Inhibition of benzo[a]pyrene-activating enzymes and DNA binding in human bronchial epithelial BEAS-2B cells by methoxylated flavonoids

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Cigarette smoking is a major risk factor in lung carcinogenesis via carcinogens such as polycyclic aromatic hydrocarbons (PAHs) and nitrosamines. In this study, we used benzo[a]pyrene (BaP) as the classic PAH compound and BEAS-2B cells, a model of normal human bronchial epithelial cells, to investigate whether 5,7-dimethoxyflavone (5,7-DMF) and 3',4'-DMF compared with resveratrol (RV) have chemopreventive properties in this cancer. Exposure of BEAS-2B cells to [1H]BaP (1μM) showed increasing binding to DNA up to 72 h of exposure, about 20-fold higher than that at 0.5 h exposure. BaP exposure also increased both CYP1A1/1B1 and microsomal epoxide hydrolase (mEH) enzyme activities with a maximum 10-fold increase at 48 h. BaP induced CYP1A1 protein and mRNA levels maximally after 48 h. In contrast, although CYP1B1 mRNA was rapidly induced, its protein expression showed a very poor response. Simultaneous treatment with BaP and 5,7-DMF, 3',4'-DMF or RV for 48 h inhibited BaP-DNA binding by ≥75%, with 3',4'-DMF being the most effective. 5,7-DMF affected CYP1A1 mRNA levels only modestly, whereas 3',4'-DMF was a potent inhibitor. The catalytic activity of CYP1A1/1B1 was reduced over 95% after exposure to 5,7-DMF, 3',4'-DMF or RV, most effectively by 3',4'-DMF. BaP-induced mEH activity was not affected by treatment with 5,7-DMF, but was significantly inhibited by 3',4'-DMF. In contrast, mEH activity was notably increased by RV. Most importantly, western blotting showed all three polyphenols dramatically reducing BaP-induced CYP1A1 protein expression. Both 5,7-DMF and 3',4'-DMF demonstrated very high, about 40-fold, accumulation in BEAS-2B cells. In summary, BaP exposure results in a high level of DNA binding in BEAS-2B cells, which is mainly mediated by induction of CYP1A1 protein, just as in the human lung. Two methoxylated dietary flavonoids with highly specific effects on BaP bioactivation block this DNA binding and CYP1A1 protein expression as effectively as RV, thus making them potential chemopreventive agents for BaP-induced lung carcinogenesis.

Introduction

Benzo[a]pyrene (BaP) is a prototypical polycyclic aromatic hydrocarbon (PAH) formed by the incomplete combustion of many organic materials and as such is ubiquitously present in our environment. Exposure to BaP results from sources such as charbroiled meats, engine exhaust and, most importantly, cigarette smoke (1), and has been linked to cancers mainly of the aerodigestive tract. Cigarette smoking, in particular, is a major risk factor in lung carcinogenesis. The poor prognosis for most lung cancer patients, in spite of much improved programs for early detection as well as introduction of many new chemotherapeutic drugs, has pointed to a great need for preventive measures, including chemopreventive measures, in this disease (1–3).

In order for chemicals such as BaP to bind to DNA, and thus initiate the carcinogenic process, these compounds need bioactivation by enzymes, such as the cytochrome P450 (CYP) 1A1 and/or 1B1 and microsomal epoxide hydrolase (mEH) (1,4,5), resulting in the formation of the ultimate carcinogen benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE).

An increasingly recognized biochemical approach is to attempt to prevent this disease at the initiation stage, potentially using dietary chemicals (1–3). Many chemopreventive approaches have been suggested, including β-carotene, which, owing to its antioxidant properties, held much promise until a series of chemopreventive trials demonstrated that it actually increased lung cancer incidence and mortality in smokers (3,6). It appears that β-carotene is an effective inducer of CYPs expressed in the human lung, most probably including the carcinogen-activating CYP1A1 (7). These events have been summarized recently to encourage continued work (8).

These observations emphasize the importance of CYP1A1, and possibly another bioactivating enzyme, namely CYP1B1, in human lung carcinogenesis. As a matter of fact, research in cultured normal human bronchial epithelial cells suggests that CYP1B1, at least at the mRNA level, may be the most important (9). This may be similar in lung tissue (10). In addition, the role of mEH together with the CYP1A1/1B1 isoforms has only been superficially investigated in bronchial epithelial cells (9).

Studies focusing on specific ways to inhibit, or preferentially inactivate, these enzymes in bronchial epithelial cells, the target site for smoke-induced carcinogenesis in human lung, would seem like an important mission. In fact, dietary polyphenols have been thought to be protective against lung cancer (11–13). Although previous cellular studies have indicated resveratrol (RV) to be a promising compound (9,14), a more recent study (15) demonstrated lack of effect in the animal model.

A study in our laboratory (16) identified a flavonoid, 5,7-dimethoxyflavone (5,7-DMF), which, compared to a variety of other flavonoids, was an unusually effective inhibitor of CYP1A1. Similarly, an analog, 3',4'-dimethoxyflavone.

Abbreviations: BaP, benzo[a]pyrene; BPDE, benzo[a]pyrene-7,8-diol-9,10-epoxide; CYP, cytochrome P450; 3',4'-DMF, 3',4'-dimethoxyflavone; DMSO, dimethyl sulfoxide; EROD, ethoxyresorufin-O-deethylase; mEH, microsomal epoxide hydrolase; PAH, polycyclic aromatic hydrocarbon; RV, resveratrol; TBST, Tris-buffered saline/Tween.

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(3′,4′-DMF) was an effective inhibitor of CYP1B1 (17). Both 5,7-DMF and 3′,4′-DMF have the potential to have adequate bioavailability (17). It is possible that exposure to these flavonoids may reduce the risk of BaP-DNA adduct formation by reducing the expression and/or the activity of BaP-bioactivating enzymes, thus, potentially decreasing adverse biological effects, such as mutagenesis and tumor formation.

The present study used cultured BEAS-2B cells, thought to be a good model of normal bronchial epithelial cells (18–20), to determine the relationship between DNA binding of BaP and its bioactivation by CYP1A1/1B1 and mEH. Of particular importance was to determine the catalytic activities and mRNA as well as protein expression of these enzymes. Further, we investigated the effects of 5,7-DMF, a major constituent of the leaves of Piper canum (21) and Leptospermum scoparium (22), and 3′,4′-DMF, a constituent in leaves of Primula veris L. (23), in comparison with RV, a polyphenol found in grapes, berries and nuts (24), on the DNA binding and expression of carcinogen-metabolizing enzymes and the possible mechanisms involved.

Materials and methods

Chemicals

5,7-DMF and 3′,4′-dimethoxyflavone (3′,4′-DMF) were purchased from Indofine Chemical Co. (Somerville, NJ). RV, 7-ethoxyresorufin, resorufin, BaP, Dl-dithiothreitol and bovine serum albumin were obtained from Sigma Chemical Co. (St Louis, MO). Phenol : chloroform : isoamyl alcohol (24), 5,7-DMF, a constituent in leaves of Primula veris L. (23), in comparison with RV, a polyphenol found in grapes, berries and nuts (24), on the DNA binding and expression of carcinogen-metabolizing enzymes and the possible mechanisms involved.

Cell culture and treatment

Human bronchial epithelial cells (BEAS-2B) obtained from American Type Culture Collection (Rockville, MD) were grown in serum-free bronchial epithelial cell growth medium (BEGM, Cambrex, Walkersville, MD) with 1% penicillin-streptomycin in uncoated flasks in a humidified atmosphere with 5% carbon dioxide at 37°C. At 3–5 days post-seeding, the cells were treated at passage 10–30. While the basal levels of BaP-DNA binding and enzyme activity varied somewhat among different passages, the magnitude of effects remained the same.

BaP-DNA binding

DNA binding was measured as described previously (16,25) in cells grown in 6-well plates incubated with 1 μM [3H]-BaP for various times or with 1 μM [3H]-BaP +/− 25 μM 5,7-DMF, 3′,4′-DMF or RV for 48 h. Medium was changed every 24 h. The cells were washed with 0.9% saline and lifted off the plastic with lift buffer (10 mM Tris, 1 mM EDTA and 0.14 M NaCl) and pelleted. Cell pellets were subsequently lysed in swell 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 0.1% Triton X-100, and then centrifuged to obtain crude nuclear pellets. Nuclei were purified by centrifugation through a 30% sucrose cushion. Nuclear protein and RNA were digested with protease K and RNase, and the samples were extracted three times with phenol : chloroform : isooxyl alcohol. DNA was precipitated with sodium acetate and cold ethanol, and the pellet was washed with ethanol and dissolved in water. DNA quantity and purity were estimated spectrophotometrically at 260/280 nm. The amount of [3H]-BaP bound to DNA was quantified by liquid scintillation spectrometry.

Catalytic activities

CYP1A1/1B1/1A2. BEAS-2B cells were treated with 1 μM BaP and/or 25 μM of the polyphenols in 6-well plates, as described in the respective figure legends, with fresh medium change every 24 h. Following treatment, cells were washed twice with fresh medium and incubated with 0.6 μM 7-ethoxyresorufin for 30 min in the presence of salicylaldehyde to inhibit conjugation enzymes (26). Ethoxyresorufin-O-deethylayation (EROD) was measured fluorometrically as resorufin formation in the cell culture medium with excitation/emission wavelengths of 530/590 nm. Resorufin was used as standard (27). The results were normalized for the amount of cellular protein in each well, as determined according to Lowry et al. (28) with bovine serum albumin as standard.

Epoxide hydrolase. BEAS-2B cells were treated with 1 μM BaP and/or 25 μM of the polyphenols in 6-well plates, as described in the respective figure legends, with fresh medium change every 24 h. Following treatment, cells were washed twice with fresh medium and intact cells were incubated with 20 μM BaP-7,8-epoxide for 30 min. Formation and release of BaP-7,8-diol into the medium was measured fluorometrically (29) with excitation/emission wavelengths of 360/420 nm. A concentration curve using BaP-7,8-dihydriodiol was used as standard. The results were normalized for the amount of protein in each well.

Western analyses

BEAS-2B cells grown in 10 cm dishes were treated with 1 μM BaP for 0.5, 2, 6, 24 or 48 h, or with 1 μM BaP +/− 25 μM 5,7-DMF, 3′,4′-DMF or RV for 48 h. After treatment, cells were washed and scraped, and cells from 12 dishes per treatment were combined, resuspended in Tris/EDTA buffer with protease inhibitors and then sonicated on ice for 3 x 10 s. Microsomal fractions were generated through differential centrifugation at 8000 and 100 000 g at 4°C. Microsomal pellets were suspended in Tris/sucrose buffer with protease inhibitors. Whole cell protein content was determined by the Lowry method and aliquots were denatured with Dl-dithiothreitol and NuPAGE® LDS sample buffer at 70°C for 10 min, and then separated by electrophoresis on 10% NuPAGE® Novex Bis–Tris gels (Invitrogen, Carlsbad, CA) under reducing conditions, transferred to nitrocellulose membranes and blocked in 5% non-fat milk in 0.1% Tris-buffered saline/Tween 20 (TBST) for 3 h. The membranes were incubated overnight with primary antibodies specific for human CYP1A1 (rabbit anti-trout CYP1A, Cayman Chemical Co., Ann Arbor, MI), or CYP1B1 (rabbit anti-human IB1, BD Gentest, Woburn, MA) or mEH (goat anti-rabbit mEH, Oxford Biomedical Research, Oxford, MI), in 5% non-fat milk/TBST, washed three times with 0.1% TBST, incubated with secondary antibodies (goat anti-rabbit IgG peroxidase conjugate, BD Gentest, or rabbit anti-goat IgG peroxidase conjugate, Chemicon, Temecula, CA), washed with 0.1% TBST, incubated with chemiluminescent substrate (KPL, Gaithersburg, MA) and exposed to ECL Hyperfilm (Amersham Biosciences, Piscataway, NJ). Baculovirus-expressed human CYP1A1 and IB1 supersomes (0.25–0.40 μg protein corresponding to 25–40 fmol) and human mEH microsomes prepared from a human B lymphoblastoid cell line (BD Gentest) were used as positive or negative controls in the same experiments. The membranes were then stripped and checked for equal protein loading by measuring β-actin levels as above, using an antibody from Sigma at a 1 : 5000 dilution.

CYP1A1 and IB1 mRNA analyses

BEAS-2B cells were seeded in 96-well plates at 5000 cells/well. At 4–5 days post seeding, the cells were treated with 1 μM BaP for various times, or with and without 25 μM 5,7-DMF or 3′,4′-DMF for 48 h. CYP1A1 and CYP1B1 mRNA levels of BEAS-2B cells were quantified with the branched DNA (bDNA) technology (16,30) using QuantiGene kits (Genospectra Co., Fremont, CA) with primers for human CYP1A1 or CYP1B1 and GAPDH. Briefly, the target mRNA was captured in coated microwells and amplified with branched oligonucleotide probes with covalently attached alkaline phosphatase. After adding the chemiluminesogenic substrate, the luminescence, proportional to the amount of target mRNA, was assayed with a plate reader. Six wells were used per treatment, and in each sample, the CYP1A1 or IB1 mRNA expression was normalized to its respective GAPDH mRNA content.

Cellular uptake of 5,7-DMF, 3′,4′-DMF and RV

BEAS-2B cells were incubated with 25 μM 5,7-DMF, 3′,4′-DMF or RV in complete medium for various times. The cells were then washed and extracted with methanol as described previously (31). The samples were analyzed by reverse-phase HPLC with 268 nm or 338 nm UV detection, respectively. The uptake was adjusted for the amount of cellular protein in each well (28).

Statistical analyses

Results were expressed as means ± SEM of at least three experiments. Comparisons among means were made using two-tailed paired ANOVA (parametric or non-parametric) followed by Dunnett’s or Dunn’s Multiple Comparison Test (InStat, v. 2.00). The level of significance for all experiments was set at α = 0.05.
Inhibition of BaP-activation by methoxylated flavonoids

Results

**Enzymatic activation of BaP and its binding to DNA**

Treatment of normal human bronchial epithelial BEAS-2B cells with 1 μM BaP for up to 72 h resulted in a dramatic time-dependent binding of 3H-BaP to DNA (Figure 1A), with the highest level of binding at 72 h (about a 20-fold increase, compared with the 0.5 h time-point). To be able to understand how this increasing DNA binding occurred, we measured the enzyme activities thought to be responsible for BaP bioactivation.

Treatment of the BEAS-2B cells with 1 μM BaP produced a large increase in CYP1A1/1A2/1B1 activity using the EROD assay (Figure 1B). Maximum induction (about a 10-fold increase) occurred at 24–72 h. As BaP is a much better substrate for CYP1A1 than 1B1 (5), the dramatic induction of EROD activity would suggest that CYP1A1 was induced. The catalytic activity of mEH, as determined by formation of the fluorescent BaP-7,8-dihydrodiol from BaP-7,8-oxide, also demonstrated a time-dependent increase upon treatment with BaP, with the highest levels about 10-fold higher than vehicle controls, found at 72 h (Figure 1C), a time-point coinciding with the maximum DNA binding (Figure 1A).

**Effect of BaP on mRNA and protein expression of bioactivating enzymes**

In preliminary experiments, the mRNA levels of CYP1A1, CYP1A2, CYP1B1 and mEH in control and BaP-treated cells were determined by semiquantitative RT–PCR. CYP1A2 mRNA was not detectable and not pursued further. mEH mRNA was clearly detectable, but there was no effect of BaP treatment (data not shown). The effect of BaP on the CYP1A1 and 1B1 mRNA levels were pursued further, using the quantitative bDNA assay (16).

CYP1A1 mRNA was expressed at a very low level in the DMSO-control BEAS-2B cells. Treatment of the cells with 1 μM BaP significantly induced the CYP1A1 mRNA at 2 h, but there was a further significant increase after 24–48 h with as much as a ~75-fold increase compared with DMSO-controls (Figure 2A). In sharp contrast, CYP1B1 mRNA was highly expressed in the DMSO-treated cells and was significantly (about 10-fold) induced after only 2 h of treatment, remaining elevated, although at somewhat lower level, through 48 h (Figure 2A).

To determine the effect of BaP on protein expression of these catalytic enzymes, we isolated the microsomal fractions from cells treated with 1 μM BaP for 2–48 h as well as DMSO-treated control cells. Western blotting analysis showed no constitutive expression of either CYP1A1 or CYP1B1 protein in the BEAS-2B cells. However, treatment with 1 μM BaP induced CYP1A1 protein expression dramatically following 24 h and, in particular, following 48 h treatment (Figure 2B). CYP1B1 protein could only be detected, at a very low level, after 48 h. Figure 2B displays one of three experiments. A low constitutive protein
expression of mEH in BEAS-2B cells, compared with hepatocytes, was not affected by treatment with BaP (data not shown).

**Effects of methoxylated flavones compared with RV on BaP-DNA binding and activation**

On the basis of previous observations (16), 5,7-DMF seemed to be a good candidate for blocking the BaP binding to cellular DNA compared with most other flavonoids. To explore the potential chemopreventive function of 5,7-DMF and 3',4'-DMF compared with RV in the BEAS-2B cells, the effect of these polyphenols on the BaP–DNA binding was determined. The cells were treated with 1 µM [3H]BaP for 48 h in the presence of 25 µM concentrations of polyphenol or with BaP alone. At these concentrations neither BaP nor the polyphenols were cytotoxic, as reflected in only minor differences in the amount of protein per well compared with vehicle controls. Also, neither 1 µM BaP nor 25 µM of the polyphenols affected the morphology of the cells, or caused the cells to detach. All three polyphenols decreased the BaP–DNA binding, with ~75% in the presence of 5,7-DMF or RV and >90% inhibition in the presence of 3',4'-DMF (Figure 3A).

The effects of 5,7-DMF, 3',4'-DMF and RV on BaP-induced CYP1A1/1B1 catalytic activity, as measured by the EROD assay, in BEAS-2B cells were investigated. The cells were treated with 1 µM BaP plus 25 µM polyphenol or BaP alone for 48 h. As shown in Figure 3B, both methoxylated flavonoids as well as RV effectively inhibited the BaP-induced EROD activity at 97% (5,7-DMF), 96% (RV) and >99% (3',4'-DMF).

As shown in Figure 3C, the addition of 25 µM 5,7-DMF to 1 µM BaP had no effect on the catalytic activity of mEH, as determined by the formation of BaP-7,8-dihydrodiol from BaP-7,8-epoxide. However, the addition of 3',4'-DMF resulted in a significant 40% inhibition. In contrast, RV resulted in a significant ~50% increase of BaP-induced mEH activity compared with BaP alone.

**Effect of methoxylated flavonoids compared with RV on BaP-induced mRNA and protein levels of bioactivating enzymes**

Simultaneous treatment with 25 µM 5,7-DMF and 1 µM BaP for 48 h resulted in a modest, non-significant reduction of CYP1A1 at the mRNA level (Figure 4A) compared with BaP treatment alone. On the other hand, 3',4'-DMF was an extremely potent inhibitor of CYP1A1 mRNA, resulting in message levels even below the constitutive levels. RV has been shown previously to inhibit BaP-induced CYP1B1 mRNA expression in BEAS-2B cells (14). The mEH mRNA levels were not affected by treatment with BaP or flavonoids (RT–PCR data—not shown).

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**Figure 3.** Effect of methoxylated flavonoids and RV on BaP-induced DNA binding (A), catalytic activity of CYP1A1/1B1 (B) and catalytic activity of mEH (C) in BEAS-2B cells. Cells were treated with 1 µM BaP in the presence or absence of 5,7-DMF, 3',4'-DMF or RV (25 µM) for 48 h. Values are represented as means ± SEM (n = 3). Significantly different from BaP treatment, **P < 0.01; ***P < 0.001.

**Figure 4.** Effects of 5,7-DMF and 3',4'-DMF on BaP-induced mRNA expression (A), and effects of 5,7-DMF, 3',4'-DMF and RV on protein expression (B) of CYP1A1. BEAS-2B cells were exposed to DMSO or 1 µM BaP and/or 25 µM polyphenol alone for 48 h. Data are represented as means ± SEM (n = 3–6). Significantly different from respective BaP-induced treatment, **P < 0.001. Western blots were repeated three times with 75–100 µg protein per well, and β-actin was used as the loading control.
On the protein level, the BaP-induced CYP1A1 enzyme was effectively abolished by a 48 h co-treatment with 25 µM 5,7-DMF, 3',4'-DMF or RV (Figure 4B). None of these three polyphenols affected CYP1A1 protein on their own. The effect of the polyphenols on the CYP1B1 protein levels was not measured because of the extremely low levels present even after BaP treatment. No effect on protein expression of mEH by itself or in combination with 1 µM BaP treatment in BEAS-2B cells was observed (data not shown).

**Accumulation of 5,7-DMF, 3',4'-DMF and RV in BEAS-2B cells**

To test whether the two methoxylated polyphenols accumulated in the BEAS-2B cells, uptake studies were done with 25 µM 5,7-DMF, 3',4'-DMF and RV, with up to 60 min exposure to each polyphenol. An intracellular concentration of about 6000 pmol/mg protein was reached for 5,7-DMF and 3',4'-DMF at 60 min (Figure 5). The intracellular polyphenol content corresponded to an about 30- to 50-fold accumulation by the cells compared with the surrounding buffer. For comparison, the uptake of 25 µM RV resulted in about 2-fold higher cellular uptake compared with the flavones. No decline in 5,7-DMF concentrations was observed when the uptake study was extended to 24 h, and no metabolites or degradation products were observed in the HPLC tracings.

**Discussion**

Lung cancer, which is related to tobacco smoking, is the leading cause of cancer death in the Western world and accounts for nearly one-third of all cancer deaths in the United States. The incidence and mortality rate for lung cancer have continued to increase over the last 25 years (32). According to the American Cancer Society, an estimated 172,570 new cases of lung cancer will be diagnosed in the United States in 2005. Furthermore, an estimated 75% of these patients will die within 2 years, and epidemiological studies have shown that the 5-year survival rate of patients diagnosed with lung cancer is less than 15%. These numbers have not improved over the past 10 years. In addition to smoking cessation, the prevention of lung cancer through interactions with molecular events occurring at the cancer initiation stage has become one important focus. Chemoprevention via phytochemicals as a means of inhibiting chemically induced carcinogenesis is a potentially beneficial area of research (1–3).

Resveratrol (RV), a polyphenol present in grapes, red wine, peanuts and other dietary sources, is one such potentially cancer-preventive agent. In immortalized human bronchial epithelial BEAS-2B cells, thought to be a good model of the normal human bronchial epithelium (18), RV effectively inhibited BaP-induced CYP1A1 and 1B1 mRNA expression, BaP metabolism and BaP–DNA adduct formation, the latter using 32P-post-labeling (9,14). From these studies, RV appeared to have potential utility as a lung cancer chemopreventive agent. However, in a subsequent study, RV was shown to be inactive in an in vivo animal model (15), consistent with very poor bioavailability in animals (33) and also in humans (34). Clearly, there is a need for other non-toxic chemopreventive agents with better bioavailability.

Our present study expanded the BEAS-2B cell studies mechanistically to include time-courses for the effect of BaP on CYP1A1/1B1 catalytic activity, CYP1A1 and 1B1 mRNA expression, and DNA binding, the latter using radiolabeled BaP. Our study, importantly, also examined the CYP1A1 and 1B1 protein expression, both of critical importance considering function and for comparisons to the in vivo situation (35). In addition, we examined the effect of BaP on mEH, previously not considered critically important. The most important addition to the field was an examination of two new potential chemopreventive polyphenols, 5,7-DMF and 3',4'-DMF, compared with RV.

The dramatic increase in the BaP–DNA binding with increasing time of BaP exposure through 72 h in these cells is worth noting (Figure 1A). It clearly demonstrates that the molecular events preceding the BaP–DNA binding are rapid. Although it has been assumed that the bay region dihydrodiol epoxide formation is the dominant mechanism for covalent binding of BaP to DNA, other mechanisms contribute, in particular the formation of a radical cation by CYP peroxidase (36). However, this was beyond the scope of the present study. The greatest increase in DNA binding occurred from 0.5 to 6 and 24 h, suggesting an inductive response. This was also the time at which the greatest increase in EROD activity was seen (Figure 1B). This large increase in EROD activity is consistent with induction of CYP1A1 rather than CYP1B1 protein, as this assay is much more sensitive for the CYP1A1 isoform (37).

The basal mRNA levels were much higher for CYP1B1 than for CYP1A1, as observed previously (9). The time-courses for their induction by BaP were distinctly different. CYP1B1 mRNA showed a very early maximum induction response at 2 h after BaP treatment with the levels maintained at induced, albeit slightly lower, levels for 48 h (Figure 2A). For CYP1A1 mRNA there was also an early increase at 2 h, but a very slow further increase over the 48 h time-course. At 48 h its levels were similar to those of CYP1B1 mRNA. This difference suggests that CYP1B1 transcription is regulated differently than CYP1A1 in BEAS-2B cells. In this respect, it should be noted that CYP1B1 is uniquely regulated by cAMP in addition to the AhR (38).

At the protein level, the basal levels of both proteins were undetectable (Figure 2B). After BaP treatment, the CYP1A1 protein was strongly expressed, but not until after 24 h, and even more after 48 h, in parallel with maximum CYP1A1 mRNA expression (Figure 2A). Importantly, this also coincided with the EROD activity (Figure 1B). For CYP1B1,
the protein, after repeated efforts, could only be detected at a low level after 48 h treatment with BaP. This low and, compared with mRNA, delayed expression of CYP1B1 protein in BEAS-2B cells has no clear explanation. Highly complex mechanisms of transcriptional and post-transcriptional control of CYP1B1 expression have been described previously (39,40). In contrast, in human oral epithelial cells, identical treatment with BaP greatly induced CYP1B1 protein in parallel with its mRNA (17), indicating cell-specific regulation of this enzyme. To the best of our knowledge, there is no prior information on CYP1A1 and 1B1 protein expression in cultured human bronchial epithelial cells, and the in vivo tissue data have been highly controversial. However, the recent study by Kim et al. (35), using highly specific antibodies, showed 8.6 times higher levels of CYP1A1 than CYP1B1 protein in the lungs of cigarette smokers. Thus, the BEAS-2B cells appear to be a good model of the human lung for these studies.

The 10-fold increase in mEH activity after BaP treatment (Figure 1C) was a surprising observation. The assay used in our studies, that is, the conversion by mEH of BaP-7,8-epoxide to the BaP-7,8-diol, the precursor of the final BaP bioactivation step, should be the most appropriate assay for this enzyme activity. In other cell types, including human hepatic Hep G2 cells, foreign compounds have been shown to activate mEH slightly (41). Our parallel studies in Hep G2 cells, using the same treatment conditions as in BEAS-2B cells, showed an about 2-fold increase in mEH activity (data not shown), indicating that mEH in human bronchial epithelial cells may respond differently to BaP than in other cell types. Thus, mEH might be a more important contributor to BaP-induced lung carcinogenesis than thought previously.

The three potential chemopreventive polyphenolics studied, that is, 5,7-DMF, 3',4'-DMF and RV, were all effective inhibitors of BaP-DNA binding at 25 μM concentration, with 3',4'-DMF being the most potent compound (Figure 3A). A similar pattern was seen for the effects of these compounds on BaP-induced EROD activity, with all compounds being remarkably potent (Figure 3B). 5,7-DMF had no effect on the mEH activity, whereas RV caused a 50% increase in activity (Figure 3C), already observed as a trend in a previous study, when exposure of cells to RV caused an increase in mEH mRNA (9). In contrast, 3',4'-DMF was an inhibitor of the mEH activity. While mEH in general is a detoxifying enzyme, in the context of BaP metabolism it has the opposite effect, that is, mEH is a necessary component in bioactivating this procarcinogen (42). Such inhibition of mEH, not shown previously, could contribute to a chemopreventive effect of this flavonoid.

With respect to the molecularly specific effects of 5,7-DMF, 3',4'-DMF and RV on CYP1A1 and 1B1, RV has been shown previously to inhibit the mRNA expression of both isozymes (9). For CYP1A1 mRNA, 5,7-DMF produced an inhibition that, however, was not statistically significant, whereas 3',4'-DMF was a highly potent inhibitor (Figure 4A). Most interesting as well as important for a potential chemopreventive effect, all three polyphenols prevented BaP-induced CYP1A1 protein expression (Figure 4B). Because of the very low CYP1B1 expression even after BaP induction (Figure 2B), the effect of polyphenols on this protein could not be determined. This inhibitory effect of 3',4'-DMF and RV on CYP1A1 mRNA and protein may be due to their AhR antagonist properties (43,44), or it may be due to a post-translational event, as has been shown for RV (45).

The effect of 5,7-DMF on the CYP1A1 protein expression may be secondary to the slight decrease in the CYP1A1 mRNA, and may also be regulated through the AhR (Figure 4A). However, it may also include other cellular mechanisms (17), such as effects on the methylation of the CYP1A1 promoter involved in tobacco smoking-induced CYP1A1 expression in the lung (46).

Considering the potential utility of these polyphenols as chemopreventive agents in lung cancer, RV has already been shown in an animal model to be ineffective (15), probably because of its poor bioavailability (33), a large problem also in humans due to extensive presystemic sulfate and glucuronic acid conjugation (34). However, it might also be due to a different cellular response in the animal model used. For example, whereas RV showed clear chemopreventive responses in human bronchial epithelial cells (9,14), it had no such effects in oral epithelial cells (17). The effect of cell context on the biological responses to polyphenols must be considered (47). Both methoxylated flavones seem equally or more effective than RV in our cell study. In addition, being non-hydroxyl-containing flavonoids, they should not be subject to either sulfation or glucoronidation; thus, they may have a high oral bioavailability (17). The uptake of both 5,7-DMF and 3',4'-DMF by the BEAS-2B cells was very high, with an about 40-fold accumulation factor. Also, very important, 5,7-DMF was metabolically stable in the BEAS-2B cells for at least 24 h (data not shown). Thus, in contrast to RV, once reaching the bronchial epithelial cells, the methoxylated flavones may have a long duration of action. The potential for direct inhibition of the CYP1A1 protein also exists, as the IC₅₀ for 5,7-DMF has been shown to be as low as 0.8 μM (16). Like RV, 3',4'-DMF and 5,7-DMF are natural products used in herbal extracts (21–24,48). This presents opportunities to test these compounds in vivo in humans, initially focusing on their bioavailability.

Other potential chemopreventive targets for these polyphenols, not addressed in this study, include the induction of detoxifying enzymes, such as glutathione transferases. This was addressed in a preliminary study of another class of natural products, also in BEAS-2B cells (49).

In summary, this study has expanded our knowledge of normal human bronchial epithelial cells in culture as a model of BaP-induced bioactivating enzymes and DNA binding. The identification of CYP1A1, rather than CYP1B1, at the catalytically active protein level as the main CYP1 isoform induced, mirrors the in vivo situation in the lungs of cigarette smokers. The observation of a high activation of mEH by BaP is a novel finding. Two methoxylated flavones were identified as effective chemopreventive agents and their mechanism(s) of action partially deduced. These flavonoids with potentially high bioavailability appear promising for clinical use, and are the subject of continuing studies.

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References


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