

Influence of probiotics on rat liver biotransformation enzymes

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Abstract

OBJECTIVES: The aim of the study was to find, whether probiotic *Escherichia coli* Nissle 1917 O6:K5:H1 (EcN) influence the amount and activity of cytochromes P450 (CYP) in rat liver.

DESIGN: Live bacterial suspension of EcN was applied to the female Wistar rats in single dose or for 14 days consecutively. The bacterial lipopolysaccharide (LPS) isolated by phenol extraction from the EcN was given to the rats for 14 days as well. Control rats were treated with the saline solution daily for 14 days. Relative amount of CYP2C6, CYP2C9 (corresponding to rat CYP2C11), CYP3A1 and CYP1A2 protein expression in rat liver microsomes was determined by Western blotting. For the determination of six CYP activities (corresponding to human CYP1A2, CYP2A6, CYP2B6, CYP3A4, CYP2C9 and CYP2D6) fluorescence, luminescence or absorbance detection was used.

RESULTS: The data presented show that the changes of the total content of the CYP enzymes in rat liver are not significant after administration of the probiotic for 1 or 14 days as well as of the LPS. Western blots revealed a slight increase in CYP2C6 protein expression; level of another rat CYP2C protein (readings with anti-human CYP2C9 antibody corresponding to the rat CYP2C11) as well as of CYP1A2 was elevated after administration of LPS; a small decrease was observed with CYP3A1 protein. Changes in activities of CYP forms are not significant, only the activity of rat CYP2C forms in liver microsomal samples of rats given free LPS appeared to exhibit a small, but significant tendency to increase.

CONCLUSION: The results show that the p.o. administration of probiotics to rat does not markedly influence the rat hepatic CYP enzymes.

Abbreviations & Units:

CYP	- cytochrome P450
EcN	- probiotic <i>E. coli</i> Nissle 1917 O6:K5:H1
EFC	- 7-ethoxy-4-(trifluoromethyl)-coumarin
ETR	- 7-ethoxyresorufin
HPLC	- high performance liquid chromatography
LPS	- bacterial lipopolysaccharide

INTRODUCTION

The processes of absorption in the digestive tract are a key step in the oral delivery of a drug. Orally administered drugs have to pass epithelial cell layer consisting of enterocytes (in the case of small intestine) or colonocytes (in the colon). Intestinal cells have a variety of specific features as tight junctions, influencing paracellular permeability, or a presence of a variety of drug transporters or enzymes metabolizing xenobiotics which can effectively affect the entry of drugs into the bloodstream (Krizkova *et al.* 2008).

Another factor which can affect the absorption of drug from gastrointestinal tract is bacterial composition of the gut. Gut bacterial (commensal) microflora consists of about hundred trillions of cells of more than five hundred different bacterial species which can be divided as being either beneficial or potentially pathogenic due to their metabolic activities and fermentation end-products (Guarner & Malagelada, 2003). On the other hand, probiotics – contrary to the commensal microorganisms – can be defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (www.fao.org/es/ESN/Probio/probio.htm). The effects of probiotics should be hence beneficial including e.g. prevention of antibiotic-associated diarrhea, eradication of *C. difficile* infection and enhancement of intestinal immunity (Preidis & Versalovic, 2009). However, being present in the intestine, the probiotics could in principle also interfere with processes determining bioavailability of orally administered drugs, namely, with drug biotransformation by enzymes acting in the gastrointestinal tract and in the liver.

Among enzymes involved in drug metabolism, cytochromes P450 are the most important being responsible for more than three quarters of known biotransformation pathways during the first phase of drug metabolism (Evans & Relling, 1999). The aim of this work was to prove whether the presence of probiotic bacteria (*Escherichia coli* strain Nissle 1917) in the gut may influence the amount and activity of cytochrome P450 (abbrev. CYP) enzymes. The probiotic *E. coli* strain Nissle 1917 has been reported to maintain remission of ulcerative colitis and pouchitis or to prevent colitis in different murine models of colitis (Rembacken *et al.* 1999; Schultz *et al.* 2004). To date, several reports on the influence of probiotic bacteria and of the lipopolysaccharide endotoxin derived from them on activities of liver CYP enzymes were published (reviewed in Yang & Lee, 2008). However, the doses applied as well as the

route of administration did not correspond to the usual route in the man being intravenous or intraperitoneal instead of oral. Hence, there is a need of obtaining the data on possible effect of probiotics on drug metabolizing enzyme activities which will help in evaluating the possibility of probiotic-drug interaction in the man.

MATERIAL AND METHODS

Material. All reagents and chemicals were obtained from Sigma-Aldrich (Prague, Czech Republic) if not stated otherwise. The CYP3A1 and CYP2C6 antibodies were purchased from Abcam (Cambridge, UK). Anti-rat CYP1A2 antibody was obtained from Daichi Pure Chemicals (Tokyo, Japan). Anti-human CYP2C9 antibody was acquired from BD Gentest (Woburn, MA). The chemiluminescence kit for Western blotting (Immun Star) was purchased from Bio-Rad (Hercules, CA), and the nitrocellulose membrane was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Luciferin-ME EGE was obtained from Promega (through East Port Scientific, Prague, CZ), Miniprotean electrophoresis and Western blotting apparatus was purchased from Bio-Rad (Hercules, CA). The TECAN Infinity absorbance/fluorescence/luminescence reader (Tecan, Vienna, Austria) was used for detection of the respective spectral data. HPLC-UV analyses were performed on Shimadzu LC-20A Prominence (Kyoto, Japan).

Methods

Preparation of samples. Live bacterial suspension of *E. coli* Nissle 1917 O6:K5:H1 (abbrev. EcN) was applied to female Wistar rats (10¹¹ CFU/dose, orally). Four rats were stressed by oral application of the saline solution daily for 14 days. This group was used as control. The probiotic was applied daily to four animals for 14 days; four rats were given the probiotic only once, the last day of the experiment. To evaluate the effect of bacterial lipopolysaccharide (abbrev. LPS), the LPS isolated by phenol extraction from the same *E. coli* strain Nissle was given orally to fourth group of animals as an EcN-free supernatant containing 150 µg of free LPS per mL. The LPS was applied to three rats daily for 14 days.

After 14 days the rats were sacrificed and livers were removed and weighted. The liver samples were frozen in liquid nitrogen and stored at -70 °C until used. The protocol of the experiment was approved by the institutional Ethics Committee. For preparation of liver microsomes, the liver samples were rinsed in cold 0.25 M sucrose in 1 mM EDTA (pH 7.4). The tissue was then homogenized and subjected to differential centrifugation to obtain the microsomal fraction according to standard procedures (Bruyere *et al.* 2009).

Determination of total protein and cytochrome P450. Total protein content was determined by bicinchoninic acid method with a standard BCA Protein Assay kit (Pierce, Rockford, IL). Determination was done in three

parallels using a calibration curve using bovine serum albumin as a standard. Differences between determinations were below 15%. Cytochrome P450 content was determined by the method described by Omura & Sato, (1964).

Western blotting. Microsomal proteins from liver (10 µg) were separated on 8%-SDS (w/v) polyacrylamide gel electrophoresis and then transferred electrophoretically onto nitrocellulose membranes according to the method of Towbin *et al.* (1979). Immunodetection of CYP was achieved by anti-rat CYP1A2, anti-rat CYP2C6 and anti-rat CYP3A1 as the primary antibodies. For detection of CYP2C11, the anti-human CYP2C9 antibody was used. The bands were visualized with respective peroxidase-conjugated secondary antibodies and their relative intensity was evaluated with Elfoman (Semecky Inc., Prague, Czech Republic) software.

Determination of activities of individual CYP forms. The activities and relative amounts of selected CYP enzymes were followed by established enzymological techniques based on analogy between human and rat forms and their respective substrates: 7-ethoxyresorufin *O*-deethylation (substrate of CYP1A2) (Chang & Waxman, 1998, Siskova *et al.*, 2008); 7-ethoxy-4-(trifluoromethyl)-coumarin *O*-deethylation (substrate of CYP2B6) (Donato *et al.* 2004); diclofenac 4'-hydroxylation (substrate of CYP2C9) (Crespi *et al.* 1998), coumarin 7-hydroxylation (substrate of CYP2A6) (Soucek, 1999); luciferin-ME EGE 6'-dealkylation (substrate of CYP2D6) (Promega Technical Bulletin No. 325, <http://www.promega.com>); and testosterone 6β-hydroxylation (substrate of CYP3A4) (Guengerich *et al.* 1986). Tecan Infinite reader was used for fluorescence detection of products of 7-ethoxyresorufin *O*-deethylation (excitation at 535 nm, emission at 585 nm), 7-ethoxy-4-(trifluoromethyl)-coumarin *O*-deethylation (excitation at 410 nm, emission at 510 nm), coumarin 7-hydroxylation (excitation at 360 nm, emission at 465 nm) and for luminescence detection of product of luciferin-ME EGE 6'-dealkylation. The metabolites of diclofenac 4'-hydroxylation and testosterone 6β-hydroxylation were measured by an HPLC with UV detection using C-18 analytical reversed phase column (250 x 4 mm i.d., 5 µm particle size, Merck, Darmstadt, Germany). To the determination of metabolite of diclofenac 4'-hydroxylation was used mobile phase consisted of two solvents, A (2 mM perchloric acid in an acetonitril in a ratio 7:3 (v/v)) and B (methanol) with the following gradients: 0 min, 30% B; 20 min, 100% B; 22 min, 100 % B; 23 min, 30% B; 33 min, 30% B. The flow rate was set at 1 ml/min. The temperature of oven was set at 50°C and absorbance was monitored at 280 nm. Testosterone 6β-hydroxylation was measured in an isocratic mode. Mobile phase contained 64% methanol and the flow rate was set at 1 ml/min. The temperature of oven was set at 30°C and absorbance was monitored at 245 nm; 50 µl of samples were injected. Enzyme activities were expressed both as nmol product/min/mg

microsomal protein and as nmol product/min/nmol total CYP, i.e. as specific CYP activity.

RESULTS AND DISCUSSION

Total content of protein and of the CYP enzymes; Western blot analyses of protein expression of individual CYP forms. Application of probiotic *Escherichia coli* Nissle 1917 either once, or for 14 consecutive days as well as application of LPS-containing supernatant did not lead to significant changes in the protein content in livers of experimental animals (**Tab. 1**). Similarly, also the total content of the CYP enzymes did not change markedly; the specific content of cytochrome P450 enzymes expressed as ratio of total CYP to total protein content shows only a tendency to decrease in samples from rats given *E. coli* for 14 days or from rats to which the LPS was administered (**Fig. 1**). Hence, the main functional parameters of the liver microsomal drug metabolizing CYP system do not indicate any significant influence of probiotic which could alter the ability of these systems to metabolize concomitantly administered drugs.

Western blots have shown minor, however detectable changes in expression of CYP proteins (Fig. 2A, B, C). The expression of rat CYP2C forms, CYP2C6 and human CYP2C9-like rat protein, according to literature data (Wang *et al.* 2009) and according to BLAST (www.

Table 1. The protein content in rat liver microsomes obtained after treatment with bacterial lipopolysaccharide daily for 14 days (Free LPS) and *E. coli* Nissle 1917 in single dose (1xEcN) or daily for 14 days (14xEcN). Control rats were treated with the saline solution daily for 14 days.

	Protein [mg/ml]	
	Average	S.D.
Saline solution	33.30	4.33
Free LPS	33.89	3.44
1xEcN	33.37	5.36
14xEcN	30.60	4.11

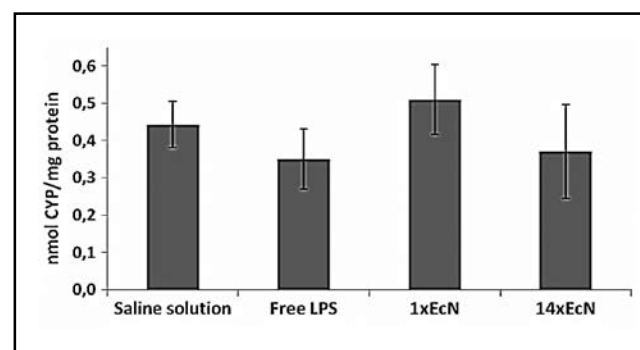


Figure 1. The total content of the cytochrome P450 (CYP) is expressed as nmol CYP/mg protein. The rats were treated with bacterial lipopolysaccharide daily for 14 days (Free LPS) and *E. coli* Nissle 1917 in single dose (1xEcN) or daily for 14 days (14xEcN). Control rats were treated with the saline solution for 14 days. The bars express the ± S.D. (N=4).

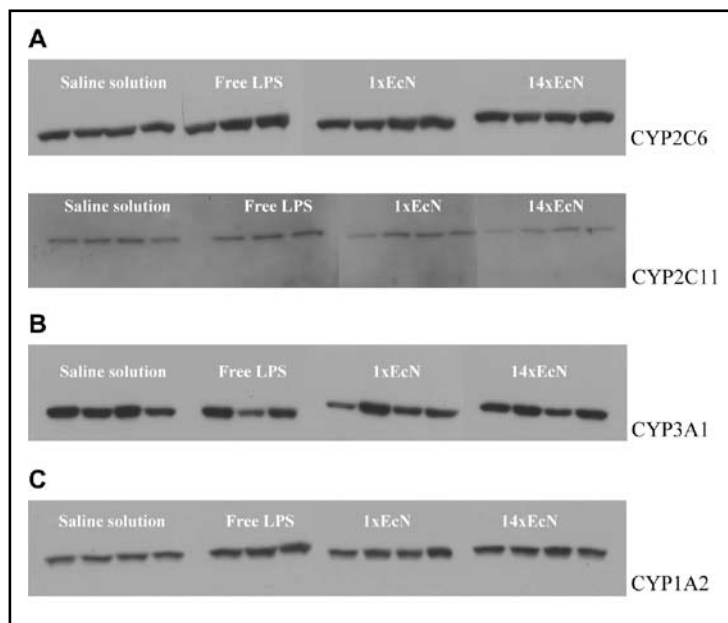


Figure 2. Western blots of expression of cytochromes P450 (CYP) from rat liver. Expression of CYP2C6 and human CYP2C9-like rat protein CYP2C11 (A), CYP3A1 (B) and CYP1A2 protein (C) are shown. The rats were treated with bacterial lipopolysaccharide daily for 14 days (Free LPS), and *E. coli* Nissle 1917 in 1 dose (1xEcN) or daily for 14 days (14xEcN). Control rats were treated with the saline solution for 14 days.

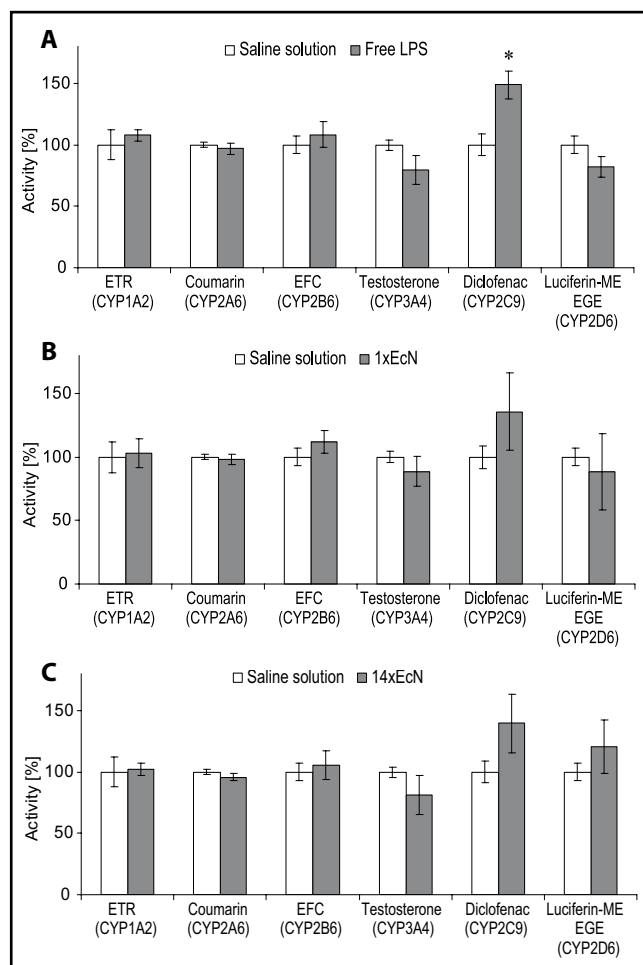


Figure 3. Specific activities of CYP enzymes in rat liver microsomes after treatment with bacterial lipopolysaccharide daily for 14 days (Free LPS; A) and *E. coli* Nissle 1917 in a single dose (1xEcN; B) or daily for 14 days (14xEcN; C). Control samples were obtained by treatment with the saline solution for 14 days. The activity of rat CYP2C form (corresponding to human CYP2C9) significantly increased vs. control (***) $p < 0.05$ after application free LPS (A). The results presented as means \pm S.D., $N = 4$; ETR, 7-ethoxyresorufin; EFC, 7-ethoxy-4-(trifluoromethyl)-coumarin.

ncbi.nlm.nih.gov/BLAST) structurally corresponding to CYP2C11 (Fig. 2A), exhibits a slight increase in expression in samples obtained after administration of the LPS, and, in the case of CYP2C6, a slight increase in protein expression also after administration of the probiotic strain in comparison to control.

For the CYP3A1 protein (Fig. 2B), a small degree of decrease is observed for all samples (after treatment with LPS, the probiotic for 1 or 14 days). On the contrary, an apparent increase of CYP1A2 protein (Fig. 2C) can be traced after administration of LPS; application of probiotic for 1 or 14 days caused only minor changes relatively to the controls. In a summary, the differences in expression of CYP proteins after administration of probiotic or LPS are not uniform, some forms exhibit a slight increase in expression (CYP2C proteins, CYP1A2), other a minor decrease (CYP3A1).

Enzyme activities of liver microsomal CYP forms. Fig. 3A shows all six activities determined (using substrates prototypic of the corresponding human CYP forms) in liver microsomal samples obtained after treatment of experimental animals with bacterial LPS. A relatively significant increase ($p < 0.05$) of activity has been observed with diclofenac, substrate of human CYP2C (2C8, 2C9, 2C10, 2C18, 2C19) as well as of rat CYP2C forms (2C6, 2C11) (Masubuchi *et al.* 2001; Bruyere *et al.* 2009). The increase in this CYP2C activity is in line with an observed increase in CYP2C protein expression (Fig. 2A). Similarly, also a decrease in the CYP3A4 prototypic activity (testosterone 6 β -hydroxylation) is in line with the results of Western blots (expression of rat CYP3A1, Fig. 2B); a slight but nonsignificant increase in the CYP1A2 prototypic activity (7-ethoxyresorufin O-deethylation) most probably reflects a corresponding increase in expression of the CYP1A2 protein (Fig. 2C).

Fig. 3B shows all six activities determined in liver microsomal samples again, but obtained after treatment of experimental animals with only single dose of EcN. Changes of CYP activities are not significant even in the case of diclofenac (the substrate of human CYP2C8, CYP2C9, CYP2C10, CYP2C18 and CYP2C19 as well as of rat CYP2C6 and CYP2C11) (Masubuchi *et al.* 2001; Bruyere *et al.* 2009). A tendency of a decrease of the CYP3A4 prototypic activity (testosterone

6 β -hydroxylation) is in line with the results of Western blots (Fig. 2B). A slight increase in the CYP1A2 prototypic activity (7-ethoxyresorufin O-deethylation) is correspondent with an increase in expression of the CYP1A2 protein (Fig. 2C).

Fig. 3C represents similar changes of activities of CYP enzymes as described in the former cases. These samples were obtained after application of EcN to experimental animals daily for 14 days. The greatest change of CYP activity is with diclofenac 4'-hydroxylation (substrate of rat CYP2C6 and CYP2C11 protein); however, this change is statistically not significant. The figure shows also a decrease of the CYP3A4 prototypic activity (testosterone 6 β -hydroxylation) and a very slight increase of the CYP1A2 prototypic activity (7-ethoxyresorufin O-deethylation) which is in line with an observed increase of levels of expressed CYP1A2 protein (Fig. 2C).

In a summary, six substrates of CYP enzymes was used to find changes of specific rat CYP activities after administration of bacterial LPS for 14 days, and EcN in only single dose or daily for 14 days consecutively. The activity of rat CYP2C forms (CYP2C6 and CYP2C11) in liver microsomal samples of rats given LPS containing supernatant appeared to exhibit an increase. A tendency of a decrease in the CYP3A4 activity and of the CYP3A1 protein expression is apparent for testosterone 6 β -hydroxylation. This conclusion is important for evaluation of possible changes in drug metabolism after intake of the probiotics in human food showing that there is most likely either little or no influence of probiotics to liver drug metabolism by CYP enzymes.

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