5,7-Dimethoxyflavone downregulates CYP1A1 expression and benzo[a]pyrene-induced DNA binding in Hep G2 cells

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The objective of this study was to examine the ability of dietary polyphenols to inhibit cytochrome P450 (CYP) 1A1 expression and activity and benzo[a]pyrene (BaP) DNA binding, with the main emphasis on prevention of chemical-induced hepatic carcinogenesis. For this purpose we used Hep G2 cells, a good model of the normal human hepatocyte for CYP1A1 cell signaling. First, when these cells were exposed to a low concentration (1 μM) of BaP, DNA binding occurred, which dramatically increased after 6 h of treatment. BaP also dramatically induced CYP1A1 activity, protein expression and mRNA levels, the likely reason for the marked increase in DNA binding. Second, we screened 25 polyphenols with highly varying chemical structures for maximum ability to inhibit CYP1A1 activity in the Hep G2 cells. Highly varying responses were obtained, ranging from a 10-fold induction by some polyphenols to almost complete inhibition, in particular by 5,7-dimethoxyflavone (DMF), a flavonoid found in some tropical plants. Third, we examined the ability of DMF to inhibit DNA binding of BaP and the mechanisms involved. DMF (2–20 μM) inhibited BaP-induced DNA binding. DMF also inhibited BaP-induced CYP1A1 activity, CYP1A1 protein expression and mRNA levels. Moreover, DMF directly inhibited the catalytic activity of recombinant CYP1A1 with an IC50 of 0.8 μM. In conclusion, DMF was a highly potent inhibitor of BaP-induced DNA binding and CYP1A1 protein expression and activity in the Hep G2 cells. These properties may make DMF an effective chemoprotectant in chemical-induced liver cancer.

Introduction

Exposure to environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) is associated with the development of numerous cancers in humans (1,2). Although cancers of the lung, larynx, and head and neck appear to be the major sites for PAH-induced carcinogenesis (3,4), recent studies have also added the liver (5–8), the latter being the focus of the present study. Benzo[a]pyrene (BaP), a prototypical environmental PAH, present in cigarette smoke, charbroiled meat and industrial waste by-products (3,7), is mainly metabolized by human cytochrome (CYP) 1A1/1B1 and epoxide hydrolase to carcinogenic BaP 7,8-diol-9,10-epoxides, which covalently binds to cellular DNA to start the carcinogenic process (2,9).

In contrast, some detoxification enzymes, such as glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs), can eliminate the genotoxic metabolite of these tobacco-related carcinogens and decrease the risk of cancer (10,11). Similarly, protective effects are exerted by DNA repair enzymes (4). However, at least for the liver, the most important enzyme promoting BaP-induced carcinogenesis appears to be CYP1A1 (12).

Whereas the constitutive levels of CYP1A1 in human liver are low (12,13), BaP increases its own DNA binding by induction of CYP1A1, which is regulated through the ligand-activated aryl hydrocarbon receptor (AhR) (2,9). After AhR translocation, including binding to the aryl hydrocarbon nuclear translocator (ARNT), the CYP1A1 gene is transcriptionally activated. Thus, inhibition of CYP1A1 would seem like a promising way to prevent BaP-induced liver cancer.

Cancer chemoprevention, defined almost 30 years ago (14), uses natural or synthetic agents to prevent or suppress carcinogenic progression. After some setbacks, owing to the dismal lack of progress in cancer treatment modalities, this approach is on the rise again. However, as recently emphasized by Tsao et al. (15), this has to be based on a solid foundation of mechanistic studies. Polyphenols are a large class of potential cancer chemopreventive compounds, which have multiple modes of action (16–18).

The importance of inducible CYP1A1 in BaP bioactivation and carcinogenesis of the liver is the basis of the present study. Finding dietary polyphenols, which could downregulate as well as inhibit CYP1A1, was the primary objective.

In the present study, we first established the DNA binding of BaP and the catalytic activity, protein and mRNA expression of CYP1A1 after exposing the human Hep G2 cells to a low (1 μM) concentration of BaP. In these respects we also evaluated the contribution by CYP1A2 and 1B1. Secondly, we screened 25 polyphenols for CYP1A1 inhibiting activity in the same cell line. 5,7-Dimethoxyflavone (DMF) was found to have outstanding inhibitory ability. Thirdly, we examined the molecular mechanisms involved in this inhibitory effect, finding potent effects of DMF both on CYP1A1 protein expression and catalytic activity.

Materials and methods

Chemicals

DMF, diosmetin, 5-hydroxy-7-methoxylavone and 7-hydroxy-5-methylflavone were purchased from Indofine Chemical Co. (Somerville, NJ). Acacetin, apigenin, chrysin, isoflavonoids, quercetin, genistein, (−)-epigallocatechin-3-gallate, naringenin, diosmin, (+)-catechin, luteolin,
kaempferol, resveratrol, ethoxyresorufin, resorufin and Williams’ Medium E were obtained from Sigma Chemical Co. (St Louis, MO). 3-Methylcholanthrene (3MC) was purchased from Eastman Kodak Co. (Rochester, NY). Fetal bovine serum was obtained from Atlas Biologicals (Norcross, GA), [G-3H]BaP (76 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ).

Cell culture and treatment

Hep G2 human hepatoma cells obtained from American Type Culture Collection (Rockville, MD) were maintained in Williams’ Medium E with 10% fetal bovine serum, 10% penicillin/streptomycin solution in a humidified 37°C incubator with 5% carbon dioxide. At 4–6 days after seeding in 6-well plates, the cells (90% confluent) were treated with 25μg polyphenols or 1μM BaP in the presence or absence of DMF in growth medium for the times and concentrations indicated in the figures. Vehicle dimethyl sulfoxide (DMSO, 0.1% of final volume) was used as a control in all experiments. The cells were used at passage 10–25. The basal levels of BaP DNA binding and enzyme activity varied somewhat between different passages, but the magnitude of effects was the same.

BaP DNA binding formation

The binding of BaP to cellular DNA was measured using a previous method (19). Hep G2 cells in 6-well plates were treated with 1 μM [3H]BaP for 10 s (control) to 48 h. In the inhibition experiments, the cells were cotreated with 1 μM [3H]BaP and DMF (2 and 20 μM) for 6 h. After treatment, the cell layers 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 then lysed in 0.5% Triton X-100 with protease inhibitors (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 RNAase and the samples were extracted repeatedly with phenol/chloroform. DNA was then precipitated, and washed with cold ethanol. The amount and purity of DNA in the dissolved pellet were measured by UV and the amount of [3H]BaP bound to DNA was quantified by liquid scintillation spectrometry.

CYP1A1 catalytic activity (EROD assay)

Hep G2 cells in 6-wells were treated several different ways, as indicated in the figure legends. In each case, the medium was changed every 24 h. Following the treatments, the cells were washed with fresh medium and incubated with 0.6 μM ethoxyresorufin for 30 min in the presence of salicylaldehyde to inhibit conjugation enzymes (20). The formation of resorufin was measured in the cell culture medium by fluorometry with excitation at 530 nm and emission at 580 nm. The results were adjusted for the amount of cellular protein in each well, as measured by the Lowry assay (21).

Western blotting analysis of CYP1A1, 1A2 and 1B1

Hep G2 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 for 6 h. After the 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 for 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 Bi-Tris gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose and blocked with 5% milk in 0.1% TBST (Tris-buffered saline with 1% Tween-20) for 3 h. The membranes were incubated overnight with primary antibodies to CYP1A1 (rabbit anti-trout 1A from Biosense Laboratories in 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. Baclovirus-expressing human CYP1A1 and 1B1 (supersomes) were used as positive or negative control in the same experiments (BD Gentest, Bedford, MA).

CYP1A1 and 1B1 mRNA analysis

Hep G2 cells cultured in 96-wells plate were treated with BaP and/or DMF as mentioned above for western blotting analysis. The quantitative detection of CYP1A1 and 1B1 mRNA from cells used branched DNA (bDNA) technology (22) with primers for human CYP1A1, CYP1B1 and GAPDH (QuantGenekits, 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 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 CYP1A1 activity

Recombinant CYP1A1 (BD Gentest, Bedford, MA) (5 nM) was incubated with 5 μM ethoxyresorufin and 0.1–100 μM of DMF (dissolved in DMSO, final concentration 0.1%) in 0.1 M sodium phosphate buffer (pH 7.4) containing 10 mM MgCl2. The reaction (1 ml) was initiated with 1 mM NADPH, and stopped by ice after a 15-min incubation at 37°C. The resorufin fluorescence was measured as described above.

Data analysis

Statistical differences between different treatments were determined using two-tailed unpaired ANOVA with a multicomparison (Dunnett) posttest (InStat). The results were expressed as mean ± SD for at least triplicate determinations.

Results

BaP activation and DNA binding

To be able to determine the potential preventive effects of polyphenols on BaP DNA binding and the mechanisms involved, we first had to establish the factors governing this binding. Treatment of Hep G2 cells with a low concentration of BaP (1 μM) for up to 48 h resulted in a dramatic DNA binding (Figure 1A). The effect was highly time-dependent and the highest level of binding was found at 6 h (70-fold increase, compared with the 10-s control), then decreased somewhat at 24 h and went back to the highest point at 48 h. Interestingly, a particularly sharp increase was observed between the 2 and 6 h treatments, suggesting an induction process. Similar to the DNA binding, 1 μM BaP increased CYP1A1 activity, as measured by the EROD assay (Figure 1B). The greatest increase in CYP1A1 activity (~40-fold, compared with vehicle-treated control) was observed after 6 h of treatment and it declined through 48 h. Consistent with the observation in Figure 1A, a great increase in EROD activity was found between 2 and 6 h of treatment.

To determine the effect of BaP on CYP1A1 protein expression, we isolated microsomes from cells treated with 1 μM BaP for 2–48 h as well as vehicle-treated control cells. Western blotting analysis showed no constitutive expression of CYP1A1 protein in the Hep G2 cells. However, 1 μM BaP induced CYP1A1 protein expression dramatically following 6 h treatment and then declined by 24 h. Interestingly, there was no CYP1A1 protein expression by 48 h (Figure 1C). The bDNA assay was used to determine the mRNA levels of CYP1A1 following BaP treatment. BaP (1 μM) significantly induced the CYP1A1 mRNA level as early as after 2 h of treatment with an ~10-fold increase compared with control and kept a high level though 48 h (Figure 1D).

In contrast, CYP1B1 protein (Figure 1C) was not constitutively expressed in the Hep G2 cells. Interestingly, CYP1B1 was also not induced by 1 μM BaP. To examine this further, CYP1B1 mRNA was also determined, but could not be detected (data not shown). When probing for CYP1A2 protein a low constitutive level of expression could be detected, which was induced along with CYP1A1 by 1 μM BaP (Figure 1C).

Pattern of CYP1A1 inhibition or induction by polyphenols

Since it is clear from the above findings that the key enzyme in the bioactivation of BaP in the Hep G2 cells is CYP1A1, a large number of polyphenols were tested for their ability to inhibit this enzyme activity, using the EROD assay (Figure 2A). These experiments were done with 72 h exposure to the polyphenols to detect inductive as well as inhibitory
Fig. 1. BaP-induced DNA binding (A), EROD activity (B), CYP1A1, 1B1 and CYP1A2 protein expressions (C) as well as CYP1A1 mRNA level (D) in the Hep G2 cells. Cells were exposed to 1μM BaP for 0–48 h. Values in A, B and D are mean ± SD (n = 3). Significantly different from 0.1% DMSO-treated control, *P < 0.05; **P < 0.01." 10 s exposure to BaP as a control.

Fig. 2. Effects of polyphenols (25 μM) or 3MC (2 μM) on EROD activity in the Hep G2 cells after 72 h of treatment (A). Effects of chrysin (20 μM) (B) or DMF (20 μM) (C) treatment for various times on EROD activity. Values are mean ± SD (n = 6). Significantly different from 0.1% DMSO-treated control, *P < 0.05; **P < 0.01.
effects. Most of these polyphenols increased CYP1A1 activity, with apigenin, chrys, galangin, isorhamnetin, diosmin, diosmetin and luteolin being almost as potent inducers of EROD as the prototype CYP1A1 inducer 3MC. However, a few polyphenols showed apparent inhibitory effects, including resveratrol, 5-hydroxy-7-methoxyflavone and, in particular, DMF, the latter reducing the basal control EROD activity of the Hep G2 cells virtually down to zero. The time-courses (0–72 h) of the inhibitory effect of DMF and the inductive effect of its close structural analog chrysin are shown in Figure 2B and C, respectively. The inducing effect of chrysin was, thus, not maximal until after 72 h. On the other hand, the inhibitory effect of DMF was expressed throughout the entire time-course.

**Effects of DMF on BaP-induced DNA binding**

Based on our observations (Figure 2), DMF seemed to be a good candidate for blocking the BaP binding to cellular DNA. To explore the potential chemopreventive function of DMF in BaP-induced cancer, the effect of DMF on BaP DNA binding was determined. The concentrations of DMF used, 2–20 μM, had no cytotoxic effects (data not shown). Both 2 and 20 μM DMF blocked BaP-induced DNA binding, with ~50% inhibition after exposure to 2 μM DMF and 90% inhibition following 20 μM DMF treatment (Figure 3).

**Effects of DMF on BaP-induced CYP1A1 activity, protein expression and mRNA level in Hep G2 cells and on recombinant CYP1A1 microsomes**

As shown in Figure 2C, DMF effectively inhibited the basal EROD activity in the Hep G2 cells. DMF also effectively inhibited the BaP-induced EROD activity (Figure 4A), with a potency similar to the effect on BaP DNA binding (Figure 4A), with a potency similar to the effect on BaP DNA binding. The mechanism of the inhibitory effect of DMF on EROD activity
was then examined at the protein level. As shown in Figure 4B, the BaP-induced CYP1A1 protein was effectively abolished by a 6-h cotreatment with 20 \( \mu \)M DMF. When examining the effect of 20 \( \mu \)M DMF on CYP1A1 mRNA using the bDNA assay, ~70% reduction was observed after the same treatment, Figure 4C. However, that DMF directly inhibited the CYP1A1 activity as well is something that could not be ruled out. For these experiments, we used recombinant CYP1A1 protein to determine the direct inhibitory effect of DMF on CYP1A1 activity as measured by the EROD assay. As seen in Figure 4D, DMF is a potent inhibitor of the CYP1A1 enzyme activity with an IC\(_{50}\) of ~0.8 \( \mu \)M.

**Discussion**

 Hepatocellular carcinoma (HCC) is an environmentally related cancer, with the highest mortality rate in developing countries (5,8). Epidemiological studies have demonstrated that chronic hepatitis B virus infection, consumption of dietary aflatoxins and, more recently, cigarette smoking are important risk factors for the development of HCC (5,7). PAHs, which are well-known carcinogenic compounds in cigarette smoke as well as in many other sources, have been found to form DNA adducts in human liver tissue (7) with PAH hepatitis B virus infection, consumption of dietary aflatoxins (5,8). Epidemiological studies have demonstrated that chronic hepatocellular cancer, with the highest mortality rate in developing countries.

Consistent with this finding, the present study demonstrated that BaP, a major PAH procarcinogen, formed DNA adducts in Hep G2 cells, which were dramatically increased after 6 h of treatment. Uninduced Hep G2 cells express very low CYP1A1 activity and no detectable CYP1A1 protein, very similar to normal human hepatocytes (6,13). However, our study showed that a low concentration of BaP (1 \( \mu \)M) induced both CYP1A1 catalytic activity and protein levels in the Hep G2 cells as early as 6 h after treatment, which is presumably responsible for the dramatic increase in DNA binding. Similarly, BaP was found to induce CYP1A1 activity and protein expression in primary human hepatocytes (6,13). The decreased CYP1A1 protein expression at 24 h and absence of a protein band at 48 h in our study has no clear explanation, unless CYP1A1 protein has a short half-life in the Hep G2 cells. The effect of BaP on CYP1A1 protein expression has not been clearly shown, previously, in liver-derived cells. In contrast, the CYP1A1 mRNA levels peaked from 6--48 h of treatment, which seems contradictory to a previous study, also in the Hep G2 cells, reporting on rapid degradation of CYP1A1 mRNA after induction by TCDD, not BaP (23). Based on these observations, it is strongly indicated that BaP interacts with the CYP1A1 enzyme at the transcriptional level in the Hep G2 cells and presumably also in the normal human liver.

Both CYP1A1 and CYP1B1 are importantly involved in the activation of BaP (2). Our data demonstrated clearly that CYP1A1, and not CYP1B1, was inducible by BaP in the Hep G2 cells. Also, CYP1A1 is the most important bioactive enzyme in the metabolism of BaP in the human liver (12,13). Based on these observations, CYP1A1 should be an important target in chemopreventive strategies for HCC. The lack of expression of CYP1B1 in the Hep G2 cells in our study, both at the protein and mRNA levels, under basal conditions as well as after treatment with inducer, is similar to most reports (24--28), although some studies have shown CYP1B1 expression as well as induction (29--31). The reason for this major discrepancy between studies is unknown.

The mechanism of the inhibitory effect of DMF on BaP-induced DNA binding appears to be 2-fold. First, BaP induces CYP1A1 protein and its catalytic activity as well as CYP1A1 mRNA; DMF clearly inhibits this transcriptional activation by decreasing CYP1A1 catalytic activity as well as protein and mRNA expression. Second, whether CYP1A1-induced or not, DMF can also directly inhibit CYP1A1 protein catalytic activity, as determined with recombinant protein. Both the downregulation and the direct inhibition of CYP1A1 were accomplished with low micromolar concentrations of DMF. These dual effects may be a clear advantage in chemoprevention in hepatic carcinogenesis. Thus, downregulation of the bioactivating CYP1A1 expression would possibly lead to a relatively sustained effect, whereas the direct inhibitory effect will be more transient, varying with the concentration of DMF at the CYP1A1 protein.

These effects are encouraging in our search for additional chemopreventive molecules. It is remarkable that two so seemingly similar compounds as DMF and chrysin can have such differing effects, i.e. the 5,7-dimethoxy compound is a potent inhibitor of the CYP1A1 protein whereas the 5,7-dihydroxy compound is a potent inducer, although the latter has not yet been rigorously established. Thus, methylation of flavonoids seems to be an important feature. The polyphenols (Figure 2) showed a potency rank order for CYP1A1 inhibition of DMF > 7-hydroxy-5-methylflavone > 5-hydroxy-7-methoxyflavone > 5,7-dihydroxyflavone (chrysin). Further supporting the importance of methylation is a study showing that 3',4'-dimethoxyflavone appears to be an Ah antagonist (32), i.e. should likely reduce CYP1A1 catalytic activity and protein expression like DMF in the present study. A structure-activity study of methoxylated flavonoids is currently in progress in our laboratory, attempting to define further these beneficial relationships.

Interactions between potential cancer chemopreventive polyphenols and CYP1A1 expression have been extensively studied in various ways in the past. Some studies have focused on specific polyphenols in attempting to seek out their mechanism(s) of interactions (33--35). Others have been screening among multiple polyphenols, focusing on their effects on the Ah in luciferase expression systems (36--38) or using the EMSA assay (39). Similar to the present study, a limited study in this laboratory (40) used the EROD assay in the Hep G2 cells. In view of our goal to develop polyphenols as chemopreventive agents in liver cancer, our approach in this study was simple, rapid and effective. The 72-h treatment used in this study is important, as it permits the detection not only of inhibitors but also of potentially detrimental inducers. For example, whereas chrysin is a direct inhibitor of CYP1A1 activity (41), a long-term treatment, as shown in this study, results in induction. Also, it is a considerable advantage to pursue screenings of further polyphenols useful as chemopreventive agents in liver carcinogenesis in a cell in which mechanistic studies can also be carried out. Future studies will include the ability of polyphenols to inhibit BaP-DNA binding. Although experimentally more difficult, this will bring us closer to the cancer preventive action.

Polyphenols have been shown in this study and by others to directly inhibit CYP1A1 activity (41,42), commonly demonstrated to be a competitive-type of inhibition, and to affect CYP1A1 transcription (34,35). However, the mechanism of the very effective decrease of CYP1A1 transcription by DMF treatment is not clear. Experiments attempting to invoke
antagonism of the Ah were negative (data not shown). An alternative mechanism, as explored for resveratrol (43), is an effect of DMF on CYP1A1 mRNA stability. This is presently under investigation. There are many other potential mechanisms of cancer chemopreventive effects by polyphenols. These include induction of detoxifying enzymes, such as UGTs (44) and GSTs (45). These observations point to BaP-DNA covalent binding (Figure 4A) as a more important functional assay.

DMF is a major constituent of the leaves of a Malaysian Piper species and has been used to treat a variety of conditions (46). Moreover, many other polymethoxylated flavonoids, e.g. tangeretin and nobiletin, are present in large amounts in citrus fruits (47,48), and, are thus, already a part of our daily diet. Their utility as chemopreventive agents should be further explored. This should include studies of their bioavailability, i.e. their ability to reach their multiple targets in hepatocytes or other cells. Whereas chrysin has a very rapid metabolism by conjugation (49) and therefore very low bioavailability in humans (50), preliminary studies indicate that DMF is much more metabolically stable, i.e. has a better chance of reaching the systemic circulation, including the liver, in sufficient concentrations to exert biological effects.

As shown in Figure 1C, CYP1A2 protein was induced along with CYP1A1 by 1 μM BaP. Based on previous comparisons (2,51), the contribution by CYP1A2 to BaP bioactivation is considerably less than by CYP1A1. Also, CYP1A2 might have contributed to our EROD activities but to a minor extent (52). As CYP1B1 was not detected in the Hep G2 cells either at the protein or mRNA levels, its contribution to BaP bioactivation as well as EROD activity should be minimal.

In summary, this study has demonstrated an extraordinary ability of the PAH BaP to induce and promote its own DNA binding in hepatocytes, presumably accelerating the hepatocarcinogenesis process. This study has also identified, after screening a group of polyphenols, one naturally occurring compound, DMF, inhibiting the BaP-DNA binding utilizing a combination of two mechanisms. Further studies following this lead may be able to define clinically useful molecules effective in chemoprevention of liver cancers.

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