Role of dihydromyricetin in cytochrome P450-mediated metabolism and carcinogen activation

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Abstract

OBJECTIVES: Dihydromyricetin (DHM) is a flavonoid, which has been shown to antagonize effects of ethanol intoxication. As a potential pharmacological agent, its biological interactions with enzymes metabolizing foreign compounds should be tested. Thus, the aim of this study was to analyze the influence of DHM on the induction and metabolic activity of selected cytochromes P450 (CYPs).

METHODS: After flavonoid administration by oral gavage to stomach the CYP expression at protein and mRNA levels was determined in rat liver and small intestine. The effects of flavonoids on CYP1A1/2, CYP1A2 or CYP2B1/2 enzyme activities in microsomes were measured using marker activities of these enzymes. Flavonoid-mediated inhibition of recombinant CYP1A2 was also assayed with luciferin-ME substrate. The flavonoid interaction with aryl hydrocarbon receptor (AhR) was assayed by reporter luciferase activity in Hep2G cells.

RESULTS: The value of half maximal inhibitory concentration of DHM for CYP1A1/2, CYP1A2, and CYP2B1 were determined to be 4.1, 14.2, and 98.5 mmol.L⁻¹, respectively. With the exception of a weak induction of CYP2B1 and CYP1A2 in the middle part of small intestine and in the liver, respectively, DHM did not affect the CYP expression at protein levels. On the contrary, real-time PCR revealed elevated expression of CYP1A1 and CYP1A2 mRNA in proximal part of the small intestine while decreased in the middle part. In the study utilizing the HepG2 cells, DHM showed only an additive effect on the benzo[a] pyrene-mediated activation of Ah receptor.

CONCLUSIONS: Dihydromyricetin doesn't significantly interfere with metabolic activity of CYP1A1/2 and CYP2B1 enzymes.
INTRODUCTION

Flavonoids are chemicals produced by plants as secondary metabolites. They are indispensable in plant protection against microbial infection or herbivores and serve also as pigments and olfactants (Hodek et al. 2002). Their possible biological impacts on human body are still questionable (Hodek et al. 2009). The most investigated properties of flavonoids are associated with the reduced risk of cardiovascular diseases, osteoporosis, other age-related degenerative diseases and cancer (Morris & Zhang 2006). Dihydromyricetin (DHM) is a flavonoid found in high concentrations in Ampelopsis grossedentata. Besides anti-cancer activity (Lu et al. 2015), DHM has been shown to provide a unique antagonistic effect against an ethanol intoxication and addiction (Shen et al. 2012). Having this therapeutic potential, DHM is presumed to be supplied in high doses into the organism. On account to this, there is a need to reveal DHM biological impacts, especially on enzymes involved in the metabolism of xenobiotics.

The main organ involved in processing of foreign compounds absorbed in gastrointestinal tract is the liver. By the first-pass elimination process, hepatic enzymes modify hydrophobic molecules by the cascade of two biotransformation pathways into more polar and easily eliminated compounds. Final products show usually a lower biological toxicity, but some intermediate metabolites can be reactive and cause cytotoxicity, genotoxicity or mutagenicity. Cytochromes P450 (CYPs) are the key enzymes participating in the so called phase I of xenobiotic biotransformation. The activity of this hemoprotein could be modulated by diverse mechanisms, from which the most common one is the induction by xenobiotics, including flavonoids (Hodek et al. 2002). They influence the level of transcription, which is controlled via ligand-activated nuclear receptors or transcriptional factors. For instance, the gene expression of a CYP1 family is mediated by aryl hydrocarbon receptor (AhR), which is a ligand-activated transcriptional factor dimerizing with AhR nuclear translocator. As a heterodimer, AhR is binding to specific DNA target sequences, called xenobiotic-response elements (XRE) and by this mechanism AhR is able to initiate the transcription (Pavek & Dvorak 2008). This molecular pathway explains the induction effect of polycyclic aromatic hydrocarbons (PAH), which are specific AhR ligands. PAH represent not only the example of CYP1 inducers, but simultaneously, they are frequently also the substrates of CYP1A1, which might be metabolically activated to reactive intermediates (metabolites) possessing the (geno)toxic effects. Benzo[a]pyrene (BaP) is a PAH found in smoke originated from incomplete combustion of organic material, such as compounds present in the cigarette smoke or in the charred food. By successive oxidation via CYP1A1 BaP may form reactive intermediates forming covalent adducts with DNA (Phillips 1999; Stiborova et al. 2013).

The modulation of CYP activities results in changes in their substrate metabolism. Flavonoids are compounds, which may affect the CYP system as inducers, inhibitors or activators. For example, the transcription of CYP1A1/2 can be induced by tangeretin while CYP2B family has flavanone as a specific inducer (Hodek et al. 2002). Myricetin was found to be an inhibitor of the CYP3A4-mediated metabolism of several substrates (Ho et al. 2001). In this study, we investigated the influence of DHM on induction of CYP1A1/2 and CYP2B1 in rats in vivo, in hepatocellular carcinoma cell line, Hep2G, and in the microsomal systems containing these CYPs.

MATERIAL AND METHODS

Chemicals

High-Capacity cDNA Reverse Transcription Kits and Gene Expression Master Mix (Applied Biosystems, USA), λ DNA EcoRI + Hind III (Fermentas, USA), Trizol® Reagent (Invitrogen, USA.), Prime & Probes β-actin and Prime & probes CYP1A1 (Life Technologies, USA), blue Gel loading Dye (New England BioLabs, USA), ethanol, isopropanol, EDTA, chloroform, NADPH, boricinonic acid, resorufin, 7-ethoxyresorufin, 7-methoxyresorufin, 7-pentoxysresorufin, 5-bromo-4-chloro-3'-indophosphate/nitro-blue tetrazolium (BCIP/NBT), anti-chicken IgG alkaline phosphatase conjugate (Sigma Chemical Co., USA) were used in the experiments. Dihydromyricetin was provided from APIchem (Shanghái, PRC) and MYR from TCI (Tokyo Chemical Industry Co., Japan). Specific chicken anti-CYP1A1/2 and anti-CYP1A2 antibodies were prepared by Prof. Hodek as described (Hodek et al. 2013). The hepatocellular carcinoma (HepG2) cells were purchased from the European Collection of Cell
Cultures (ECACC, UK). The HepG2 cells were cultivated in antibiotic-free DMEM medium supplemented with 10% fetal bovine serum and 1% non-essential amino acids at 37°C in humidified 5% CO2 incubator. 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 (150–200 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 and MYR, were dissolved in sunflower oil and administered per os by gastric gavages in four doses (0.5 mL, 60 mg.kg–1 body weight) in four consequent days. Animals of all experimental groups were sacrificed 24 hrs after the last treatment. The control group was treated with sunflower oil only. Microsomes were prepared from 3 sections of dissected small intestine (proximal, middle, and distal parts) and the whole liver, as described elsewhere (Krizkova et al. 2008, Hodek et al. 2011; Stiborova et al. 2013). Microsomal fractions were stored at –80°C before use. Protein concentrations in the microsomal fractions were determined using the bicinchoninic acid protein assay with bovine serum albumin as the standard (Weichelman et al. 1988). The concentration of CYP was estimated according to Omura & Sato (1964) based on the absorption of the of reduced CYP complex with carbon monoxide.

Western blot analysis
The CYP1A1, 1A2 and 2B1 proteins were detected by Western blotting on the PVDF, Immobilon-P membrane (Millipore, USA) using specific chicken anti-CYP1A1/2, anti-CYP1A2 and anti-CYP2B1 antibodies (20 μg.mL–1). For sodium dodecyl sulfate (SDS)-electrophoresis (10% polyacrylamide gel), 60 μg protein/well of small intestine and 15 μg protein/well of liver microsomes was applied. Western blotting was carried out as described earlier (Krizkova et al. 2008). 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
Microsomal samples, in which the CYP1A1/2 were induced with BaP and CYP2B1 by phenobarbital were analyzed for specific activity of CYP1A1/2, CYP1A2 or CYP2B1, by monitoring dealkylation of 7-ethoxyresoruﬁn (EROD), 7-methoxyresoruﬁn (MROD) or 7-pentoxyresoruﬁn (PROD), respectively, according to the method described by Burke & Mayer (1974). Final mixtures (175 μL) in 100 mmol.L–1 potassium phos-
Interaction of DHM with cytochromes P450 showed as mean of two independent experiments. The concentration of DMSO used in the culture medium was maintained at 0.1% (v/v) in all performed experiments. The experimental data were analysed using standard statistical methods ANOVA and Student’s t-test.

RESULTS

The impact of DHM on the CYP induction was analyzed in microsomal fractions of small intestine and liver after the oral premedication of rats with flavonoid. The expression of CYP1A and 2B families in microsomal samples at the protein levels was determined by Western blotting, and at mRNA levels by real-time PCR method in rat tissues. Also HepG2 cells were employed to determine the influence of DHM on the activation of AhR receptor. The DHM mediated inhibition of specific catalytic activities (EROD, MROD and PROD) of CYP1A and 2B families. The inhibitory effect of DHM on CYP1A2 was also confirmed with the recombinant human enzyme instead of microsomal samples.

Enzyme inhibition assays

MYR and DHM mediated inhibition was assayed using microsomal fractions containing elevated levels of CYP1A1/2 and CYP2B1 by their induction with BaP and phenobarbital. Figures 1 and 2 show the inhibition of microsomal CYP activities by increasing concentrations of DHM or MYR relative to activity of flavonoid un-affected controls (100%). By plotting the data into semi-logarithmic graphs, half maximal inhibitory concentration (IC50) values for each flavonoid were determined. For inhibition of CYP1A1/2, CYP1A2 and CYP 2B1, IC50 values of 4.1, 14.2 and 98.5 mmol.L–1 were determined, respectively. Interestingly, a low but significant increase (p<0.001) in CYP2B1/2 activity was found at 20–30 mmol.L –1 DHM concentrations. The stimulation of activity of NADPH:CYP oxidoreductase by DHM was not detected in this microsomal sample (data not shown).

Induction studies

At first an ex vivo gene reporter assay based on the HepG2 cell line was used to analyze the influence of DHM and MYR on AhR receptor (see Figure 4). This
study showed that neither DHM nor MYR presence in the AhR receptor reporter assay significantly activated XRE. Then flavonoids were applied simultaneously with BaP, the compound known as AhR ligand. Under this setting, DHM showed only an additive effect on BaP. Addition of MYR produced only a moderate synergistic effect on the BaP activation of AhR receptor. Results in Figure 4 show the dependence on the flavonoid concentration.

Next experiments were carried out in \textit{in vivo} with rats treated with DHM or MYR. The induction of CYP1A1/2 and CYP2B1 at their protein levels was investigated in rat microsomal fractions using the Western blot method (see Figures 5 and 6). Dihydromyricetin increased the expression of CYP2B1 in the middle part of the small intestine, only. Myricetin did not induce CYP2B1 in any organ examined, but noticeable induction of CYP1A1 was detected in the liver. In the case of DHM animal treatment, the flavonoid effect on expression levels of CYP1A1 and CYP1A2 mRNAs was determined. Results in Table 1 show an increased mRNA expression of CYPs of both families in the proximal part of small intestine. In any other organ examined no influence of DHM on CYP1A1 and CYP1A2 mRNA expression was detected.
Interaction of DHM with cytochromes P450

Tab. 1. Expression of CYP1A1/2 mRNA in the liver and the small intestine (si) after treatment of rats with DHM.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>CYP1A1</th>
<th>CYP1A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>liver</td>
<td>1.10</td>
<td>0.88</td>
</tr>
<tr>
<td>si - p</td>
<td>5.69 **</td>
<td>16.12 ***</td>
</tr>
<tr>
<td>si - m</td>
<td>0.13 *</td>
<td>ND</td>
</tr>
<tr>
<td>si - d</td>
<td>0.26</td>
<td>ND</td>
</tr>
</tbody>
</table>

Experimental animals (rats) were exposed to DHM and then mRNA was isolated from their liver and the small intestine divided into three parts – proximal (p), middle (m) and distal (d). Presented results from a real-time PCR method were measured in triplets and then evaluated in the program REST2009. Statistical significance of noticeable results is *p<0.05, **p<0.01, ***p<0.005. Values bellow the detection limit are marked ND – non-detectable. Data are expressed as fold increase over the control normalized on the internal standard (β-actin).

DISCUSSION

DHM, also called amelopsin, is a natural flavonoid known for several health promoting properties; it provides e.g. anti-oxidant, anti-bacterial and hepatoprotective effects (Zhang et al. 2007). In addition, DHM counteracts the alcohol intoxication and addiction as shown in studies with experimental animals (Shen et al. 2012). Because of that activity, DHM is getting to be popular and available on the market as a dietary supplement. Moreover, it is likely that DHM will be used in a clinical practice. This flavonoid is considered as nontoxic compound but the knowledge on its impact on biochemical pathways in the living organism is rather limited. Thus, the present study was undertaken to investigate the influence of DHM on CYP1A1/2 and CYP2B1, activity changes of which may impact e.g. carcinogen activation or drug metabolism.

To get the complex insight into the interactions of DHM with CYPs, the in vivo and in vitro experiments were carried out. First, the direct influence of DHM on the CYP enzyme activity was analyzed with a recombinantly expressed CYP or with CYPs induced in microsomal samples. Second, studies focused on the ability of DHM to induce expression of the selected CYPs were conducted. HEPG2 cells were used to find possible interactions of DHM with AhR. Then after, the CYP mRNA and protein levels were determined in samples isolated from animals pretreated with the flavonoid. MYR was included in the study because of its similar structure to DHM.

In addition to flavonoid inhibition studies with rat microsomal CYPs, human recombinant CYP1A2, which is involved in drug metabolism and activation of ingested carcinogens in liver, was used, too. The comparison of results shown in Figures 1, 2 and 3, indicates that DHM and MYR inhibit human recombinant CYP1A2 at much lower concentrations than in the microsomal samples. This discrepancy was probably caused by the differences between both experimental setups, namely in the CYP concentrations and substrates used. Moreover, the microsomal fraction is much more complex than the CYP1A2 membrane system in the respect of protein constituents of the sample. Regardless the conditions used, in both systems, MYR was a significantly more potent inhibitor than DHM. Evaluating the results of the flavonoid inhibition of CYP specific substrate metabolism (EROD, MROD and PROD) it is clear that CYP1A1/2 enzyme activities were efficiently inhibited only by MYR. This finding is in accordance with results of related studies (Master et al. 2012; Schwarz et al. 2011).

Additional experiments were performed to assess the induction effect of DHM and MYR on the expression of proteins, which are regulated via AhR. The reporter system based on cell line HepG2 was employed. None of flavonoids used notably stimulated the activity of AhR. Moreover, in this study BaP was used as a known inducer of CYP1A enzymes. BaP is a carcinogenic agent, which induces the enzymes of a CYP1 family and stimulates CYP-mediated metabolism of BaP. The biotransformation of BaP results into less toxic compounds as well as dangerous reactive intermediates. When MYR or DHM were applied simultaneously with BaP a marked synergistic effect was developed by MYR, while DHM produced an additive effect, only. However, this stimulatory effect of DHM on BaP-mediated activation of AhR was seen neither in vivo by Western blots nor in vitro by CYP activities determined in microsomal samples of the liver and small intestines in our previous studies (Hodek et al. 2014). In a distal part of small intestines, the 32P-postlabeling analysis of BaP-DNA adduct formation revealed increased levels when BaP was administrated simultaneously or after DHM (Hodek et al. 2014). However, it should be noted that cell culture systems are hardly comparable with in vivo conditions. Diverse mechanisms such as chemical absorption, distribution and metabolism in other sites than the liver or small intestines might be involved in the entire biotransformation fate of the tested compounds in vivo.

The finding that DHM does not induce transcription of a CYP1A gene in the liver was confirmed also by mRNA expression analysis. On the contrary, the pre-treatment of rats with DHM increased levels of CYP1A1 and CYP1A2 mRNA in a proximal part of the small intestine. In the rest of small intestine the induction by DHM was not observed. These results may reflect the major absorption of DHM in the proximal part of small intestines. In contrast to mRNA analysis, results of Western blots revealed a slight induction of CYP2B1 in the liver (Figure 6) and in the middle part of the small intestine (Figure 5) of DHM pre-treated animals. But no induction was determined in the proximal or distal parts of the small intestine. The other flavonoid, MYR, slightly induced CYP1A2 (Figure 6) in the
liver, that is, however, in contradiction to results of the HepG2-based study. In general, none of the Western blots show an extensive induction of CYPs tested.

Taking together, flavonoids DHM and MYR were not found to be strong inducers of certain CYPs of 1A and 2B families. From the point of view of the CYP inhibition, DHM is only a weak inhibitor of CYPs tested in comparison to a higher inhibitory potency of MYR. Otherwise, MYR and DHM exhibited comparable low induction effects, especially in XRE activation assay. However, when MYR or DHM were used together with BaP, higher levels of XRE stimulation were detected in the presence of MYR. Even though both flavonoids do not significantly interfere with examined CYP activities, the MYR synergy with BaP should be reconsidered in future studies.

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