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Induction of carbonyl reductase 1 (*CBR1*) expression in human lung tissues and lung cancer cells by the cigarette smoke constituent benzo[a]pyrene

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Abstract

Carbonyl reductase 1 (*CBR1*) reduces various xenobiotic carbonyl substrates to corresponding alcohol metabolites. Here we demonstrated that benzo[a]pyrene (B[a]P), a potent pro-carcinogen and predominant polycyclic aromatic hydrocarbon (PAH) compound in cigarette smoke and air pollutants, upregulates *CBR1* gene expression *in vitro* and *in vivo*, and that a proximal xenobiotic response elements (*XRE*) motif ($-_{122}XRE$) mediates the induction effect of B[a]P. First, we observed 46% and 50% increases in *CBR1* mRNA and *CBR1* protein levels, respectively, in human lung tissue samples from smokers compared to never-smokers. Second, we detected 3.0-fold ($p < 0.0001$) induction of *CBR1* mRNA and 1.5-fold ($p < 0.01$) induction of *CBR1* protein levels in cells of the human lung cancer cell line A549 incubated with 2.5 μ M B[a]P for 24 hours. Third, results from experiments with *CBR1* promoter constructs indicated that a proximal *XRE* motif ($-_{122}XRE$) mediates induction of reporter activity in response to B[a]P. Furthermore, we detected enhanced nuclear translocation of aryl hydrocarbon receptor (AhR) following B[a]P exposure in A549 cells. Finally, we demonstrated increased binding of specific protein complexes to $-_{122}XRE$ in nuclear extracts from B[a]P-treated cells and the presence of the AhR/Arnt complex in the specific nuclear protein $-_{122}XRE$ complexes.

Keywords

Human Carbonyl Reductase 1 (*CBR1*); benzo[a]pyrene; cigarette smoke metabolism

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Conflict of interest

The authors declare that there are no conflicts of interest.

1. Introduction

Among the three carbonyl reductases (CBRs) found in humans (CBR1, CBR3, and CBR4), of particular interest is CBR1, a monomeric cytosolic enzyme consisting of 277 amino acids with a molecular mass of 30,375 Da (Malatkova et al., 2010). CBR1 is an ubiquitously distributed enzyme and is found in high levels in the epidermis, liver, and epithelial cells of the gastrointestinal tract (Wermuth, 1981; Wirth and Wermuth, 1992). The frequent exposure of these organs to exogenous compounds is consistent with the role of CBR1 in xenobiotic detoxification (Wermuth et al., 1986).

Examples of CBR1 substrates include the antipsychotic drug haloperidol as well as the anticancer anthracyclines doxorubicin and daunorubicin (Gonzalez-Covarrubias et al., 2009). The latter are reduced into their primary alcohol metabolites, doxorubicinol and daunorubicinol, respectively, which are cardiotoxic, have less tumor cell killing activity, and contribute to the capricious pharmacology that is characteristic of anthracycline drugs (Minotti et al., 2004). Quinones such as benzo- and naphthoquinones, as well as K-region-containing *ortho*-quinones derived from polycyclic aromatic hydrocarbons (PAH) such as benzo[*a*]pyrene (B[*a*]P), are also good CBR1 substrates (Wermuth et al., 1986). B[*a*]P is one of the best characterized and most insidious of the 81 compounds in cigarette smoke that have been classified as carcinogens by the International Agency for Research on Cancer (IARC) (Hecht, 1999). B[*a*]P is highly carcinogenic due to its potential to initiate the formation of DNA adducts (Hockley et al., 2007). The complex toxicodynamics of B[*a*]P are mediated through the aryl hydrocarbon receptor (AhR) pathway. Upon ligand binding, activated AhR translocates into the nucleus to form heterodimeric complexes with the aryl hydrocarbon receptor nuclear translocator protein (ARNT). AhR-ARNT complexes interact with specific DNA sequences known as xenobiotic response elements (*XREs*) to activate the transcription of a battery of target genes (Denison and Nagy, 2003).

We recently showed that human *CBR1* is regulated by the AhR/*XRE* pathway (Lakhman et al., 2007). In tandem with evidence of increased CBR1 activity in lung tumors compared to paired normal lung tissue (Schlager and Powis, 1990), we performed our current studies based on the hypothesis that B[*a*]P present in cigarette smoke might modulate *CBR1* expression via the AhR pathway (Lakhman et al., 2007). First, we compared the expression of *CBR1* mRNA and CBR1 protein in the lungs of smoker and never-smoker donors. Next, we assessed the ability of B[*a*]P to induce *CBR1* mRNA expression in cultures of the human lung adenocarcinoma-derived cell line A549. Then, we determined the impact of B[*a*]P on the gene reporter activities of engineered *CBR1* promoter constructs harboring and lacking a functional *XRE*. Furthermore, we checked whether B[*a*]P enhances nuclear translocation of aryl hydrocarbon receptor (AhR) in A549 cells. Finally, we sought to assess AhR-*XRE* binding interactions in nuclear extracts from A549 cells treated with B[*a*]P. Our findings provide insight into the mechanisms underlying the regulation of *CBR1* by the AhR pathway and its potential implications in the detoxification of cigarette smoke.

2. Materials and methods

2.1. Human Lung Samples

The Institutional Review Board of the State University of New York at Buffalo approved this research. Lung samples (0.5 – 5 g) from smoker ($n = 16$) and never-smoker ($n = 12$) donors were procured from the Cooperative Human Tissue Network (CHTN). Smoking status (yes/no) was obtained from detailed medical records. For 56% of smoking donors, we had additional information about their smoking habits with a mean consumption of 1.2 packs per day for an average of 34.4 years. Tissue procurement protocols included the following criteria: no current diagnosis of congestive obstructive pulmonary disease/

emphysema and/or lung cancer, no evidence of sepsis, and no history of chemotherapy and/or radiation within the last year. Lung tissue samples were harvested either from surgery within 10 hours or autopsy within 15 hours post-mortem. Lung samples were frozen immediately after recovery and stored in liquid nitrogen until further processing. Tissue samples were processed following standardized procedures to isolate RNA and DNA as described (Gonzalez-Covarrubias et al., 2009). Cytosols were obtained by differential ultracentrifugation as described (Mordente et al., 2003).

2.2. Real-time quantitative RT-PCR of *CBR1* mRNA in human lung tissue

Total RNA (100 ng) from human lung samples was reverse-transcribed and amplified by using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA) with the following primers: 5'-TCAAGCTGAAGTGACGATGA-3' (forward) and 5'-GGTGCACCTCCCTTCTTTGTA-3' (reverse). *CBR1* mRNA levels were determined by the comparative quantitation method as previously described (Gonzalez-Covarrubias et al., 2009). Individual β -actin mRNA levels were used for normalization. Experimental samples and standards for calibration curves were analyzed in quadruplicate. The relative amount of *CBR1* mRNA in each sample was automatically calculated with a comparative quantitation algorithm (iQ5 Optical System Software version 2.0, Bio-Rad, Hercules, CA). *CBR1* mRNA levels present in human lung tissue were expressed relative to the normalized *CBR1* mRNA content of a sample from a never-smoker.

2.3. Quantitative immunoblotting of *CBR1* in human lung tissue

CBR1 protein levels were quantitated as described previously (Gonzalez-Covarrubias et al., 2009). In brief, lung cytosols (35 μ g) and recombinant *CBR1* standards (0.03, 0.05, 0.10, 0.15, and 0.30 μ g; Abcam Inc., Cambridge, MA) were loaded into 12% precast polyacrylamide gels (Invitrogen, Carlsbad, CA) and separated by electrophoresis. Protein blots were probed with a specific polyclonal anti-human *CBR1* antibody (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-human β -actin antibody (1:3000; Santa Cruz Biotechnology, Inc.) and a secondary goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:10,000; Sigma-Aldrich, St. Louis, MO). Immunoreactive bands were visualized with the ECL plus western blotting detection system (GE Healthcare, Chalfont St. Giles, UK). *CBR1* band intensity values (pixels/mm²) were quantified with a ChemiDoc XRS gel documentation system (Bio-Rad Laboratories). Cytosolic *CBR1* levels were calculated by interpolation from the calibration curves of recombinant *CBR1*. Detection of *CBR1* was linear (range 0.03 – 0.30 μ g; $r^2 > 0.93$).

2.4. Cell culture and reagents

A549 cells (human lung adenocarcinoma-derived cell line, CCL-185) were obtained from the American Type Culture Collection (Manassas, VA). Common cell culture reagents were purchased from Invitrogen (Carlsbad, CA). A549 cells were routinely cultured in 10 cm² tissue culture dishes using F-12K medium (ATCC) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cultures were grown and maintained at low passage numbers ($n < 12$) using standard incubator conditions of 37°C, 5% CO₂, and 95% relative humidity.

2.5. Real-time quantitative RT-PCR of *CBR1* mRNA in A549 cells

A549 cells (70 – 80% confluence) were incubated with 1 μ M and 2.5 μ M B[a]P (Indofine Chemical Company, Inc., Hillsborough, NJ) for 24 hours. Transcription inhibitory studies were conducted in cell cultures incubated with B[a]P in the presence of 10 μ M actinomycin D (Enzo Life Sciences, Farmingdale, NY). RNA was extracted from cell lysates using TRIzol (Invitrogen) according to the manufacturer's instructions. *CBR1* mRNA levels were

analyzed as described above (Section 2.2). *CBR1* mRNA values were expressed relative to the normalized *CBR1* mRNA content of vehicle DMSO-treated cells

2.6. Quantitative immunoblotting of CBR1 in A549 cells

To assess the influence of B[a]P exposure on CBR1 protein expression, A549 cells (80% confluence) were treated for 24 hours with DMSO or 2.5 μ M B[a]P in the presence or absence of 10 μ M cyclohexamide (Enzo Life Sciences), a translation inhibitor. Total protein was extracted from cell lysates using an All-in-One Purification Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's instructions. CBR1 protein levels in whole cell lysates were quantified using the protocol described in Section 2.3. CBR1 levels in each sample were calculated by interpolation from the calibration curves of recombinant CBR1. Detection of CBR1 was linear (range 0.03 – 0.30 μ g; $r^2 > 0.96$).

2.7. Transient transfections and luciferase activity assays

A549 cells were plated in 48-well plates 24 to 48 hours prior to transfection. Reporter gene constructs comprising *CBR1* promoter sequences of 413-bp (with or without the deletion of $_{-122}XRE$) upstream of the *CBR1* translation initiation codon (Lakhman et al., 2007) and empty vector, pGL3-Basic (Promega, Madison, WI), were co-transfected with the human thymidine kinase promoter-driven pRL-TK plasmid (*Renilla* luciferase, Promega) into 70% confluent cell cultures using Lipo D293 (SignaGen Laboratories, Gaithersburg, MD). 24 hours post-transfection, cells were treated with 2.5 μ M B[a]P. After 24 hours, cultures were washed once with phosphate-buffered saline solution and cells were lysed with freshly diluted passive lysis buffer (Promega, 100 μ l/well) by incubating the plates at room temperature on a shaker at 200 rpm for 60 minutes. Luciferase reporter gene activities were determined with the Dual-Luciferase Reporter Assay System (Promega) per the manufacturer's instructions. Light intensity was measured in a Synergy HT luminometer equipped with proprietary software for data analysis (BioTek, Winooski, VT). Light intensity values from cell cultures transfected with the pGL3-basic vector were used to correct for background. Corrected firefly luciferase activities were normalized to *Renilla* luciferase activities and expressed as fold increases with respect to the values obtained from pGL3-basic empty vector. Three independent experiments were performed in triplicate to evaluate reproducibility.

2.8 Quantitative immunoblotting of AhR in cytosolic and nuclear extracts of A549 cells

Cytosolic proteins and nuclear extracts from A549 cells exposed to DMSO or 2.5 μ M B[a]P for 6 hours were prepared with the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative immunoblotting of AhR protein in the two distinct cellular fractions were conducted with slight modifications to the protocol described in Section 2.3 In brief, 20 μ g of cytosolic protein and 9 μ g of nuclear extract were loaded into 12% precast polyacrylamide gels (Thermo Fisher Scientific) and separated by electrophoresis. Protein blots were sequentially probed with a specific polyclonal anti-human AhR antibody (1:1000; Santa Cruz Biotechnology, Inc.) and a secondary goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:10,000; Sigma-Aldrich) prior to chemiluminescent imaging. Protein blots were stripped with BlotFresh Western Blot Stripping Reagent (Ver. II) (SignaGen Laboratories, Rockville, MD) according to the manufacturer's instructions and then cut in half. One half of each blot containing cytosolic protein was re-probed with anti-human β -actin antibody and secondary antibody as described above, while the other half harboring nuclear extracts was re-probed with a specific polyclonal anti-human histone H1 antibody (1:400; Santa Cruz Biotechnology, Inc.) and a secondary goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:10,000; Sigma-Aldrich).

2.9. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts from A549 cells were prepared with the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific). The $_{-122}XRE$ oligonucleotides: 5'-CTCAGCGGCCGGCGTGTAACCCACGGG-3' ($_{-122}XRE-CBR1$ forward) and 5'-CCCGTGGGTTACACGCCCGGCCGCTGAG-3' ($_{-122}XRE-CBR1$ reverse) were synthesized and purified by Invitrogen. Additionally, modified $_{-122}XRE-CBR1$ oligonucleotides, $_{-122}XRE-CBR1$ m-f ($_{-122}\Delta XRE$ forward): 5'-CTCAGCGGCCGGGTAACCCACGGG-3' and $_{-122}XRE-CBR1$ m-r ($_{-122}\Delta XRE$ reverse): 5'-CCCGTGGGTTACCGGCCGCTGAG-3' were constructed.

A specific antibody for human AhR, e.g. H-211x (sc-5579x, rabbit IgG), and normal rabbit IgG suitable for supershift EMSA analysis (sc-2027x) were purchased from Santa Cruz Biotechnology. EMSA were performed with the LightShift Chemiluminescent Kit (Thermo Fisher Scientific) by following the manufacturer's instructions. In brief, $_{-122}XRE-CBR1$ forward and $_{-122}XRE-CBR1$ reverse oligonucleotides were labeled with Biotin 3' End DNA Labeling kits (Thermo Fisher Scientific). Equimolar amounts of these biotin-labeled oligos were annealed to generate double-stranded probe. Similarly, equimolar amount of unlabeled $_{-122}XRE-CBR1$ forward and $_{-122}XRE-CBR1$ reverse were annealed to generate double-stranded "wild type" competitor, and equimolar amount of unlabeled $_{-122}XRE-CBR1$ m-f and $_{-122}XRE-CBR1$ m-r were annealed to generate double-stranded "mutant" competitor. Double-stranded probes (100 pmole) were incubated with nuclear extracts (6 μ g) for 45 minutes at 4°C. Competition studies were performed by adding a 50-fold molar excess of double-stranded "wild type" competitor or double-stranded "mutant" competitor to the reaction mixtures.

EMSA with anti-AhR antibody (H-211x) or with isotype control antibody (normal rabbit IgG, sc-2027x) were performed by first incubating double-stranded $_{-122}XRE-CBR1$ probe with nuclear extracts for 15 minutes followed by a 30 minute incubation with 3 μ g of antibody at 4°C. In all cases, incubation mixtures (20 μ l per binding reaction) were analyzed by electrophoresis on 5% non-denaturing polyacrylamide gels with 0.5X Tris/borate/EDTA buffer at 4°C. DNA-protein complexes were visualized with a Molecular Imager® Gel Doc™ XR+ System (Bio-Rad).

2.10. Data analysis

Data for all experiments except for the EMSA studies were evaluated using unpaired Student's *t*-tests with differences considered to be significant at $p < 0.05$. For EMSA analyses, where appropriate, we utilized *t*-tests with a Bonferroni correction to account for multiple comparisons where differences were considered significant at $p < 0.005$ ($\approx 0.05/10$). Statistics were computed with Excel 2007 (Microsoft Office; Microsoft, Redmond, WA), GraphPad Prism (GraphPad Software Inc., version 4.03, San Diego, CA), and SigmaPlot version 9.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. *CBR1* mRNA and *CBR1* protein expression is elevated in the lungs of cigarette smoking donors

For the current study, we obtained lung tissue samples from individuals with a history of smoking ($n = 16$) and from donors who had never smoked ($n = 12$). Due to the relatively high amounts of B[a]P contained in cigarette smoke (20 – 40 ng/cigarette) (Hecht, 1999), we anticipated that chronic smoking might have a cumulative effect on expression of the *CBR1* gene via AhR-mediated induction. Real-time quantitative RT-PCR (real-time qRT-PCR) analyses revealed that *CBR1* mRNA levels varied widely in lung samples from smokers

(30.0-fold; *CBR1* mRNA range, 0.1 – 3.0 relative fold). *CBR1* mRNA levels varied 2.7-fold in lung samples from never-smokers (*CBR1* mRNA range, 0.6 – 1.6 relative fold). Statistical comparisons demonstrated that *CBR1* mRNA expression was higher in lung samples from smokers than in samples from never-smokers (*CBR1* mRNA_{smokers} = 1.5 ± 0.7 relative fold versus *CBR1* mRNA_{never-smokers} = 1.0 ± 0.3 relative fold; $p < 0.05$; Figure 1A). In line, cigarette smoking status also significantly impacted CBR1 protein levels (CBR1 protein_{smokers} = 2.87 ± 1.12 ng/ μ g versus CBR1 protein_{never-smokers} = 1.95 ± 1.04 ng/ μ g; $p < 0.05$; Figure 1B).

3.2. B[a]P increases *CBR1* mRNA levels in A549 cells

Earlier work by our group and others demonstrated that *CBR1* gene expression is induced by β -naphthoflavone, a prototypical AhR ligand (Forrest et al., 1990; Lakhman et al., 2007). Coupled with our observations of elevated *CBR1* mRNA in the lungs of smokers, we evaluated the effect of B[a]P, another strong AhR ligand, on *CBR1* transcript levels in A549 cells. Real-time qRT-PCR analyses in A549 cells exposed to 1 μ M and 2.5 μ M B[a]P showed 2.6-fold ($p < 0.0001$) and 3.0-fold ($p < 0.0001$) increases in *CBR1* mRNA levels, respectively, relative to *CBR1* expression levels in vehicle-treated control cells (Figure 2A). Parallel B[a]P incubation studies in MCF-7 cells, a human breast adenocarcinoma-derived cell line, demonstrated similar augmentation of *CBR1* transcript levels (not shown).

3.3. B[a]P induces *CBR1* gene expression by transcriptional activation

To determine if the observed increase of *CBR1* mRNA level in B[a]P-exposed A549 cells is attributable to transcriptional activation or to modulation of mRNA turnover mediated by B[a]P, we incubated A549 cell cultures simultaneously with B[a]P and actinomycin D, an antineoplastic antibiotic known to inhibit transcription by DNA intercalation (Sobell, 1985). The addition of 10 μ M actinomycin D knocked down *CBR1* mRNA expression in all samples to the same level as the DMSO control, demonstrating that B[a]P induces *de novo* synthesis of *CBR1* mRNA (Figure 2B).

To pinpoint potential regulatory sites involved in the up-regulation of *CBR1* expression by B[a]P, we performed dual-luciferase assays using a group of *CBR1* gene promoter constructs. First, A549 cells in culture were transfected with firefly luciferase reporters (pGL3-basic or pGL3-*CBR1*-413, 250 ng/well) together with internal control plasmid pRL-TK (50 ng/well) and then exposed to 2.5 μ M B[a]P or to vehicle DMSO for 24 hours. Luciferase activity in A549 cells transfected with the -413bp construct (-413*CBR1*) increased 1.4-fold ($p < 0.001$) with the addition of B[a]P compared to vehicle (Figure 3). The AhR pathway has been demonstrated to regulate expression of target genes via conserved XREs in the gene promoter region (5'-T_GNGCGTG-3') (Hankinson, 1995). The *CBR1* promoter contains a conserved XRE (-122XRE) -122 bp upstream of the translation initiation codon (+1ATG) (Lakhman et al., 2007). Deletion of the proximal -122XRE from the -413*CBR1* construct nearly abrogated the inductive effect of B[a]P on *CBR1* gene promoter activity ($p > 0.05$; Figure 3).

3.4. CBR1 protein expression is augmented by B[a]P treatment in A549 cells

To assess if induction of *CBR1* gene transcription by B[a]P exposure in A549 cells produces a downstream increase in CBR1 protein level, we performed quantitative immunoblotting analyses of cell lysates. A 1.5-fold induction of CBR1 protein levels was observed in A549 cells incubated with 2.5 μ M B[a]P compared to vehicle-treated cells ($p < 0.01$; Figure 4A and inset). Simultaneous treatment with 10 μ M cyclohexamide, a translation inhibitor, essentially nullified B[a]P-mediated induction of CBR1 expression ($p > 0.05$; Figure 4B and inset).

3.5. B[a]P enhances nuclear translocation of AhR in A549 cells

To confirm B[a]P-mediated activation of the AhR pathway, we checked the occurrence of nuclear translocation of AhR following exposure to B[a]P. We conducted quantitative immunoblotting analyses of cytosolic and nuclear proteins from A549 cells 6 h after B[a]P treatment. We observed a 1.5-fold induction of AhR protein levels in the nuclear fraction along with a 78% decrease of AhR protein levels in the cytoplasmic fraction of A549 cells incubated with 2.5 μ M B[a]P compared to vehicle-treated cells (Figure 5).

3.6. Binding of nuclear proteins to *CBR1*₋₁₂₂*XRE* is enhanced in A549 cells exposed to B[a]P

Nuclear extracts from A549 cells treated with vehicle or with 2.5 μ M B[a]P were incubated with biotin-labeled *-122XRE-CBR1* oligonucleotides and examined by EMSA followed by densitometric analysis. Seven distinct DNA-nuclear protein complexes (6 prominent and one non-prominent) were detected (Figure 6A). Nuclear extracts from B[a]P-treated cells exhibited more intense bands for DNA-protein complexes A (the non-prominent complex, $p < 0.05$) and B ($p < 0.0001$) than extracts from vehicle-treated cells, while the formation of the other five DNA-protein complexes (including DNA-protein complex C and DNA-protein complex D) showed no significant difference between nuclear extracts from B[a]P-treated cells and vehicle-treated cells ($p > 0.05$; Figure 6A and B, and not shown).

The specificity of the DNA-protein complexes was examined by performing competition experiments with unlabeled *-122XRE-CBR1* oligonucleotides. Incubations with a 50-fold molar excess of “wild type” (wt) competitor diminished the formation of DNA-protein complexes A ($p < 0.0002$), B ($p < 0.0002$), C ($p < 0.0002$), and D ($p < 0.0002$) by 65%, 88%, 85%, and 81%, respectively (Figure 6A and C). Similar competition experiments with the “mutant” (mt) competitor showed relatively less impact on the formation of DNA protein complexes A, B, C, and D than that observed for the “wild type” competitor. That is, competition using the mt oligonucleotide resulted in decreases in band intensity of 7% (complex A), 48% (complex B), 19% (complex C), and 21% (complex D), respectively (Figure 6A and C). Competition experiments with the “mutant” (mt) competitor caused no significant change on the formation of any of the three non-specific DNA protein complexes compared to the “wild type” competitor ($p > 0.005$; Figure 6A, densitometric analysis not shown). Taken together, these results suggest that protein(s) in complexes A, B, C, and D may specifically bind to *-122XRE-CBR1*.

The presence of AhR in the *-122XRE* binding complexes was examined by performing EMSA experiments with the anti-AhR antibody H-211x. Addition of H-211x to the binding reactions was expected to either yield larger DNA-protein complexes evident as “supershifted” EMSA bands or to diminish the formation of DNA-protein complexes containing AhR (Dougherty and Pollenz, 2008; Vogel et al., 2007). Results from our EMSA experiments are in line with the latter possibility (Figure 7). The anti-AhR antibody (3 μ g) markedly decreased formation of DNA-protein complexes A, B, C, and D, while 3 μ g of the isotype control normal rabbit IgG exerted no effect. These trends were consistent across three separate gels. Collectively, these results suggest that AhR was present in specific DNA-protein complexes A, B, C, and D (Figure 7).

4. Discussion

Previous work in our lab revealed that the AhR pathway mediates *CBR1* expression through conserved *XRE* elements in the gene promoter region (Lakhman et al., 2007). We have also observed 2.3-fold induction of *CBR1* mRNA expression in MCF-7 cells following incubation with cigarette smoke condensate, the particulate component of cigarette smoke

(not shown). These findings led us to hypothesize that the potent smoke-derived pro-carcinogen B[a]P may up-regulate the expression of *CBR1* in human lung tissues and lung cancer cells via the AhR pathway.

In consequence, the first component of our study was determination of *CBR1* mRNA and CBR1 protein expression in the lungs of smokers and never-smokers. We observed that the exposure to B[a]P imparted by smoking was sufficient to augment *CBR1* mRNA and CBR1 protein levels in smokers compared to non-smokers (1.5-fold; Figure 1A and B).

Our second aim was to characterize the ability of B[a]P to induce *CBR1* gene expression. Exposure of A549 cells to 1 μ M and 2.5 μ M B[a]P resulted in increased *CBR1* mRNA expression levels in a dose-dependent manner (Figure 2). Simultaneous supplementation of cells with 10 μ M actinomycin D, a potent transcription inhibitor, effectively eliminated up-regulation of *CBR1* transcripts by B[a]P, indicating that B[a]P promotes *de novo* transcription of the *CBR1* gene. CBR1 protein levels were also significantly induced by B[a]P treatment (1.5-fold; Figure 3). Similar studies were performed to measure the mRNA expression levels of *CYP1A1*, a known AhR target gene. The results in A549 cells (1 μ M B[a]P incubation produced a 4.7-fold increase in *CYP1A1* mRNA expression compared to DMSO controls; not shown) confirmed the activation of the AhR pathway. Experiments with engineered *CBR1* promoter constructs suggested that a proximal *XRE*_(-122XRE) mediates *CBR1* induction in response to B[a]P exposure, since deletion of *XRE*_(-122XRE) from the *CBR1* reporter construct significantly suppresses induction of luciferase activity by B[a]P (Figure 4). In similar gene reporter experiments with MCF-7 cells (not shown), B[a]P treatment enhanced the promoter activity of the -413 bp construct by 2.0-fold ($p < 0.0001$) relative to the vehicle DMSO whereas deletion of *XRE*_(-122XRE) abrogated the induction of promoter activity by B[a]P ($p < 0.002$). These findings suggest that *XRE*_(-122XRE) mediates induction of *CBR1* promoter activity in different cellular contexts.

Pilot *in vivo* investigations in *Ahr*-knockout mice corroborate our *in vitro* findings. *Ahr*-knockout mice dosed with a single ip injection of 500 mg/kg B[a]P demonstrated significantly increased *Cbr1* mRNA (4.0-fold; $p < 0.012$) and Cbr1 protein (3.2-fold; $p < 0.008$) expression in the liver compared to knockout animals. In line, hepatic Cbr activity for the prototypical quinone substrate menadione (vitamin K3) was 1.6-fold higher in *Ahr*-proficient mice compared to *Ahr*-deficient animals ($p < 0.015$). These initial efforts suggest that the AhR pathway is critical to the *in vivo* induction of *Cbr1* in response to B[a]P.

Our findings that 2.5 μ M B[a]P caused enhanced translocation of AhR into the nucleus of A549 cells (Figure 5) and that formation of the prominent specific DNA-protein(s) complex B was significantly increased in EMSA with nuclear extracts from B[a]P-treated A549 cells (compared to those from vehicle-treated A549 cells) lend support to the notion that B[a]P enhanced binding of AhR-containing specific nuclear protein complexes to the proximal *XRE* motif (*XRE*_(-122XRE-CBR1)) in the promoter of *CBR1* in A549 cells (Figure 6A and B). Furthermore, EMSAs with an anti-AhR antibody suggested that AhR was present in DNA-protein(s) complex B (Figure 7). Nevertheless, additional experimental approaches such as DNA pull-down and chromatin immunoprecipitation assays are needed to further confirm the *XRE*_(-122XRE-CBR1)-AhR interaction.

We acknowledge that the induction of *CBR1* by B[a]P may be affected by additional signaling pathways. It is also possible that cigarette smoking might impact *CBR1* expression levels through these alternative mechanisms. For instance, it has been reported that cigarette smoking activates both the NF-E2-related factor 2 (Nrf2) (Iizuka et al., 2005) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (Shen et al., 1996) pathways. Interestingly, we previously identified the existence of a binding site for NF- κ B on *CBR1*

(Lakhman et al., 2007). Experiments are needed to determine whether conserved motifs in the *CBR1* promoter mediate gene expression through other signaling pathways.

Analyses of the more than 4000 compounds in cigarette smoke have identified around 80 carcinogens, most of which require metabolic activation to exert their cancer-causing effects. Among the most prominent, by both percent content and carcinogenicity, are 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) and B[a]P (Hecht, 1999). Interestingly, Atalla et al. have shown that CBR1 activity plays a predominant role during the metabolism of NNK in human liver and lung (Atalla et al., 2000). Using systematic inhibitory studies in human liver and lung cytosols, they concluded that CBR1 activity accounts for the majority of the initial deactivation of NNK in those organs (Maser et al., 2000). CBR1 catalyzes the reduction of NNK into the N-nitroso alcohol metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNAL is glucuronidated and excreted in the urine in variable amounts (Hoffmann and Maser, 2007; Maser, 2004). Thus, CBR1 is involved in a complex balance of competing bioactivation and detoxification pathways which could have significant implications on the carcinogenicity of NNK. We propose that the extent to which B[a]P-mediated induction of *CBR1* expression occurs may in turn modulate the detoxification rate of NNK by promoting its reduction into the less toxic NNAL metabolite. Interestingly, a recent study by Wang et al. comparing gene expression profiles between individuals with or without lung cancer, stratified by smoking history, lends further support to this notion. The authors found that *CBR1* mRNA expression was significantly higher ($p < 0.05$) in the bronchial epithelium of smokers compared to never-smokers. Furthermore, they reported the existence of considerably greater *CBR1* mRNA levels in smokers without cancer than in smokers with cancer (Wang et al., 2010). It is tempting to speculate that the observed increased levels of *CBR1* expression may have contributed to greater inactivation of NNK, thereby conferring a degree of protection against carcinogenesis in smokers without cancer. Our findings support their observations and provide a potential mechanistic explanation for smoking-related *CBR1* induction. Studies are ongoing in our laboratory to test the impact of variable *CBR1* expression on the toxicodynamics of B[a]P and NNK in the lung and liver.

Mounting evidence suggests that lung cancer risk has a heritable component (Matakidou et al., 2005). Individual susceptibility to lung cancer may be impacted by polymorphisms in genes such as *CBR1* which encode proteins responsible for the detoxification of carcinogens in cigarette smoke. *CBR1* contains a number of common polymorphisms. For instance, we recently identified a single-nucleotide polymorphism (SNP) in the 3'-untranslated region of *CBR1* (*CBR1* 1096G>A, rs9024) that significantly impacts hepatic CBR1 at the mRNA, protein, and activity levels (Gonzalez-Covarrubias et al., 2009). *CBR1* polymorphisms have the potential to tip the delicate balance of activation versus detoxification of NNK thus imparting additional vulnerability or protection to the lungs of smokers. Future work is needed to explore the relationship between *CBR1* SNPs and lung cancer risk.

One final consideration is the potential impact of cigarette smoking and its associated B[a]P-mediated induction of *CBR1* on cancer therapy. Recently, we reported that increased expression of *CBR1* mRNA, protein, and activity in the hearts of cancer patients with Down syndrome might contribute to the risk of anthracycline-related cardiotoxicity (Kalabus et al., 2010). Additionally, it is well-appreciated that the alcohol metabolites of the parent anthracyclines doxorubicin and daunorubicin have almost negligible antineoplastic effects (Dessypris et al., 1986; Dorr et al., 1991). Therefore, cigarette smoking during anthracycline-based chemotherapy regimens could reduce treatment efficacy and exacerbate cardiotoxic side effects.

5. Conclusions

Our study demonstrates that *CBR1* is induced by B[a]P via the AhR pathway. Our work provides the foundation for further efforts in characterizing the influence of polymorphic *CBR1* expression on lung cancer susceptibility and treatment recommendations.

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Abbreviations

CBR	carbonyl reductase
PAH	polycyclic aromatic hydrocarbon
B[a]P	benzo(<i>a</i>)pyrene
AhR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
XRE	xenobiotic response element
EMSA	electrophoretic mobility shift assay
real-time qRT-PCR	real-time quantitative reverse transcription-polymerase chain reaction
NNK	4-methylnitrosamino-1-(3-pyridyl)-1-butanone
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
Nrf2	NF-E2-related factor 2
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
SNP	single-nucleotide polymorphism.

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- Carbonyl reductase 1 (CBR1) reduces a wide range of xenobiotic carbonyl substrates
- *CBR1* mRNA levels were higher in lungs from smokers than in samples from never-smokers
- The smoke carcinogen benzo[*a*]pyrene (B[*a*]P) induces *CBR1* expression in A549 cells
- B[*a*]P increases binding of protein complexes to a xenobiotic response element (*XRE*)

Figure 1. *CBR1* mRNA (A) and CBR1 protein (B) expression in human lung tissues of smokers ($n = 16$) and never-smokers ($n = 12$). Relative mRNA and protein levels were calculated as described in *Materials and Methods*. Each circle depicts the average of three replicates. Horizontal lines indicate group means. *: $p < 0.05$ (Student's t -test).

Figure 2.

Effect of B[a]P on *CBR1* mRNA expression in A549 cells. Relative mRNA levels were calculated with the comparative quantitation method (see *Materials and Methods* for details) in cells incubated with B[a]P (A) and B[a]P plus actinomycin D (B). Results shown are mean $\pm$ SD from triplicate exposures. ****: $p < 0.0001$ (Student's *t*-test).

Figure 3.

Effect of B[a]P on gene reporter activities of the promoter constructs -413 CBR1 (A) and $-413\text{ CBR1} - \Delta-122\text{ XRE}$ (B). Luciferase activities were measured as light intensity with a dual-luciferase assay (see *Materials and Methods*). For each construct, normalized luciferase activities were expressed as fold increases with respect to the values from control incubations (DMSO), which were set arbitrarily at 1. Data represent the mean $\pm$ S.D. from three independent experiments performed in triplicate. ***: $p < 0.001$ (Student's *t*-test).

Figure 4.

CBR1 protein expression in A549 cells exposed to B[a]P (A) and B[a]P plus cyclohexamide (B). Protein quantification was performed following immunodetection of β -actin-normalized CBR1 (insets) by interpolation from calibration curves of recombinant CBR1. **: $p < 0.01$ (Student's *t*-test).

Cytoplasmic fraction of A549 cells

Nuclear fraction of A549 cells

Figure 5.

Effect of B[a]P on nuclear translocation of the AhR transcription factor. AhR protein levels were determined in cytosolic and nuclear fractions from A549 cells treated with vehicle (DMSO, 0.1% v/v) or B[a]P (2.5 μ M) by quantitative immunoblotting (see *Materials and Methods* for details). Images are representative and data shown are the mean of three independent experiments.

Figure 6.

Formation of specific nuclear protein-DNA complexes as evidenced by EMSA with the double stranded $_{122}XRE-CBR1$ oligonucleotides (A). Nuclear extracts from vehicle- (DMSO, 0.1% v/v) and B[a]P- (2.5 μ M) treated A549 cells were incubated with the double stranded biotin-labeled $_{122}XRE-CBR1$ oligonucleotides. Competition experiments with a 50-fold molar excess of the unlabelled “wild type” (wt) and “mutant” (mt) $_{122}XRE-CBR1$ oligonucleotides evidenced the formation of four specific complexes (see text for details). Densitometric analysis was performed for each of the four complexes A, B, C, and D (B and C). EMSA experiments were repeated three times to evaluate reproducibility. For Figure 6B, asterisks indicate significant differences between band intensity values (*, $p < 0.05$; ****, $p < 0.0001$). For Figure 6C, asterisks indicate significant differences between band intensity values (*, $p < 0.005$; **, $p < 0.001$).

Figure 7.

Analysis of nuclear protein-DNA complexes by EMSA in the presence of anti-AhR antibody H-211x, or in the presence of isotype control antibody sc-2027x (A). Nuclear extracts from B[a]P- (2.5 µM) treated A549 cells were incubated with the double-stranded biotin-labeled -122*XRE-CBR1* oligonucleotides in the presence or absence of 3 µg antibody as described in the text. These EMSA experiments were repeated three times to evaluate reproducibility.