Tobacco smoke induces CYP1B1 in the aerodigestive tract

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Several members of the P450 family, including cytochrome P450 1B1 (CYP1B1), can convert tobacco smoke (TS) procarcinogens, including benzo[a]pyrene (B[a]P), to carcinogenic intermediates. In this study we investigated the effects of TS condensate and B[a]P on the expression of CYP1B1 in vitro and in vivo. CYP1B1 mRNA and protein were induced by both TS condensate and B[a]P in cell lines derived from the human aerodigestive tract. Treatment with TS condensate stimulated binding of the aryl hydrocarbon receptor (AhR) to an oligonucleotide containing a canonical xenobiotic response element (XRE) site and induced XRE–luciferase activity. These findings are consistent with prior evidence that polycyclic aromatic hydrocarbons, known ligands of the AhR, stimulate CYP1B1 transcription by an XRE-dependent mechanism. To determine whether these in vitro findings applied in vivo, both murine and human studies were carried out. Short-term exposure to TS induced CYP1B1 in the tongue, esophagus, lung and colon of experimental mice. In contrast, CYP1B1 was not induced by TS in the aorta of these mice. Levels of CYP1B1 mRNA were also elevated in the bronchial mucosa of human tobacco smokers versus never smokers (P < 0.05). Taken together, these results support a role for CYP1B1 in TS-induced carcinogenesis in the aerodigestive tract.

Introduction

Cigarette smoke, a complex chemical mixture containing more than 4000 different compounds, is widely recognized as an important cause of cancer (1). More than 100 carcinogens, mutagens and tumor promoters have been identified in cigarette smoke. The polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines and aromatic amines are among the best-characterized carcinogens in cigarette smoke (1). Benzo[a]pyrene (B[a]P), a prototypic PAH, has been implicated in the pathogenesis of several human malignancies, including cancers of the aerodigestive tract (1,2). PAHs are activated to reactive metabolites by the cytochrome P450 monooxygenase system, specifically the CYP1 family (3).

Cytochrome P450 1B1 (CYP1B1) is a relatively recently identified member of the CYP1 family gene that has been detected in a variety of human tissues, including the respiratory and gastrointestinal tracts (4–7). Several lines of evidence suggest that CYP1B1 plays a role in carcinogenesis. CYP1B1 is commonly overexpressed in human malignancies (8–10) and activates a variety of carcinogens, including PAHs (11–14). For example, CYP1B1 catalyzes both the formation of dihydrodiols of specific PAHs and their subsequent oxidation to carcinogenic dihydrodiol epoxides (15). In addition to being a substrate for CYP1B1, PAHs induce CYP1B1 gene transcription through binding to and activation of the aryl hydrocarbon receptor (AhR) (16,17). A reduced incidence of PAH-induced lymphomas and tumors was demonstrated in mice engineered to be CYP1B1 deficient (18,19). Polymorphisms of CYP1B1 have been associated with an elevated risk of several malignancies, including tobacco smoke (TS)-related head and neck squamous cell carcinoma (20,21).

Given the potential significance of CYP1B1 in TS-induced carcinogenesis, we investigated the mechanism by which TS induced CYP1B1 in cell lines derived from the human aerodigestive tract. These in vitro studies were complemented by experiments that evaluated the effects of TS on levels of CYP1B1 in the aerodigestive tract and bronchial mucosa of mice and humans, respectively. Evidence is presented suggesting that TS condensate induced the transcription of CYP1B1 by an AhR–dependent mechanism. Consistent with this finding, increased levels of CYP1B1 were detected in the aerodigestive tracts of mice exposed to TS and in the bronchial mucosa of human subjects who smoked cigarettes. Taken together, these data support a role for CYP1B1 in TS-mediated carcinogenesis.

Materials and methods

Abbreviations: AhR, aryl hydrocarbon receptor; B[a]P, benzo[a]pyrene; CYP1B1, cytochrome P450 1B1; DMEM, Dulbecco’s modified Eagle’s medium; PAHs, polycyclic aromatic hydrocarbons; SSPE, sodium chloride, sodium phosphate, EDTA buffer; TS, tobacco smoke; XRE, xenobiotic response element.

Materials

Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM and LipofectAMINE 2000 were from Life Technologies Inc. (Grand Island, NY). Keratinocyte basal and growth media were from Clonetics Corp (San Diego, CA). Poly(dI -dC), B[a]P and o-nitrophenyl-β-D-galactopyranoside were from Sigma Chemical Co. (St Louis, MO). [32P]CTP, [32P]ATP and the ECL western blot detection system were from NEN (Boston, MA). Random priming kits were from Roche Molecular Biochemicals (Indianapolis, IN). CYP1B1 antibody was provided by Dr Craig B. Marcus at the University of New Mexico. The anti-AhR antibody was obtained from Santa Cruz Biotechnology (San Diego, CA). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). Reagents for the luciferase assay were from Analytical Luminescence (San Diego, CA). The 18S rRNA cDNA was from Ambion Inc. (Austin, TX). pSVβgal was from Promega (Madison, WI). Oligonucleotides were synthesized by Sigma Genosys (Woodlands, TX).

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Cells and culture conditions
H212 and SCC450 cell lines were derived from human non-small cell lung (adenocarcinoma) and esophageal squamous cell cancers, respectively (22,23). Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. MSKLeuk1 cells were derived from a dyplastic oral leukoplasia lesion (24). These cells were routinely cultured in keratinocyte growth medium. In all experiments cells were grown in basal medium for 24 h before treatment. All treatments were carried out in serum-free medium.

Preparation of tobacco smoke condensate
Marlboro cigarettes were batch purchased and stored at −20°C. Before use they were incubated overnight at room temperature under a controlled humidity of 65%. Cigarettes were smoked in a Borgwardt piston-controlled apparatus (model RG-1) using the FTC standard protocol. The protocol includes a puff volume of 35 ml and puff duration of 2 s, with a puff frequency of 1/min. The aqueous smoke fractions from one cigarette correspond to 8 puffs of smoke drawn into a 5 ml volume of sterile phosphate-buffered saline. This smoke-buffered saline represents whole mainstream smoke, abbreviated as TS. The final concentration of TS in the cell culture medium is expressed as puffs/ml.

Western blotting
Cell lysates were prepared by treating cells with lysis buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM phenylmethylsulfonfyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor and 10 μg/ml leupeptin). Lysates were sonicated for 4 min and centrifuged at 10,000 g for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry (25).

After UV cross-linking, membranes were transferred to nylon-supported membranes. The resolved proteins were transferred to nitrocellulose sheets. The nitrocellulose membrane was then incubated with anti-CYP1B1 antisera (26). Secondary antibody to IgG conjugated to horseradish peroxidase was used. The blots were probed with the ECL western blot detection system according to the manufacturer’s instructions.

Electrophoretic mobility shift assay
Nuclear extracts were prepared from cells using established methods (27). For binding studies, oligonucleotides containing the human xenobiotic response element (XRE) were synthesized: 5′-GATGTGCGTCTTCTCAGCGAATCTCG-3′ (sense); 5′-CGAGTTTCGTTAAAGACCTGATC-3′ (antisense). The complementary oligonucleotides were annealed in 10 mM Tris (pH 7.6), 50 mM NaCl and 1 mM EDTA. The annealed oligonucleotide was phosphorylated at the 5′-end with 32P-ATP using T4 polynucleotide kinase. The binding reaction was performed by incubating 5 μg of nuclear protein in 10 mM Tris–HCl (pH 7.5), 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl and 0.01 mg/ml poly(dI–dC) in a final volume of 10 μl at 25°C. The labeled oligonucleotide gel was added to the reaction mixture and allowed to incubate for an additional 20 min at 25°C. To determine the identity of the protein in the binding complex, 2 μl of polyclonal antibodies were added after incubation of the probe, nuclear extract and binding buffer. The reaction mixture was left on ice for 60 min before electrophoresis. For competition assays, cold competitor oligonucleotides (50-fold excess) were preincubated with nuclear extract and binding buffer for 15 min on ice before the addition of labeled probe. The binding reactions were then incubated on ice for an additional 15 min before electrophoresis. The samples were electrophoresed on a 4% non-denaturing polyacrylamide gel. The gel was then dried and subjected to autoradiography.

Transient transfection assays
SCC450 cells were seeded at a density of 5 × 104 cells/well in 6-well dishes and grown to 50–60% confluence. In each well, cells were co-transfected with 1.8 μg of the XRE-luciferase plasmid pGudLuc1 (a gift of Dr Michael S. Denison, University of California at Davis) and 0.2 μg of pSVβgal using 2 μg of LipofectAMINE 2000 as per the manufacturer’s instructions. After 12 h incubation, the medium was replaced with serum-free medium for 24 h and then treated with vehicle or TS condensate for 12 h. The activities of luciferase and β-galactosidase were measured in cellular extract.

Marine smoke exposure
Mice were secured in fitted polycarbonate chambers prior to placement into a 12 port nose-only exposure chamber (CH Technologies, NJ). The system contains a filtrona holder and valveless reciprocating pump as well as an electronic control system. Mice were treated with either room air or TS for 1 h at a total suspended particulate dose of 200 mg/m3. Tissues were harvested 18 h later and stored at −80°C until analysis. The Institutional Animal Care and Use Committee of Weill Medical College of Cornell University approved this study.

Bronchoxoscopy
Ten human volunteers who never smoked (n = 5) or smoked heavily (n = 5) were recruited for fiberoptic bronchoxoscopy. Smokers were active smokers with a history of at least 20 pack years. Informed consent was obtained for each subject. Bronchoxoscopy was then performed under conscious sedation through the oropharynx. Bronchial biopsies were obtained with forceps, snap frozen in liquid nitrogen and stored at −80°C until analysis. The above protocol was approved by the Institutional Review Board of Weill Medical College of Cornell University.

Analysis of CYP1B1 expression in tissues
Total cellular RNA was isolated from both human and murine frozen tissue specimens using the RNeasy Mini-kit with on-column DNA digestion using RNase-free DNase according to the manufacturer’s instructions. Reverse transcription was performed using 1 μg RNA per 50 μl reaction. The reaction mixture contained 1× PCR Buffer II, 2.5 mM MgCl2, 500 μM dNTPs, 50 U RNase inhibitor, 125 U MuLV reverse transcriptase and 2.5 μM random hexamers. Samples were incubated for 10 min at room temperature, then cycled at 42°C for 15 min and 95°C for 10 min. CYP1B1 expression was then determined by RT–PCR. An aliquot of 2 μl of cDNA was subjected to 30 cycles of PCR in a 25 μl reaction mixture (1× PCR Buffer II, 2 mM MgCl2, 400 μM dNTPs, 2.5 U Taq polymerase and upstream and downstream primers). Primers used were: human CYP1B1, forward 5′-AACGTCATGATGCCGCTGTGT-3′, reverse 5′-GCGGCAGAACGTCTCCACATC-3′; CYP1B1, forward 5′-CTCGAGAAGGTGAGAAACC-3′, reverse 5′-AAAGGCTTTGCCAGTTGT-3′; β-actin, forward 5′-GGTCACCCACACTGTTCCCAT-3′, reverse 5′-GGATGCACAGGACTCATGC-3′. Thermal cycling conditions were: 95°C for 2 min, followed by 30 s at 95°C, 30 s at 62°C and 45 s at 72°C for 30 cycles and then 72°C for 10 min. Importantly, the signal for each product was linear with the amount of RNA analyzed. PCR products were electrophoresed on a 1% agarose gel with ethidium bromide and photographed under UV light. The identity of each PCR product was confirmed by direct sequencing (28). A computer densitometer (Chem Doc; Bio-Rad) was used to quantify the density of the different bands. Data are expressed in arbitrary units.

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Statistics
Comparisons between groups were made with the Student’s t-test. A difference between groups of P < 0.05 was considered significant.

Results
Tobacco smoke condensate or B[a]P induce CYP1B1 in cell lines derived from the human aerodigestive tract
Immunoblot analysis was carried out to determine whether TS condensate or B[a]P induce CYP1B1. As shown in Figure 1, treatment with either TS condensate or B[a]P caused a marked increase in levels of CYP1B1 protein in cell lines derived from the human lung (Figure 1A), oral cavity (Figure 1B) and esophagus (Figure 1C). To determine whether regulation was pre-translational, steady-state levels of CYP1B1 mRNA were determined by northern blotting (Figure 2). Consistent with the immunoblot findings, treatment with TS condensate or B[a]P led to a marked increase in levels of CYP1B1 mRNA.

CYP1B1 is an AhR-dependent gene. Experiments were performed to evaluate the effects of TS condensate on AhR-dependent gene expression. Increased binding of nuclear protein to a canonical XRE site was detected following
treatment with either TS condensate or B[a]P (Figure 3A). The increase in binding to the XRE was competed by incubating nuclear extract from TS condensate- or B[a]P-treated cells with an excess of XRE cold probe (Figure 3A). To identify the transcription factor present in the binding complex, we utilized an antibody to the AhR. Preincubation of the reaction mixture with this antibody abrogated TS condensate- or B[a]P-induced binding to the XRE (Figure 3B). To determine the functional significance of TS condensate-mediated increased binding of the AhR to the XRE, transient transfections were performed. Treatment with TS condensate led to a dose-dependent increase in XRE-luciferase activity (Figure 3C). Taken together, these results suggest that treatment with TS induced AhR signaling resulting in the induction of CYP1B1.

Increased levels of CYP1B1 were detected in the aerodigestive tract of mice exposed to TS and in the bronchial mucosa of humans who smoke cigarettes

Based on the in vitro findings described above, we investigated whether exposure to TS would induce CYP1B1 in the aerodigestive tracts of mice. Mice were exposed to TS for 1 h and then killed 18 h later. RT-PCR was carried out to assess levels of CYP1B1 mRNA in the tongue, esophagus, colon, lung and aorta. Exposure to TS resulted in increased amounts of CYP1B1 in tongue, esophagus, colon and lung, but not aorta (Figure 4). Interestingly, the extent of CYP1B1 induction was greater in the proximal (tongue and esophagus) than in the distal (colon) gastrointestinal tract.

To determine whether a similar effect occurred in humans, we investigated whether levels of CYP1B1 mRNA were increased in the bronchial mucosa of cigarette smokers versus non-smoking human volunteers. Amounts of CYP1B1 were increased by ~100% in the bronchial mucosa of smokers (Figure 5).

Discussion

In this study we have shown that TS, a rich source of carcinogenic PAHs, induced CYP1B1 both in vitro and in vivo. The induction of CYP1B1 by TS is consistent with prior evidence that PAHs, known ligands of the AhR, stimulate the transcription of CYP1B1 by an XRE-dependent mechanism (29,30). However, our data do not enable us to exclude the involvement of other regulatory mechanisms (31,32). In experimental mice, inhalation of TS resulted in increased amounts of CYP1B1 in tongue, esophagus, colon and lung, but not aorta. Notably, the inductive effects of TS were greater in the proximal than in the distal gastrointestinal tract, suggesting the importance of topical exposure. The significance of topical exposure is supported by the absence of CYP1B1 induction in the aortas of TS-exposed mice. In all likelihood, the failure to observe induction of CYP1B1 in the aorta reflects the comparatively low level of PAH achieved in the blood (as compared with the level achieved topically in the aerodigestive tract) following inhalation of TS for 1 h. A less likely explanation is that the regulation of CYP1B1 is different in vascular compared with epithelial tissues. Importantly, the results observed in vitro and in experimental animals translated to humans. More specifically, increased levels of CYP1B1 were also observed in the bronchial mucosa of humans who smoked cigarettes heavily compared with never smokers. This finding is consistent with previous evidence of increased CYP1B1 in bronchial epithelial cells from cigarette smokers (33).
It is important to consider the possible implications of TS-mediated induction of CYP1B1. As mentioned above, CYP1B1 converts a broad array of carcinogens to active metabolites which can form DNA adducts. Thus, several chemical classes of carcinogens, e.g. PAHs, arylamines and nitroaromatics, are activated to mutagenic derivatives by CYP1B1 (11–14). Possibly, TS-mediated induction of CYP1B1 will amplify the mutagenic effects of these carcinogens. The

Fig. 3. Treatment with tobacco smoke condensate or B[a]P stimulates binding of the aryl hydrocarbon receptor to the XRE, resulting in activation of transcription. SCC450 and MSKLeuk1 cells were treated with vehicle (control), TS condensate (0.03 puffs/ml) or B[a]P (250 nM) for 2 h. (A) An aliquot of 5 μg of nuclear protein from TS condensate or B[a]P-treated SCC450 and MSK Leuk1 cells was incubated with a 32P-labeled oligonucleotide containing the human XRE consensus site. (B) An aliquot of 5 μg of nuclear protein from TS condensate or B[a]P-treated SCC450 cells was incubated with antibodies to AhR or goat IgG for 1 h. Subsequently, the reaction mixture was incubated with a 32P-labeled oligonucleotide containing the human XRE consensus site. In the cold chase experiments, nuclear extract from TS condensate-treated cells was incubated with a 32P-labeled oligonucleotide containing the human XRE consensus site and a 50-fold excess of unlabeled oligonucleotide containing the human XRE consensus site. In (A) and (B) the protein–DNA complex that formed was separated on a 4% polyacrylamide gel. (C) SCC450 cells were co-transfected with 1.8 μg XRE-luciferase and 0.2 μg of pSVβgal. After transfection, cells were incubated for 24 h with serum-free medium and then treated with vehicle or TS condensate (0.0075, 0.015 or 0.03 puffs/ml). Reporter activities were measured in cellular extracts 12 h later. Luciferase activity represents data that have been normalized with β-galactosidase activity. Columns, means; bars, SD; n = 6.
potential significance of this idea is underscored by the finding that B[a]P diol epoxide, a mutagen formed by CYP1B1, causes adducts along exons of the \( p53 \) gene that correspond to \( p53 \) mutational hot-spots in human lung cancer (2). Notably, other members of the \( CYP1 \) gene family, such as CYP1A1, can also be induced by PAHs and activate TS carcinogens (3,16). It is likely, therefore, that multiple members of the \( CYP1 \) gene family contribute to TS-induced carcinogenesis. This redundancy is an important consideration in attempting to develop chemopreventive strategies to reduce TS-induced mutagenesis in active smokers. Even if selective inhibitors of CYP1B1 could be developed (34), these drugs might be less effective than agents that induce Phase II detoxifying enzymes and thereby reduce DNA adduct formation. CYP1B1 has been reported to be overexpressed in most human malignancies, with minimal expression in normal cells (8–10). Active efforts are underway to exploit this difference in developing new strategies to treat cancer. One approach is to identify prodrugs that undergo activation by CYP1B1 in tumor cells resulting in cell killing (35). A second approach is to target CYP1B1 as a tumor antigen (36). Preclinical trials of a CYP1B1 DNA vaccine have been carried out.

Fig. 4. Exposure to tobacco smoke induces CYP1B1 in the aerodigestive tract of mice. Mice were treated with room air (control) or tobacco smoke for 1 h at a total suspended particulate dose of 200 mg/m\(^3\). Eighteen hours later the mice were killed and tissues harvested. (A) Total cellular RNA was extracted from tongue, esophagus, colon, lung and aortic tissue and reverse transcribed. Levels of CYP1B1 and \( \beta \)-actin mRNA from three control (lanes 1–3) and three smoke-exposed (lanes 4–6) mice were assessed by RT-PCR. STD represents a CYP1B1 positive control. No bands were observed when cDNA was omitted from the PCR reaction or when reverse transcriptase enzyme was not included in the reverse transcriptase reaction. (B) Results of the data shown in (A) expressed in arbitrary units. Elevated levels of CYP1B1 were detected in the tongue, esophagus, colon and lungs of tobacco smoke-exposed mice. Columns, means; bars, SD; * \( P < 0.05 \), ** \( P < 0.01 \).
and shown to destroy cancer cells (36). Clinical trials targeting CYP1B1 have been initiated. We also note that several widely used anticancer agents, including docetaxel and tamoxifen, are inactivated by CYP1B1 (35,37). Because cigarette smoking can induce CYP1B1 in normal tissues and possibly tumors, it will be important to determine whether the efficacy or toxicity of these different types of anticancer therapy are different in active smokers compared with never smokers. In the field of chemoprevention, treatment with \( \beta \)-carotene increased the risk of lung cancer in smokers (38). The results of this study provide a mechanistic rationale for evaluating whether the efficacy or toxicity of certain cancer treatments differ in smokers compared with non-smokers.

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