Preferential induction of CYP1B1 by benzo[a]pyrene in human oral epithelial cells: impact on DNA adduct formation and prevention by polyphenols

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The roles of CYP1A1 and 1B1 in tobacco smoke carcinogen, e.g. benzo[a]pyrene (BaP), induced DNA binding and their inhibition by the dietary polyphenol 5,7-dimethoxyflavone (DMF), compared with 3',4'-dimethoxyflavone (3',4'-DMF) and resveratrol, were investigated in the human oral epithelial squamous cell carcinoma (SCC)-9 cells. A low concentration of BaP (1 \( \mu \)M) dramatically induced BaP-DNA adduct formation (~40-fold) in a time-dependent manner, while it only increased CYP1A1/1B1 activities, as measured by ethoxyresorufin O-deethylolation, ~3-fold. Furthermore, BaP induced both CYP1B1 and CYP1A1 mRNA and protein expression, as determined by the branched DNA assay and western blot analysis, but with considerably higher levels of CYP1B1. Combined treatment of SCC-9 cells with 1 \( \mu \)M BaP and 20 \( \mu \)M DMF inhibited BaP-DNA adduct formation. The mechanism of this appearance seemed to be direct inhibition of CYP1B1 enzyme with a \( K_i \) value of 0.58 \( \mu \)M, a highly potent inhibition considering the high cellular uptake of DMF in the SCC-9 cells. DMF also inhibited CYP1A1, but not CYP1B1 protein, and mRNA expression in the cells. In an extension to other polyphenols, the structural analog 3',4'-DMF, in contrast to DMF, inhibited the expression of CYP1B1 both at the mRNA and protein levels. Surprisingly, in contrast to previous studies in other cell types, resveratrol had no effect on CYP1B1 in the SCC-9 cells. Based on this study, CYP1B1 mRNA may be an early biomarker of oral cancer, being a sensitive signal for tobacco-carcinogen exposure. Methoxylated dietary flavonoids, e.g. DMF and 3',4'-DMF, may be potent chemoprotectants by direct inhibition of CYP1B1/1A1 function and/or their protein expression.

Introduction

Tobacco smoking is an important cause of human oral squamous cell carcinoma (SCC) (1,2). The presence of tobacco-related carcinogen–DNA adducts in oral squamous cells of smokers compared with non-smokers has strengthened the link between the development of oral cancer and smoking (3–5). Tobacco smoke components, including polycyclic aromatic hydrocarbons (PAHs), \( N \)-nitrosamines and aromatic amines, are thought to be potent carcinogens responsible for many cancers, most notably lung cancer (6). Metabolic activation of tobacco-associated PAHs like benzo[a]pyrene (BaP) and the DNA adducts formed have been postulated to be central to the carcinogenic process of PAH-induced cancers, including oral cancer (6,7). Two cytochrome P450 (CYP) enzymes, i.e. CYP1A1 and 1B1 are mainly involved in bioactivation of BaP, with a small contribution from CYP1A2, resulting in the formation of cellular DNA adducts (8,9).

In a previous study, we found that BaP induced CYP1A1 activity as well as protein and mRNA expression, which resulted in dramatically increased BaP–DNA adducts in human hepatoma (Hep G2) cells (10). In addition, we found that the dietary flavonoid 5,7-dimethoxyflavone (DMF) inhibited BaP-induced DNA binding through its effect on both, CYP1A1 expression as well as directly on this enzyme activity. These properties suggested DMF as an effective chemoprotectant in BaP-induced liver cancer. CYP1B1 was not an important contributor in BaP-induced liver carcinogenesis, neither was CYP1A2. A recent study found that tobacco smoke induced CYP1B1 mRNA in the aerodigestive tract tissues of mice, in particular of tongue and esophagus, suggesting a key role of CYP1B1 in tobacco-induced carcinogenesis in the aerodigestive tract (11). However, the role of CYP1A1 was never investigated in this study, nor was that of CYP1A2. Also, the protein expressions of these three isoforms were not examined.

In the present study, we investigated the effects of BaP on DNA binding as well as on CYP1A1, 1A2 and 1B1 activities and expression in the human oral epithelial SCC-9 cells, which are derived from the tongue (12). The BaP-induced DNA binding and CYP isoform expression were characterized in the presence and absence of DMF, in comparison with the polyphenols 3',4'-DMF and resveratrol. Furthermore, the direct effect of DMF on CYP1B1 recombiant enzyme and the uptake of DMF in the SCC-9 cells were also determined.

Materials and methods

Chemicals

DMF and 3',4'-DMF were purchased from Indofine Chemical Co. (Somerville, NJ). Ethoxyresorufin, resorufin, resveratrol and Williams’ medium E were obtained from Sigma Chemical Co. (St Louis, MO). Fetal bovine serum was obtained from Atlas Biologicals (Norcross, GA). [\( ^{3} \)H]BaP (76 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ).

Cell culture and treatment

Human oral SCC-9 cells obtained from American Type Culture Collection (Rockville, MD) were maintained in DMEM/F12 with 10% fetal bovine serum, hydrocortisone and penicillin/streptomycin solution in a humidified 37°C incubator with 5% carbon dioxide. After seeding in six-well plates for 2–3 days, 90% confluent cells (passages 15–30) were treated with 1 \( \mu \)M BaP...

Abbreviations:

AhR, aryl hydrocarbon receptor; BaP, benzo[a]pyrene; bDNA, branched DNA; DMF, 5,7-dimethoxyflavone; DMSO, dimethyl sulfoxide; EROD, ethoxyresorufin O-deethylolation; HBSS, Hanks’ balanced salt solution; PAH, polycyclic aromatic hydrocarbon; SCC, squamous cell carcinoma.

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in the presence or absence of DMF, 3′,4′-DMF or resveratrol in growth medium for the times and concentrations indicated in the figure. Vehicle dimethyl sulfoxide (DMSO; 0.1% of final volume) was used as a control in all experiments.

BaP–DNA binding formation

The binding of BaP to cellular DNA was measured using a method described previously (13,14). SCC-9 cells in six-well plates were treated with 1 μM [3H]BaP (10 μCi/ml medium) for 30 min (control) to 72 h. In the inhibition experiments, the cells were cotreated with 1 μM [3H]BaP and 20 μM DMF for 24 h. After treatments, the cell layers were washed with 0.9% saline and lifted off the plastic with lysis buffer (10 mM Tris, 1 mM EDTA and 0.14 M NaCl) and pelleted. The cell pellets were then lysed in swivel buffer (100 mM HEPES, 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA and 0.1 mM EGTA) and spun down to get crude nuclear pellets. The nuclei were purified by centrifugation through a 30% sucrose cushion. Nuclear protein and RNA were digested with proteinase K and RNase, and the samples were extracted repeatedly with phenol–chloroform. DNA was then precipitated and washed with ice-cold ethanol. The quantity and purity of DNA in the dissolved pellet were measured by ultraviolet spectroscopy and the amount of [3H]BaP bound to DNA was quantified by liquid scintillation spectrometry.

Ethoxyresorufin O-deethylation (EROD) assay

SCC-9 cells grown in 100 mm dishes were treated with 1 μM BaP and/or DMF, 3′,4′-DMF or resveratrol in growth medium, as indicated in the figure legends. Following the treatments, the cells were washed with fresh medium and incubated with 0.6 μM ethoxyresorufin for 120 min in the presence of salicylamide to inhibit conjugation enzymes (15,16). The formation of resorufin was measured in the cell culture medium by fluorometry with excitation at 530 nm and emission at 590 nm. The results were adjusted for the amount of cellular protein in each well, as measured by the Lowry assay (17).

Western blot analysis of CYP1A1, 1A2 and 1B1

SCC-9 cells grown in 100 mm dishes were treated with 1 μM BaP for 0, 2, 6, 24 and 48 h, or with 1 μM BaP in the presence or absence of 20 μM DMF, 3′,4′-DMF or resveratrol for 24 h. After these treatments, the cells were washed and scraped into tubes, resuspended in Tris/EDTA buffer with protease inhibitors and sonicated. The microsomal fraction was isolated by differential centrifugation at 4°C at 10 000 and 100 000 g. Microsomal pellets were resuspended in Tris/sucrose with protease inhibitors. After denaturing with sample buffer (+β-mercaptoethanol), the microsomal proteins (24 μg) were separated by electrophoresis on 10% NuPAGE Novex Tris gels (Invitrogen, Carlsbad, CA), transferred to nitrocellulose and blocked with 5% milk in 0.1% TBST (Tris-buffered saline with 0.1% Tween-20) for 3 h. The membranes were incubated overnight with primary antibodies to CYP1A1 (rabbit anti-trout 1A from Biosense Laboratories, Bergen, Norway, specific for human 1A1), CYP1A2 (rabbit anti-human 1A2 from Research Diagnostics, Flanders, NJ) or CYP1B1 (rabbit anti-human 1B1, BD Gentest, Woburn, MA), washed with 0.1% TBST, incubated with secondary antibodies (goat anti-rabbit IgG peroxidase conjugate, BD Gentest, Woburn, MA), washed and incubated with chemiluminescent substrate (KPL, Gaithersburg, MA) and exposed to ECL film. Baculovirus-expressed human CYP1A1, CYP1B1 and CYP1A2 (supernatons) were used as positive or negative control in the same experiments (BD Gentest, Bedford, MA). All blots were done at least three times.

CYP1A1 and 1B1 mRNA analysis

SCC-9 cells cultured in 96-well plates were treated with BaP and/or DMF, 3′,4′-DMF or resveratrol as described above for western blot analysis. The quantitative detection of CYP1A1 and 1B1 mRNA in cells lysed used branched DNA (bDNA) technology (18) with primers for human CYP1A1, CYP1B1 and GAPDH (QuantGene kits, Genospectra Co., Fremont, CA). Briefly, the target mRNA was captured in coated microwells and amplified with branched oligonucleotide probes with covalently attached alkaline phosphatase. After the addition of chemiluminescent substrate, the luminescence, as measured with a plate reader in the luminescence mode, was directly proportional to the amount of target mRNA. Six wells were used for each treatment, with each sample normalized to its GAPDH mRNA content.

Inhibition of recombinant CYP1B1 activity

Recombinant CYP1B1 (BD Gentest, Bedford, MA) (1.67 nM) in 0.1 M sodium phosphate buffer (pH 7.4) containing 10 mM MgCl2, was incubated with ethoxyresorufin (0.1, 1 and 2 μM) and DMF (0, 0.1 and 1 μM), dissolved in DMSO, final concentration 0.1%. The reaction (500 μl) was initiated with 1 mM NADPH, and stopped by adding 500 μl ice-cold methanol, after a 15 min incubation at 37°C. The resorufin fluorescence was measured as described above.

Time-dependent uptake of DMF in the SCC-9 cells

SCC-9 cells were grown in complete medium in six-well plates until confluent, then washed twice with warm Hanks’ balanced salt solution (HBSS) and incubated twice with warm HBSS (2 ml/well) for 30 min. The cells were incubated with 1 ml of 20 μM DMF in HBSS for 5 s to 30 min at 37°C and rinsed three times with ice-cold saline (0.9%). DMF was extracted by shaking the plates twice for 10 min with 1 ml of methanol (19). The combined extracts were evaporated to dryness under N2 gas and reconstituted with 500 μl of mobile phase. The uptake of DMF was analyzed by reverse-phase HPLC, using a Millennium HPLC system (Waters Corp., Milford, MA) with a photodiode array detector (Model 996) and a Symmetry C18 column (3.9 × 150 mm, Waters). The mobile phase consisted of 55% methanol in 0.3% trifluoroacetic acid with a flow rate 0.9 ml/min with detection at 260 nm. Quantification was done by peak area measurement in comparison with standard 20 μM DMF.

The results were adjusted for the amount of cellular protein in each well, as measured by the Lowry assay (17).

Data analysis

Statistical differences between different treatments were determined using two-tailed unpaired ANOVA with a multi comparison (Dunnett) post-test (InStat). The results were expressed as means ± SD for at least triplicate determinations. The apparent inhibitory constant (Ki) value was calculated by a Dixon plot (20).

Results

Effects of BaP on BaP–DNA binding, EROD activity, and CYP1A1, 1B1 and 1A2 expression

The formation of carcinogen–DNA adducts is a crucial step in the carcinogenic process. We measured the levels of the DNA adducts after a low concentration (1 μM) of BaP treatment from 0.5 to 72 h in the SCC-9 cells (Figure 1A). BaP-induced DNA adduct formation in a time-dependent manner, with a 40-fold increase at 72 h compared with 0.5 h. This not only demonstrated that BaP was metabolically activated in the SCC-9 cells, but also that BaP accelerated its own binding, most notably from 6 to 24 h. Consistent with this observation, the O-deethylation of ethoxyresorufin in the SCC-9 cells was significantly increased after treatment with BaP from 2 through 48 h compared with vehicle control, reaching the highest level after 24 h (3-fold increase compared with baseline) (Figure 1B).

To explore which CYP isoforms were responsible for the increased BaP–DNA binding and EROD activity after BaP treatment, we isolated microsomes from the SCC-9 cells treated with 1 μM BaP for 2–48 h and ran western blot analysis using antibodies to CYP1A1, 1B1 and 1A2 with β-actin as loading control (Figure 1C). No cross-reactivity among these three antibodies was found in the present study. None of the three—CYP1A1, 1B1 1A2—was constitutively expressed in the SCC-9 cells. BaP-induced CYP1B1 protein expression as early as after 6 h treatment, with the strongest expression at 24 h. Consistent with this finding and especially the catalytic activity data (Figure 1B), the bDNA assay showed a significant increase in CYP1B1 mRNA levels by BaP treatment as early as after 24 h, with the highest expression at 24 h (Figure 1D).

In agreement with our previous study that CYP1A1 was induced by BaP in Hep G2 cells (10), 1 μM BaP also induced CYP1A1 protein expression in the SCC-9 cells (Figure 1C), with faint bands following 24 and 48 h treatment. Consistent with this, the mRNA levels of CYP1A1 were also induced by 1 μM BaP treatment (Figure 1D). However, after BaP treatment, the levels of CYP1B1 protein as well as mRNA were much higher than those of CYP1A1.

CYP1A2 protein could not be detected in the SCC-9 cells, even after BaP treatment (Figure 1C).
Effects of DMF on BaP-induced DNA binding, EROD activity and cytochrome P450 1A1/1B1 expression

In our previous study, we found that one naturally occurring flavonoid, DMF, had a dramatically inhibitory effect on BaP-induced DNA binding and CYP1A1 expression in Hep G2 cells (10). The effect of DMF on BaP-induced DNA binding in the SCC-9 cells was determined similarly. The BaP–DNA adduct formation after treatment of SCC-9 cells with 1 mM BaP for 24 h was significantly lower (70% inhibition) following cotreatment with 20 mM DMF (Figure 2).

To examine the inhibitory mechanism of DMF on BaP–DNA binding, the EROD activity was determined after treatment of SCC-9 cells with 1 mM BaP for 24 h was significantly lower (~70% inhibition) following cotreatment with 20 μM DMF (Figure 2).

The effects of DMF on BaP-induced CYP1B1 and 1A1 protein expression and mRNA levels were further determined in the SCC-9 cells. Consistent with our previous results in Hep G2 cells (10), DMF (20 μM, 24 h) inhibited BaP-induced CYP1A1 expression both at the protein and the mRNA levels in SCC-9 cells (Figure 3B and C). However, DMF did not significantly affect BaP-induced CYP1B1 protein or mRNA levels, the latter determined both by the bDNA assay and by RT–PCR (data not shown).

Effect of DMF on recombinant CYP1B1 activity

Based on the observations in Figure 3, DMF might directly interact with the CYP1B1 protein and inhibit its enzymatic activity. The direct effect of DMF on CYP1B1 was verified using the recombinant enzyme. DMF was a potent inhibitor of recombinant CYP1B1 as determined by the EROD assay, with a Kᵢ value of ~0.58 μM, as shown in a Dixon plot in Figure 4A. DMF appeared to show a mixed-type of inhibition indicated by the double reciprocal plot (Figure 4B).
Time-dependent uptake of DMF in the SCC-9 cells

As shown in Figure 4, DMF was a potent direct inhibitor of CYP1B1 with a low $K_i$ value. However, it was not clear whether DMF could enter oral cells in humans. The time-dependent uptake of DMF into the SCC-9 cells was therefore determined (Figure 5). Rapid and extensive uptake of DMF was found after 20 μM DMF treatment, with a high initial uptake of 2600 pmol/mg protein at 1 min. The maximum uptake was reached at 10 min. The accumulation factor, i.e., cellular concentration versus the concentration in the uptake buffer, was ~20. Thus, the cellular concentration of DMF was as high as 400 μM.
Effects of other polyphenols on BaP-induced EROD activity and CYP1B1 mRNA and protein expression

To further explore the chemopreventive effects of the dietary polyphenols and the mechanisms involved, we determined the effects of 3',4'-DMF and resveratrol on BaP-induced CYP1B1 activity and expression. Interestingly, these two compounds have been demonstrated to have effects on CYP1A1 expression in other cell types (21,22). In the present study, 20 μM 3',4'-DMF significantly inhibited BaP-induced EROD activity, with ~70% inhibition (Figure 6A). In contrast to DMF, 3',4'-DMF also inhibited BaP-induced CYP1B1 mRNA levels (Figure 6B) and protein expression (Figure 6C). Surprisingly, 20 μM resveratrol did not significantly affect BaP-induced EROD activity or CYP1B1 mRNA expression and only slightly affected CYP1B1 protein expression.

Discussion

Exposure to PAHs is associated with the development of oral cancer in humans, with the most commonly affected sites being tongue and floor of the mouth (2,5). Therefore, human oral epithelial SCC-9 cells, which are SCC cells derived from the tongue (12), were used as the model cells in the present study.

In these cells, a low concentration of BaP (1 μM), a prototypical environmental PAH, dramatically increased BaP–DNA binding in a time-dependent manner with as much as a 40-fold increase at 72 h, compared with 0.5 h. This increase of BaP–DNA binding in the SCC-9 cells is consistent with the higher levels of PAH–DNA, including BaP–DNA, adducts in human oral (3–5) as well as non-oral tissues from smokers compared with non-smokers (23–25). Furthermore, the time-dependent increase in BaP–DNA binding, in particular between 6 and 12 h, is consistent with the results of Figure 5, which shows that DMF was effectively transported into the cell in a time-dependent manner (Figure 6).
CYP1A1 and 1B1 are the main enzymes involved in the activation of BaP (9,26). It was, thus, reasonable to assume that CYP1A1 and/or CYP1B1 were induced by BaP in the SCC-9 cells. However, 1 µM BaP only showed a moderately inductive effect on the EROD activity (3-fold increase at 24 h, using a 2 h incubation of cells with ethoxyresorufin rather than 30 min incubation, as in most cases), a marker reaction mainly of CYP1A1. The weak effect of BaP on EROD activity, as compared with the DNA binding, suggested that the induction might be mainly due to CYP1B1 for which ethoxyresorufin is a rather poor substrate. As CYP1A2 is considered to play much less of a role in BaP bioactivation than CYP1A1 and 1B1 (9,26), we assumed that CYP1B1 is more important than CYP1A1 and 1A2 in BaP-induced DNA binding in the oral SCC-9 cells.

Further experiments with the bDNA assay provided direct evidence for this assumption. In these experiments, 1 µM BaP treatment resulted in induction of CYP1B1 mRNA to much higher levels than those of CYP1A1 and no detectable effect on CYP1A2. Western blot analysis confirmed these observations with higher levels of CYP1B1 than of CYP1A1 in the SCC-9 cells. These findings are consistent with one very recent study showing tobacco smoke-induced CYP1B1 mRNA in the human bronchial mucosa and the aerodigestive tract of mice, in particular in the tongue and esophagus (11). The latter study did, however, not examine the effect on CYP1A1 or CYP1A2 mRNA, neither did it examine the catalytic activity or the levels of any of these proteins. Our results are also in agreement with a study showing that levels of CYP1B1 protein were increased in human lung tissue from smokers and ex-smokers versus non-smokers (27). However, the results are very different from our previous study showing that CYP1A1, not CYP1B1, was induced by 1 µM BaP in human Hep G2 cells under very similar conditions (10). Interestingly, similar observations that CYP1B1, but not CYP1A1, mRNA induced by environmental dioxins was found in human white cells in vivo (28). Thus, the constitutive and PAH-inducible expressions of human CYP1A1 and 1B1 appear to be highly cell-type-specific.

CYP1A1 and 1B1 have been known to be regulated by the ligand-activated aryl hydrocarbon receptor (AhR), with cross-talk of AhR and estrogen receptor regulation for CYP1B1 (28). Thus, the constitutive and PAH-inducible expressions of human CYP1A1 and 1B1 appear to be highly cell-type-specific.

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shown to act as an AhR antagonist in human hepatoma (22,49) and bronchial epithelial cells (50), but, interestingly, inhibited CYP1A1 via an AhR-independent post-transcriptional pathway in breast cancer cells (51). In an extension of the study by Berge et al. (52) from human bronchial epithelial cells to an in vivo model, resveratrol was without effects on either BaP–DNA binding or CYP1A1 and 1B1 expression. In our present study in the SCC-9 cells, 3′,4′-DMF behaved as an AhR antagonist, decreasing both the mRNA and protein of CYP1B1. Surprisingly, resveratrol was devoid of any effect, even including the EROD assay, as in the study above in mice (52). Taken together, these observations demonstrate that the effect of the dietary polyphenols on BaP bioactivation depends very much on the cell type. However, if taking chemical structure as well as cell context into consideration, selected polyphenols have the potential to act as very powerful inhibitors at the initiation stage in preventing oral carcinogenesis.

In summary, the present study demonstrates that tobacco-associated BaP-induced DNA binding of BaP depended highly on CYP1B1, and also CYP1A1, in human oral SCC-9 cells, although the levels of the mRNA/protein highly favoured CYP1B1. These results are dramatically different from our previous study in human liver Hep G2 cells, showing that BaP almost exclusively induced CYP1A1 expression. Inducible CYP1B1 might be an early biomarker of oral cancer, being a sensitive signal for tobacco-carcinogen exposure. A natural product, DMF, which was highly accumulated in oral epithelial cells, was found to inhibit BaP-induced DNA binding in the SCC-9 cells. The mechanism of the inhibitory effect of DMF is most likely a direct inhibition of the CYP1B1 protein and downregulation of CYP1A1 transcription. In extended studies, we demonstrated that a structural analog of DMF, i.e. 3′,4′-DMF, downregulated both CYP1B1 mRNA and protein expression, whereas resveratrol, being shown to be effective in other cell types, had no activity in the SCC-9 cells. In vivo studies to verify the chemopreventive effects of DMF, 3′,4′-DMF and other dietary polyphenols on oral carcinogenesis are now in progress.

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