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Differential Induction of CYP1A1 and CYP1B1 by Benzo[*a*]pyrene in Oral Squamous Cell Carcinoma Cell Lines and by Tobacco Smoking in Oral Mucosa

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Abstract

Polyaromatic hydrocarbons, including benzo*[a]*pyrene (BP), are major tobacco carcinogens. Their carcinogenic effects require metabolic activation by cytochrome p450 (CYP) enzymes. Relative CYP isoform expression is related to tissue-specific tobacco-related squamous cell carcinoma (SCC) susceptibility. There have been conflicting reports regarding relative CYP1A1 and CYP1B1 oral expression, and information regarding CYP1B1 expression in oral tissues is limited.

OBJECTIVE—To quantify BP- and tobacco-induced CYP1A1 and CYP1B1 expression in oral SCC cells and oral mucosa.

STUDY DESIGN—Real-time qPCR was performed to measure 1) BP-induced CYP1A1 and CYP1B1 mRNA expression in seven oral/other head and neck SCC cell lines 2) CYP1A1 and CYP1B1 mRNA expression in gingiva from 22 smokers and 24 nonsmokers.

RESULTS—SCC lines exhibited either similar induction of both isoforms or preferential CYP1A1 induction (CYP1A1-to-CYP1B1 ratios 0.8-4.3). In contrast, gingival tissues from smokers exhibited preferential CYP1B1 induction. Marked interindividual variation in CYP1A1 and CYP1B1 expression was observed among smokers.

CONCLUSIONS—*In vitro* conditions may not account for factors that modulate expression *in vivo*. Interindividual variation in inducible CYP1A1 and CYP1B1 expression may account in part for variation in tobacco-related oral SCC risk.

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CONFLICT OF INTEREST STATEMENT
None declared.

Keywords

oral cancer; CYP1A1; CYP1B1; tobacco; squamous cell carcinoma

INTRODUCTION

Tobacco use is a major risk factor for oral squamous cell carcinoma (SCC).[1] Chief among the >60 carcinogens in cigarette smoke are the polycyclic aromatic hydrocarbons (PAH)--including the prototype of this chemical class benzo[a]pyrene (BP).[2, 3] BP exerts its carcinogenic effects after metabolic activation.[4, 5] Activation involves monooxygenation by cytochrome p450 (CYP) isoforms CYP1 and hydration by microsomal epoxide hydrolase. These reactions produce reactive species that form DNA adducts.[5, 6] DNA adducts may lead to cancer by causing mutations in genes essential for key functions, including apoptosis, proliferation, and differentiation.

The major CYP isoforms involved in BP bioactivation are CYP1A1 and CYP1B1. CYP1A1 is considered the classical isoform for PAH activation.[7] The discovery of CYP1B1 in 1994 has led to recent studies suggesting CYP1B1 is important in tobacco-related head and neck carcinogenesis as well. Some have suggested CYP1B1 may play an even more important role than CYP1A1 in this process[8, 9], and the CYP1B1*3 polymorphism has been identified as a susceptibility factor for head and neck SCC.[10, 11]

The literature regarding CYP1B1 expression in human oral cell lines is limited and variable. In previous studies, Wen and Walle reported preferential induction by BP of CYP1B1 over CYP1A1 in SCC-9 cells and bioengineered human gingiva.[8, 9] In contrast, in a microarray profiling study with validation by qRT-PCR to assess gene expression in response to cigarette smoke condensate, Nagaraj et al. reported similar CYP1A1 and CYP1B1 induction in oral dysplasia lines (Leuk1, Leuk2) and greater induction of CYP1A1 than CYP1B1 in oral SCC line 101A.[12]

Reports of oral CYP1B1 expression in humans also are limited. Spivack et al. demonstrated CYP1B1 mRNA induction using real-time qPCR on exfoliated buccal mucosa cells obtained from a tobacco-naïve individual who smoked four cigarettes. They also observed a positive relationship between smoking and CYP1B1 (but not CYP1A1) mRNA levels in exfoliated buccal epithelial cells from a small group of human subjects.[13]

Our underlying hypothesis is that CYP1B1 is a biomarker of tobacco use with biologic relevance to smoking-induced oral carcinogenesis. In this study, we quantified inducible CYP1A1 and CYP1B1 expression to assess whether there is preferential CYP1B1 induction. CYP1A1 and CYP1B1 mRNA expression was evaluated in oral SCC lines exposed to BP and in normal oral tissues from smokers and nonsmokers.

MATERIALS AND METHODS

Cell culture

Oral SCC lines UM-SCC-1 and UM-SCC-14A, hypopharyngeal SCC line UM-SCC-22A, and laryngeal SCC line UM-SCC-12 were provided by Prof. T. Carey (University of Michigan, MI, USA). SCC-9 (derived from oral tongue) was obtained from ATCC (Bethesda, MD, USA). These lines were maintained in DMEM/F12 with 10% fetal bovine serum (FBS) (Hyclone), hydrocortisone, and penicillin/streptomycin. Oral SCC lines UPCI:SCC040 and UPCI:SCC081 (courtesy of Dr. Susanne Gollin, University of Pittsburgh, PA, USA) were

maintained in MEM with 1% non-essential amino acids, 1% L-glutamine, 50μg/ml gentamicin and 15% FBS. Cell lines were grown in a humidified incubator (37°C, 5% CO_2).

BP treatment and CYP1A1/CYP1B1 mRNA quantification for SCC lines

Cell lines were exposed to 2μM BP or vehicle for 24h. Additionally, to demonstrate a dose response, UMSCC-14A was treated for 48h with varying BP concentrations (.01, .1, 1, 10μM) or vehicle.

Total RNA was isolated using the RNAqueous-4PCR Kit (Ambion, Austin, TX); cDNA was synthesized from 1μg total RNA using the Accuscript High-Fidelity 1st Strand cDNA Synthesis Kit (Stratagene, La Jolla, CA). Real-time qPCR was performed using an iCycler (Bio-Rad, Austin, TX). Each 20μL reaction included 10μL iQ SYBR-Green Supermix (Bio-Rad), 20pmol primers, and 1μL cDNA template. Primers were as follows:

CYP1A1: (forward)5′-GCTGACTTCATCCCTATTCT-3′

(reverse)5′-GCTCCAGGAGATAGCAGTTG-3′

CYP1B1: (forward)5′-GGCCACTATCACTGACATCT-3′

(reverse)5′-ACAGTGTCCTTGGGAATGTG-3′

β-actin: (forward)5′-GACGAGGCCCAGAGCAAGAG-3′

(reverse)5′-GTGGTGGTGAAGCTGTAGCC-3′

Samples were run in triplicate. Parameters included 95°C 1min followed by 45 amplification cycles (95°C 20s, 55°C 30s, 70°C 30s). Dissociation curve analysis was verified for each run. Products were visualized by 2% agarose-ethidium-bromide electrophoresis.

Changes in gene expression between BP-treated and untreated cells were calculated using the comparative threshold cycle method,[14] with normalization to β-actin. The relative quantification (RQ) (i.e., fold-change in expression) was expressed as $2^{-\Delta\,\Delta Ct}$. The Wilcoxon two-sample rank sum test[15] (SAS, v9.1, SAS Institute, Cary, NC) was applied to compare fold-changes in CYP1A1 and CYP1B1 mRNA expression in BP-treated versus vehicle-treated controls (alpha<0.05).

Western blot analysis

UPCI:SCC040 cells treated with 2μM BP or vehicle were harvested at 24h. Microsome isolation and Western blot analysis [with primary antibodies to CYP1A1 (1:500, rabbit anti-fish 1A, Biosense Laboratories, Bergen, Norway, specific for human 1A1) and CYP1B1 (1:1000, rabbit anti-human, gift from Dr. Craig Marcus, University of New Mexico, Albuquerque, USA)] were performed using protocols described previously.[9]

Gingival samples

Gingival tissues were obtained from patients in the periodontics and oral surgery clinics at the Medical University of South Carolina College of Dental Medicine. Subjects were receiving surgical procedures during which tissue that otherwise would have been discarded was removed. The protocol was approved by the Institutional Review Board, and patient consent was obtained. Demographic (age/gender/ethnicity), tobacco, alcohol, medication, and occupational exposure information was recorded. Upon clinical and histopathologic examination, the tissues exhibited either no pathology or nonspecific inflammation. The smoking group reported current cigarette use >1 pack/day. The nonsmoking group consisted of never smokers. Gingival samples (10-20mg) were immersed in 10 volumes RNAlater (Ambion) and stored (-20°C).

Salivary cotinine assay

Patient current smoking status was verified by Cotinine Saliva Micro-Plate EIA (OraSure Technologies, Bethlehem, PA). 1mL unstimulated whole saliva was collected and stored at 4° C for <21 days. Salivary cotinine concentrations were analyzed in duplicate per manufacturer's protocol (>10ng/mL positive for smoking).

RNA isolation and cDNA synthesis from tissue samples

Frozen tissues were thawed (37°C), placed in RLT-βME lysis buf fer, pulverized, and centrifuged (6,000*g*, 3min). RNA was isolated from supernatants using the RNeasy Plus Micro Kit (Qiagen, Valencia, CA). RNA purity and quantity was evaluated by A_{260}/A_{280} measurement (>1.8). cDNA synthesis was performed from 1μg RNA using SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA) per manufacturer's protocol.

CYP1A1/CYP1B1 mRNA quantification in tissue samples by real-time qPCR

Real-time qPCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions were prepared as described above with primers as follows:

CYP1A1: (forward)5′-GCTGACTTCATCCCTATTCT-3′

(reverse)5′-GCTCCAGGAGATAGCAGTTG-3′

CYP1B1: (forward)5′-GGCCACTATCACTGACATCT-3′

(reverse)5′-CCACGACCTGATCCAATTCT-3′

TATA-box binding protein(TBP):

(forward)5′-TTCGGAGAGTTCTGGGATTGTA-3′

(reverse)5′-TGGACTGTTCTTCACTCTTGGC-3′

Samples were run in duplicate. Along with each patient sample, a reaction was run using a constant amount UM-SCC-22A cDNA template (1μL from a single cDNA synthesis). Parameters included 95°C 10min and 50 amplification cycles (95°C 20s, 55°C 30s, 70°C 30s). Dissociation curve analysis was verified for each run. Products were visualized by 2% agarose-ethidium-bromide gel electrophoresis.

RQ was calculated using the comparative threshold cycle method[14] with normalization to TBP (a housekeeping gene) and comparison to UM-SCC-22A. Wilcoxon two-sample rank sum[15] and Kruskal-Wallis one-way ANOVA[16] tests (SAS, v9.1, SAS Institute, Cary, NC) were used to detect significant differences (*alpha*<0.05) in CYP1A1 and CYP1B1 RQs according to smoking (yes/no), age quartile (<50/50-54/55-62/>62 years), ethnicity (white/non-white), alcohol consumption (yes/no), and gender (male/female).

RESULTS

Preferential CYP1A1 induction or similar CYP1A1/CYP1B1 induction in oral/other head and neck SCC lines

All lines exhibited significant CYP1A1 and CYP1B1 induction with 24h 2μM BP exposure (Figure 1). For the oral SCC lines UM-SCC-1, UPCI:SCC040, and SCC-9, the CYP1A1-to-CYP1B1 fold-change ratios were 4.3, 2.5, and 1.8, respectively, indicating preferential CYP1A1 induction. In the remaining lines, ratios were 0.8-1.0, indicating similar induction of both isoforms. Western blot analysis of untreated and BP-treated UPCI:SCC040 confirmed induction of CYP1A1 and CYP1B1 protein expression (Figure 2).

BP dose response for CYP1A1 and CYP1B1 induction

For UM-SCC-14A, significant CYP1A1 and CYP1B1 mRNA induction (p=0.04) was evident at BP concentrations as low as 1μM (Figure 3). Previous investigators have considered the 1μM scale to represent a low and physiologically relevant BP concentration.[9]

Significantly greater CYP1B1 expression in gingiva from smokers versus nonsmokers

Subject characteristics are summarized in Table 1. Salivary cotinine analysis confirmed each patient's smoking/nonsmoking status. No patients were taking medications metabolized by CYP1A1/CYP1B1, and none reported occupational chemical exposures known to induce CYP1A1/CYP1B1.

Real-time qPCR analysis (Figure 4A-B) showed significantly greater CYP1A1 and CYP1B1 expression in smokers than nonsmokers (p<0.0001 for CYP1A1 and CYP1B1). Kruskal-Wallis ANOVA tests showed no significant differences in expression by age, gender, ethnicity, or alcohol use. Within the smoking and nonsmoking groups, there was considerable interindividual variation in CYP1A1 (smokers: mean RQ=1.00±1.66, nonsmokers: mean RQ=0.03±0.08) and CYP1B1 expression (smokers: mean RQ=10.70±8.13, nonsmokers: mean RQ=2.31±3.14).

DISCUSSION

We demonstrated preferential CYP1A1 induction or similar CYP1A1 and CYP1B1 induction in the oral/other head and neck SCC lines tested. Our results are similar to those of Nagaraj et al. (described above).[12] However, our results differ from those of Wen and Walle who reported preferential CYP1B1 induction in SCC-9 cells and bioengineered gingival tissue.[9] These authors used semiquantitative analysis based upon branched DNA technology, which may explain their differing results.

In contrast to our cell line analysis, gingival tissue analysis showed more marked CYP1B1 than CYP1A1 induction among smokers. The discordance between *in vitro* and patient study results may reflect a difference between SCC cells and normal oral mucosa or effects of BP versus whole tobacco smoke. Furthermore, *in vitro* conditions may not account for *in vivo* factors that might modulate CYP expression. Lipopolysaccharides and pro-inflammatory cytokines, including tumor necrosis factor(TNF)-α, interleukin(IL)-1β, and transforming growth factor(TGF)-β1, have been reported to downregulate CYP1A1 expression; also, TNF-α and TGF-β1 upregulate CYP1B1 expression.[5, 17-19] These observations are from studies of rat liver epithelial and stellate cells and human hepatocytes--it is unclear whether such effects also may apply to human oral tissue. However, this possibility is compelling because of the prevalence of inflammation in the oral cavity; in our study, some gingival samples indeed exhibited inflammation.

The balance between CYP1A1/CYP1B1 expression in a given tissue type is important as it relates to organ-specific susceptibility to BP toxicity and carcinogenesis. This balance is complex, and *in vitro* models do not always predict *in vivo* carcinogen sensitivity. Uno et al. showed that Cyp1a1$^{-/-}$ knockout mice exhibit increased hepatic levels of BaP-DNA adducts and greater protection from BP-mediated hepatotoxicity and death compared with Cyp1a1$^{+/+}$ mice.[20] Thus, CYP1A1 paradoxically has a protective role *in vivo* that would not be predicted by *in vitro* studies. In contrast, no such paradox has been found in Cyp1b1$^{-/-}$ knockout mice.[21-24] CYP1 enzymes are coupled to Phase II detoxification enzymes *in vivo*. It has been proposed that compared to CYP1B1, CYP1A1 is more tightly coupled to Phase II metabolism and plays a more important role *in vivo* in detoxification than toxin activation.[25]

There has been much interest in characterizing CYP1B1 expression in different normal and cancer tissue types. CYP1B1 upregulation has been reported in numerous cancers, including those arising in breast, colon, lung, esophagus, skin, brain, and testis.[26] In normal adult tissues, CYP1B1 has been detected only at low levels by Northern blot analysis in kidney, liver, eye, and brain.[27-29] Thus, investigators have regarded CYP1B1 as a biomarker of tumor development and potential therapeutic target. In our study, observed CYP1B1 induction in normal oral tissues from smokers suggests CYP1B1 indeed may be an important early biomarker related to risk of tobacco-induced oral SCC development.

Murray et al. cautioned that previous investigations of CYP1B1 expression in normal human tissues have utilized commercially obtained samples.[27] Such samples have no corresponding patient histories (e.g., tobacco, chemical, and drug exposure), and typically it is unknown whether such samples represent a single patient or pool of patients. Therefore, our study is significant in that it represents the only investigation of CYP1B1 expression in normal oral tissues obtained from well-characterized smoking and nonsmoking cohorts. Among smokers, we observed marked variation in CYP1A1 and CYP1B1 expression (RQ range 0 to 6-fold for CYP1A1 and 2- to 28-fold for CYP1B1). Interindividual variation in CYP expression is a well-known phenomenon. For instance, Wiley et al. reported significant interindividual variation in CYP1A1 and CYP1B1 expression in bronchial epithelial cells, which might account for interindividual variation in the risk of bronchogenic carcinoma.[30] Accordingly, differences in CYP1A1 and CYP1B1 inducibility and activity may account for interindividual variation in tobacco-related oral cancer risk and may relate to CYP1A1 and CYP1B1 polymorphic variants.[10, 11, 31, 32]

Unlike smokers, most nonsmokers had low or undetectable CYP1B1 expression. Nevertheless, there were some cases (n=7) in which CYP1B1 expression was detected at levels comparable to those in the smoking group. Salivary cotinine assay confirmed reported smoking status for each patient. Thus, we suspect sources of PAH exposure other than tobacco. High PAH levels have been found in grilled or smoked meats.[33-35] The ingestion of such foods has been associated with an increased risk of colorectal, gastric, and esophageal cancers, although no such association with oral cancer has been reported.[36, 37] Among our subjects, we did not find any occupational exposures or medications that might interact with CYP1A1 or CYP1B1. Nonetheless, various environmental factors (e.g., diesel exhaust, asphalt fumes, wood smoke) are sources of PAH exposure as well.[33]

We recognize that there were a limited number of patients analyzed in this study, and thus a population-based study with subjects matched by age, gender, and ethnicity would be needed to confirm our findings. Nonetheless, in the current study, the demographic composition of the smoking and nonsmoking groups was similar, and our analysis demonstrates preferential CYPB1 induction in smokers regardless of age, gender, ethnicity, and alcohol use.

In conclusion, this study represents the first well-characterized clinical investigation of tobacco-induced oral CYP1A1 and CYP1B1 expression. We demonstrate preferential induction of CYP1B1 in smokers. In contrast, with BP exposure, oral SCC cell lines show similar induction of both isoforms or preferential CYP1A1 induction. *In vitro* conditions may not include *in vivo* factors, such as modulation by inflammatory cytokines. Interindividual variation in CYP1A1 and CYP1B1 expression among smokers may account in part for interindividual variation in tobacco-related oral SCC risk. Moreover, CYP1B1 may be an important early biomarker for risk of tobacco-induced oral SCC development.

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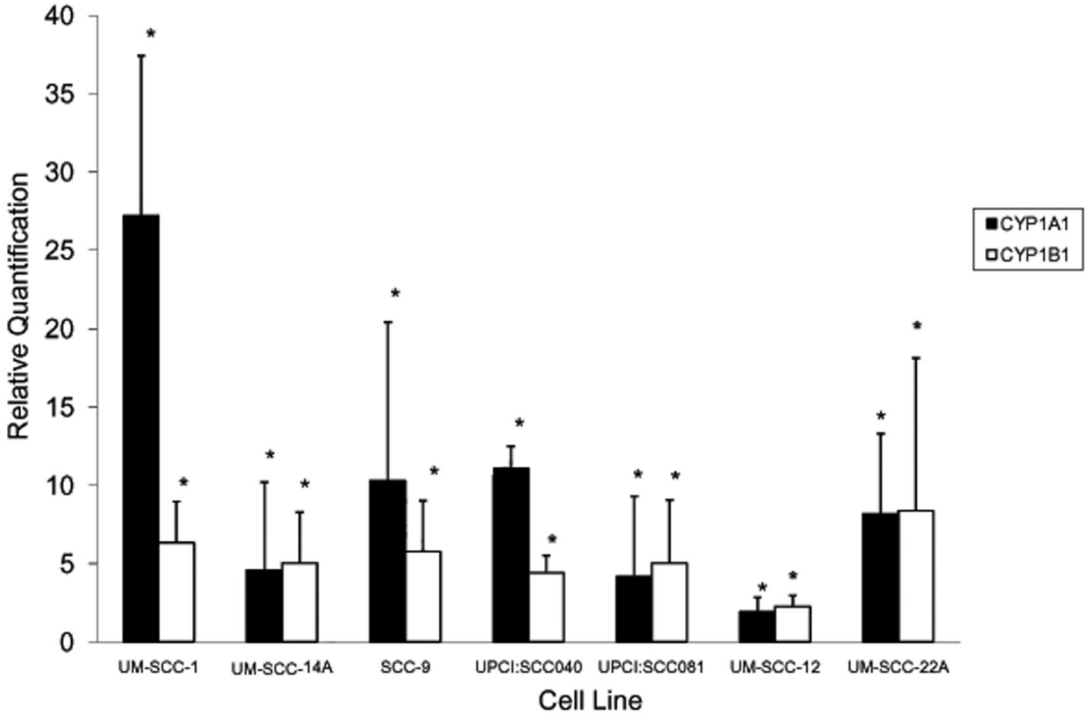

Figure 1.

CYP1A1 and CYP1B1 mRNA induction in different oral/head and neck SCC cell lines. Cell lines were exposed to 2 μM BP for 24 h. Total RNA was isolated and reverse transcribed to generate cDNA. Real-time qPCR was performed to measure CYP1A1 and CYP1B1 mRNA expression. Each sample was run in triplicate and mean values were used in subsequent calculations. The relative quantification (RQ) (or fold-change in expression between treated and untreated cells) was determined by the $2^{-\Delta\Delta Ct}$ method with normalization to β-actin. * indicates target expression is significantly greater in BP-treated versus untreated cells ($p < 0.05$).

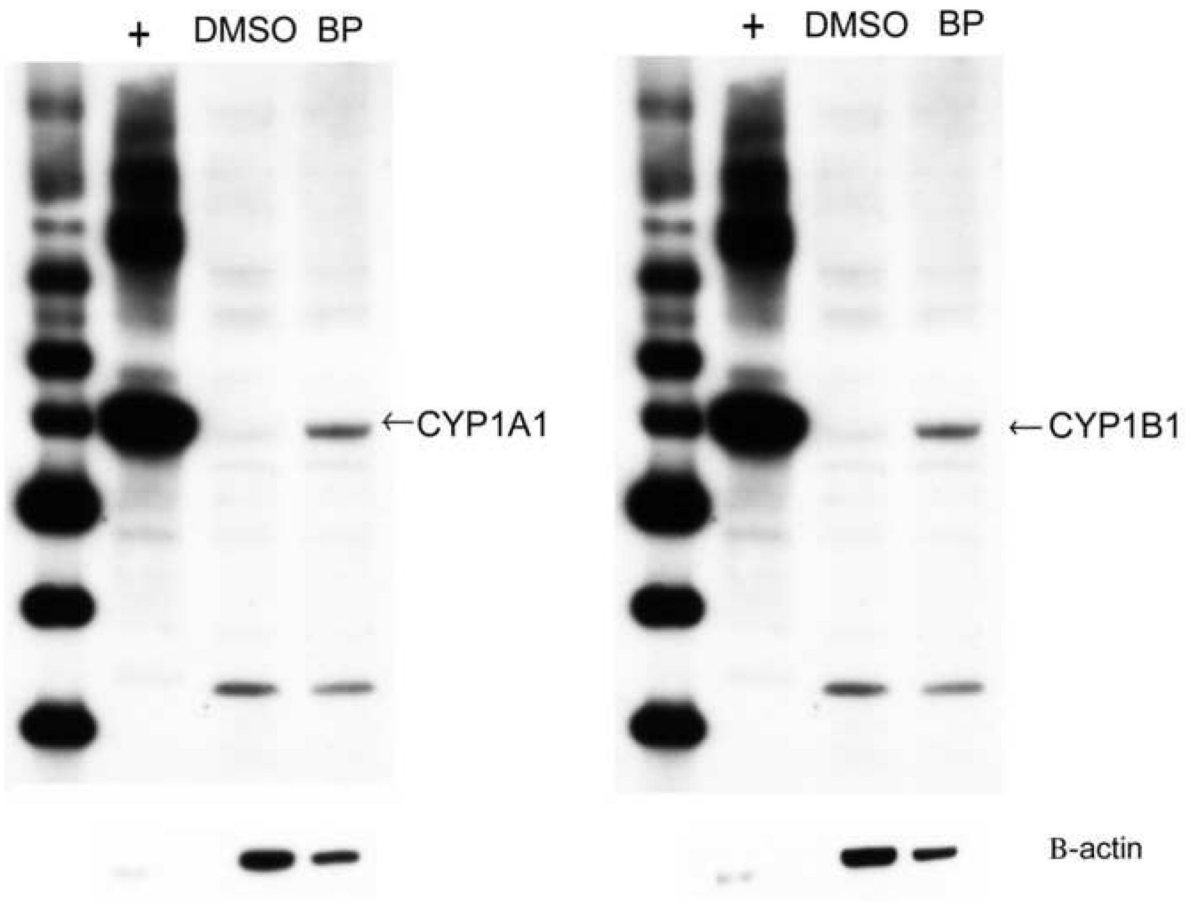

Figure 2.

Western blot analysis demonstrates induction of CYP1A1 and CYP1B1 protein expression by BP in UPCI:SCC040 cells. The cells were exposed to either 2 μM BP or DMSO (dimethylsulfoxide) vehicle (negative control) for 24 h. Microsomes were isolated, and 24 μg microsomal protein was loaded. Recombinant baculovirus-expressed CYP1A1 and CYP1B1 protein (0.025 μg) served as positive controls (+). β-actin loading control is shown below.

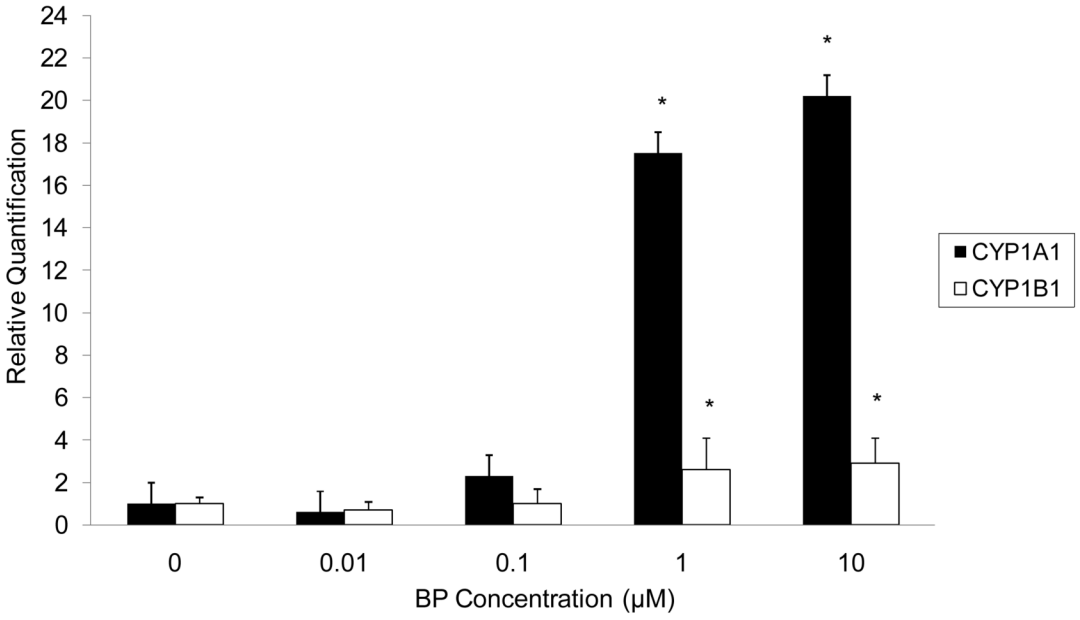

Figure 3.

CYP1A1 and CYP1B1 mRNA expression in UM-SCC-14A in response to different doses of BP. The cells were exposed to .01, .1, 1, or 10 μM BP for 48h. Vehicle-treated cells served as a negative control. Total RNA was isolated and reverse transcribed to generate cDNA. Real-time qPCR was performed to analyze the expression of CYP1A1 and CYP1B1 mRNA. Each sample was run in triplicate and mean values were used in subsequent calculations. The relative quantification (RQ) was determined by the $2^{-\Delta\Delta Ct}$ method using normalization to β-actin and comparison to the vehicle-treated control. * indicates that target expression is significantly greater in BP-treated than vehicle-treated cells ($p < 0.05$).

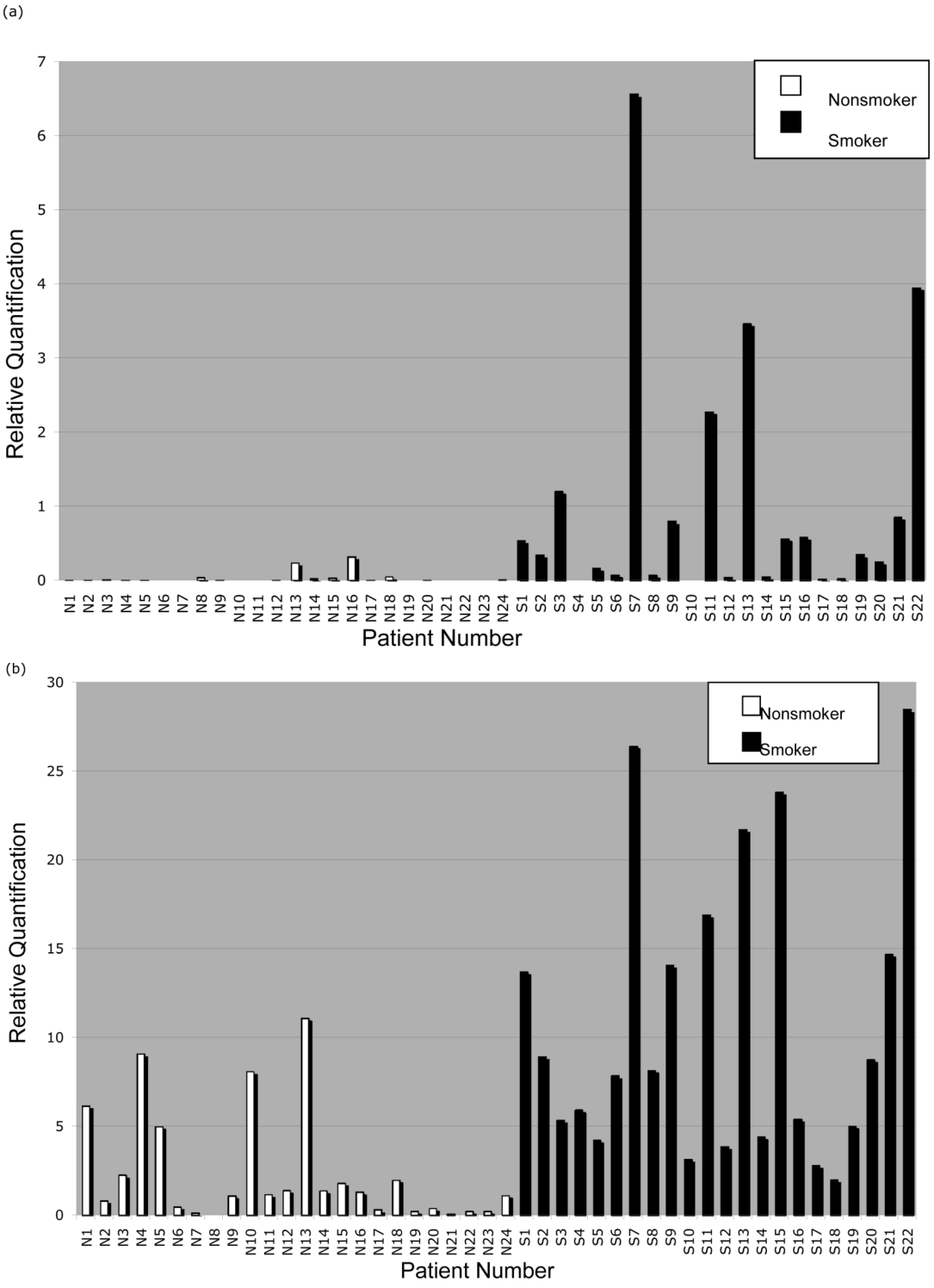

Figure 4.

CYP1A1 and CYP1B1 mRNA quantification in normal gingival tissue samples from nonsmokers and smokers. Gingival tissue samples were obtained from nonsmoking and smoking patients. Total RNA was isolated and reverse transcribed to generate cDNA. Real-time qPCR was performed to analyze the expression of (a) CYP1A1 mRNA and (b) CYP1B1

mRNA. Each patient sample was run in duplicate and mean values were used in subsequent calculations. The relative quantification (RQ) was determined by the $2^{-\Delta\Delta Ct}$ method using normalization to the housekeeping gene for TATA-box binding protein (TBP) and comparison to reactions using a constant amount of uninduced UM-SCC-22A cDNA.

Table 1

Characteristics of subjects donating normal gingival tissue samples

Characteristic	Smokers (n=22)	Nonsmokers (n=24)
Mean age (years)	51	55
Male: female ratio	1.2:1	1.2:1
Ethnicity	18 Caucasian 4 African American	18 Caucasian 6 African American
Mean tobacco exposure (pack-years)	27.9	(not applicable)