aim of this work has been to investigate the expression of CYP1B1 in normal human breast cells including selectivity of expression between ductal epithelia, and stromal fibroblasts, *in vivo* and in cell culture. These studies will be paralleled by an examination of the expression in carcinoma cell lines with various phenotypes, and in cells cultured from breast cancers. We are interested in whether CYP1B1 is sensitive to hormonal regulation and induction or suppression by OC's. The first experiments will focus on induction by 2, 3, 7, 8 tetrachlorodibenzodioxin (TCDD), the best stimulant for the Ah-receptor. This will be correlated with measurements of potential CYP1B1-dependent activities, notably metabolism of 7, 12-dimethyl benzanthracene (DMBA) and 17β-estradiol. Recent work suggests that estradiol 4-hydroxylase activity is high in breast cancers. (9) Since our measurements of CYP1B1 mRNA show no such variation, further evaluation is necessary.

A key goal of our work has been to determine whether the estrogen receptor (ER) plays a selective role in transcriptional activations mediated by the Ah receptor. To this end, we have obtained human mammary cell lines that differ primarily through presence or absence of the ER. A

critical problem here is that selection processes involved with the isolation of these lines are likely to introduce substantial adaptive secondary effects, notably the introduction of autocrine growth factors that take over the growth promoting role of ER. These selection effects are avoided here by comparison of ER^- cells with the ER^+ counterparts treated briefly with a potent ER antagonist ICI. This of course does not discriminate between direct nuclear effects of ER and secondary signalling effects of ER-enhanced factors.

A related goal of this work is to determine whether organochlorine compounds can reproduce the effects that are linked to estradiol activation of the ER. This has been made more important by the possibilty of synergism of organochlorine compounds. Our first priority here is to determine whether organochlorine compounds in human breast fat show estrogenic activity and secondly whether other biological activities associated with OC's are detectable. We are measuring estrogenic activity with a luciferase-fusion construct which is responsive to estrogens via a triple combination of estrogen response elements. Additionally we are testing for AhR activity and various OC-sensitive hepatocyte activities. This work is in progress using extracts from breast adipose tissue from individual surgical samples. We are also looking for correlations with AhR-mediated induction of CYP1A1 in adipose mRNA to test the possibility that the adipose OC's in this tissue are biologically active.

Carcinogenic activity of polycyclic hydrocarbons in human mammary epithelial cells is almost certainly mediated by formation of DNA adducts. We have established a collaboration with Dr. William Baird's laboratory at Purdue University to measure DNA adducts formed in human breast epithelia by polycyclic aromatic hydrocarbons (PAH's) 7.12-dimethyl benzanthracene (DMBA) and dibenzpyrene (DBP). In these experiments activations from basal levels of CYP1B1 are being compared with activations resulting from PAH-induction and from TCDD-induction. Adduct levels are being correlated with levels of CYP1B1 and CYP1A1 expression in the same cells.

Progress in Year 2

1. Development of Methodologies:

1A. PCR

rtPCR has been used in an attempt to quantitate expression of CYP1A1 and CYP1B1 in cultured human mammary cell lines and in primary tissues. A serial dilution of cDNA from a strongly positive source (e.g.. TCDD-induced MCF7 cells) is amplified together with samples of unknown expression. Following separation of PCR products on an ethidium bromide-containing 1.5% agarose gel, a photograph is taken and the negative scanned, digitized, and quantitated. However, we have found quantitation of digitized negatives generated by ethidium bromide-impregnated gels to be marginally successful due to a poor signal to dilution ratio. To enhance the signal to dilution ratio, we are generating digoxigenin-tailed oligomer probes specific for the PCR products of the gene of interest (i.e.. CYP1A1 or CYP1B1). The intent is to use slot blot of the PCR products, followed by hybridization with the digoxigenin-tailed probes to generate data that is more clearly quantitated.

1B. Immunoblotting

Constitutive and TCDD-inducible CYP1B1 and CYP1A1 expression has been examined in the primary cells and immortalized cell lines by immunoblot analysis, relative to expression in microsomal standards, namely the transformed human mammary carcinoma cell line, MCF-7, and

in recombinantly expressed human CYP1B1 microsomes (Gentest Corp., Woburn, MA). SDS-PAGE (3% acrylamide stacking gel, 7.5% acrylamide separating gel) and immunoblot analyses were completed according to standard protocols. The polyclonal antisera (IgG fraction) employed in the analysis, specifically recognizing either CYP1A1 or CYP1B1, have been generated in this laboratory against rat CYP1A1 and mouse recombinant CYP1B1 antigens, respectively. Immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL) method of detection.

2. Differential expression of P450 cytochromes in human breast cell cultures: variable Ah receptor expression is a determinant

2A. Epithelial cells

Microsomal CYP1B1 and CYP1A1 expression has been characterized in early passage normal primary human mammary epithelial cells (HMEC) as well as in tumor-derived HME cell lines by immunoblotting methodologies. Primary HMEC, previously obtained from patients undergoing reduction mammoplasty surgeries, were examined from 7 individuals ranging from 19 to 49 years of age. The 21T cell line series was utilized as representative tumor-derived HMEC. The 21-NT, 21-PT, and 21-MT2 cells were originally isolated from a primary infiltrating carcinoma, a primary intraductal carcinoma, and a pleural effusion of a metastatic tumor, respectively, from a 36 year old woman, thus representing three stages of tumor progression. The primary cells were expanded in culture and harvested 6 days post passage 1 for microsomal isolation. The cell lines were, likewise, harvested 6 days post plating.

CYP1B1 was shown to be the predominant constitutively expressed PAH-responsive P450 cytochrome in normal primary HMEC by immunoblot (Fig. 1) as well as PCR (Fig. 2) analyses. Immunoblotting demonstrated lower constitutive and increased TCDD-induction of CYP1B1 in the normal primary cells as compared to the tumor-derived cell lines (Fig. 3). Constitutive CYP1B1 expression in the primary HMEC's was observed to be somewhat variable between the seven indivduals, while the 21-PT, 21-NT, and 21-MT2 cell lines demonstrated similar levels of basal expression. Interestingly, TCDD-induced CYP1B1 expression was the lowest in the cells originating from the oldest donor (49 years of age) and in the 21MT-2 cell line which was reflective of the most progressive stage of tumorigenesis. TCDD-mediated CYP1A1 expression was observed to be widely variable in all cell populations, while constitutive expression of CYP1A1 was undetectable. The AhR is variably expressed in the normal primary cells and preliminary analysis demonstates that the level of expression parallels basal and TCDD-induced CYP1B1 and TCDD-induced CYP1A1 expression (Fig. 4). This analysis is currently being extended to include all seven donors.

DMBA metabolic activity analyses were completed with several of the microsomal fractions characterized in the immunoblot studies. The normal primary HMEC demonstrated low constitutuve levels of DMBA metabolism which were highly inducible by TCDD (Table 1). The tumor derived cell lines exhibited a substantially higher rate of induced metabolic activity (2- to 3-fold) relative to the normal cells. Human recombinant CYP1A1 and CYP1B1 demonstrated similar distributions of DMBA metabolites, in contrast to the rodent models in which the mouse isozymes demonstrate distinctly characteristic distribution profiles. Isozyme specific antibody inhibition studies are currently being completed in the primary cells.

PAH's often undergo metabolic activation, thereby, acquiring carcinogenic and mutagenic potential. Human CYP1B1 has been shown to metabolize DMBA to the carcinogenic precurser, the 3,4-dihydrodiol of DMBA (Table 1). We are currently examining CYP1B1-mediated PAH-DNA adduct formation in the primary HMEC in collaboration with Dr. William Baird (Purdue University, West Lafayette, IN). Cultured cells have been exposed to several PAH's, including

dibenzo(al)pyrene, (+/-)trans-DB(al)P-11,12-diol, benzo(c)phenanthrene, benzo(c)phenanthrene-3,4-diol, and DMBA, under basal (2 and 24 hour incubation) as well as TCDD-induced (2 hour incubation) conditions in order to differentiate P450-mediated metabolic activation versus PAH induction on DNA adduct formation. The cellular DNA has been isolated according to Trizol methodologies and the adduct analysis completed by the Baird laboratory. Adduct formation is being analyzed parallel to immunoblotting (microsomes) and rtPCR (RNA) analyses on cellular fractions isolated from the same cells.

2B. Fibroblasts

In rodent mammary fibroblasts (see next section) we have seen selective expression of CYP1B1 relative to CYP1A1, although also regulated through the Ah receptor. We have compared expression of CYP1B1 and CYP1A1 in mammary fibroblasts isolated from one individual (A) for whom we have also characterized expression in primary mammary epithelial cells (Fig. 4). We find a pattern of expression which is very similar to expression in rat mammary fibroblasts; significant constitutive CYP1B1 and high induction by TCDD of CYP1B1 whereas levels of CYP1A1 were over a 100-fold lower. The Ah receptor which presumably mediates this response was clearly detectable and down-regulated by TCDD treatment consistent with TCDD-activation of other cultured cells. Interestingly, CYP1B1 was only marginally detectable in human skin fibroblasts. Fibroblasts cultured from a human mammary carcinoma showed much higher basal expression of CYP1B1 but smaller induction.

This data indicates that selective expression of CYP1B1 is conserved from rodents to human, that mammary fibroblasts represent a distinct population from skin fibroblasts as evidenced by CYP1B1 expressions, and that carcinoma associated fibroblasts may represent a further distinct population. In this respect, CYP1B1 functions like other markers that distinguish these fibroblasts. This work strengthens evidence that stromal fibroblasts in the mammary gland may be affected by environmental organochlorine compounds. We believe, however, that effects on stromal signalling will be far more important than effects on metabolism of carcinogens, largely because of the low porportion of these cells *in vivo*.

2C. Role of the estrogen receptor in Ah-receptor linked induction

We have examined the effect of the estrogen receptor on the induction of CYP1A1 and CYP1B1 mediated by the AhR utilizing a number of human breast epithelial cell lines. MCF7 is a classical model of estrogen receptor positive (ER+) epithelial breast cancer cells which requires estrogen for effective proliferation. The ER- MDA-MB-231wt line was isolated from an ER- metastatic tumor, thus is less differentiated and more invasive than MCF7 cells. The S30 variant of MDA-MB-231 has the human estrogen receptor vectored into it; yet it appears and behaves in culture as the parental wt line. The T47D:A18 line is, like MCF7, an ER+ cell line. The T47D:C4:2W line was developed by selection from T47D:A18 cells by continuous growth in estrogen-free media, and is ER- at the protein level, but has the ER message.

At the message level CYP1A1 was induced by exposure of the cells to 10^{-9}M TCDD in all the cell lines examined (Fig. 5). Induction was greatest in the MCF7 and two T47D lines, weaker in the MDA-MB-231 lines. Also, at the protein level, TCDD induced an induction of CYP1A1. Again, the induction in the T47D lines was greater than in the MDA-MB-231 lines. These results support the widely accepted dogma that the AhR regulates CYP1A1 induction since it is induced at the message and protein levels by TCDD, the classic AhR agonist.

CYP1B1 was also induced by TCDD at both the message and protein levels, indicating regulation through the AhR (Fig. 5). The magnitude of induction was, however, substantially less than that of CYP1A1. In opposition to CYP1A1, CYP1B1 was constitutively expressed in all the breast epithelial cell lines examined. At the level of message, MCF7 cells were the most highly induced, and S30 cells the least induced by TCDD treatment. Interestingly, at the protein level, the T47D lines contained substantially greater amounts of protein than the MDA-MB-231 lines. The

induction, however, was greater in the MDA-MB-231 lines than the T47D lines. Further, the ER+ T47D:A18 and S30 lines appeared to have lower basal amounts of protein than the ER- C4:2W and MDA-MB-231wt lines, for an equal loading of total protein. This observation introduced the possibility that the ER plays a role in the regulation or induction of CYP1B1.

The pure antiestrogen ICI 182780 eliminates the ER by blocking shuttling of the receptor into the nucleus from the cytoplasm, thus allowing cytoplasmic degradation of the receptor. To investigate a role for the ER in regulation of CYP1A1 and CYP1B1, each of the breast epithelial cell lines was pretreated for 1 day with ICI 182780, followed by exposure to ICI or ICI + TCDD (Fig. 5). Blockade of the ER by ICI 182780 appeared not to have an effect on the induction of CYP1A1 or CYP1B1 at the message level. However, it was observed at the protein level that ER status has an opposing effect on TCDD-induced expression of CYP1A1 and CYP1B1 in all lines. The loss of ER in the T47D:C4:2W cells caused small decreases in CYP1A1 expression, and increases in CYP1B1 expression. Similarly, restoration of ER in the S30 line increased CYP1A1 expression, and decreased CYP1B1 expression, each by about two-fold. The small effects of the engineered presence/absence of ER in the S30 and C4:2W lines contrasted with a 15-25-fold lower expression of TCDD-induced CYP1A1 and CYP1B1 in MDA-MB-231 cells as compared to the T47D lines. Interestingly, at the level of basal protein expression in MDA-MB-231 cells, introduction of the ER led to suppression of basal CYP1B1 expression, which was partially reversed by treatment with ICI 182780. This trend was also observed in the T47D lines, where the basal CYP1B1 expression was greater in the ER- C4:2W line, and where ICI 182780 increased the basal CYP1B1 expression in the ER+ A18 line. It is also of interest that later passages of MCF 7 cells also exhibit diminished CYP1A1 and increased CYP1B1 expression. This alteration in ratios of CYP1A1 to CYP1B1 may be important in view of the observed selectivity of metabolism of estradiol by these P450 cytochromes (i.e. 2- versus 4-hydroxylation).

These results establish the separate regulation of CYP1A1 and CYP1B1 in cultured mammary epithelial cells. A comparison of the consequences of expression of the ER in respectively, T47D lines and MDA-MB-231 lines suggest that other factors inherent in the cell lines are more important than the ER in determining AhR activity. These observations also suggest that the ER may play a role in the basal expression of CYP1B1. A more careful examination of the effects of ICI 182780 on the basal levels of CYP1B1 protein in ER+ cells will be done to support this preliminary observation.

3. Molecular mechanisms for organochlorine induction of P450 genes in the breast

3A. Regulation of P450 expression in rat mammary cells

Previous work in this laboratory (10) has established the presence of CYP1B1 and CYP1A1 in isolated rat mammary cells. We wanted to study the expression and regulation of these P450 cytochromes in vivo. In isolated mammary glands, basal levels of CYP1B1 and CYP1A1 were lower in pregnant versus virgin rats. Upon ß-naphthoflavone treatment, however, both CYP1B1 and CYP1A1 were induced to higher levels in the pregnant animals (Fig 6). This previous work also demonstrated glucocorticoid suppression of CYP1B1 in rat mammary fibroblasts (RMF). In order to investigate the role of the glucocorticoid receptor in mediating this suppression, we have employed RU486, a potent anti-progestin with anti-glucocorticoid activity. The synthetic glucocorticoid, dexamethasone, suppressed BA-induced levels of CYP1B1 in RMF and rat embryo fibroblasts (REF), with suppression being partially relieved by treatment of the cells with RU486 (Fig. 7). Interestingly, dexamethasone also suppresses induction of CYP1B1 in rat adrenocortical cells by cAMP. As PAH induction of CYP1B1 is mediated through the Ah receptor, we analyzed cytosolic and nuclear levels of the Ah receptor in response to glucocorticoid treatment of RMF (Fig. 8). The level of Ah receptor is reduced in cells pre-treated with

dexamethasone, but the receptor still translocates to the nucleus upon BA treatment. Studies utilizing a luciferase reporter gene containing ~1 kb of the mouse CYP1B1 promoter have been initiated to examine the molecular events involved in glucocorticoid suppression of the CYP1B1 gene. Preliminary results with RMF and REF transiently transfected with this construct indicate that in RMF, TCDD- and BA-induced luciferase activities were suppressed 59 and 46%, respectively, following a 6 h pre-treatment with dexamethasone (Fig. 9-A). Luciferase activity was induced 4-fold by TCDD and more than 6-fold by BA in REF, however, dexamethasone treatment resulted in only a slight suppression of activity (Fig. 9-B). In both cell types, RU486 relieved dexamethasone suppression of luciferase activity.

3B. Regulation of CYP1B1 via 5' flanking region control elements

Understanding the regulation of CYP1B1 by OC's and estrogens depends on knowledge of the upstream regulatory region of this gene. These regulatory sequences also play a key role in determining the cell specificity of expression of CYP1B1 e.g. in mammary cells rather than hepatocytes. [Due to non-renewal of CA16265 this complementary research has been temporarily partially sustained by this grant.]. We have isolated and sequenced 2.5kb of 5' flanking region of rat CYAB1 gene and have identified the transcriptional start site. The same site was seen in mammary and embryo fibroblasts and adrenal cells. A shorter mouse seqence has also been characterized and used to construct a set of CYP1B1 promoter-luciferase reporter vectors. These have been used to establish that DNA sequences exist in this 5'-flanking region which confer glucocorticoid responsiveness in rat mammary fibroblasts. We have also carried out deletion analysis to show that TCDD-induction is mediated by a 260 base segment (-1070 to -810 bp upstream). Additionally we have shown that in embryo fibroblasts basal expression is entirely dependent on the Ah receptor and on this same enhancer region. These findings indicate that regualtion of the Ah receptor level and activity are likely to be critical for basal and induced transcription of CYP1B1 in mammary cells. Notably, these findings are consistent with glucocorticoid suppression via lowering of Ah receptor levels.

4. Assessment of P450 expression in surgically removed breast samples

Human mammary tissue has been collected in collaboration with Dr. David Mahvi (University of Wisconsin Medical School). To date, 30 normal and 10 malignant tissue samples, as well as 8 adipose specimens have been obtained from patients ranging from 25 to 78 years of age. rtPCR analysis for CYP1B1, CYP1A1, AhR, and ER has been completed on 1 adipose, 7 normal, and 10 tumor tissue samples. Relatively consistent levels (variation within a factor of 10) of CYP1B1 expression were observed in all of the normal and tumor tissue samples, irrespective of ER status (Fig. 10). The CYP1B1 expression appears to parallel AhR expression in the tissues. CYP1A1 expression was largely undetectable in the normal and tumor tissues, but was expressed in the adipose sample. Further characterization of additional samples as well as validation of PCR product generation by Southern analysis is currently being completed.

In particular we testing the possibility that CYP1A1 induction in adipose samples will correlate with the capacity of adipose solvent extracts to induce CYP1A1 in cultured mouse Hepa 1 cells. We have also taken steps to develop other cell cutures that respond to organochlorine compounds. Notably, we have obtained an estrogen-responsive luciferase construct that can be transfected into various mammary cell lines with the aim of measuring estrogenicity of organochlorine compounds in different cell types. We have also developed a rat hepatocyte culture system that responds to an entirely different hormone sensitive signalling process that has yet to be characterized. This pathway is sensitve to growth hormone, androgens and estrogens but we do not know whether mammary cells contain the necessary signalling components. In the next year we plan to correlate these biological responses with analysis of the organochlorine content of the adipose extracts.

We have continued to collaborate with Judith Weiss (Hershey Medical Center) to localize CYP1B1 in breast tissue. Preliminary results induicate a broad expression in multiple cell types but immunocytochemistry must be continued by *in situ* hybridization.

Conclusions

This work now shows that CYP1B1 is the dominant constitutive activator of PAH's and E_2 in human breast cells. Primary epithelial cultures from 7 normal human surgical samples all show constitutive CYP1B1 without the related CYP1A1 form which metabolizes PAH. Addition of the environmental organochlorine compound dioxin elevates CYP1B1 (10x) while introducing CYP1A1. Constitutive expression of CYP1B1 and induction of CYP1A1 varies 5-10 fold between cultures from different individuals apparently in proportion to variable expression of the Ah receptor which mediates dioxin effects. Studies with cultured human breast tumor cell lines indicate that loss of estrogen receptors decreases the ratio of CYP1A1 to CYP1B1. Breast stromal fibroblasts express CYP1B1 stimulated by dioxin but not CYP1A1. Normal and tumor breast tissue consistently express CYP1B1 but not CYP1A1. The regulation of transcription of CYP1B1 has been studied in cultured rat mammary cells using promoter constructs. A gene region that mediates Ah receptor/dioxin regulation has been identified. However, environmental elevation of CYP1B1 is not a contributor relative to high basal expression which may however be subject to genetic variability.

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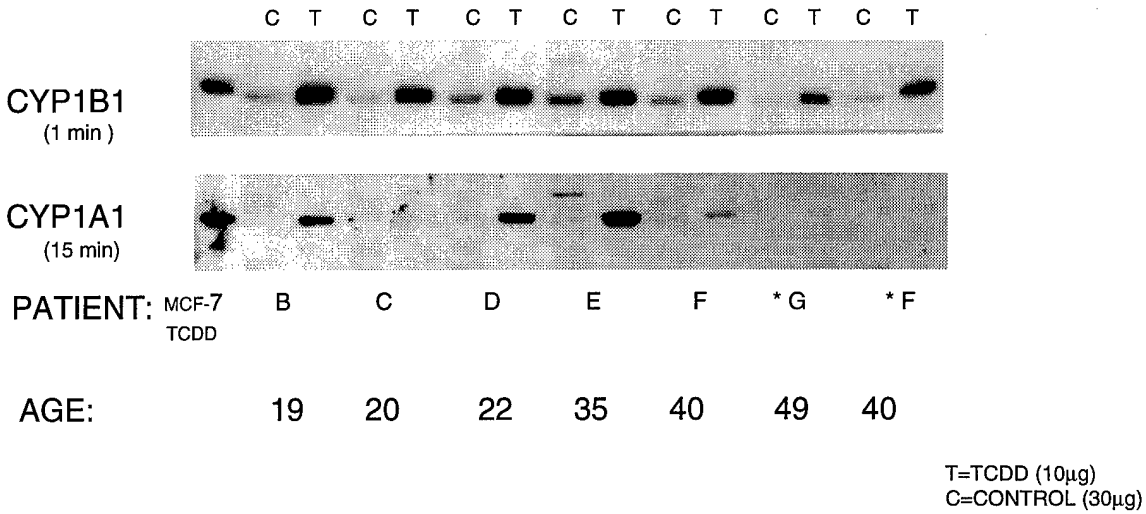

Figure 1. **Between individuals B-F, constitutive CYP1B1 is variably expressed, while the level of TCDD-induction is similar in all individuals.**

CYP1B1 is the predominant constitutively expressed PAH-metabolizing P450 in primary HMEC.

Immunoblot analysis of constitutive and TCDD-induced CYP1B1 and CYP1A1 expression in primary human mammary epithelial cells. Induced HMEC were treated with 10nM TCDD for 24h prior to harvest. Microsomal proteins were analyzed by SDS-PAGE. Immunoreactive proteins were visualized by the ECLmethod of detection (exposure times as indicated). *Indicates passage 2 cells, while all others represent passage 1 cells.

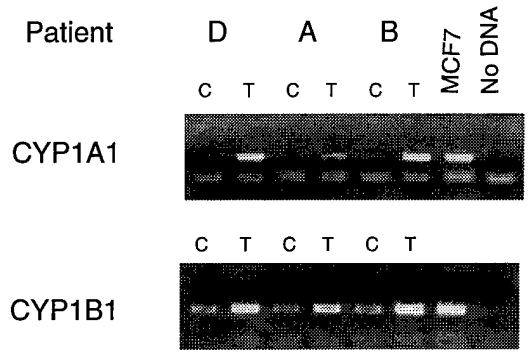

Figure 2. Expression of CYP1A1 and CYP1B1 in primary normal breast epithelial cells.
CYP1A1 and CYP1B1 RNA expression parallels protein expression in primary HMEC.
rtPCR was used to amplify message from normal breast epithelial cells. Primary normal breast epithelial cells were obtained in collaboration with Dr. Michael Gould (UW). TCDD (10 nM) exposure resulted in induction of CYP1A1 and CYP1B1in these cells. The human breast epithelial cell line MCF7, TCDD-treated, was used as a positive control. CYP1B1 is constitutively expressed in normal breast epithelial cells, and is inducible by TCDD, however, CYP1A1 is expressed only following induction with TCDD. C = Vehicle control T = TCDD exposed.

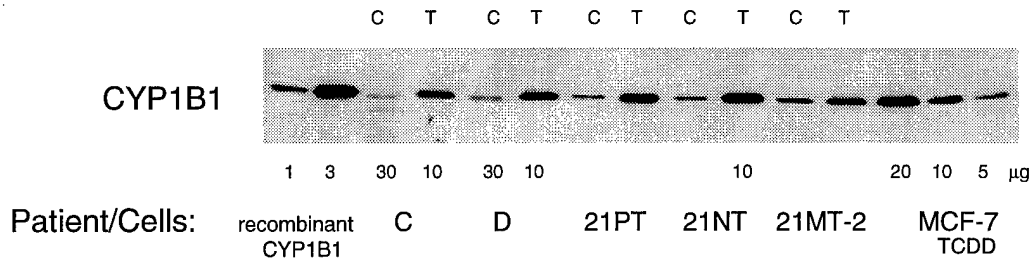

Figure 3. **Constitutive CYP1B1 expression is substantially higher in three immortalized HME cell lines as compared to primary epithelia.**

Immunoblot analysis of constitutive and TCDD-induced CYP1B1 expression in normal primary epithelia and tumor-derived epithelial cell lines of increasing tumorigenicity. TCDD-induced samples were treated with TCDD for 24h prior to harvest. Microsomal proteins (loadings as indicated) were analyzed bySDS-PAGE. Human recombinantCYP1B1 microsomes (86 pmol/mg microsomal protein) were purchased from Gentest Corp.(Woburn, MA).

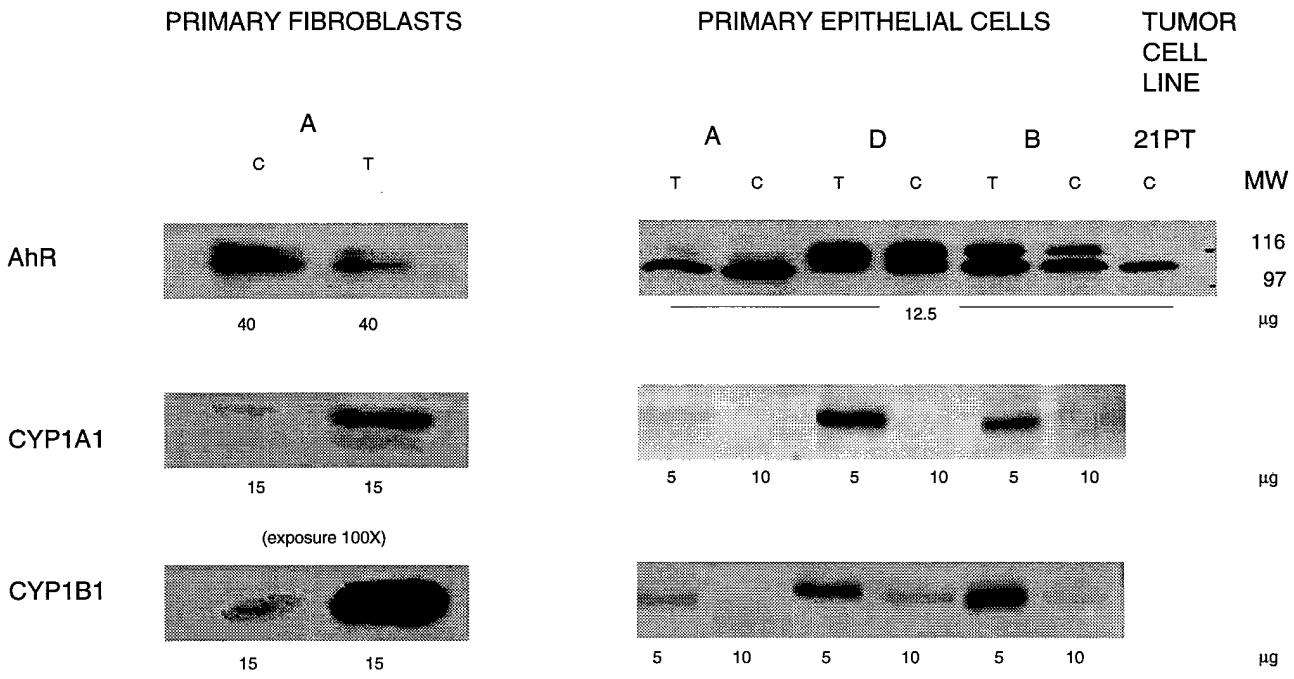

Figure 4. **Constitutive CYP1A1 expression is only barely detectable in primary HMF and HMEC.**

Basal CYP1B1 and TCDD-induced CYP1A1 expression is proportional to the level of AhR in the cell.

Immunoblot analysis of constitutive and TCDD-induced AhR, CYP1B1, and CYP1A1 expression in human primary mammary fibroblast and epithelial cells. Microsomal proteins (loadings as indicated) were analyzed by SDS-PAGE.

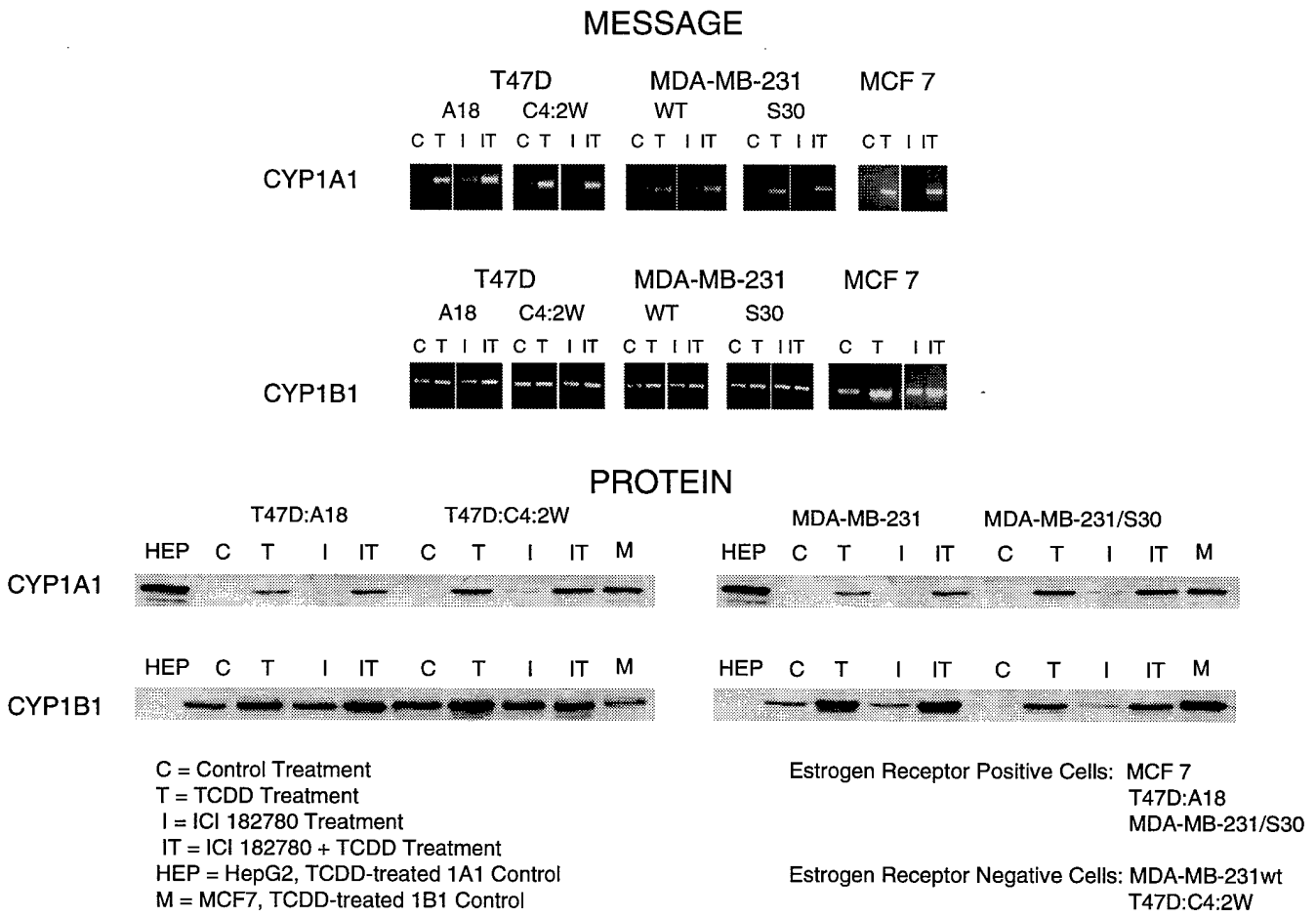

Figure 5. Expression on CYP1A1 and CYP1B1 at the message and protein levels in human breast epithelial cells treated with or without ICI 182780 and with or without TCDD.

Top: Cells were treated for 24 hours with or without 100 nM ICI 182780, followed by exposure for 24 hours to either vehicle or 10 nM TCDD. rtPCR was used to amplify CYP1A1 or CYP1B1 message from the various breast epithelial cell lines. Bottom: Microsomes were isolated from human breast epithelial cells treated as previously described above. 20 ug of microsomal protein was loaded into each well, except for HepG2 (2 ug) and MCF7 (10 ug).

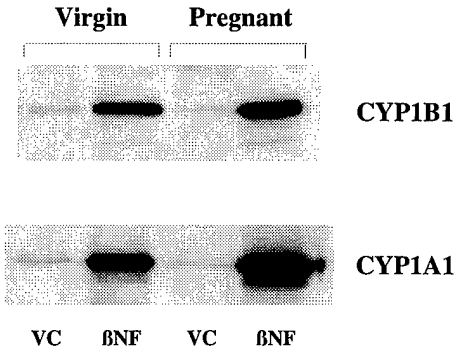

Figure 6. Immunoblot analysis of CYP1B1 and CYP1A1 expression in the rat mammary gland. Microsomal protein was isolated from ß-naphthoflavone (ßNF, 60 mg/kg, 3 days)- and vehicle (VC)-treated virgin or pregnant Sprague-Dawley rats, separated by SDS-PAGE, and transferred to nitrocelluose membranes. The membranes were incubated with polyclonal antibodies raised against recombinant mouse CYP1B1 or rat CYP1A1 and visualized by the enhanced chemiluminescence method.

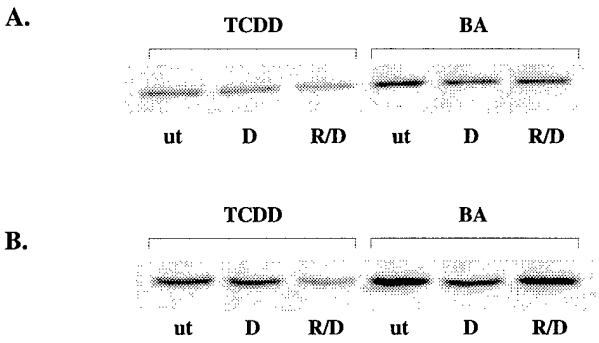

Figure 7. Immunoblot analysis of CYP1B1 expression in (A) rat mammary fibroblasts and (B) rat embryo fibroblasts in response to glucocorticoid treatment. Cells were pre-treated with 0.2% DMSO (ut), dexamethasone (1 µM, D), or in combination with RU486 (1 µM, R+D) for 24 h, then treated with TCDD (10 nM) or BA (10 µM) for 24 h. Microsomal protein was isolated, separated by SDS-PAGE, and transferred to nitrocelluose membranes. The membranes were incubated with polyclonal antibodies raised against recombinant mouse CYP1B1 and visualized by the enhanced chemiluminescence method.

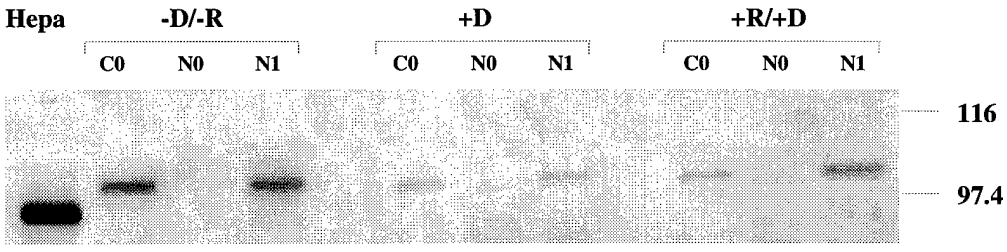

Figure 8. Immunoblot analysis of Ah receptor expression in rat mammary fibroblasts in response to glucocorticoid treatment. Cells were pre-treated with 0.2% DMSO (-D/-R), dexamethasone (1 µM, +D), or in combination with RU486 (1 µM, +R/+D) for 24 h, and lysate containing cytosol and membranes (C) or nuclei (N) were collected at 0 and 1 h following BA (10 µM) treatment. Cytosolic and nuclear protein were isolated, separated by SDS-PAGE, and transferred to nitrocelluose membranes. Cytosolic protein from the mouse hepatoma cell line, Hepa1c1c7, was included as a postive control. The membranes were incubated with polyclonal antibodies raised against mouse Ah receptor and visualized by the enhanced chemiluminescence method.

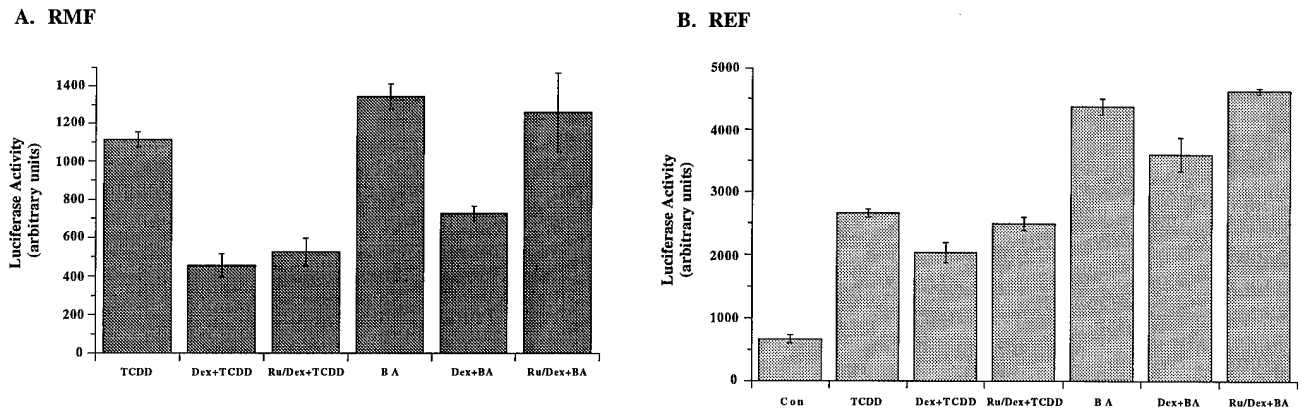

Figure 9. Glucocorticoid treatment suppresses activity of a CYP1B1 promoter-luciferase reporter gene construct in (A) rat mammary fibroblasts and (B) rat embryo fibroblasts. A construct containing ~1 kb of 5'-flanking sequence from the mouse CYP1B1 gene was transiently transfected into cells using the calcium phosphate/DNA precipitation method. The transfected cells were pre-treated with 0.2% DMSO, dexamethasone (1 μM, Dex), or in combination with RU486 (1μM, RU) for 6 h, then treated with either TCDD (10 nM) or BA (10 μM) for 18 h. Each treatment was performed in triplicate (n = 3). Cell lysates were collected and luciferase activities were assayed using a kit from Promega.

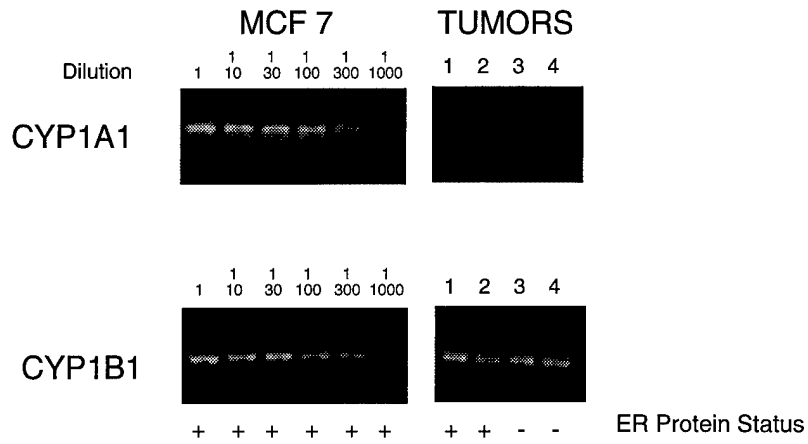

Figure 10. Expression of CYP1A1 and CYP1B1 in primary tumor tissue. CYP1B1 is consistently expressed in all primary mammary tumors, while CYP1A1 is undetectable.

rtPCR was used to amplify message from primary tumor cells, and levels of expression were compared to message from TCDD-induced MCF7 cells. Tumors were obtained in collaboration with Dr. David Mahvi, M.D. (UW Hospital). Tumor 1: 44 year old, ER protein positive infiltrating tubular carcinoma; Tumor 2: 41 year old, ER protein positive infiltrating ductal carcinoma; Tumor 3: 45 year old, ER protein negative, infiltrating ductal carcinoma; Tumor 4: 31 year old, ER protein negative, poorly differentiated infiltrating ductal carcinoma. CYP1B1 is constitutively expressed in these primary tumors, whereas CYP1A1 is not.

TABLE 1

REGIOSELECTIVITY OF DMBA METABOLISM OF HUMAN AND RODENT CYP1B1 AND CYP1A1

Enzyme Source	DMBA Metabolites (pmol/mg microsomal protein/h)						
	Dihydrodiols				Phenols[f]		Total
	5,6-	8,9- / 10,11-	3,4-	7-OHMMBA	A	B	Actitity
Primary Epithelia[a]							
Control	1.0 *(14)*[e]	3.5 *(48)*	0.3 *(4)*	0	2.5 *(34)*	0	7.3
TCDD	80 *(24)*	137 *(43)*	16 *(5)*	0	89 *(28)*	0	322
Hu. rCYP1B1[b]	61 *(20)*	93 *(31)*	14 *(5)*	0	123 *(41)*	9 *(3)*	299
Hu. rCYP1A1	124 *(15)*	365 *(45)*	48 *(6)*	17 *(2)*	245 *(30)*	6 *(<1)*	805
Mo. Cyp1b-1[d] (10T1/2)	14 *(<1)*	710 *(34)*	298 *(14)*	0	924 *(45)*	113 *(5)*	2059
Mo. Cyp1a-1[d] (Hepa-1)	92 *(6)*	696 *(49)*	28 *(2)*	316 *(22)*	273 *(19)*	21 *(2)*	428

[a] Patient D. Similar proportions of individual metabolites were identified in microsomal fractions isolated from 2 additional individuals.

[b] Human recombinant CYP1B1 microsomes (Gentest Corp., Woburn, MA; 86 pmol/mg microsomal protein) supplemented with 100nM EH.

[c] Human recombinant CYP1A1, as previously described in this laboratory.

[d] Mouse Cyp1b-1 and Cyp1a-1, as previously described in this laboratory.

[e] Per cent distribution of metabolites.

[f] DMBA phenols A include 2- and 3-OH DMBA; phenol B includes 4-OH DMBA.

Abstract P-272

ENDOCRINE REGULATION OF CYTOCHROME P4501B1 IN THE RAT MAMMARY

P.B. Brake, L. Zhang, and C.R. Jefcoate, Environmental Toxicology Center and Department of Pharmacology, University of Wisconsin (Madison, WI USA)

Cytochrome P4501B1 (CYP1B1) is expressed and regulated in a cell-specific manner by endogenous steroid and peptide hormones and ubiquitous environmental contaminants. It is regulated in rat adrenocortical (RAC) cells primarily by cAMP (6-fold) and shows smaller increases with TCDD (2-fold), while in rat embryo fibroblasts (REF) CYP1B1 is regulated by TCDD, but insensitive to cAMP. Primer extension analysis indicates that *CYP1B1* is transcriptionally initiated from the same site following TCDD treatment in rat mammary fibroblasts (RMF) and by cAMP in RAC cells. We have demonstrated the presence of CYP1B1 in the rat and human mammary gland as the major constitutive polycyclic aromatic hydrocarbon (PAH)-metabolizing species. In cultures of isolated rat mammary cells, CYP1B1 and CYP1A1 exhibit cell-type specific expression. A constitutively expressed CYP1B1 is stimulated by TCDD, through the Ah receptor, in isolated RMF, while CYP1A1 is induced by TCDD and BA in isolated rat mammary epithelial cells. Constitutive levels of CYP1B1 and CYP1A1 are lower in the mammary glands of pregnant compared to virgin rats. However, upon ß-naphthoflavone treatment, both CYP1B1 and CYP1A1 were induced to higher levels in pregnant animals. The synthetic glucocorticoid, dexamethasone, suppressed PAH-induced levels, in a dose-dependent mannaer, of CYP1B1 in RMF and REF, with suppression of BA-induced CYP1B1 being partially relieved by treatment of the cells with the anti-progestin, RU486, both at the protein level and mRNA level. Interestingly, dexamethasone also suppresses induction of CYP1B1 in RAC by cAMP. Following pre-treatment of both RMF and REF with dexamethasone, levels of cytosolic Ah receptor were lowered, although preliminary cell proliferation assays indicate that dexamethasone does not inhibit cell growth significantly during this pre-treatment period. Both RMF and REF were transiently transfected with a luciferase vector containing a TCDD-responsive, mouse CYP1B1 promoter construct. Luciferase activity was induced 4-fold by TCDD and more than 6-fold by BA in REF. In RMF, TCDD- and BA-induced luciferase activities were suppressed 59 and 46%, resepectively, following a 6 h pre-treatment with dexamethasone, while in REF, this same treatment resulted in only a slight suppression of activity.
(Supported by NIH Grant P30 CA14520 and DAMD Grant 17-94-J-4054)

Presented at the 11th International Symposium on Microsomes and Drug Oxidations, UCLA, Los Angeles, CA, USA, July 21-24, 1996.

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Presenting Author Michele Larsen Title Research Associate

Organization/Institution University of Wisconsin

Address 1300 University Ave

City/State Madison, WI Zip Code 53706 Country USA

Business Telephone _____ FAX _____ E-Mail mllarsen@facstaff.wisc.edu

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CHARACTERIZATION OF CYP1B1 AND CYP1A1 EXPRESSION IN NORMAL PRIMARY AND TUMOR-DERIVED HMEC

M. Larsen, K. Sukow, S. Eltom, W. Angus, and C. Jefcoate, *Environmental Toxicology Center and the Department of Pharmacology, University of Wisconsin* (Madison, WI, USA).

CYP1B1 and CYP1A1, the major PAH metabolizing P450 cytochromes, have been shown to metabolically activate 7,12-dimethylbenz(a)anthracene (DMBA) in a cell-type selective manner in rat mammary fibroblasts and rat mammary epithelial cells, respectively [Christou *et al.* (1995) *Molec. Cell. Endocrinol.* **115**: 41-50.]. Conversely, constitutive CYP1B1 as well as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-inducible CYP1B1 and CYP1A1 expression has been identified in the transformed human mammary MCF-7 carcinoma cell line [Christou *et. al.* (1994) *Carcinogenesis* **15**: 725-732.]. We have characterized CYP1B1 and CYP1A1 expression in early passage normal primary human mammary epithelial cells (HMEC) from six individuals and in the 21T cell line series (21PT, 21NT, and 21MT-2), isolated from an ER-/PR- tumor, representing HMEC of different stages of tumor development [Band *et al.* (1990) *Cancer Res.* **50**: 7351-7357.]. The primary cells and the 21T cell lines demonstrated low constitutive levels of DMBA metabolism which were highly inducible by TCDD. The tumor-derived cell lines exhibited a substantially higher rate of induced metabolic activity (2- to 3-fold) relative to the normal cells. rtPCR analysis of RNA and immunoblot analysis of microsomal proteins each demonstrated lower constitutive and increased TCDD-inducible expression of CYP1B1 in the normal primary HMEC as compared to the tumor cell lines. Similar levels of TCDD-inducible CYP1A1 expression were observed in the normal and tumor-derived cell populations, while constitutive CYP1A1 was undetectable in all of the cells. The 21PT, 21NT, and 21MT-2 cell lines exhibited variable levels of ER expression which correlated with TCDD-responsive CYP1B1 and CYP1A1 expression, whereby increased P450 expression was observed in cells exhibiting elevated levels of ER. The normal primary HMEC failed to express detectable ER by PCR methodologies despite showing extensive TCDD induction. Comparisons of cellular AhR expression are being completed by immunoblot and PCR analyses in all cell populations. This data suggests a complex mechanism of regulation of CYP1B1 and CYP1A1 expression which differs between primary and immortalized HMEC.

(Supported by DAMD17-94-J-4054)