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Estrogen Receptor α Increases Basal and Cigarette Smoke Extract-Induced Expression of *CYP1A1* and *CYP1B1*, but not *GSTP1*, in Normal Human Bronchial Epithelial Cells

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Abstract

Gender-specific estrogen receptor α (ER α) expression may plausibly influence lung carcinogenesis in females. Initial genome-wide microarray studies confirmed that carcinogen metabolism genes (*CYP1A1*, *CYP1B1*) were those most responsive to cigarette smoke extract (CSE) in normal bronchial epithelial (NHBE) cells. These two genes encoding phase I bioactivating enzymes and the *GSTP1* gene encoding a phase II deactivating enzyme were then tested for induction by ER α . NHBE cells (native ER α ⁺) were transfected with wild-type ER α -adenoviral constructs, and then exposed to CSE, 17 β -estradiol (E₂), and/or the ER α inhibitor, ICI 182,780. The expression levels of *CYP1A1*, *CYP1B1* and *GSTP1* were then determined by RNA-specific quantitative RT-PCR and immunoassay. ER α increased the basal expression of *CYP1B1* 4.04-fold (p<0.01) at the mRNA level and 6.5-fold at the protein level. ER α also increased the CSE-induced mRNA expression of *CYP1B1* 2.26-fold (p<0.01), but not the protein expression. ER α did not alter the *CYP1A1* mRNA levels, but did increase protein expression 2.0-fold (p<0.01) on CSE exposure, and 6.2-fold (p<0.01) upon E₂ exposure. These effects could be inhibited by ICI 182,780. ER α did not alter the expression of *GSTP1*. ChIP assay confirmed ER α binding to *CYP1B1* promoter near the transcription start site. These results suggest that ER α regulates the *CYP1B1* expression at a transcriptional level, and *CYP1A1* expression at a translational level. These data raise the possibility that inter-gender differences in expression of ER α that are known to exist in human lung may contribute to inter-individual expression differences in *CYP1A1* and *CYP1B1*, and to differences in carcinogen metabolism and mutation.

Keywords

estrogen receptor; *CYP1A1*; *CYP1B1*; *GSTP1*; cigarette smoke extract

Abbreviations

CYP1A1/1B1, cytochrome P450 1A1/1B1; GSTP1, glutathione S-transferase pi; CSE, cigarette smoke extract; NHBE, normal bronchial epithelial cell; ChIP, Chromatin Immunoprecipitation;

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ER α , estrogen receptor α ; AD-CON: adenovirus control vector; AD-ER: estrogen receptor α recombinant adenovirus expression vector; DMSO: dimethyl sulfoxide; XRE, xenobiotic response element; ERE, estrogen response element; AhR, aromatic hydrocarbon receptor; ARNT, aromatic receptor nuclear translocator; PAH, polycyclic aromatic hydrocarbon; E2, 17 β -estradiol

INTRODUCTION

There is a significant literature suggesting a possible enhancement of female susceptibility to lung cancer (1–8), although recent studies with contrary epidemiological findings have been reported (9,10). Higher levels of polycyclic aromatic hydrocarbon (PAH) metabolite-DNA adducts occur in woman when smokers are divided into quartile groups according to adducts per pack year (11). Although dosimetric factors more precise than simple pack-years have not been thoroughly studied as they relate to female lung cancer risk, there remains support in the epidemiologic literature for the position that woman are at higher risk for lung cancer, and convincing evidence that they have different prognoses once diagnosed (12–14), at any given level of smoking.

Estrogen receptors α and β (ER α , ER β) are ligand-binding transcription factors, and members of the nuclear receptor family (15,16). ERs bind estrogen response elements (EREs) encoded in genomic DNA, as well as other DNA binding proteins, and they classically activate transcription in an estrogen-dependent manner. The patterns of expression of two ER forms in human lung tissue has been described (17): ER α is expressed more often in the lungs of women than men, while ER β is expressed with approximately equal frequencies in the lungs of men and women. Recent studies suggest that ERs can directly interact with the aromatic hydrocarbon receptor (AhR) and aromatic receptor nuclear translocator (ARNT) (18,19). The AhR/ARNT complex is also a ligand-dependent transcription factor; PAH-activated AhR heterodimerizes with ARNT and activates the transcription of carcinogen- and estrogen-metabolism target genes, such as *CYP1A1* and *CYP1B1*, through xenobiotic response elements (XREs) (20,21). This literature implies that ERs plausibly affect the regulation of carcinogen metabolism gene expression.

Because tobacco-induced expression of phase I bioactivating and phase II deactivating carcinogen metabolism enzymes in human lung has been suggested in observational studies to demonstrate wide inter-individual and inter-gender variation (22,23), as well as putative corollary changes in mutagenesis, we have explored the possibility that qualitative differences in expression of ER α underlie some of this inter-individual variability in quantitative carcinogen-metabolism gene expression. In the current study, we experimentally replicate the gene-environment interaction present in normal human bronchial epithelial (NHBE) cells exposed to tobacco smoke during early initiation of lung carcinogenesis. We confirm, by genome-wide expression microarray scan, that the most CSE-responsive genes are members of the *CYP1* family, and we demonstrate the impact of experimental ER α co-expression on the CSE-induced expression of *CYP1A1*, *CYP1B1*, and *GSTP1* native transcripts in NHBE cells. We further explore potential mechanisms by which ER α enhances *CYP1B1* expression, using reporter constructs and chromatin immunoprecipitation assay (ChIP).

MATERIALS AND METHODS

Microarray scan for CSE responsive genes

Total RNA was prepared from NHBE cells (RNeasy®, Qiagen, Valencia, CA) that had been exposed to 1.0 μ g/ml CSE and DMSO as control for 18 h. After gel and capillary electrophoresis (Agilent Bioanalyzer® 2100, Palo Alto, CA) confirmation of total RNA integrity from two separate experimental NHBE exposures, biotinylated cRNA was generated using a

MessageAmp^{TM} aRNA Kit (Ambion, Austin, TX) with minor modifications to the manufacturer's instructions. This cRNA was hybridized to Human U133 Plus 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA) in replicate samples, in the Wadsworth Center Microarray Core Facility. Pooled data were checked against results of individual runs for reproducibility, and were analyzed using GeneTraffic® (Iobion, La Jolla, CA) software.

Constructs

Recombinant adenovirus constructs: The human ER α construct with a mutation at Gly400Val (24) was reverted to wild type by site-directed mutagenesis, whereby oligonucleotide-directed PCR mutagenesis (QuikChange® multi site-directed mutagenesis kit, Stratagene, La Jolla, CA) generated a wild-type ER construct, using the synthetic oligonucleotide: 5'-CCATGGAGCACCCAGGGAAGCTACTGTTTGC-3' (the changed nucleotide is underlined). The high-fidelity PCR was performed by PfuTurbo DNA polymerase (Stratagene) to introduce *KpnI* and *SalI* sites into the 5' and 3' ends of the ER sequence, using forward primer: 5'-TACTATGGTACCCGGCCACGGACCATG-3' and reverse primer: 5'-TACTATGTCGACGCCAGGAGCTCTCAG-3' (the underlined portions are introduced restriction sites). PCR product was purified from an agarose gel, digested with *KpnI* and *SalI*, and inserted into the *KpnI/SalI* site of pAdTrack-CMV vector (Stratagene) to generate an AdTrack-ER expression construct. The desired base changes, inducing reversion of the coding sequence to that of wild-type ER α , were verified by direct cycle sequencing of the entire ER insert. Recombinant adenoviruses were produced by AdEasy XL Adenoviral vector system (Stratagene). Each construct contained a green fluorescent protein (GFP) coding sequence downstream from the CMV promoter, for tracking of transfection efficiency. Briefly, AdTrack-ER was linearized with *PmeI* and was transformed into BJ5183-AD-1 cells. The recombinant adenovirus ER construct (R-ER) was identified by *PacI* digestion. Similarly, a recombinant adenovirus control construct (R-CON) was created by transforming linearized AdTrack-CMV vector into BJ5183-AD-1 cells. Recombinant adenoviruses, AD-ER and AD-CON, were produced by transfection of *PacI* digested R-ER or R-CON into AD-293 cells. The wild type ER sequence insert was further confirmed by PCR and sequencing. *CYP1B1* promoter luciferase reporter construct was described previously (25). Estrogen response element (ERE) Luciferase reporter construct and Xenobiotic response element (XRE) Luciferase reporter construct were generous gifts of Drs. E. Bessette and L. Kaminsky (26,27).

Cell culture

NHBE cells were primary cultures obtained from a commercial source (BioWhittaker, Inc., Walkersville, MD). ER α -negative background was confirmed by RT-PCR and Western blot (data not shown). NHBE cells were maintained in BEGM (Bronchial Epithelial Cell Growth Medium) medium (BioWhittaker, Inc.) containing BEBM (Bronchial Epithelial Cell Basal Medium) supplemented with 52 μ g/ml bovine pituitary extract, 0.5 μ g/ml hydrocortisone, 0.5 ng/ml human epidermal growth factor, 0.5 ng/ml epinephrine, 10 μ g/ml transferrin, 5 μ g/ml insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodothyronine, 50 μ g/ml gentamicin, and 50 ng/ml amphotericin-B, at 37 °C in a humidified 5% CO₂ atmosphere. All experiments were performed on cells from a single female donor, lot, aliquot and passage.

Adenoviral transfection

Adenoviral transfections were performed in triplicate in 24-well or 6-well plates. Each well was plated with 1×10^5 cells (for 24-well plate) or 5×10^5 cells (for 6-well plate) on passage 4, for 24 h before transfection. The virus of AD-ER or AD-CON, 0.02 ml/cm² of 1:320 dilution normalized to obtain approximately 90% transfection efficiency (as tracked by GFP fluorescent microscopy) was incubated with NHBE cells for 2 h, and the media was then replaced with growth medium (BEGM). These ER- transfectants were ready for use after 24-h incubation.

Reporter construct transfection

For ERE-Luc, XRE-Luc and p1B1-Luc luciferase reporter construct studies, transfection was performed on NHBE transfected with AD-ER or AD-CON for 24 h in 24-well plates using 0.2 µg of each plasmid, together with 0.2 µg of reference PRL-TK vector (Promega, Madison, WI) expressing renilla luciferase constitutively; the latter serves as an internal control of reporter construct transfection efficiency. DNA was combined with 1 µl of LipofectAmine 2000 in 50 µl of Opti-MEM reduced serum medium (Invitrogen, Carlsbad, CA).

In vitro exposure

For quantitative mRNA transcript studies of NHBE, carried out 24 h after the initial AD-ER transfection, the medium of NHBE was replaced with growth medium (BEGM) containing CSE (1.0 µg/ml media), 1.0 ng/ml media 17β-estradiol, and/or 0.1 µM of the ERα inhibitor ICI 182,780, dissolved in 100% dimethyl sulfoxide (DMSO). Cells were harvested after an additional 3, 12, or 18 h of exposure, for total RNA extraction. Control cells were exposed to 0.1% (v/v) DMSO vehicle only. For protein studies, cells were harvested after an additional 24 h of exposure, which was chosen on the basis of optimum GFP expression. CSE was purchased from Murty Pharmaceutical Inc. (Lexington, KY). It was prepared using a Phipps-Bird 20-channel smoking machine designed for Federal Trade Commission testing. Kentucky standard cigarettes 1R3F (University of Kentucky, KY) were smoked, and the particulate matter was trapped onto filters. The amount of CSE obtained was determined by weight increase of the filter. CSE was dissolved from the filter in dimethyl sulfoxide by sonication to yield 4% solution and stored at -20 °C. The average yield of CSE was 21 mg per cigarette including 1.3 mg nicotine.

For reporter construct studies, after initial transfection incubation for 5 h, the medium was replaced with growth medium (BEGM) containing CSE (Murty Pharmaceuticals, Inc.), 17β-estradiol, and/or the ERα inhibitor ICI 182,780, dissolved in DMSO as above, and cells were harvested after an additional 24 h (the previously determined maximal-effect time point). In each condition, the final concentration of DMSO was adjusted to 0.1% (v/v). The cells were lysed by Passive Lysis Buffer (Promega). The activities of firefly and renilla luciferases were determined by Dual-Luciferase reporter assay system (Promega). Firefly luciferase activity was then numerically normalized to renilla luciferase activity.

RNA-specific Universal Reverse Transcription and PCR

Total RNA was prepared by RNeasy Mini Kit (Qiagen). RT was performed by universal RT primer as previously described (28,29), avoiding genomic DNA-encoded false positives in the RT-PCR that are yielded by pseudogene-encoded sequences (*e.g.*, *GSTP1* and *36B4*).

RT was performed with 2.0 µg of total RNA, using Superscript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD) as follows. RNA template was added to 1 µl of RT primer (100 µM universal RT primer (URT); Table 1), 1 µl of dNTP mix (each base, 10 mM) and DNase/RNase-free water, to a volume of 13 µl. The solution was incubated at 65 °C for 5 min and then cooled to 4 °C. A master mix containing 4 µl of 5x first-strand buffer (Gibco/BRL Life Technologies, Gaithersburg, MD) and 2 µl of 0.1 mM DTT per RT sample was prepared, and was added to each sample. The sample was then incubated at 42 °C for 2 min. SuperScript II Reverse Transcriptase (Gibco/BRL Life Technologies) was added (1 µl), and the samples were incubated at 42 °C for 50 min, followed by a further incubation at 70 °C for 15 min.

Quantitative PCR was then performed in the LightCycler® (Roche, Indianapolis, IN) thermocycler using *CYP1A1*, *CYP1B1*, *GSTP1*, *PgR*, *HEM45*, *36B4* and 28S RNA-specific primers (Table 1). RT-PCR target transcript results were normalized to expression levels of

the internal reference housekeeping gene *36B4* which is relatively unaffected by hormonal factors (30), or to 28S ribosomal RNA.

Western immunoblotting

Microsomes for the analysis of CYP1A1 and CYP1B1 proteins were prepared as previously described (31). For analysis of ER α and *GSTP1*, cultured cells were lysed in SDS sample buffer described previously (32). These microsomal and cell-lysate samples were subjected to electrophoresis in 10% acrylamide NuPAGE Novex Bis-Tris denaturing gels (Invitrogen) as recommended by the manufacturer. Proteins were blotted onto 0.2 μ m Nitrocellulose membranes (Invitrogen), and blots were blocked in Blotto B (1% bovine serum albumin, 1% non-fat dry milk, 0.05% Tween20 in PBS) for 1 h at room temperature. Blots were then probed with goat polyclonal anti-rat CYP1A1/2 antibody (Daiichi, Tokyo, Japan) diluted 1:1000 in Blotto B; ER α -specific antibody HC-20 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:100; rabbit polyclonal anti-human CYP1B1 antibody (33) diluted 1:400; or rabbit polyclonal anti-human GSTP1 antibody (Oxford Biomedical Research, Inc., MI) diluted 1:500 in blocking buffer. Blots were then probed with HRP-conjugated anti-rabbit IgG or anti-goat IgG (Sigma), at 1:10,000 dilution. Immunoreactive proteins were detected by using the ECL western blotting reagent (Amersham Biosciences, Piscataway, NJ). Proteins were detected by enhanced chemiluminescence on Kodak X-OMAT film (Sigma-Aldrich) and quantified by densitometry with a Molecular Dynamics scanning laser densitometer.

Chromatin Immunoprecipitation (ChIP) Assay

NHBE cells were transduced with AD-ER or AD-CON as described above, then exposure to 1nM E2 and 1 μ g/ml CSE for 18 hours and ChIP assay was carried out essentially using the method of Braunstein and coworkers (34) briefly, treated NHBE Cells were cross-linked by addition of 1% formaldehyde to the medium for 10 min. Crude nuclei prepared by hypotonic lysis were resuspended in 100 μ l SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), sonicated under conditions that reduced DNA length to between 200 and 1000 base pairs, and debris removed by centrifugation. The chromatin solution was diluted 10-fold in IP buffer (34) and precleared for 45' at 22–25 °C on protein A beads (Pierce) preadsorbed with sonicated single-stranded DNA. The chromatin solution was then incubated with anti-ER α antibody (1 μ l; Santa Cruz) for 45 min at 22–25 °C, and immune complexes collected with protein A beads preadsorbed with sonicated single-stranded DNA. Following washes and elution (34), cross-links were reversed by heating at 65 °C for 4–5 hr; DNA was recovered by phenol extraction and ethanol precipitation. Specific sequences in the immunoprecipitates were detected by PCR. The forward and reverse primers for region encompassing the putative ERE sequence (35) were 5'- TGGAGGTGGCTGTGATGA-3' and 5'- CACAACTGGAGTTCGCAGAA -3', respectively. PCR analyses were performed using the Advantage-GC Genomic PCR kit (Clontech, Palo Alto, CA) as follows: after an initial denaturation at 95 °C for 1 min, denaturation at 95 °C for 10 s, touchdown annealing at 62 °C, 30s for 5 cycles, 58 °C, 30s for 35 cycles, and extension at 72 °C for 30s, for a total of 40 cycles. A final extension step at 72 °C for 7 min terminated the reaction. The PCR product was electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Statistical analyses

For the microarray studies, normalization and statistical analysis were performed with GeneTraffic UNO v2.8 software (Iobion). The set of four arrays was normalized using the Robust Multichip Analysis algorithm (RMA) (36), and probe sets were excluded from further analysis if the normalized intensity value did not reach 200 in at least two (50%) of the hybridizations. Unpaired t-tests were performed on the RMA-transformed data, utilizing the variance stabilization and Benjamini-Hochberg multiple testing (37) correction options. For

quantitative RT-PCR studies, triplicate samples under various conditions were compared by t-test, using MS-Excel software. Figure error bars depict standard deviations, and differences were considered significant if $p < 0.05$.

RESULTS

Genome-wide scan for CSE induction

Initial genome-wide expression studies employing the Affymetrix human U133 plus 2.0 GeneChip suggested that five genes were significantly induced > 2 -fold (Table 2) on NHBE exposure to cigarette smoke extract for 18 h. *CYP1A1* and *CYP1B1* were the most highly CSE-induced genes among 36,000 genes included in this GeneChip. All known cytochrome P450, glutathione S-transferase, sulfotransferase and UDP glycosyltransferase family enzymes are included on this latest version GeneChip. The results of quantitative real-time RT-PCR (data not shown) confirmed this CSE induction; vector-only transfected NHBE cells(ER-) were increased 4.45 fold for *CYP1A1* and 7.94 fold for *CYP1B1* by 18 h of CSE exposure at 1.0 $\mu\text{g/ml}$. *GSTP1* expression was unchanged by CSE exposure.

Expression and function of ER α

NHBE cells were transfected with AD-ER or AD-CON. The expression of ER α was demonstrated by Western immunoblotting (Figure 1). We chose a 1:320 dilution in this study, because at that titer, the transfection efficiency was about 90% by green fluorescent protein (*GFP*) integrated in the adenoviral expression constructs, in preliminary titration studies. The expressed ER α drove a positive control ERE reporter construct expression in an estradiol dose-dependent manner (Figure 2). As additional positive controls, the expressed ER α enhanced the expression of the known, ER-regulated *PgR* 7.47-fold and that of *HEM45* 9.04-fold (Figure 3). These results collectively demonstrate that transfected ER α was expressed and was functional.

Effect of AD-ER transfectionion on *CYP1A1*, *CYP1B1*, *GSTP1* mRNA Expression

NHBE cells were transfected with AD-CON or AD-ER for 24 h, and were then exposed to combinations of DMSO, CSE, 17 β -estradiol (E2) and ICI 172,780. mRNA levels of *CYP1A1*, *CYP1B1*, *GSTP1* and *36B4* after 0, 3, 12 and 18-hour exposure. Maximal expression was observed at 3-hour exposure. CSE could induce mRNA expression of *CYP1A1* and *CYP1B1* 4.3 to 6.8-fold in both AD-ER and AD-CON transfected cells, but not that of *GSTP1*. This result is consistent with the gene expression microarray (Figure 4). Compared to ER- cells, ER α significantly increased *CYP1B1* basal expression 4.04-fold ($p < 0.01$) with medium alone, and CSE-induced expression 2.26-fold ($p < 0.01$) (Figure 4a). Estradiol at 1nM affected neither the basal nor the CSE-induced *CYP1B1* expression, in either ER- or ER+ cells (Figure 4a). The enhancer effect of ER α was inhibited by 0.1 μM ICI 182,780 (Figure 4a). ER α did not alter the mRNA expression of *CYP1A1* and *GSTP1* (Figure 4b, 4c). Similar results were observed at 12 and 18-hour exposure (data not shown). We also checked the expression of *CYP1B1* at lower transfectionion titer (1:640), where transfection efficiency was 80% (versus 90% with 1:320) and results were predictably consistent with those reported at 1:320 titer, but of lower magnitude, due to lower transfection efficiency (Figure 4d).

Effect of AD-ER transfectionion on *CYP1A1*, *CYP1B1*, *GSTP1* protein Expression

CYP1A1, *CYP1B1*, *GSTP1* protein was detected by Western immunoblot in the treated NHBE cells as described above. Results showed that CSE induced *CYP1A1* and *CYP1B1* protein expression in both AD-ER and AD-CON transfected cells, but not *GSTP1* expression (Figure 5). Compared to ER- cells, ER α increased the basal expression of *CYP1B1* by 6.5-fold ($p < 0.01$; Figure 5a), but not the CSE-induced *CYP1B1* protein level. ER α also increased *CYP1A1*

protein expression by 2.0-fold ($p < 0.01$) upon CSE exposure, and by 6.2-fold upon E2 exposure ($p < 0.01$; Figure 5b). GSTP1 protein expression was not affected by either CSE or by ER α status (Figure 5c).

Binding of ER α to CYP1B1 promoter

A putative ERE sequence in the human *CYP1B1* gene between -63 and -49 (Figure 6A) has recently been reported (35). The sequence (AGGTCGCGCTGCCCT) is different from the consensus ERE (AGGTCAANNNTGACCT, from the *Xenopus* vitellogenin A2 gene) (38) in two bases. To confirm that ER α is associated with the putative ERE on the human *CYP1B1* gene in NHBE cells, chromatin immunoprecipitation (ChIP) assays were performed. DNA was extracted from NHBE cells transfected with AD-ER or AD-CON and exposure to 1nM E2 and 1 μ g/ml CSE for 18 h after cross-linking treatment and incubated with specific anti-ER α antibodies. PCR was performed with a primer set for the region (-235 to +46), which includes the putative ERE. In AD-ER transduced NHBE cells, immunoprecipitants obtained with anti-ER α antibodies generated a distinct PCR product with the primer set, whereas in AD-CON transduced NHBE cells, no distinct band was observed (Figure 6B). These results indicated that ER α binds to a region containing the putative ERE, and suggest that ER α directly regulates the *CYP1B1* promoter activity.

DISCUSSION

We have shown by genome-wide expression microarray scan, that *CYP1B1* and *CYP1A1* are the two genes that are most highly responsive to tobacco smoke in normal primary isolates of bronchial epithelial cells. We then proceeded to confirm ER α regulation of these two phase I genes, but not the phase II *GSTP1* genes, in several relevant exposure contexts, using RNA-specific quantitative RT-PCR, and Western immunoblots. Finally, we detected a putative corresponding estrogen response element (ERE) in the *CYP1B1* promoter.

For the medium-only control, ER α increased basal expression of *CYP1B1* for both mRNA and protein by 4.04- and 6.50-fold, respectively. The ER α -induced increment for DMSO vehicle controls was less, at 2.26-fold. Both conditions suggest that ER α effects on this pathway can be qualitatively independent of ligand. For mRNA, the reason for the difference between the ER α -induced increments in medium only *versus* DMSO vehicle controls is a matter of speculation, with no direct studies available. However, DMSO at 4% is known to release ribosomes from mRNA transcripts (39), albeit the relevance to the lower DMSO concentrations employed in this study (0.1%) is unclear. Whether a similar release phenomenon occurs with transcription factor binding as that for observed for ribosomes is unknown.

In CSE-induced expression of *CYP1B1*, ER α increased mRNA expression only, but not protein. It could be speculated that this indicates saturation of protein translation, as CSE induced *CYP1B1* expression by 31-fold on protein level. The saturated expression was also observed in expression of *CYP1B1* promoter luciferase reporter constructs when CSE concentration reached 10 μ g/ml (data not shown). Further studies are warranted to clarify the kinetics of transcriptional and translational regulation of this gene.

ChIP assays demonstrated that ER α could bind to the putative ERE sequence. This confirms a mechanism by which ER α likely affects *CYP1B1* expression at the transcriptional level. Additionally, it has been reported that some estrogen-regulated genes are indirectly regulated by the cooperation of Sp1 and ER within a GC-box and ERE half site (40,41). In the human *CYP1B1* gene promoter, two Sp1 binding sequences have been reported to be located at -84 to -89 and -68 to -73, (42) that are near the putative ERE (-49 to -63). It will be important, in future studies, to clarify whether Sp1 and ER cooperatively regulate the transcription of the *CYP1B1* gene.

ER α did not alter the *CYP1A1* mRNA levels, but did increase protein expression 6.2-fold ($p < 0.01$) upon E₂ exposure and 2.0-fold ($p < 0.01$) on CSE exposure. This result suggests that ER α regulates *CYP1A1* expression at the translational level. While the mechanism is still unclear, it is known that the effects of ER α status on the expression of *CYP1A1* and *CYP1B1* differ in a tissue-specific manner (43–46). ER α as a transcriptional factor is well known, but as a translational factor is not defined. Therefore, it will be of some significance to explore how ER α regulates *CYP1A1* expression at this translational level.

There is a large literature confirming the critical role of the two phase I enzymes encoded by these genes both for PAH and 17 β -estradiol bioactivation. It has been shown that *CYP1B1* is the more abundant species in human lung (47); the phase II enzyme expressed in highest abundance in human lung is *GSTP1* (22,48). This study confirms that ER α has a potential role in significantly enhancing the expression of key bioactivating genes (i.e., *CYP1A1*, *CYP1B1*) in the carcinogen metabolism pathway. The implication is that phase I (*CYP1A1*, *CYP1B1*) metabolism enhancement, in the absence of similar, coordinate enhancement of the phase II deactivation gene (*GSTP1*), may serve as a mechanism for enhanced female gender-related susceptibility to tobacco- or estrogen-mediated mutations.

Classically, ER α binds to estrogen response elements (EREs) and activates transcription of estrogen target genes in an estrogen-dependent manner (49–53). Given recent confirmation of AhR ligand mediation of ER-mediated estrogen signaling by direct ER-AhR(18) or ER-Arnt(19) interaction, results from the current study reinforce the data from our prior studies that those individuals who express ER in lung (17) demonstrate enhanced expression of *CYP1B1* (22).

To demonstrate if ER-AhR cross-talk was also involved in the ER α enhanced *CYP1B1* expression, XRE luciferase reporter assay and *CYP1B1* promoter luciferase assay were performed (data not shown). These luciferase reporter assay results did not demonstrate the ER α enhancement effect seen in the native *CYP1B1* expression, indicating that ER-AhR cross-talk are not apparently involved in this ER α mediated enhancement of *CYP1B1* expression.

A putative ERE motif (–63 and –49) in the *CYP1B1* promoter has been recently confirmed (35), and can up-regulate the *CYP1B1* reporter construct expression mediated by ER α . Our chromosome immunoprecipitation (ChIP) assay did confirm the ER α binding to this putative ERE sequence in *CYP1B1* promoter. However, ER α did not increase the *CYP1B1* promoter (containing 1.5kb of native sequence, and the putative ERE) activity, as measured in a luciferase reporter construct expression (data not shown). This indicates that the elements in other regions of *CYP1B1* promoter may be cooperatively involved in ER α regulation of *CYP1B1*. There are three reported components in the promoter (42): essential elements located between nt-164 and nt+25; enhancer elements are located between nt 1500 and nt 1356 and between nt 1022 and 835; negative regulatory elements are located between nt 1356 and 1022 and between nt 835 and 164. Increasing the size of the promoter sequence analyzed may aide the identification of the essential promoter structure for ER α regulation, even though the ER α binding site demonstrated by ChIP was within the 1.5kb fragment studied. Alternatively, epigenetic or other regulatory features that are not well-represented in reporter constructs may be at play.

A strength of the current study is the use of non-malignant, non-transformed primary lung epithelial cells that are likely to reflect, with reasonable accuracy, the cellular environment for the earliest steps in carcinogen bioactivation in the native epithelium of the human target organ being studied, as compared to already-transformed or malignant cells. Corollary attempts were made to employ relevant concentrations of estradiol (0.1 nM, equivalent to post-menopausal, and 1.0 nM, equivalent to premenopausal plasma levels) and of CSE (0.1 and 1.0 μ g/ml; only

estimates of bronchial and alveolar lining fluid concentrations currently exist) that are reasonably representative of human *in vivo* exposure conditions. ER α was over-expressed in NHBE cells (Figure 1), compared with the native level in MCF7 cells, when 90–95% of NHBE cells were transduced. This overexpression was due to strong CMV promoter directly upstream of the ER α coding sequence, and not due to over-transfection (data not shown).

In summary, we have demonstrated ER α enhancement of expression of two phase I carcinogen and estradiol bioactivating enzymes, without corollary enhancement of the lung's main phase II deactivating enzyme. This could be hypothesized to confer female gender related susceptibility to carcinogen- or estrogen-mediated malignancies. Mechanisms at play for this ER α mediated up-regulation may include direct promoter region binding. These data additionally suggest that sequence or structure that is further 5' upstream, epigenetic structure, higher order protein-protein interactions, or translational regulation may separately, or in aggregate, play important roles in ER α up-regulated *CYP1B1* and *CYP1A1* expression, and therefore in individual carcinogen bioactivating pathway phenotypes.

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Fig. 1. Detection of ER α in AD-ER and AD-CON transfected NHBE cells by Western immunoblotting. Two titers of the recombinant adenovirus (1:160 and 1:320) were used to transfect NHBE cell. Whole cell lysate was prepared 24 hours after transfection. 20 μ g protein was loaded on each lane. An extract of MCF-7 cell was used as positive control. The antibody HC-20 was used to locate ER α . The ~80-kd band is a nonspecific band detected by the HC-20 polyclonal antibody.

Fig. 2.

The effect of expressed ER α on ERE-luciferase reporter construct (ERE-Luc) expression. NHBE cells were transfected with AD-CON or AD-ER adenovirus for 24 hours, then transfected ERE-luciferase construct and exposure to 0.1% DMSO, 17 β -estradiol (E2) and ICI 182,780. Luciferase activity was determined after additional 24-hour incubation. The reporter luciferase (firefly) activity was normalized to activity of renilla luciferase co-transfected with reporter constructs. ER-: NHBE cells transfected with AD-CON adenovirus; ER+: NHBE cells transfected with AD-ER adenovirus; RLU: Relative luciferase unit; Data are shown as mean value $\pm$ SD (n=3). All comparisons are of ER+ versus ER- in the same exposure: *: p<0.05; **: p<0.01.

Fig. 3.

Effect of expressed ER α on the expression of *PgR* and *HEM45*. NHBE cells were transfected with AD-CON or AD-ER adenovirus and then exposed to 0.1% DMSO, 1nM 17 β -estradiol (E2) for 3 hours. The mRNA levels of *PgR* (a), *HEM45* (b) were determined by Quantitative RT-PCR and normalized to 28S housekeeper gene expression. Data are shown as mean value $\pm$ SD (n=3). All comparisons are of ER+ versus ER- in the same exposure: *: p<0.05; **: p<0.01.

Fig.4.

Effect of expressed ER α on the expression of *CYP1A1*, *CYP1B1* and *GSTP1*. NHBE cells were transfected with AD-CON or AD-ER adenovirus for 24 hours and then exposed to 0.1% (v/v) DMSO as vehicle control; 1nM 17 β -estradiol (E2); 1.0 μ g/ml CSE; 1.0 μ g/ml CSE+1nM 17 β -estradiol and 1.0 μ g/ml CSE+1nM 17 β -estradiol +0.1 μ M ICI182,780 for additional 3 hours. The mRNA levels of *CYP1B1* (4a), *CYP1A1* (4b), and *GSTP1* (4c) were determined by Quantitative RT-PCR and normalized to 36B4 housekeeper gene expression. In figure 4d, CYP1B1 expression was observed when NHBE cell was transfected with lower dose (1:640) of AD-CON or AD-ER adenovirus. Data are shown as mean value $\pm$ SD (n=3). All comparisons are of ER+ versus ER- in the same exposure: *: p<0.05; **: p<0.01.

Figure 5.

Effect of expressed ER α on the protein expression of *CYP1A1*, *CYP1B1* and *GSTP1* in NHBE cells. NHBE cells were transfected with AD-ER and AD-CON for 24 hours, then exposure to

0.1% DMSO, 1nM 17 β -estradiol (E2); 1.0 μ g/ml CSE; 1.0 μ g/ml CSE+1nM 17 β -estradiol and 1.0 μ g/ml CSE+1nM 17 β -estradiol +0.1 μ M ICI182,780 for additional 24 hours. Microsome (for CYP1A1, CYP1B1) and cell lysate were prepared. (a) immunoblotting for CYP1B1. 10 μ g microsomal protein was loaded for each lane. (b) immunoblotting for CYP1A1. 10 μ g microsomal protein was loaded for each lane. (c) immunoblotting for GSTP1. 10 μ g cell lysate protein was loaded for each lane. Data are shown as mean value $\pm$ SD (n=3). All comparisons are of ER+ *versus* ER- in the same exposure: *: p<0.05; **: p<0.01.

Figure 6.

Chromatin immunoprecipitation (ChIP) assay of promoter region of *CYP1B1* gene. (A): the amplified promoter region of *CYP1B1*. The underlined is the putative estrogen response element (ERE) binding site. (B): NHBE cells were transfected with AD-ER or AD-CON as described above, then exposure to 1nM E2 and 1ug/ml CSE for 18 hours. DNA-protein was cross-linked by treatment with formaldehyde, and immunoprecipitated with anti-ER α antibody. After the reversal of cross-linking, DNA was purified and amplified by PCR using *CYP1B1* promoter specific primers. The PCR product was electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. M: 100-bp DNA ladder marker. Anti-ER α : immunoprecipitated with anti-ER α antibody. Input: not immunoprecipitated with anti-ER α antibody.

Table 1

Primers for quantitative RT-PCR.

	Primer	Sequence	Annealing Temperature
RT	URT	5'-AAC GAG ACG ACG ACA GAC TTT TTT TTT TTT TTT TTT TTT A/C/G A/C/G A/C/G/T-3'	42 °C
<i>CYP1A1</i>	UPF2	5'-GAG ACA ACA GGT AAA ACA GGG-3'	59 °C
	UR	5'-AAC GAG ACG ACG ACA GAC-3'	
<i>CYP1B1</i>	DSF	5'-GCC ACT ATC ACT GAC ATC T-3'	59 °C
	DSR	5'-CTT GCC TCT TGC TTC TTA TT-3'	
<i>GSTP1</i>	UF2	5'-TCT CCT TCG CTG ACT ACA AC-3'	59 °C
	UR	5'-AAC GAG ACG ACG ACA GAC-3'	
<i>36B4</i>	UF1	5'-GAC AAT GGC AGC ATC TAC AA-3'	59 °C
	UR	5'-AAC GAG ACG ACG ACA GAC-3'	
<i>PgR</i>	Forward	5'-TGC GAG GTC ACC AGC TCT TG-3'	55 °C
	Reverse	5'-TTT GCC CTT CAG AAG CGG AC-3'	
<i>HEM45</i>	Forward	5'-GAG CGC CTC CTA CAC AAG AG-3'	55 °C
	Reverse	5'-AAG CCG AAA GCC TCT AGT CC-3'	
<i>28S</i>	Forward	5'-TTG AAA ATC CGG GGG AGA C-3'	55 °C
	Reverse	5'-ACA TTG TTC CAA CAT GCC AG-3'	

:RT: reverse transcription; : acidic ribosomal phosphoprotein P0; : progesterone receptor gene; : interferon stimulated gene; : 28S ribosomal RNA gene.

Table 2

Genes significantly induced over two fold by CSE-induction using expression microarray.

UniGene Symbol	Fold change	Std Dev	p-value	Probe set
<i>CYP1A1</i>	7.94	0.13	<0.01	205749_at
<i>CYP1B1</i>	7.36	0.4	<0.01	202437_s_at
<i>NICE-1</i> [†]	2.93	0.3	<0.05	220620_at
<i>ECG2</i> [‡]	2.62	0.17	<0.01	223720_at
<i>LOC120224</i> [§]	2.45	0	<0.01	230323_s_at

[†]: chromosome 1 open reading frame 42[‡]: esophagus cancer-related protein 2[§]: Hypothetical protein BC016153

This table represents probe sets that have passed the following criteria:

1. Statistically significantly changed (CSE versus DMSO vehicle control) at p-value of 0.05 when an unpaired t-test was employed, using the Benjamini-Hochberg multiple testing correction.
2. Probe sets had to have normalized intensity values of at least 200 on at least two arrays.