ORIGINAL ARTICLE

Effects of probiotic *Escherichia coli* Nissle 1917 on expression of cytochromes P450 along the gastrointestinal tract of male rats

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Abstract

OBJECTIVES: The aim of the study was to find whether probiotic *Escherichia coli* Nissle 1917 O6:K5:H1 (EcN) influences the expression of cytochromes P450 (CYP) in the rat intestine. **DESIGN:** Live bacterial suspension of EcN was administered to healthy male Wistar rats daily for 7 days. Control group of rats was stressed by oral application of the saline solution daily for 7 days as well. Sections of the duodenum, jejunum, ileum, caecum and colon have been taken from each experimental animal. With all individual samples, microsomal fraction has been prepared and expression of selected CYPs was determined by Western blotting. The levels of expression of CYPs were also evaluated by mRNA using real-time PCR. **RESULTS:** It was found that there are changes in expression of CYP enzymes studied along the intestine. CYP1A1, 2B1/2 and 2E1 are present mainly in the duodenum and jejunum; on the other hand, CYP2C6 is expressed mainly in the caecum and colon. CYP3A was found all over the rat intestine. The results show that there are no prominent differences between control samples and samples with EcN, only the expression of CYP3A protein in the duodenum appears to exhibit a clear tendency to decrease. In the case of the colon, a significant increase in the expression of CYP3A (most likely CYP3A1) after treatment of rats with EcN was found. **CONCLUSION:** This *in vivo* study revealed that the levels of colon CYP3A could be significantly increased in rats treated with probiotic EcN. On the contrary, the expression of CYP3A in the duodenum decreased. However, the changes in the expression of CYP enzymes are probably not as extensive to be clinically important in man; hence, most likely the probiotic EcN has little influence on the intestinal drug metabolism by CYP enzymes.

Abbreviations:

CYP - Cytochrome P450
EcN - Probiotic *E. coli* Nissle 1917 O6:K5:H1
LPS - Bacterial lipopolysaccharide

INTRODUCTION

The epithelial lining of the intestine is a complex and dynamic tissue that is responsible for maintaining the barrier and absorptive functions of the gastrointestinal tract (Kojima *et al.* 2004). Probiotics are microbial organisms which, when they are administered in adequate amounts, have beneficial effects on the gastrointestinal tract (Schlee *et al.* 2007).

Escherichia coli are microorganisms which can be pathogenic (causing a wide range of gastrointestinal infections) and nonpathogenic (used as a probiotic against a variety of intestinal disorders) (Hancock *et al.* 2010). Both nonpathogenic and pathogenic *E. coli* strains are able to colonize the gut and are well adapted to the conditions found in the large intestine. *Escherichia coli* Nissle 1917 with serotype O6:K5:H1 (abbrev. EcN) is a typical example of a nonpathogenic strain which is marketed under the name Mutaflor (Grozdanov *et al.* 2004). These gramnegative bacteria possess a lipopolysaccharide (LPS) which is a key component of their outer membrane. LPS chemical structure comprises three regions: a lipid (called lipid A), an internal oligosaccharide bound to lipid A by 3-deoxy-D-manno-octulosonic acid, and specific O-chain bound to the internal oligosaccharide (Brabetz *et al.* 2000; Pupo & Hardy 2009). However, the EcN has a special O6 antigen oligosaccharide corresponding to the so-called biological repeating unit which is assembled on a lipid carrier and then polymerized in the O-antigen. A defect in the O-antigen polymerase gene may result in the inability of the enzyme to produce a polysaccharide and thus give rise to a semirough-type LPS, like LPS of EcN. (Grozdanov *et al.* 2002).

According to the literature data, probiotics can ameliorate the function of liver in humans with hepatic cirrhosis (Lata *et al.* 2006), they can assist in a therapy of a habitual constipation and diverticular illness of colon and in a therapy of a mycotic infection, nonspecific intestinal inflammation and they are suitable to a regeneration of an inestinal microflora after therapy by antibiotics (Stibůrek *et al.* 2009). Probiotics have a wide spectrum of the use; however, they could, in principle, also interfere with processes determining bioavailability of orally administered drugs, namely, with drug biotransformation by cytochrome P450 (CYP) enzymes. These enzymes are monooxygenases metabolizing xenobiotics and endogenous substrates (Anzenbacher & Anzenbacherová 2001; Hodek *et al.* 2009) and they in fact comprise the major drug-metabolizing enzyme system in humans, accounting for the metabolism of many clinically useful medications (Bai & Liu 2005). The CYP enzymes are localized mainly in the liver as well as in the other tissues and organs such as the intestine (Paine et al 2006; Křížková *et al.* 2008). Therefore the aim of this work was to prove whether the presence of probiotic bacteria EcN in the rat gut may influence the expression of intestinal CYP enzymes.

MATERIAL & METHODS

Material

All reagents and chemicals were obtained from Sigma-Aldrich CZ (Prague, Czech Republic) if not stated otherwise. Other chemicals as sodium chloride, potassium chloride, hydrochloric acid, potassium hydroxide, EDTA, sucrose and methanol, which were used for isolation of microsomal fraction and Western blotting, were purchased from Lach-Ner (Neratovice, Czech Republic). Glycerol that was used as a cryoprotectant was obtained from Merck (Prague, Czech Republic). Protease inhibitor cocktail tablets were obtained from Roche (Mannheim, Germany). Mouse anti-rat CYP3A1, 2B1/2 and 2C6 monoclonal antibodies were purchased from Abcam (Cambridge, UK). Goat anti-rat CYP1A1 polyclonal and rabbit anti-human CYP2E1 monoclonal antibodies were obtained from Daiichi Pure Chemicals (Tokyo, Japan). Rabbit anti-rat Villin polyclonal antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The chemiluminiscence 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) and 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.

Preparation of samples

Live bacterial suspension of probiotic *E. coli* Nissle 1917 O6:K5:H1 (abbrev. EcN) was administered (10^{10} CFU/dose, orally) to male Wistar rats (body weight 400–580 g). Four rats were stressed by oral application of the saline solution daily for 7 days. This group was used as the control one. The probiotic was applied daily to six animals for 7 days as well.

After 7 days the rats were sacrificed. The duodenum, jejunum, ileum, caecum and colon were removed and weighted. The intestinal samples were frozen on the dry ice and stored at –70 °C until used. The protocol of the experiment was approved by the institutional Ethics Committee. For preparation of intestinal microsomes, the intestinal samples were rinsed in cold 0.25 M sucrose in 1 mM EDTA (pH 7.4) with a phenylmethanesulfonyl fluoride and a protease inhibitor cocktail. The tissue was then homogenized and subjected to differential centrifugation to obtain the microsomal fraction according to standard procedures (Lake 1990).

Determination of total protein

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 with human serum albumin as a standard. Differences between determinations were below 15%. Content of CYP was determined by the method described by Omura and Sato (1964).

Western blotting

Microsomal proteins from all various parts of the intestine (35 μg) obtained from all experimental rats 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 CYP1A1, anti-rat CYP2C6, anti-human CYP2E1, anti-rat CYP2B1/2 and anti-rat CYP3A1 as the primary antibodies. For reference, a villin was detected using a polyclonal antibody (1:500). The bands were visualized with respective peroxidase-conjugated secondary antibodies and their relative intensity evaluated with Elfoman (Semecky Inc., Prague, Czech Republic) software.

RNA isolation and real-time PCR procedures

A sample of about 30 mg of each rat intestinal tissue was stabilized in RNA later® (Quiagen, Germantown, MD, USA) and subsequently homogenized with homogenizer Diax 900, Heidolph, Kelheim, Germany). The sample was then applied onto the QIAshredder columns to eliminate tissue microparticles. RNA was isolated with use of RNeasy® Plus Minikit (Quiagen, Germantown, MD, USA) enabling degradation of contaminating genomic DNA. One microgram of each isolated RNA was subjected to reverse-transcription using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) with random hexamer primers. New synthesized cDNA was applied for real-time PCR using Light Cycler® 480 SYBR Green Master I mix in a Light Cycler 480 (Roche, Mannheim, Germany) with the following thermal cycling conditions: preincubation for 10 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 58 °C for 15 s and 72 °C for 15 s for denaturation, annealing and elongation. All samples for real-time PCR were prepared in triplicates. Rat primers were designed in our laboratory and synthesized by Invitrogen (LifeTechnologies, division Prague, Czech Republic). Absolute quantification method was applied for obtaining gene expression data. The following primer sequences were used:

CYP2B1/2 Fw: 5′-TCC CAG GGA GCC CCA CTG GAT CCC A-3′
CYP2B1/2 Rev: 5′-GAA CCC AGA GAA GAA CTC AAA CAC CTG G-3′
CYP2C6 Fw: 5′-GCC TTG TGG AGG AAC TGA GG-3′
CYP2C6 Rev: 5′-GCA CAG CCC AGG ATA AAC GT-3′
CYP2E1 Fw: 5′-CCA AGG GTA CAG TTG TGA TTC CAA C-3′
CYP2E1 Rev:5′-CAA CAC ACA CAC GCT TTC CTG CAG A-3′
CYP3A1 Fw: 5′-GTG CTC CTC TAC GGA TTT GGG A-3′
CYP3A1 Rev: 5′- TCC ACA TCG AAT TTC CAT AAA CCC-3′

RESULTS AND DISCUSSION

Application of probiotic EcN to rats for 7 consecutive days led to a result showing no changes in the protein content in all parts of the intestine of experimental animals (Figure 1).

Representative Western blots have shown that the expression of CYP enzymes differed through individual parts of the intestine (Figure 2). The expression of rat CYP3A was found all over the intestine (from duodenum to colon) of all experimental animals. The relative content of imunoreactive CYP3A protein in the duodenum of rats after treatment with EcN was lowered to approximately 60 % compared with control samples.

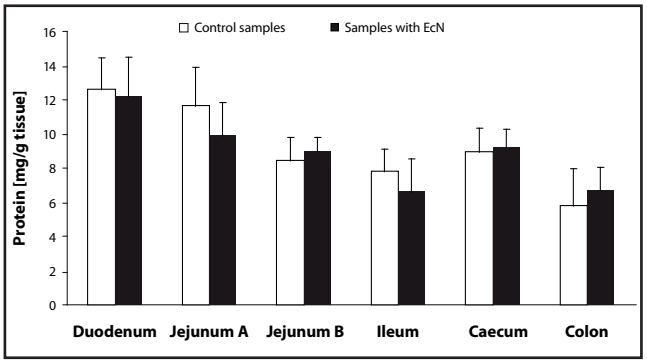

Fig. 1. The protein content in the intestinal microsomes in rats after treatment with probiotic *Escherichia coli* Nissle 1917 (EcN). Control rats were treated with the saline solution. The results are presented as means ± S.D.; N ≥ 4.

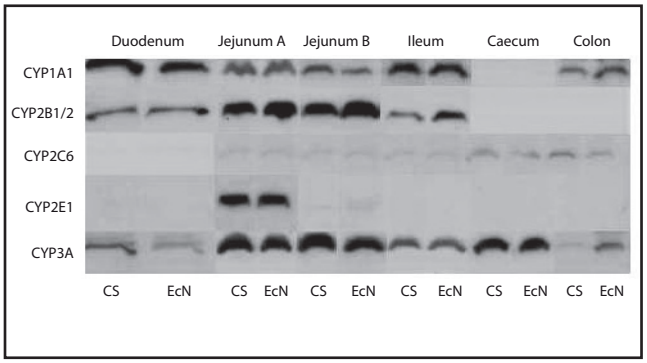

Fig. 2. Representative Western blots of all analyzed P450 enzymes. CYP3A: two immunoreactive bands were detected after incubation with CYP3A1 antibody used. The double band was detected in all samples as well as in the liver (data not shown), suggesting cross-reactivity with other isoforms of the CYP3A family. For data evaluation, the density of both bands was taken and named CYP3A. For reference, a villin was detected using a polyclonal antibody (data not shown).

This result has not been confirmed by real-time PCR method, because the whole duodenum (very small part of the intestine) was used for Western blotting experiment. The changes in the expression of CYP3A in the jejunum (the longest part of intestine, therefore it was divided into two parts, A and B), ileum and caecum after administration of probiotic EcN were not markedly changed and these findings were also supported by results obtained by real-time PCR method (Figure 3). On the other hand, a statistically significant change in protein expression was found in the case of the CYP3A enzyme in the colon of rats pretreated with EcN (Figure 2). The CYP3A form level was increased on average by about 50 % in comparison to the CYP3A protein level in control samples. Also, real-time PCR analysis detected significantly higher levels of CYP3A1 mRNA in these samples (Figure 3).

The expression of cytochrome P450 2B1/2 was detected by Western blotting mainly in the duodenum, jejunum and ileum; its presence in the caecum and colon was not detected (Figure 2). The expression of CYP2B1/2 in rats after treatment with probiotic EcN was slightly increased compared with control samples. This statistically nonsignificant change was however not proved by real-time PCR method, where no prominent changes were detected (Figure 4).

Similarly, the CYP2E1 enzyme is present mainly in the duodenum and jejunum. It was also detected in the ileum, but in the lower extent (Figure 2). No significant changes in the expression of CYP2E1 were found in the duodenum, jejunum and ileum in rats after administration of EcN in comparison to control samples. The same results were obtained by Western blotting as well as by real-time PCR method (Figure 5).

In case of the CYP2C6, a small decrease in protein level was observed only in the caecum (after treatment with probiotic EcN) (Figure 2). The caecum is the major site of expression of the CYP2C6 enzyme, however, the change in the expression of CYP2C6 was detectable, but not significant. A nonsignificant change in the expression of CYP2C6 was also confirmed in the real-time PCR experiment (Figure 6).

Expression of the CYP1A1 enzyme was also studied in all parts of the intestine. The CYP1A1 protein was detected in the duodenum, jejunum, ileum, and colon by Western blotting (Figure 2). No significant changes in the expression of CYP1A1 were found after treatment of rats with probiotic EcN. Changes in the expres-

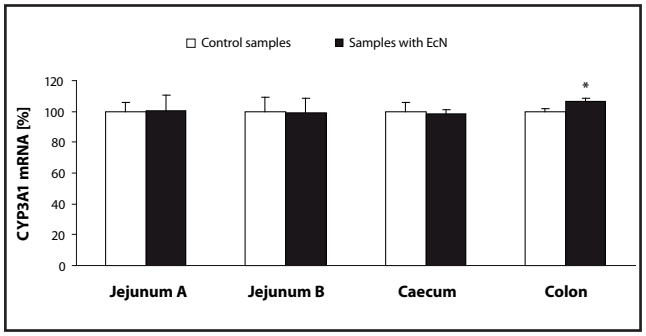

Fig. 3. The CYP3A1 mRNA level in the rat jejunum, caecum and colon after treatment of probiotic *Escherichia coli* Nissle 1917 (EcN) daily for 7 days. Control rats were treated with the saline solution for 7 days as well. The CYP3A1 mRNA level in the colon in rats after administration of EcN significantly increased vs. control (* $p<0.05$). The results are presented as means ± S.D.; N ≥ 4.

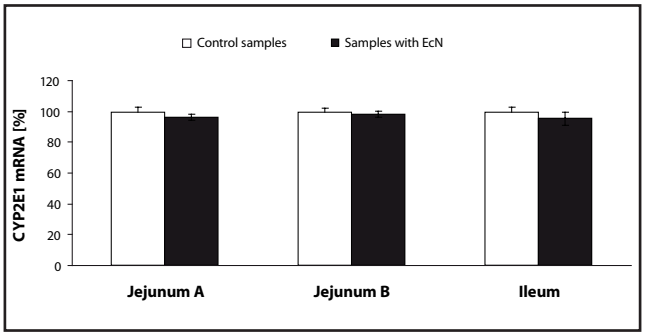

Fig. 5. The CYP2E1 mRNA level in the rat jejunum and ileum after treatment of probiotic *Escherichia coli* Nissle 1917 (EcN) daily for 7 days. Control rats were treated with the saline solution for 7 days as well. The results are presented as means ± S.D.; N ≥ 4.

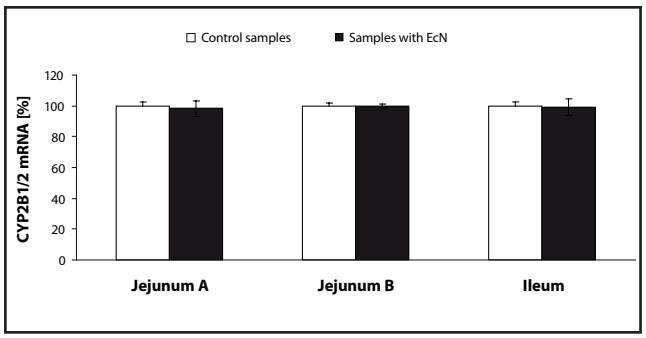

Fig. 4. The CYP2B1/2 mRNA level in the rat jejunum and ileum after administration of *Escherichia coli* Nissle 1917 (EcN) daily for 7 days. Control rats were treated with the saline solution for 7 days as well. The results are presented as means ± S.D.; N ≥ 4.

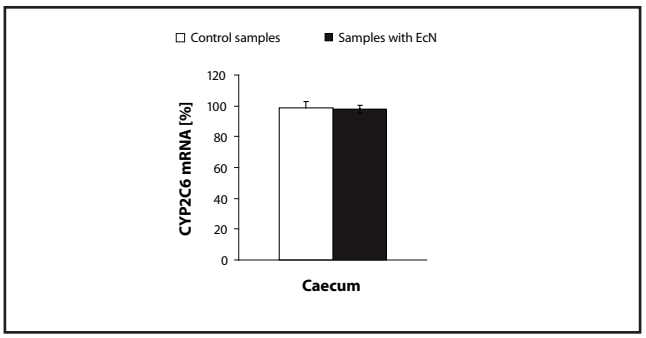

Fig. 6. The CYP2C6 mRNA level in the rat caecum after administration of probiotic *Escherichia coli* Nissle 1917 (EcN) daily for 7 days. Control rats were treated with the saline solution for 7 days as well. The results are presented as means ± S.D.; N ≥ 4.

sion of CYP1A1 were not measured by real-time PCR method either.

It should be noted here that the experiments were performed *in vivo* with rat experimental model and this is why the results have shown in principle a great variability. The greatest differences among the biological samples within one group were with expression of the CYP1A1. Despite the variability among the biological samples, the tendencies and statistical significance are clear. For example, a measurable decrease in the expression of CYP3A in the duodenum and as well as an increase in CYP3A expression in the colon after treatment of rats with probiotic EcN could be seen. In fact the rat CYP3A forms (3A1, 3A2, 3A9, 3A18 and 3A23) are similar to human CYP3A4 and CYP3A5 (Matsubara *et al.* 2004). The human CYP3A4 is known as the most important CYP enzyme of drug metabolism in humans (Anzenbacher & Anzenbacherová 2001). The human CYP3A4 is a major CYP enzyme present in critical tissues such as the liver, gastrointestinal tract, brain (Tanaka 1999). Therefore, there could be a risk of potential drug interaction of probiotic with concomitantly administered drugs metabolized by this enzyme. However, the changes in the expression of CYP3A form (most likely CYP3A1) in the duodenum and colon found in this work will have probably no relevance in clinical practice. In addition, the question whether any other factors (such as age or sex) influence an action of probiotic EcN in the gut (for example by change in the expression of CYP enzymes) has not been answered yet. These questions are very important, because nowadays the consumption of probiotics became more popular among people of all age.

In conclusion, in this work, the influence of probiotic EcN on the expression of intestinal CYP1A1, 2B1/2, 2C6, 2E1 and 3A was studied. This *in vivo* study revealed that treatment of rats with EcN did not have a prominent influence on the expression of intestinal CYP enzymes. However, the results indicate that the levels of colon CYP3A1 could be significantly increased in rats treated with probiotic EcN. On the contrary, the expression of CYP3A in the duodenum apparently decreased. This conclusion is important for evaluation of possible changes in the intestinal drug metabolism after administration of the probiotics in human food.

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