Benzo[a]pyrene phenols are more potent inducers of CYP1A1, CYP1B1 and COX-2 than benzo[a]pyrene glucuronides in cell lines derived from the human aerodigestive tract

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Introduction

Exposure to polycyclic aromatic hydrocarbons is an important cause of cancer because these molecules are activated by selected enzymes, including P-450s and cyclooxygenase (COX) to carcinogenic metabolites. For example, benzo[a]-pyrene (B[a]P), a component of tobacco smoke, undergoes metabolic biotransformation to electrophilic intermediates that react with cellular DNA and protein. The extent to which a given exposure to carcinogens produces adducts between reactive metabolites and cellular components in vivo depends on the balance between rates of oxidation of the parent compound and rates of detoxification of reactive metabolites via conjugation (1). Thus, induction of mono-oxygenase enzymes (2) or inhibition of conjugating enzymes (1,3) enhances the covalent binding of B[a]P metabolites to DNA.

The cytochrome P-450 family of enzymes including CYP1A1 and CYP1B1 is widely recognized for catalyzing oxidative reactions and activating xenobiotics to reactive metabolites that are carcinogenic (4–6). COX is capable of co-oxidizing B[a]P; the peroxyl radicals formed during arachidonic acid metabolism catalyze the epoxidation of B[a]P to its mutagenic products (7,8). In this regard, COX-2, an inducible form of COX, may be particularly important because it is frequently over-expressed in intraepithelial neoplasia of the aerodigestive tract (9,10). Previous studies have suggested a role for each of these enzymes in carcinogenesis of the upper aerodigestive tract (11–13).

The UDP-glucuronosyltransferases (UGTs) are a large multigene family of phase 2 enzymes that catalyze the detoxification of many xenobiots and their metabolic intermediates. Several studies have demonstrated that UGTs protect against the carcinogenic effects of tobacco smoke and B[a]P. The addition of UDP-glucuronic acid to the Ames test is associated with a reduction of B[a]P-induced mutagenicity (14). Reduced glucuronidation of B[a]P metabolites correlated with increased covalent binding to hepatic DNA and micrometal protein in UGT-deficient rats (15). Importantly, this concept has been extended to humans. Several human UGTs detoxify B[a]P metabolites including 3-hydroxy-benzo[a]pyrene (3-OH-B[a]P), 7-hydroxy-benzo[a]pyrene (7-OH-B[a]P), 9-hydroxy-benzo[a]pyrene (9-OH-B[a]P) (16–19). In studies examining polymorphisms in the UGT1A7 gene, three allelic variants of wild-type UGT1A7 were identified (16,20). The enzymes encoded by the three variant alleles are less active in glucuronidating B[a]P phenols than the wild-type enzyme (16). Individuals with any of the predicted low-activity UGT1A7 genotypes were found to be at markedly increased risk of developing smoking-related orolaryngeal cancer (20). Similarly, Elahi and colleagues have shown recently that a non-conservative missense polymorphism in codon 139 of the UGT1A10 gene is associated with risk for orolaryngeal cancer (21). Although the precise mechanism linking low-activity UGT1A7 or UGT1A10 to tobacco smoke-induced cancer is uncertain, reduced glucuronidation may result in increased levels of B[a]P phenols in target tissues. If B[a]P phenols like other aryl hydrocarbons induce CYP1A1, CYP1B1 and COX-2 (22–24), this would be predicted to increase the risk of tobacco smoke-induced carcinogenesis.

This study was designed to explore the effects of B[a]P phenols on levels of carcinogen-activating enzymes in cell lines derived from the human aerodigestive tract. We show that hydroxylated metabolites of B[a]P induce CYP1A1, CYP1B1 and COX-2. In contrast, the corresponding glucuronide conjugates of B[a]P caused less induction of each of these enzymes. Taken together, these results may help to explain why individuals with low-activity UGT genotypes are at increased risk for tobacco smoke-related orolaryngeal cancer.

Materials and methods

Materials

Keratinocyte growth media (KGM) was obtained from Clonetics (San Diego, CA). DMEM and FBS were from Life Technologies (Grand Island, NY). Enzyme immunoassay reagents for assays of prostaglandin E2 (PGE2) were from Cayman (Ann Arbor, MI). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). DNA and RNA were prepared using kits from Qiagen (Chatsworth, CA). The 18S rRNA cDNA was from Ambion (Austin, TX). [3H]-[3-2]CTP was from DuPont NEN (Boston, MA). Random priming kits were from Boehringer-Mannheim Biochemicals (Indianapolis, IN). B[a]P was from Sigma Chemical (St Louis, MO). 3-OH-B[a]P, 7-OH-B[a]P and 9-OH-B[a]P, as well as 3-benzo[a]pyrene-β-d-glucopyranosiduronic acid (B[a]P-3-O-Gluc), 7-benzo[a]pyrene-β-d-glucopyranosiduronic acid (B[a]P-7-O-Gluc) and 9-benzo[a]pyrene-β-d-glucopyranosiduronic acid (B[a]P-9-O-Gluc) (all used as standards), were obtained from the National Cancer Institute Chemical...
Human liver microsomes with a final protein concentration of 10 mg/ml were incubated with 0.1-2.5 μM 7- or 9-OH-PGE2 for 2 h at 37 °C. The activities of luciferase and radio-receptor binding were measured by enzyme immunoassay. Production of PGE2 was calculated and normalized to protein concentrations.

Preparation of 3-, 7- and 9-OH-[3H]-BP glucuronidases

Total cellular RNA was isolated from cell monolayers using an RNA isolation kit from Qiagen. Ten micrograms of total RNA per lane were electrophoresed on 15% polyacrylamide gels as described by Laemmli (30). The resolved proteins were transferred to nitrocellulose sheets as detailed by Towbin et al. (31). The nitrocellulose membrane was then incubated with primary antisera. Second antibody to IgG conjugated to horseradish peroxidase was then used. The proteins were then detected by incubation with ECL reagents according to the manufacturer’s instructions. The results shown are representative of individual experiments carried out a minimum of three times.

Northern blotting

Total cellular RNA was isolated from cell monolayers using an RNA isolation kit from Qiagen. Ten micrograms of total RNA per lane were electrophoresed in a formaldehyde-containing 1.2% agarose gel and transferred to nylon-supported membranes. After baking, membranes were pre-hybridized for 4 h and then hybridized in a solution containing 50% formamide, 5× sodium chloride-sodium phosphate-EDTA buffer (SSPE), 5× Denhardt’s solution, 0.1% SDS and 100 μg/ml single-stranded salmon sperm DNA. Hybridization was carried out for 16 h at 42 °C with radiolabeled cDNA probes for human COX-2 (a gift from Dr Stephen M. Prescott, University of Utah, Salt Lake City, UT) or human CYP1A1 (Oxford Biomedical Research, Oxford, MI). To verify equivalency of RNA loading in the different lanes, the blot was stripped of radioactivity and re-hybridized to determine levels of 18S rRNA. COX-2, CYP1A1 and 18S RNA probes were labeled with [32P]dCTP by random priming. Membranes were washed twice for 20 min at room temperature in 2× SSPE-0.1% SDS, and twice for 20 min in 0.1× SSPE-0.1% SDS at room temperature. Washed membranes were then subjected to autoradiography.

Plasmids

The XRE luciferase plasmid pGudLuc6.1 was a kind gift of Dr Michael S. Denison from the University of California at Davis (Davis, CA). The dominant negative Ah receptor (AhR) construct pEh-AhR-L678A was a gift of Dr Gary H. Peredew from Pennsylvania State University (University Park, PA) (32). pSVβgal was obtained from Promega (Madison, WI).

Transient transfection assays

MSK-Leuk1 cells were seeded at a density of 8×10^5 cells/well in 6-well dishes and grown to 60% confluence. For each well, 2 μg of plasmid DNA were introduced using LipofECTAMINE 2000 as per the manufacturer’s instructions. After 4 h of incubation, the medium was replaced with basal medium. The activities of luciferase and β-galactosidase were measured in cellular extracts as described previously (33).

Patient samples

Specimens were obtained from six healthy non-smoking human volunteers. The buccal mucosa was examined and then anaesthetized with topical cetacaine spray. A 3 mm punch was used to biopsy the mucosa and the specimen was immediately snap frozen and stored at −80°C. The study was approved by the Committee on Human Rights in Research of Well Medical College of Cornell University.

Analysis of CYP1B1 expression

Total cellular RNA was isolated from frozen buccal mucosa 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, MgCl2 2.5 mM, dNTP (250 mM each), RNAse inhibitor (1 U/ml), MuLV reverse transcriptase (2.5 U/ml) and random hexamers (2.5 mM). 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. Two microliters 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, Taq polymerase 2.5 U and upstream and downstream primers). Primers used were: CYP1B1 forward 5'-AACGT-CATGAGTGGCCTGTGTG-3', reverse 5'-GGCCGGTAAGTCTCCAAACATC-3'; and β-actin forward 5'-GGTCACCCACACTGTGCCCAT-3', reverse 5'-GGATGCCACAGGACTCTGCA-3'. Thermal cycling conditions were: 95°C for 2 min; 30 s at 95°C, 30 s at 62°C and 45 s at 72°C for 20 cycles; and 72°C for 10 min. The PCR product was electrophoresed through 1% agarose with ethidium bromide in 1× TBE, and photographed under UV light. The identity of the CYP1B1 PCR product was confirmed by direct sequencing.

Statistics

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

Results

We investigated whether B[a]P or its hydroxylated metabolites (3-OH-B[a]P, 7-OH-B[a]P, 9-OH-B[a]P) induced CYP1A protein. As shown by western blotting (Figure 1A and C), B[a]P and its hydroxylated metabolites caused marked induction of CYP1A protein in two human oral epithelial cell lines. In contrast, the corresponding B[a]P glucuronides (B[a]P-3-O-G, B[a]P-7-O-G, B[a]P-9-O-G) caused little to no induction of CYP1A. To determine whether regulation was pre-translational,
northern blotting was performed (Figure 1B and D). Consistent with the western blot findings, the B[a]P phenols were much more potent inducers of CYP1A1 mRNA than the corresponding B[a]P glucuronides in both cell lines.

Recently, CYP1B1 has been postulated to be important in the pathogenesis of head and neck cancer (34). As a first step in evaluating this question, we determined whether CYP1B1 was specific for CYP1A and B[a]P glucuronides in human oral epithelial cells. Cells isolated and 10^6 M B[a]P phenols or their respective glucuronides for 12 h. In (A) and (C), cellular lysate protein (100 g/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose. The immunoblots were sequentially probed with antibodies specific for CYP1A and β-actin. In (B) and (D), total cellular RNA was isolated and 10 μg of RNA was added to each lane. The northern blots were hybridized sequentially with probes that recognized CYP1A1 mRNA and 18S ribosomal RNA, respectively.

Fig. 1. B[a]P phenols are more potent inducers of CYP1A than B[a]P glucuronides in human oral epithelial cells. Cells [(A) and (B), MSK-Leuk1 cells; (C) and (D), 1483 cells] were treated with vehicle, 1 μM B[a]P, 1 μM B[a]P phenols or their respective glucuronides for 12 h. In (A) and (C), cellular lysate protein (100 μg/lane) was loaded onto a 10% SDS-polyaclamyd gel, electrophoresed and subsequently transferred onto nitrocellulose. The immunoblots were sequentially probed with antibodies specific for CYP1A and β-actin. In (B) and (D), total cellular RNA was isolated and 10 μg of RNA was added to each lane. The northern blots were hybridized sequentially with probes that recognized CYP1A1 mRNA and 18S ribosomal RNA, respectively.

protein in cell lines derived from both the head and neck and lung (Figure 5). Analysis of COX-2 expression in the H2122 cell line demonstrated that this induction was dose-dependent for B[a]P as well as its hydroxylated metabolites (Figure 5C). Northern blot analysis demonstrated that COX-2 mRNA was also induced by treatment with B[a]P and its hydroxylated metabolites (Figure 5D). As was the case for CYP1A1 and CYP1B1, each of the B[a]P phenols was a more potent inducer of COX-2 than the corresponding glucuronide (Figure 6).

Differences in uptake of the conjugated versus unconjugated B[a]P hydroxylated metabolites into cells might explain why B[a]P glucuronides caused less induction of CYP1A1, CYP1B1 and COX-2 than B[a]P phenols. As shown in Table I, there were minimal differences in the uptake of B[a]P-3-O-G compared with its unconjugated 3-OH-B[a]P counterpart in the 1483 cell line. In contrast, a large difference in uptake
between the two compounds was observed in the MSK-Leuk1 cell line, with the level of 3-OH-B[a]P being 3.5-fold higher than that observed for B[a]P-3-O-G.

Discussion

Several UGTs including UGT1A7 and UGT1A10 are expressed in aerodigestive tract tissues (20,35--37) and were shown to detoxify tobacco smoke carcinogens (16,25). Recent studies have implicated UGT genetic variants in risk for aerodigestive tract cancers. Zheng and colleagues have reported that individuals with any of the predicted low-activity UGT1A7 genotypes who smoked had an increased risk of orolaryngeal cancer compared with those subjects with the wild-type genotype (20). In contrast, there was no association between UGT1A7 genotype and risk of development of orolaryngeal cancer in never smokers (20). A link between polymorphisms of UGT1A7 and cancers of the proximal digestive tract (36), colon (38) and liver (39) has also been reported. Similarly, Elahi and colleagues have found recently that a non-conservative missense polymorphism in codon 139 of the UGT1A10 gene is associated with risk for orolaryngeal cancer (21). Together, these studies implicate glucuronidation as an important pathway for tobacco smoke carcinogen detoxification in the aerodigestive tract.

The primary purpose of the current study was to investigate the mechanistic basis for the increased risk of orolaryngeal cancer associated with variant UGT genotypes. B[a]P phenols, which comprise a major form of B[a]P metabolites, are among the B[a]P metabolites that are glucuronidated by UGTs 1A7 and 1A10 (16,18,19). Glucuronides undergo biliary or renal elimination. It seems likely, therefore, that the levels of B[a]P metabolites will increase in the mucosa of individuals with low-activity UGT genotypes who smoke tobacco. If B[a]P metabolites such as B[a]P phenols induce carcinogen-activating enzymes, this would be predicted, in turn, to increase the risk of cancer.

CYP1A1, CYP1B1 and COX-2 convert tobacco carcinogens to reactive metabolites that form DNA adducts. Previous studies have demonstrated the expression of both CYP1A1 and COX-2 in the oral cavity (13,40). To the best of our knowledge, the present study is the first to show that CYP1B1 is also expressed in oral mucosa.

Each of the B[a]P phenols examined in this study induced CYP1A1, CYP1B1 and COX-2 in cell lines derived from the human aerodigestive tract including the oral cavity. Importantly, B[a]P glucuronides caused less induction of each of these enzymes. We considered the possibility that differences in cellular uptake of B[a]P phenols versus the
corresponding glucuronide conjugate could explain the observed differences in enzyme induction. For MSK-Leuk1 cells, differential uptake of 3-OH-B[a]P and its glucuronide conjugate may explain at least, in part, the finding that B[a]P phenols were more potent inducers of xenobiotic metabolizing enzymes than B[a]P glucuronides. In contrast, no difference in uptake between the two compounds was observed in 1483 cells, suggesting that glucuronide conjugate formation can induce a protective effect by directly altering B[a]P metabolite-induced activities within a cell. Differences in binding to the AhR could potentially explain why B[a]P phenols are more potent inducers of xenobiotic metabolizing enzymes than B[a]P glucuronides. Regardless of the underlying mechanism, these findings are potentially important for understanding the link between reduced glucuronidation and tobacco smoke-induced orolaryngeal cancer. In individuals with low-activity UGT genotypes who smoke tobacco, B[a]P phenol-mediated induction of xenobiotic metabolizing enzymes should amplify the effect of a given dose of B[a]P on tumor initiation. An important next step will be to determine whether levels of CYP1A1, CYP1B1 and COX-2 are higher than normal in the buccal mucosa of smokers with low-activity UGT genotypes.

In addition to potentially increasing the activation of carcinogens, B[a]P phenol-mediated induction of COX-2 could predispose to carcinogenesis by other mechanisms. Enhanced synthesis of COX-2-derived PGs favors tumor growth by stimulating cell proliferation (41), promoting angiogenesis (42,43), increasing invasiveness (44,45) and inhibiting apoptosis (46,47). Therefore, selective COX-2 inhibitors may have a role in protecting against tobacco smoke-induced carcinogenesis in individuals with low-activity UGT genotypes.

In summary, our study provides a mechanism for the increased risk of orolaryngeal cancer in smokers with low-activity UGT genotypes. We have demonstrated that B[a]P phenols are more potent inducers of CYP1A1, CYP1B1 and COX-2 than the corresponding B[a]P glucuronides. These results support the concept of developing chemopreventive agents that induce UGTs such as UGT1A7 and UGT1A10.

Fig. 5. B[a]P and its hydroxylated metabolites induce COX-2 protein and mRNA. (A) MSK-Leuk1 cells were treated with vehicle (control), 5 μM B[a]P or 5 μM B[a]P phenols for 12 h. (B) 1483 cells were treated with vehicle, 1 μM B[a]P or 1 μM B[a]P phenols for 12 h. (C) H2122 cells were treated with vehicle, B[a]P or B[a]P phenols for 8 h. In (A-C), cellular lysate protein (100 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose. The immunoblots were sequentially probed with antibodies specific for COX-2 and β-actin. (D) H2122 cells were treated with 1 μM and 10 μM B[a]P or B[a]P phenols for 12 h. Total cellular RNA was isolated and 10 μg of RNA was added to each lane. The northern blot was hybridized sequentially with probes that recognized COX-2 mRNA and 18S rRNA, respectively.
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**Fig. 6.** B[a]P phenols are more potent inducers of COX-2 than B[a]P glucuronides. (A) MSK-Leuk1 cells were treated with vehicle (control), 1 μM B[a]P phenol or the same concentration of the corresponding glucuronides for 12 h. (B) H2122 cells were treated with vehicle, 1 μM B[a]P phenols or the same concentration of the corresponding glucuronides for 24 h. Cellular lysate protein (100 μg/lane (A); 30 μg/lane (B)) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose. The immunoblots were sequentially probed with antibodies specific for COX-2 and β-actin.

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*P < 0.001, Student’s t-test (two-sided).

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**References**


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