CHARLES UNIVERSITY IN PRAGUE

Faculty of Science

Department of Biochemistry

Effects of chemopreventive compounds on cytochrome P450s

Summary of Ph.D. Thesis

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Introduction

According to the World Health Organization statistics, cancer is one of the leading causes of death in the human population worldwide for more than 50 years. Moreover, colorectal and gastrointestinal tract cancers are one of the main types of cancer leading to overall cancer mortality. Prevention consisting in a healthy lifestyle and a natural diet is suggested to be one of the main approaches to reduce cancer risk. In recent years, the consumption and use of dietary supplements containing concentrated chemopreventive phytochemicals increased dramatically. Flavonoids, as the most popular representatives of chemopreventive compounds, present in foods (fruits, vegetables, herbs, beverages) and dietary supplements have the potential to modulate the activity of xenobiotic-metabolizing enzymes [Hodek et al., 2002].

Among proteins interacting with flavonoids, cytochrome P450s (CYPs), monooxygenases metabolizing xenobiotics (e.g. drugs, carcinogens), play the most prominent role. The two members of CYP1A subfamily, CYP1A1 and CYP1A2, are involved in the activation of procarcinogens, such as polycyclic aromatic hydrocarbons, aromatic amines and heterocyclic amines [Eaton et al., 1995; Rendic and Di Carlo, 1997]. Higher CYP1A1 expression and activity seem to be associated with the risk of lung and colorectal cancer. Moreover, CYP1A2 is responsible for metabolizing many frequently used drugs, such as phenacetin, caffeine, imipramine, and it also activates procarcinogens. The enzymes of the CYP1A subfamily can be induced by aromatic hydrocarbons. The activation involves a specific receptor called the Ah receptor. After the binding of an inducing agent to the receptor, a heterodimer with the activated Ah-receptor nuclear transporter is formed, which then binds to a xenobiotic response element and functions as a transcriptional enhancer to stimulate the gene transcription [Ortiz de Montellano, 2005].

Polycyclic aromatic hydrocarbons (PAHs) and heterocyclic amines (HCAs) are groups of potential carcinogens activated by CYP1A subfamily.

PAHs are formed during the incomplete combustion or pyrolysis of organic matter and during various industrial processes. Humans are exposed to PAHs by various means; the primary routes of exposure are the inhalation of polluted air, wood smoke, and tobacco smoke, as well as the ingestion of contaminated water and foods normally containing microgram quantities of PAHs.

Dietary exposure to heterocyclic amines is considered to be a potential human cancer risk. These compounds are produced during the cooking of red meat, poultry, and fish under normal household conditions [Sinha et al., 2000]. They are formed in relatively high concentrations by the condensation of creatinine with amino acids. The role of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and other HCAs in human cancer causation is of interest, in particular for cancers of colon and breast [Snyderwine, 1994; Nagao et al., 1994]. However, the molecular principle of the carcinogen activation in organisms has not been entirely elucidated yet, mainly in the context of interactions of carcinogens or other xenobiotics in organisms.

Aims

The aim of our study is to expand the current knowledge of chemopreventive compounds and their role in the process of carcinogenesis. The rising consumption of dietary supplements containing these compounds (e.g. flavonoids) evokes concerns regarding their unlimited consumption, as their side effects are not sufficiently known. Our research is directed at their effects not as inhibitors of the carcinogen activating enzymes, but as inducers, whose effects, for example the induction persistance, due to repetitive or single dose *p.o.* administration or sequential exposure of the organism to chemopreventive compounds and carcinogens, are being omitted. In order to address these effects of chemopreventive compounds on cytochrome P450s, several specific objectives had to be accomplished.

- To establish and optimize the immunodetection of CYP1A in microsomal samples (liver, small intestine) using a chicken anti-rat CYP1A1 and CYP1A2, as well as the assay of CYP1A1 and CYP1A2 specific activities with marker substrates.

- To screen various groups of chemopreventive compounds for their ability to induce CYP1A1 and CYP1A2 in rat liver and small intestine, the two main organs responsible for xenobiotic metabolism and carcinogen activation, after *p.o.* administration of chemopreventive compounds.

- To examine the relevant effects on the expression of CYPs and their specific activities in different time and dose regimens of the chemopreventive compound treatment.

- To carry out a sequential study, which comprises the administration of an inducing or non-inducing carcinogen, preceded by the administration of a chemopreventive compound, and to determine the DNA-adducts formation using ^{32}P-postlabelling.

Results and discussion

To investigate the induction effects of chemopreventive compounds on CYP1A properly, the research was conducted in several stages. At first, a wide variety of chemopreventive compounds was tested, namely curcumins, stilbenes, organosulphur compounds, and flavonoids. The levels of protein expression and activity of CYP1A1 and CYP1A2 was determined to evaluate the ability of chemopreventive compounds, *p.o.* administered to rats, to induce CYP1A in rat small intestine and liver. These two organs are the main sites of xenobiotic metabolism. The induction effects on CYP1A1 and CYP1A2 were examined in different time and dose regimens by optimized Western blotting analysis with primary chicken antibodies, treated to eliminate their binding to keratin contamination, and specific activity measurements using 7-ethoxyresorufin and 7-methoxyresorufin as specific substrates for CYP1A1 and CYP1A2, respectively.

A well-known inducer of CYP1A subfamily, β-naphthoflavone (BNF), was used as a reference compound in the whole study. In both tissues, liver and small intestine, β-naphthoflavone proved its strong inducing capacity. Thus, β-naphthoflavone served as a positive control throughout the study.

Regimen I

The induction effects of a wide variety of chemopreventive compounds on CYP1A1 and CYP1A2 after 5 day treatment were examined. As shown in ***Figure 1***, in both tissues, the most efficient non-flavonoid inducer of CYP1A1 was diallyl sulphide, which also induced CYP1A2 in liver (***Fig. 1B***). Generally, in small intestine, the most effective flavonoid inducers of EROD and MROD activities and the CYP protein level were aglycones (β-naphthoflavone, flavone, flavanone, morin).

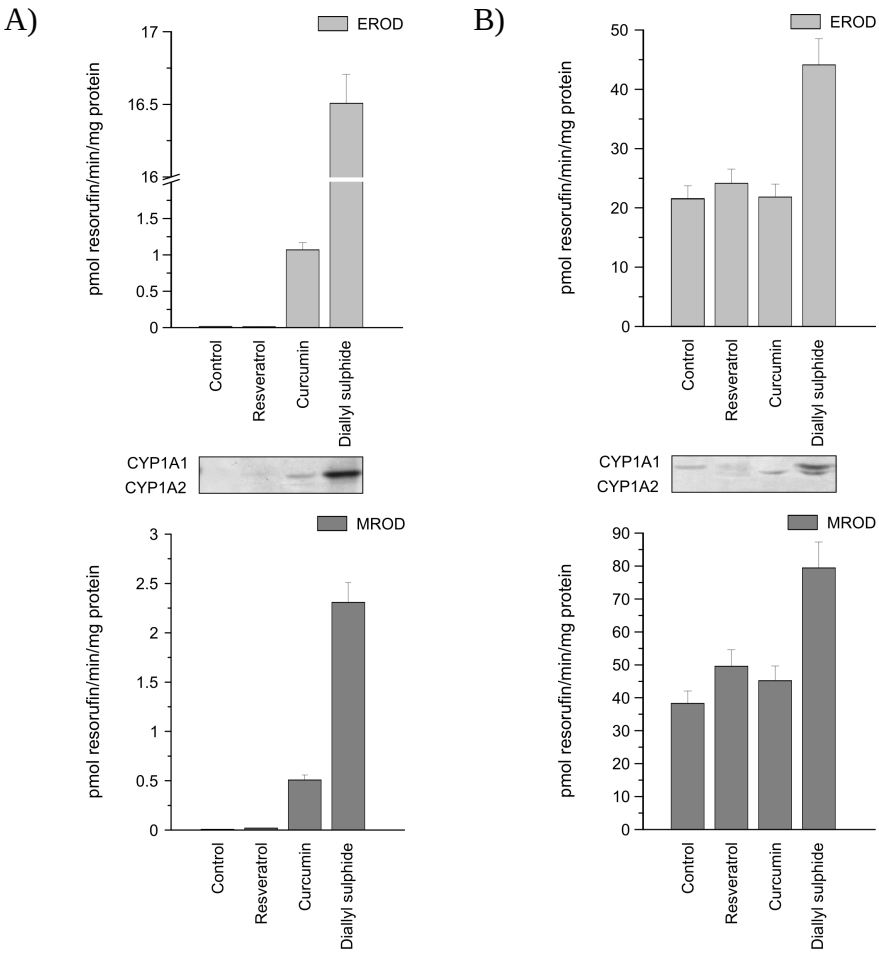

Figure 1 Effects of non-flavonoid treatment in small intestine (A) and liver (B). EROD and MROD activities of CYP1A1 and CYP1A2, respectively, were determined in rat microsomes from liver and the proximal part of small intestine after exposure to non-flavonoid chemopreventive compounds (60 mg/kg body weight) for 5 days. Immunodetection of CYP1A1/2 in liver and the proximal part of rat small intestine.

Moreover, small intestine, as an organ highly exposed to xenobiotics, was dissected into two or three parts. As shown in ***Figure 2***, the higher CYP1A1 level and activity in the proximal part of small intestine compared to the distal part was observed after the β-naphthoflavone, morin, and rutin treatment. However, the quercetin and isoquercitrin administration caused the highest increase in the middle part of small intestine (data not shown).

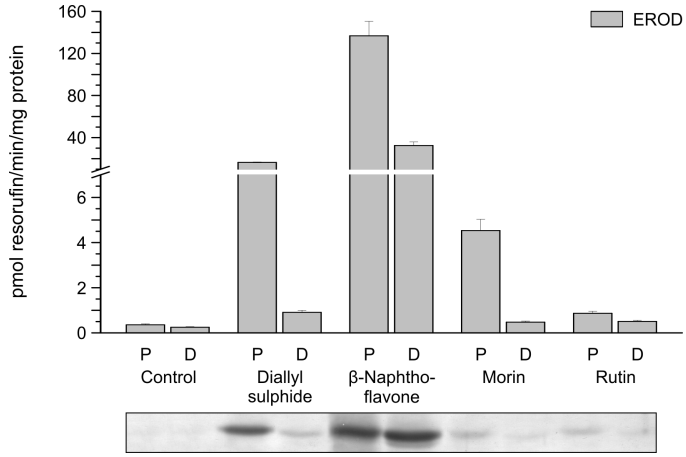

Figure 2 Effects of chemopreventive compound treatment along small intestine. EROD activity of CYP1A1 was determined in the proximal (P) and distal (D) part of rat small intestine after exposure to chemopreventive compounds (60 mg/kg body weight) for 5 days. Immunodetection of CYP1A1 in the proximal and distal part of rat small intestine.

Regimen II

A further point of view included a single dose administration to determine the induction capability of chemopreventive compounds in a low dose. The persistence of the elevated CYP levels was also investigated. Therefore, the relevant effects on CYP expression and both specific activities were evaluated 24, 48 and 72h after the treatment, in small intestine and liver of rats *p.o.* administered with the selected compounds (***Fig. 3*** and ***Fig. 4***).

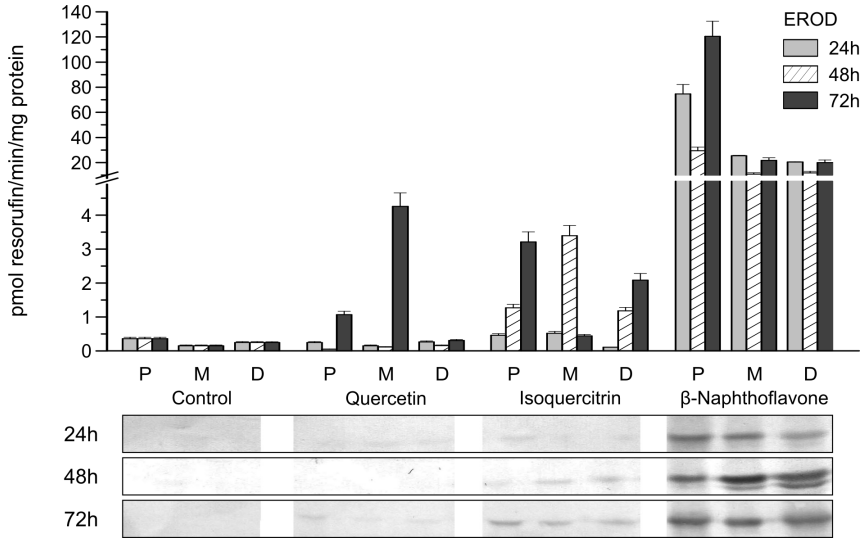

Figure 3 Effects of flavonoid treatment in small intestine in different times after the treatment. EROD activity of CYP1A1 was determined in the proximal (P), middle (M) and distal (D) part of rat small intestine after a single dose treatment with flavonoids (60 mg/kg body weight) 24, 48 and 72 hours after the treatment. Immunodetection of CYP1A1 in the proximal, middle and distal part of rat small intestine.

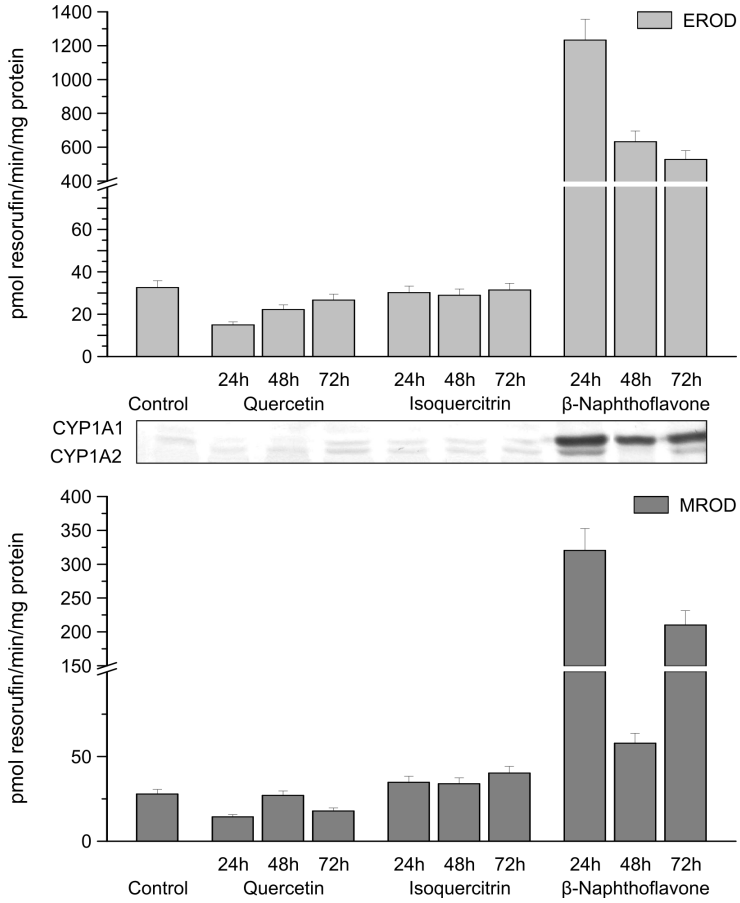

Figure 4 Effects of flavonoid treatment in liver in different times after the treatment. EROD and MROD activity of CYP1A1 and CYP1A2, respectively, were determined in rat liver after a single dose treatment with flavonoids (60 mg/kg body weight) 24, 48 and 72 hours after the treatment. Immunodetection of CYP1A1/2 in rat liver.

The results obtained with intestinal parts suggest the important role of the compound structure and its metabolism in the process of bioavailability to humans.

Regimen III

In subsequent experiments, the inducer (β-naphthoflavone) and common carcinogens (benzo[a]pyrene or PhIP) were administered in successive steps. Based on the results with β-naphthoflavone in the experiments mentioned above, a delay of 72h was chosen, between individual administrations. The time delay was chosen in such a way so as to prevent any potential inhibition of activating enzymes, evoked by the presence of chemopreventive compounds, while the induction effects would still persist.

Figure 5 shows that in small intestine, the administration of β-naphthoflavone increased EROD activity and also the CYP1A1 level in all the intestinal parts at a low dose of PhIP (PhIP1, 50 mg/kg). Contrary to that, the high dose of PhIP (PhIP2, 150 mg/kg) caused a loss of EROD activity in β-naphthoflavone pretreated rats, though the protein levels did not change markedly. Similarly to the administration of both, BNF and PhIP2, the combination of β-naphthoflavone with benzo[a]pyrene did not additively increase the protein level and activity of CYP1A1 compared to the sole compound administration.

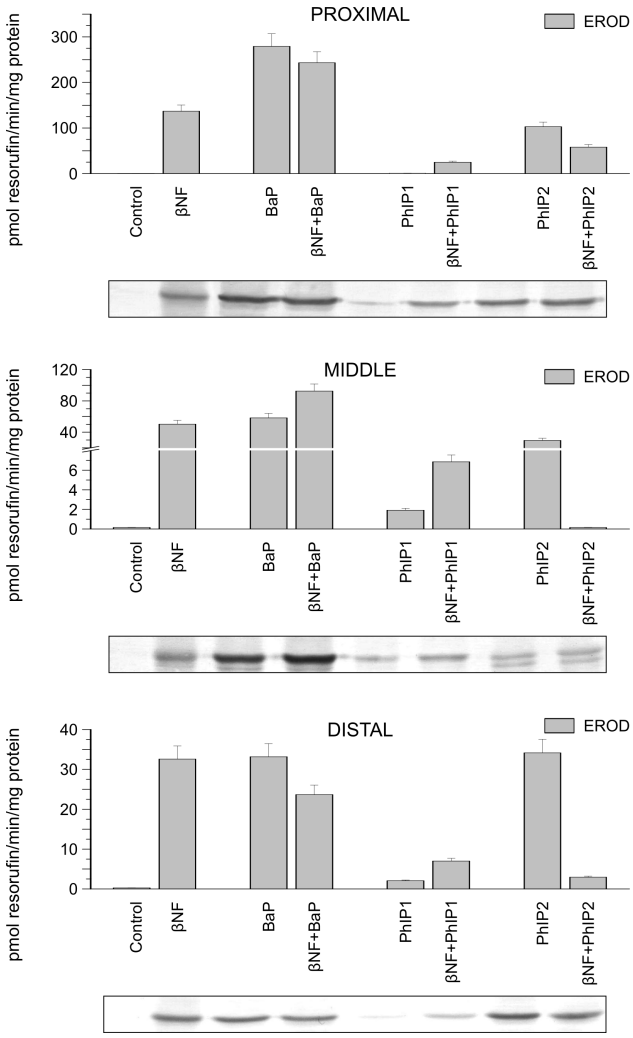

Figure 5 EROD activity of CYP1A1 in small intestine. Microsomes were isolated from the proximal, middle and distal part of rat small intestine after exposure to β-naphthoflavone and/or carcinogens. Immunodetection of CYP1A1 in the proximal, middle and distal part of rat small intestine.

In liver (***Fig. 6***), the combination of β-naphthoflavone and benzo[a]pyrene administration led to a strong increase of EROD and MROD activity compared to separately administered compounds, namely for CYP1A2. This indicates potential synergistic effects of these two compounds. However, no marked effect of the β-naphthoflavone treatment in combination with PhIP was observed, as was the case in small intestine.

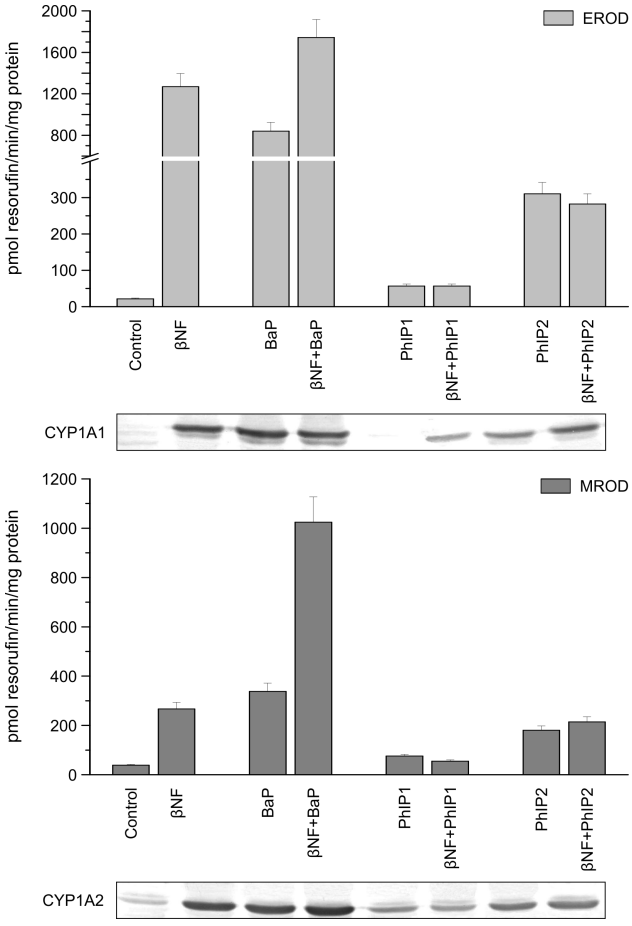

Figure 6 EROD and MROD activity of CYP1A1 and CYP1A2, respectively, in liver. Microsomes were isolated from rat liver after exposure to β-naphthoflavone and/or carcinogens. Immunodetection of CYP1A1 and CYP1A2 in liver microsomes.

Carcinogen-DNA adduct formation is generally believed to represent an important genotoxic step in the initiation of carcinogenesis. Therefore, the level of carcinogen-DNA adducts is another marker for risk assessment of cancer development. Carcinogens, benzo[a]pyrene and PhIP, undergo enzymatic biotransformation *in vivo* leading to both activation and/or detoxification of

the carcinogen. The level of BaP-DNA adducts was evaluated by ^{32}P-postlabelling in the proximal and distal parts of small intestine (***Tab. 1***). The β-naphthoflavone pretreatment markedly increased the amount of BaP-DNA adduct 1 (adduct derived from 9-hydroxy-BaP) in the proximal part of small intestine. The higher CYP expression in the proximal part of small intestine than in the distal part is in accordance with our findings that the total content of BaP-DNA adducts in the proximal part is nearly twice as high as in the distal part.

Table 1 Benzo[a]pyrene-DNA adduct levels in small intestine.

	RAL/10^8 nucleotides			
DNA sample Parts of small intestine	Spot 1	Spot 2	Spot 3	Total
BaP proximal	3.85	2.82	4.39	11.06
βNF+BaP proximal	5.15	2.97	3.96	12.08
BaP distal	4.40	1.56	1.64	7.60
βNF+BaP distal	4.02	1.85	2.46	8.33

β-Naphthoflavone and/or benzo[a]pyrene were administered to rats and DNA adducts were determined in the proximal and distal part of small intestine. RAL, "relative adduct labelling"

Spot 1 – adduct derived from 9-hydroxy-BaP

Spot 2 - the major dGp adduct (dG-N^2-BPDE)

Spot 3 – unknown adduct

The results obtained by ^{32}P-postlabelling correlate well with the immunodetection and determined specific activities, where the BNF pretreatment did not increase these measurements markedly in comparison to the sole BaP administration. Benzo[a]pyrene is known to be strong inducer of CYP1A subfamily, and thus it is possible that the maximal level of induction has already been exceeded.

PhIP-DNA adducts will be determined shortly by recently developed online column-switching liquid chromatography tandem mass spectrometry technique [Singh et al., 2010].

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