Effect of dihydromyricetin on benzo[a]pyrene activation in rats

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Submitted: 2014-09-23 Accepted: 2014-11-08 Published online: 2014-11-30

Key words: interaction; induction; activation; carcinogen; cytochrome P450; dihydromyricetin; flavonoid

Abstract

OBJECTIVES: Flavanol dihydromyricetin (DHM) has been shown to counteract acute ethanol (EtOH) intoxication and reduce excessive EtOH consumption. Since this flavonoid is being considered for human use, the in vivo study of DHM interactions with the cytochrome P450 (CYP) multienzyme system in the respect of metabolic activation of a model food-born carcinogen, benzo[a]pyrene (BaP), is of high importance. Flavonoids of known properties, alpha-naphthoflavone (ANF) and beta-naphthoflavone (BNF) were included into the study to compare their and DHM effects on BaP-DNA adduct formation.

METHODS: The flavonoids were administered by oral gavage either 72 hrs prior or simultaneously with a single dose of BaP to experimental rats. The expression of CYP1A1/2 enzymes was examined based on the enzymatic activity with a marker substrate, 7-ethoxyresorufin, and on Western blots. The nuclease P1 version of the ³²P-postlabeling assay was used to detect and quantify covalent DNA adducts formed by BaP.

RESULTS: Treatment of rats with a single dose of DHM or ANF prior to or simultaneously with BaP did not produce an increase in levels of CYP1A1 and in formation of BaP-DNA adducts in liver. BNF, a known inducer of CYP1A1, showed a synergistic effect on BaP-mediated CYP1A1 induction and BaP activation in liver. Contrary to that, in small intestine the stimulatory effect of BNF on both parameters was not detected. Animal pre-treatment with DHM or ANF before BaP administration resulted in a significant elevation of BaP-DNA adducts, namely in the distal part of small intestine, while the CYP1A1 mediated 7-ethoxyresorufin-O-deethylation (EROD) was decreased markedly. It is important to note that under all regimens of animal treatment, DHM or ANF produced the higher inhibitory effect on the BaP-DNA adduct formation and BaP-induced EROD activity of
CYP1A1 when administered simultaneously than sequentially with BaP. Our data show that DHM or ANF did not enhance the BaP-activation leading to BaP-mediated genotoxicity (the formation of BaP-DNA adducts) in rat liver, however, in small intestine the pretreatment of rats with these flavonoids may enhance BaP genotoxicity.

CONCLUSIONS: The data indicate that the intake of DHM prior to or simultaneously with the administration of BaP may increase the risk of a BaP-induced tumorigenesis in small intestine.

Abbreviations:
AHR - aryl hydrocarbon receptor
ANF - α-naphthoflavone
BaP - benzo[a]pyrene
BCIP - 5-bromo-4-chloro-3'-indolyphosphate
BPDE - BaP-7,8-dihydridiol-9,10-epoxide
BNF - β-naphthoflavone
B.w. - body weight
B5 - cytochrome b5
CYP - cytochrome P450
CYPOR - NADPH:cytochrome P450 oxidoreductase
dG-N2-BPDE - 10-(deoxyguanosin-N2-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydro-benzo[a]pyrene
DHM - dihydromyricetin
DMSO - dimethyl sulfoxide
EROD - 7-ethoxyresorufin-O-deethylation
EtOH - ethanol
GST - glutathione S-transferase
hrs - hours
mEH - microsomal epoxide hydrolase
MS - microsomes
NADPH - nicotinamidadeninedinucleotide phosphate (reduced)
NBT - nitro-blue tetrazolium
p.o. - per os
PAH - polycyclic aromatic hydrocarbons
P-gp - P-glycoprotein
RAL - relative adduct labeling
SD - standard deviation
SDS - sodium dodecyl sulfate
UT - untreated animals

INTRODUCTION

Dihydromyricetin, (2R,3R)-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-dihydrochromen-4-one (DHM) depicted in Figure 1, is credited with hepatoprotective properties (Hodek, 2012). Moreover, it is highly effective in counteracting acute EtOH intoxication, in reducing excessive EtOH consumption via antagonizing acute EtOH-induced potentiation of γ-aminobutyric acid receptors and in increasing activity of alcohol and acetaldehyde dehydrogenases (Shen et al. 2012; Chen et al. 2006). This compound found in Japanese Raisin Tree, *Hovenia dulcis* (Hyun et al. 2010), belongs to numerous groups of flavonoids, which are of a great interest since they provide a wide variety of biological activities (Hodek, 2012). In addition to their powerful antioxidant properties, they are suggested to be anti-cancer agents acting at various stages of carcinogenesis. The flavonoids are, because of their natural origin, generally accepted as health-promoting compounds and safe for the human use. Their extensive intake as food supplements may, however, cause potential threats resulting e.g. from altered metabolism of endogenic compounds and from flavonoid pro-oxidant activity (Hodek et al. 2009a). Much less attention is being paid to flavonoid-drug or flavonoid-carcinogen interactions. These interactions frequently arise from flavonoid abilities to modulate catalytic activity of xenobiotic metabolizing enzymes such as cytochromes P450 (CYPs). Flavonoids are inhibitors, inducers and even activators of CYPs. α-Naphthoflavone (ANF, Figure 1), may serve as a good example of versatile activities of flavonoids. ANF represents a well documented inhibitor of metabolic reactions mediated by CYPs of a 1A subfamily (Bauer et al. 1995), but at the same time, ANF acts as a positive modulator stimulating catalytic activity of CYP3A4 (Hodek et al. 2002). After the oral administration of ANF, high plasma concentrations (reached within 30 min) allow the modulation of CYP activities (Wang and Morris, 2008). However, at its lower concentrations, inefficient e.g. for CYP1A1 inhibition, ANF can initiate the expression of biotransformation enzymes (Ferguson, 2001). It has been shown that ANF increases the rate of the electron transfer from NADPH:cytochrom P450 oxidoreductase (CYPOR) to appropriate electron acceptors such as CYPs (Hodek et al. 2009b). Since in the endoplasmic reticulum one CYPOR molecule provides electrons for many CYPs, any raise in the CYPOR activity can stimulate the CYP1A1-mediated metabolism. Moreover, ANF was shown to induce cytochrome b5 (B5), which similarly to CYPOR, supplies the electrons for CYP catalyzed reactions (Blaich et al. 1987) and most likely acts as a positive modulator of several CYPs (Borek-Dohalska et al. 2001; Hodek et al. 2002). Furthermore, ANF activates epoxide hydrolase, the enzyme which is essential for formation of an ultimate carcinogen from BaP (Glatt et al. 1983).

Another flavonoid, β-naphthoflavone (BNF), a structural analogue of ANF (see Figure 1), significantly elevates protein and mRNA levels of the CYP1A1 and 1A2 in rat liver. In hepatic microsomes of BNF-treated animals, the CYP1A1-mediated 7-ethoxyresorufin O-deethylation (EROD) was increased by almost two orders of magnitude compared to the untreated control (Canivenc-Lavier et al. 1996). The CYP1A1 induction by BNF proceeds via an aryl hydrocarbon receptor (AHR), which binds as a heterodimer with the AHR nuclear transporter to DNA motifs, known as AHR response elements (Nebert et al. 2004). However, the process of the BNF-mediated CYP induction is rather slow; in rat small intestine the maximal induction of CYP1A1 was reached approx. 24 hrs after the BNF application (Zhang et al. 1997).

In the animal study administered flavonoids might be entirely converted to their metabolites and conju-
gates in the gastrointestinal tract (Wang and Morris, 2008). Thus the effect attributed to flavonoid is in fact caused by some of the flavonoid derivatives or degradation products. Orally administered xenobiotics are possibly absorbed to blood in their unchanged form and/or after the first-pass intestinal metabolism, further metabolized in liver by CYPs and/or conjugation enzymes, and via bile returned back as glucuronides and sulfates to small intestine, where they are exposed to intestinal microflora (Wang and Morris, 2008; Hodek, 2012). Hydrolytic microbial enzymes, such as sulfatase and glucuronidase, are of a particular interest since they may release parent metabolites from their conjugates. These metabolites can be reabsorbed into the enterocytes, and thus circulate between the gut lumen and the epithelial cells (Nanno et al. 1986). Thus, an enormous metabolic potential of intestinal microflora is involved in the overall metabolic fate of ingested compounds (Meinl et al. 2009; Bezirtzoglou, 2012). As CYPs are frequently involved in carcinogen activation, the effect of flavonoids on these enzymes should be always considered and carefully examined (Hodek et al. 2009b).

Benzo[a]pyrene (BaP) is a chemical carcinogen that is commonly used as a model carcinogenic compound in animal studies. Humans are exposed to BaP mostly in food e.g. in smoked or flame-exposed meat and/or in a cigarette smoke (Phillips, 1999). BaP has been found to cause the development of tumors in lung, skin and liver. The metabolic activation of BaP to reactive species binding to DNA proceeds similarly to other polycyclic aromatic hydrocarbons (PAH) mainly by CYP1A1 in combination with microsomal epoxide hydrolase (mEH), while its detoxification is mainly catalyzed by conjugation enzymes such as UDP-glucuronosyltransferase and glutathione S-transferase (Zhang et al. 2013; Fang et al. 2001; Aimova et al. 2008). The scheme in Figure 2 shows the pathways leading to formation of BaP derivatives covalently binding the DNA molecule (Stiborova et al. 2014). First, CYP1A1 oxidizes BaP to an epoxide that is then converted to a dihydrodiol by mEH (i.e. BaP-7,8-dihydrodiol); then further bio-activation by CYP1A1 leads to the ultimately reactive species, BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) that can react with DNA, forming adducts preferentially at guanine residues. The 10-(deoxyguanosin-N2-yl)-7,8,9,10-tetrahydrobenzo[a]pyrene (dG-N2-BPDE) adduct is the major product of the reaction of BPDE with DNA in vitro and in vivo (Bauer et al. 1995; Arlt et al. 2008; 2012). BaP is, however, oxidized also to other metabolites such as other dihydrodiols, BaP-diones and hydroxylated metabolites (Bauer et al. 1995; Indra et al. 2013; Stiborova et al. 2014). Of them, BaP-9-ol is a precursor of 9-hydroxy-BaP-4,5-epoxide that can form another adduct with deoxyguanosine in DNA (Figure 2) (Schoket et al. 1989; Nesnow et al. 1993; Fang et al. 2001; Phillips, 2005; Stiborova et al. 2014).

However, the role of CYP1A1 in BaP metabolism seems to be more complex, since this CYP has also been shown to detoxify BaP in liver and gastrointestinal tract of mice (Uno et al. 2004; Arlt et al. 2008; 2012). 3-Hydroxy-BaP formed by CYP1A1 is considered the major detoxification product of BaP oxidation (Huang et al. 1986). In addition to mEH, B5 plays a significant role in CYP1A1 dependent activation of BaP. This hemoprotein may, besides CYPOR, alternatively provide electron(s) required for CYP1A1 enzyme activity. Recently, the involvement of B5 in the CYP1A1-mediated activation of BaP in vivo has been described (Stiborova et al. 2014). Moreover, the electron transfer from reduced B5 to CYP is even faster than that from CYPOR (Schenkman and Jansson, 2003).

Treatment of animals with BaP results in an increase in the expression of CYP1A1 protein and its enzyme activity. In this respect, BaP is a prototypic PAH inducing CYP1A subfamily via the AHR mechanism. Although in vitro experiments carried out with CYP1A1-induced liver microsomes indicate a clear connection between CYP1A1 levels and DNA adduct formation, the in vivo studies with BaP-treated animals do not show such straightforward link (Uno et al. 2004; Arlt et al. 2008; 2012; Hodek et al. 2011). In animal studies, numerous additional factors, e.g. BaP transport and organ distribution, BaP-induction of conjugation enzymes participating in BaP detoxification, intestinal microflora possessing an enormous metabolic capacity, possibly contribute to the overall metabolic fate of BaP (Buesen et al. 2003; Gupta et al. 1989; Hughes and Rowland, 2000).

In the present study, the causal relation between the DHM exposure and activation of BaP, was investigated. The rat was used as an animal model. For comparison, ANF and BNF were also included for testing. To mimic the human exposure, tested compounds were admin-
istered to rats by oral gavage. Impact of flavonoids on BaP activation was determined based on BaP-DNA adduct formation in conjunction with CYP1A1 metabolic activity.

MATERIAL AND METHODS

Chemicals

α-Naphthoflavone (7,8-benzoflavone), β-naphthoflavone (5,6-benzoflavone), NADPH, bicinechonic acid, resorufin, 7-ethoxyresorufin, 7-methoxyresorufin, and anti-chicken IgG alkaline phosphatase conjugate, 5-bromo-4-chloro-3’-indolyphosphate/nitro-blue tetrazolium (BCIP/NBT) tablets were purchased from Sigma Chemical Co., (St. Louis, MO). Dihydromyricetin was provided from APIchem (Shanghai, PRC). Specific chicken anti-CYP1A1/2 and anti-CYP1A2 antibodies were prepared by Dr. Hodek as described (Hodek et al. 2013b). All chemicals used in the experiments were of analytical grade purity or better.

Animal experiments and preparation of microsomes.

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic). Male Wistar rats (150 g, AnLab, Czech Republic), were housed in groups of 3 in wire cages at 22 °C with a 12 hrs light/dark period and ad libitum diet (ST-1 diet from Velaz, Czech Republic) and water access. Tested flavonoids, DHM, ANF, BNF, and carcinogen, BaP, were dissolved in sunflower oil and administered p.o. by gastric gavages (1 ml) in two regimens: either the particular flavonoid (120 mg/kg b.w.) in a single dose 72 hrs prior the application of BaP (150 mg/kg b.w.) or the flavonoid (120 mg/kg b.w.) simultaneously with BaP (150 mg/kg b.w.). Animals of all experimental groups were sacrificed 24 hrs after the treatment with BaP. The control group was treated with 1 ml of sunflower oil only. Microsomes (MS) were prepared from 3 sections of dissected small intestine (proximal, middle, distal parts) and the whole liver, as described elsewhere (Krizkova et al. 2008, Hodek et al. 2011). Microsomal fractions were stored at –80 °C before use. Protein concentrations in the microsomal fractions were determined using the bicinechonic acid protein assay with bovine serum albumin as the standard (Weichelman et al. 1988). The concentration of CYP was estimated according to Omura and Sato (1964) based on the absorption of the of reduced CYP complex with carbon monoxide.

Western blot analysis

The CYP1A1 and 1A2 proteins were detected by Western blotting on the Immobilon-P membrane (Millipore, Bedford, MA) using specific chicken anti-CYP1A1/2 and anti-CYP1A2 antibodies (30 μg/ml). For sodium dodecyl sulfate (SDS)-electrophoresis (10% polyacryl-
amido gel), 30 μg protein/well of small intestine and liver microsomes was applied. Western blotting was carried out as described earlier (Krizkova et al. 2009). Visualization was performed using an anti-chicken IgG alkaline phosphatase-conjugated antibody (diluted 1:2000) and BCIP/NBT substrate tablets (10 mg) for alkaline phosphatase.

**Enzyme assays**

Each microsomal sample was analyzed for specific CYP1A1 activity by monitoring 7-ethoxyresorufin O-deethylation (EROD), according to the method described by Burke and Mayer (1974). Briefly, incubation mixtures, contained in the final volume (150 μl) 100 mM potassium phosphate buffer (pH 7.4): 0.5 mM NADPH, 0.5 mg of microsomal protein and 2.2 μM 7-ethoxyresorufin (from 1 mM stock in dimethyl sulfoxide, DMSO). The reaction was initiated by adding NADPH. The formation of resorufin was monitored by its fluorescence (excitation and emission wavelengths of 530 and 585 nm, respectively) in 1 min. intervals for 10 minutes at room temperature on luminescence spectrometer (PerkinElmer LS-55 equipped with 96-well plate reader). The dealkylation rate was estimated on the basis of a resorufin standard curve.

**32P-postlabeling analysis of BaP-DNA adduct**

The DNA from liver and small intestine was isolated by standard phenol/chloroform extraction. DNA adducts formed from BaP were determined by 32P-postlabeling analysis using the nuclease P1 enrichment version (Stiborova et al. 2001, 2003, 2013, 2014), and thin-layer chromatography (TLC) was performed as described (Arlt et al. 2008). After chromatography, TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, USA) and DNA adduct levels (RAL, relative adduct labeling) were calculated as described (Stiborova et al. 2001, 2003; 2013, 2014). Results were expressed as DNA adducts/10⁸ nucleotides.

**RESULTS**

To examine the effect of DHM on BaP genotoxicity, the DNA adduct formation from activated BaP was analyzed in the liver and small intestine of rats treated with these compounds. In addition to DHM or ANF and BNF, prototype CYP1A1 inhibitor and inducer, respectively, were tested, too. Flavonoids, DHM, ANF and BNF, were administered either 72 hrs before BaP animal treatment or simultaneously with BaP. In both cases, animals were sacrificed 24 hrs after BaP exposure. The induction of CYP1A1/2 was evaluated in microsomal samples of liver and small intestine based on its specific metabolic activity (EROD) as well as by immunodetection of CYP1A1 protein expression.

**The modulation of CYP1A1 activity and DNA adduct formation in liver**

First, the induction of CYP1A1 was examined in liver microsomes. Figure 3 shows that only BNF pre-treatment of BaP-administered animals significantly increased EROD activity compared to animals exposed solely to BaP. This observation suggests the synergistic effect of BNF on BaP-mediated CYP1A1 induction. In contrast to these results, when ANF or DHM were applied simultaneously with BaP a profound drop in EROD activities was detected. In that regimen both flavonoids reversed the effect of BaP. Likewise, ANF when administered 3 days before BaP decreased the EROD activity, too. The application of DHM at this sequential regimen, however, did not exert any inhibition, but EROD activity was moderately stimulated (in comparison to BaP alone). These results are in agree-

![Figure 3](image-url) **Figure 3.** Determination of EROD in liver microsomes. Microsomal samples were prepared from animals exposed to BaP alone or in combination with DHM, ANF and BNF administered either 72 hrs before BaP animal treatment (BNF-BaP, ANF-BaP, DHM-BaP) or simultaneously with BaP (BaP+ANF, BaP+DHM). Control animals were treated with sunflower oil only (UT). Each value represents the mean of two separate analyses.

![Figure 4](image-url) **Figure 4.** Immunodetection of CYP1A1/2 in liver and intestinal microsomes. Microsomal samples were prepared from liver (line A), proximal (line B) and distal (line C) parts of small intestine of animals exposed to BaP alone or in combination with DHM, ANF and BNF. The flavonoids were administered either 72 hrs before BaP animal treatment (BNF→BaP, ANF→BaP, DHM→BaP) or simultaneously with BaP (BaP+ANF, BaP+DHM). Control animals were treated with sunflower oil only (UT). Blots were developed with chicken antibody recognizing CYP1A1 and 1A2.
ment with immunodetection of CYP1A1 protein in liver microsomes (see Figure 4). When animals were treated solely with a single dose of BNF, DHM or ANF (no BaP), the BNF application only resulted in a large increase of EROD activity (~63% of BaP induction), while DHM showed the only slight increase in EROD activity and ANF had no effect on this CYP1A activity (data not shown).

Second, to reveal the effect of DHM on BaP activation, the DNA adduct formation by BaP, was examined. Treatment of rats with BaP resulted in formation of three BaP-derived DNA adducts (assigned as adduct spots A, B, and C in Figure 5) detectable by $^{32}$P-postlabeling in the liver and the proximal and distal parts of the small intestine. Two of these adducts were tentatively identified to be dG-N$^2$-BPDE (adduct spot A) (Arlt et al. 2008; 2012) and the adduct derived from a reaction of deoxyguanosine in DNA with 9-hydroxy-BaP-4,5-epoxide (adduct spot B) (Figure 2) (Schoket et al. 1989; Nesnow et al. 1993; Fang et al. 2003; Stiborova et al. 2014). This BaP metabolite is bound to the exocyclic amino group of a guanine residue, and the site of attachment is most likely either the 4 or the 5 position on the BaP-4,5-epoxide (Fang et al. 2001). The structure of another BaP-DNA adduct (adduct spot C) has not still been characterized.

Results of analysis of liver DNA from rats exposed to flavonoids and BaP in both regimens are presented in Figure 6. Of all tested flavonoids, only BNF in combination with BaP enhanced the formation of BaP-DNA adducts above the level elicited by BaP alone. ANF reduced BaP-DNA adducts formation, when applied sequentially or in combination with BaP. Likewise, DHM in either regimen also inhibited adduct formation by BaP and this effect was even more efficient compared to ANF. This finding does not correspond to the results of CYP1A1 induction detected as EROD activity.

**The modulation of CYP1A1 activity and DNA adduct formation in small intestine**

The small intestine was cut into three parts, proximal, middle and distal. The CYP1A1 induction and BaP-DNA adducts were examined in proximal and distal parts to get the figure of the flavonoid – BaP interactions along the organ reflecting short and extensive exposure of xenobiotics to metabolic capacity of small intestine.

The BaP induction of CYP1A1 with/without flavonoids was assayed as EROD activity in small intestine microsomal samples (see Figure 7). Pre-treatment with BNF did not increase the BaP-mediated induction of CYP1A1 either in the proximal or distal part of small intestine, but *vice versa* a slight drop in EROD activity was detected. When other flavonoids, ANF or DHM, were also applied before BaP, they markedly reduced CYP1A1-mediated metabolism, whereas ANF was more effective than DHM in both parts of small intestine. The simultaneous administration of ANF or DHM with BaP elicited even higher reduction of EROD activity in comparison to respective samples from sequential treatment. In general, the flavonoid abilities to lower the BaP induction of CYP1A1 noticeable decline from the proximal to distal part of small intestine, with only the exception of the microsomal sample from the proximal part of animal pre-treated with DHM (prior to BaP). The immunodetection of CYP1A1 in microsomal samples of small intestine does not show any marked differences in the protein expression (Figure 4).

Tissues from the proximal and distal parts of small intestine were examined for the levels of BaP-DNA adducts formed in the liver and small intestine of rats exposed to BaP assayed by $^{32}$P-postlabeling (BaP-DNA adducts formed in the liver are shown). Spots were identified as 10-(deoxyguanosin-N$^2$-yl)-7,8,9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene (A), adduct derived from a reaction of deoxyguanosine in DNA with 9-hydroxy-BaP-4,5-epoxide (B), and another BaP-DNA adduct of unknown structure (C).
adducts (see Figure 8). In both sections of small intestine BNF did not significantly induce the BaP-DNA adduct formation. Only a slight stimulatory effect of BNF on BaP-DNA adduct levels was found. The amount of BaP-DNA adducts was significantly higher (>2 folds) in the distal than in the proximal part of the small intestine regardless DHM or ANF administration regimen used. Moreover, in the distal part of small intestine the BaP-DNA adduct formation was stimulated, particularly with ANF, to levels substantially exceeding those obtained for BaP applied solely. On the contrary, in the proximal part of small intestine, only the pre-treatment of rats with ANF exerted a slight stimulatory effect, but in all other cases flavonoids inhibited the BaP-DNA adduct formation (in comparison to animals treated solely with BaP).

DISCUSSION

The present study is focused on the effect of DHM, the natural flavonoid, which is considered as a candidate compound for counteracting an acute EtOH intoxication and reducing excessive EtOH consumption in humans, on genotoxicity of BaP. Although DHM is a compound occurring in various plants and thus is generally believed safe for humans, its excessive consumption as a medication or food additives may raise health risk issues. Beside the acute toxicity of DHM, its possible interactions with the CYP multienzyme system, which is frequently affected by compounds of foreign origin, should be examined. As CYPs are involved in metabolism of chemical carcinogens the intake of DHM might modulate the carcinogen activation, e.g. food-derived carcinogen BaP (Hodek et al. 2009a).

In addition to DHM, two synthetic flavonoids, ANF and BNF, were included into the study. While BNF is a prototypical CYP1A1 inducer of CYP expression acting via the AHR pathway (Wiebel 1980), ANF is a powerful inhibitor of CYP1A1-mediated metabolic reactions (Bauer et al. 1995). However, besides the inhibition of CYP1A1, ANF might also stimulate CYP-dependent metabolism and consequently BaP genotoxicity in several ways.

The present experiments were undertaken to examine two situations, the first one, where the flavonoid was administered prior to the intake of a carcinogen BaP and the second one, where both compounds were applied simultaneously. The 72 hrs delay between administration of flavonoid and BaP was chosen to allow effective metabolism and a potential excretion of compounds used for the animal pre-treatment. Under such conditions the residual flavonoid concentration should not be inhibitory while the flavonoid elicited induction of CYPs persists, as shown in our previous work (Hodek et al. 2011). In the experiments of the present study the flavonoid-BaP interactions were evaluated based on the induction of CYP1A1, which plays a prominent role in BaP activation (Bauer et al. 1995) and the BaP-DNA adduct formation that is an early sign of initiated carcinogenesis (Stiborova et al. 2001, 2003, 2014).

Our experiments were performed with model animals, rats, which were treated with tested compounds...
in a single p.o. dose by gastric gavages to mimic a potential human intake. Although the liver is the major site for the metabolic activation and detoxification of PAHs in general, the intestinal epithelium plays a significant role in the first-pass metabolism of ingested xenobiotics (Kaminsky and Zhang, 2003; Thelen and Dressman, 2009). Therefore, we focused on liver and small intestine two organs, which are primarily exposed to ingested xenobiotics.

As follows from the analysis of specific EROD activity in liver microsomes, BNF is a CYP1A1 inducer enhancing the BaP induction of EROD activity. In the case of DHM or ANF at least two additional effects on BaP-induction of CYP1A1 might be regarded. These flavonoids may antagonize BaP-induction of CYP1A1 and/or inhibit CYP1A1 EROD activity. While the pretreatment of animals with DHM or ANF (72 hrs before BaP) allows manifesting namely their agonistic or antagonistic effects on AHR, simultaneous administration of DHM or ANF with BaP may result also in the inhibition of microsomal EROD activity. Since the BaP-mediated induction of CYP1A1/2 on Western blots was not significantly impaired with any of flavonoids, the changes in CYP1A1 expression were most likely not responsible for the found reduction of EROD activity in liver microsomes. Thus, in all samples with the exception of liver microsomes from DHM-pre-treated rats, the persisted flavonoids and/or their metabolites may cause the EROD activity decline. The lack of DHM impact on the EROD activity (compared to ANF) is likely caused by the presence of multiple hydroxylation of DHM, which makes it more polar and excreted readily. A slight increase in EROD activity of this sample might be attributed to possible DHM-mediated induction of CYP1A2, which also to some extent catalyzes EROD. In agreement with this assumption, a slight elevation of the CYP1A2 level on Western blots developed with anti-CYP1A2 antibody was detected for DHM (data not shown). Present data indicate that the effect of DHM on EROD activity depended on the regimen of application: while the simultaneous administration of DHM with BaP effectively reduced BaP-mediated CYP1A1 EROD activity; the activity stimulation was detected when DHM was applied 3 days before BaP.

The CYP1A1 induction in rat livers, measured as CYP1A1/2 specific EROD activity in microsomes corresponded to an increase in the formation of BaP-DNA adducts. Hence, the increase in CYP1A1 activity after pre-medication of rats with BNF resulted in stimulation of BaP activation (see Figures 3 and 6). On the contrary, the pre-treatment of rats with DHM or ANF, regardless the application regimen used, lowered the levels of BaP-DNA adducts. That is in accordance with an observed decline in the EROD activity. In the case of DHM pre-medicated animals, however, there is an obvious discrepancy between the moderate stimulation of EROD activity (above the BaP control) and a pronounced reduction of BaP-DNA adducts formation. As mentioned above, this phenomenon might be explained at least partially by the DHM induction of CYP1A2, which can, to some extent, contribute to EROD activity. While CYP1A2 exhibits EROD activity, this CYP is involved also in the BaP detoxification, giving rise to the formation of appreciable amounts of 3-hydroxy-BaP and of some dihydrodiols from BaP (Gautier et al. 1996; Bauer et al. 1995). Thus, our results show that DHM does not enhance the BaP activation leading to BaP-DNA adducts in rat liver.

Since the BaP activation might differ along length of small intestine (Hodek et al. 2011) the organ was dissected into three parts, and the proximal and distal parts were used for examination (middle part was omitted). The induction of CYP1A1 was assayed based on EROD activity and the protein expression. In contrast to liver, there is only one member of a CYP1A family, CYP1A1, in small intestine (Kaminsky and Zhang, 2003). Hence, differences in EROD activity directly reflect the CYP1A1 induction/inhibition. It should be noted that in small intestine none of BNF, DHM or ANF when applied in combination with BaP significantly induced expression of CYP1A1-mediated EROD activity more than BaP alone. The lack of the synergistic effect of BNF on BaP-mediated CYP1A1 induction is consistent with our earlier findings that either BNF or BaP already stimulated the CYP1A enzyme expression to its maximal level (Hodek et al. 2011). Interestingly, when comparing EROD activities of corresponding microsomal samples from the liver, proximal and distal parts of small intestine, the same pattern for all tissues may be found. The pre-treatment with DHM prior to BaP has always the lowest impact on BaP induced EROD activities, while the co-administration of ANF and BaP most efficiently reduced EROD activities. In addition, among the tissues there is a clear tendency showing the most pronounced differences among EROD activities in liver microsomes and relatively low of those in the distal part of small intestine. Apparently, the effect of flavonoids is getting weaker depending on the duration of xenobiotics exposure to intestinal tissue. In this respect it is also worth of noticing that EROD activities induced by BaP in liver microsomes are in average by 10–20 folds higher than those of small intestine.

Whereas in the proximal part of small intestine, likewise in liver, the BaP-DNA adduct formation was proportional to CYP1A1 induction, in the distal part of the small intestine the situation was more complex (see Figs. 7 and 8). In the proximal part of small intestine, in line with results of liver samples, the co-administration of ANF or DHM with BaP lowered BaP-mediated induction of CYP1A1 activity, thereby reducing the levels of BaP-DNA adducts. In the distal part of small intestine, however, ANF or DHM when applied in either combination with BaP caused a profound stimulation of BaP-DNA adduct formation. The highest effect was detected after the sequential application of ANF or DHM prior to BaP. The 5.4- and 3.8-
A five-fold increase in BaP-DNA adduct levels found in ANF and DHM pre-treated animals, respectively, correlated reversely with the CYP1A1-mediated EROD activity. To confirm this unexpected result, the expression of CYP1A1 at a protein and at mRNA levels was determined, too. Neither Western blots (Figure 4) nor did RT-PCR of mRNA (data not shown) show any changes in CYP1A1 expression, which could explain this extensive BaP activation in the distal part of small intestine. Our findings suggest that in this particular case ANF and DHM increase the activation metabolism of BaP via an unknown mechanism, which does not depend on the CYP1A1 induction. Similar data resulted from experiments with Cyp1a1(–/–) global- and cell-specific knockout mice. In Cyp1a1(–/–) global-knockout mice much higher levels of BaP-DNA adduct formation in liver and small intestine than those in wild-type mice were found. Therefore, CYP1A1 was presumed to be more important in the metabolic detoxification than in activation of orally administered BaP (Uno et al. 2004; Shi et al. 2010). This hypothesis, however, cannot elucidate the increased BaP adduct formation in the distal part of small intestine detected in our experiments.

To understand the differences between the proximal and distal parts of the small intestine in flavonoid-mediated BaP-DNA adduct formation, metabolic conversion of administered flavonoids should be accounted. The experiments performed by Nesnow and Bergman (1981) provide the evidence that ANF was readily metabolized to its 5,6-dihydrodiol in microsomal samples. Also other metabolites such as 7,8-dihydrodiols, and 6-, 7- and 9-hydroxy-ANF were indentified (Vyas et al. 1983). The rapid ANF oxidation, namely by CYP1A1, results in derivatives, which are less effective inhibitors of CYP1A1-mediated metabolism of BaP than ANF (Nesnow et al. 1981; Bauer et al. 1995). When orally administered, ANF because of its lipophilicity is absorbed into the enterocytes and its fraction consequently metabolized in liver while the other underwent first-pass metabolism in small intestine (Wang and Morris, 2008). Ingested ANF is gradually along the length of small intestine converted into metabolites as well as the proximal part of small intestine is exposed to conjugated forms of ANF coming in bile. Similar concept should be regarded for BaP biotransformation. In addition, intestinal microflora is involved in a cleavage of the xenobiotic conjugates. For instance, glucuronide conjugates of BaP are hydrolyzed and mutagenic derivatives can be reabsorbed into the enterocytes, and thus circulate between the gut lumen and the epithelial cells (Nanno et al. 1986).

The role of ANF, DHM and their metabolites in the activation metabolism of BaP might also be explained by their interactions with P-glycoprotein (P-gp), a membrane transporter located in the apical brush border of the enterocytes (Yeh et al. 1992). The function of P-gp is to pump xenobiotics from the enterocytes back into the intestinal lumen and protect cells against toxic exogenous substances. As shown with Caco-2 cells, BaP and/or its metabolites are transported by intestinal cells back into the gut lumen (Buesen et al. 2003). The expression of this transporter and its activity might be modulated by BaP and/or ANF as well as by their metabolites (Wang et al. 2011). Using the intestinal Caco-2 cells exposed to BaP, the increase in P-gp expression was demonstrated (Sugihara et al. 2006). In addition, flavone, a flavonoid lacking hydroxyl groups like ANF, increases the P-gp expression in mice intestinal epithelial cells in vivo (Lohner et al. 2007). By analogy, ANF, flavonoid of a similar structure to flavone, might induce the P-gp expression in rat small intestine, too. In such case ANF would affect BaP genotoxicity via P-gp in addition to already mentioned inhibition of CYP1A1. This deduction would, however, valid for the proximal part of small intestine where the major portion of ingested ANF stays unchanged.

In the distal part of small intestine ANF is expected to be largely converted to metabolites, which are obviously not capable of inducing P-gp expression, but may modulate its transporter activity. The possibility is suggested that derivatives of ANF might affect the P-gp efflux of BaP metabolites by transporter inhibition and thus prolong the persistence of genotoxic compounds in the cell. For instance, flavonoids having a hydroxyl group on position 5 and 3, and the 2–3 double bonds have been demonstrated to inhibit efflux of carcinogens from normal cells (Bansal et al. 2009). Although neither the pattern of ANF metabolites formed in vivo, nor their impact on P-gp transport activity, has been investigated in rat small intestine, the described structure-activity relationship for the interaction of P-gp with flavonoid might give a clue. In this respect, ANF metabolites fit well with the structural requirements for the P-gp inhibition.

Thus, to understand results of the oral administration of flavonoids with BaP in our experiments a complex interplay between metabolizing enzymes and transporters, especially in small intestine, should be considered. In the proximal part of small intestine the parent flavonoids possibly inhibit CYP1A1-mediated activation of BaP and do not alter BaP-mediated induction of P-gp and its activity, while in the distal part the flavonoid metabolites lacking the CYP1A1 inhibitory ability may block P-gp transport activity. The enhanced level of BaP-DNA adducts thus results from prolonged exposure of BaP and/or its metabolites to intracellular activation enzymes. This suggestion clearly describes situation in the distal part of small intestine when ANF and BaP were applied simultaneously. Moreover, for genotoxic consequences of a sequential intake of ANF prior to BaP, the overall metabolic fate of both compounds (flavonoid and BaP) should be regarded. The delay of 72 hrs allows the second-pass metabolism of the flavonoid resulting in e.g. ANF-conjugates, which are returned back to small intestine via bile. The gradual microbial cleavage of ANF-conjugates, e.g. by gluc-
urinary, along the small intestine may release ANF hydroxy-derivatives inhibiting the efflux activity of P-gp at the time when BaP genotoxic metabolites were formed. To prove this concept, additional experiments are needed, namely those with P-gp and flavonoid metabolites.

In conclusion, the results of the present study indicate a causal and organ dependent link between the BaP activation and exposure of experimental animals to flavonoids. While in liver the BNF animal pre-treatment enhanced DNA adduct formation and DHM and ANF were associated with lowered BaP-induced EROD activity of CYP1A1. Depending on the organ examined, the BaP induction of CYP1A1 might be protective against 85–106.


REFERENCES


