The effect of probiotic *Escherichia coli* strain Nissle 1917 lipopolysaccharide on the 5-aminosalicylic acid transepithelial transport across Caco-2 cell monolayers

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Abstract. The object of this study was to investigate the effect of probiotic *Escherichia coli* strain Nissle 1917 (EcN) (i) EcN lipopolysaccharide (EcN LPS) and (ii) bacteria-free supernatant of EcN suspension (EcN supernatant) on *in vitro* transepithelial transport of mesalazine (5-aminosalicylic acid, 5-ASA), the most commonly prescribed anti-inflammatory drug in inflammatory bowel disease (IBD).

Effect of co-administered EcN LPS (100 µg/ml) or EcN supernatant (50 µg/ml) on the 5-ASA transport (300 µmol/l) was studied using the Caco-2 monolayer (a human colon carcinoma cell line) as a model of human intestinal absorption. Permeability characteristics for absorptive and secretory transport of parent drug and its intracellularly-formed metabolite were determined. The quantification of 5-ASA and its main metabolite N-acetyl-5-amino-salicylic acid (N-Ac-5-ASA) was performed by high performance liquid chromatography.

Obtained results suggest that neither EcN LPS nor EcN supernatant had effect on the total 5-ASA transport (secretory flux greater than absorptive flux) and on the transport of intracellularly formed N-Ac-5-ASA (preferentially transported in the secretory direction). The percent cumulative transport of the total 5-ASA alone or in combination with EcN LPS or EcN supernatant did not exceed 1%.

Key words: Caco-2 cells — Probiotics — *E. coli* Nissle lipopolysaccharide — 5-Aminosalicylic acid — Drug transport

Abbreviations: 5-ASA, 5-aminosalicylic acid; N-Ac-5-ASA, N-acetyl-5-aminosalicylic acid; AP-BL, apical to basolateral; BL-AP, basolateral to apical; DMEM, Dulbecco’s modified Eagle’s medium; DPBS, Dulbecco’s phosphate buffered salt solution; EcN, probiotic *Escherichia coli* strain Nissle 1917 06:K5:H1; EcN LPS, EcN lipopolysaccharide; FBS, fetal bovine serum; HBSS, Hanks’ balanced salt solution; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HPLC, high performance liquid chromatography; IBD, inflammatory bowel disease, MES, 2- morpholinoethanesulfonic acid; MRP, multidrug resistance associated proteins; OATP, organic anion transporting polypeptide; P_{app}, apparent permeability coefficient; UC, ulcerative colitis.

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Introduction

Although the observation of the positive role of certain bacteria was firstly introduced at the beginning of the 20th century, an increasing appreciation of the role of probiotics was shown as late as the last decades of the 20th century (Martel et al. 2002a; Fioramonti et al. 2003). It was shown in vitro (Schultz et al. 2004; Zyrek et al. 2007; Štětinová et al. 2010) and in vivo (Madsen et al. 2001a; Schultz et al. 2004) that probiotics have potential to improve human health, i.e. to prevent and treat a wide variety of diseases (Martel et al. 2002b; Cukrowska et al. 2002).

However, the most of the identified benefits of probiotics relate to gastrointestinal tract (Madsen et al. 2001a; Marteau et al. 2002b), including antibiotic-associated diarrhoea (Madsen 2001b), acute infectious diarrhoea (Britton and Versalovic 2008), irritable bowel syndrome (Spiller 2008), and inflammatory bowel disease (IBD) (Brogden et al. 1989; Prakash and Markham 1999), has been chosen as a model drug for studying its interaction with probiotics. Considering mesalazine (5-aminosalicylic acid, 5-ASA) permeability, transepithelial transport mechanisms of 5-ASA (and of its intracellularly formed metabolite N-acetyl-5-aminosalicylic acid) using the in vitro model of the human intestinal barrier, Caco-2 cells monolayers.

In this study, one of the best characterized probiotics, nonpathogenic Escherichia coli strain Nissle 1917 O6:K5:H1 (EcN) exhibiting a serum-sensitive, semirough phenotype (Grozdanov et al. 2002) was used. As for EcN efficacy, there are several clinical trials comparing the oral EcN and 5-ASA formulations in maintenance of remission of UC. In these trials, EcN showed efficacy and safety equivalent to the gold standard 5-ASA (Kruis et al. 1997, 2004, Rembacken et al. 1999, Faubion 2000). However, in spite of the increasingly recognized role of probiotics, very little is known about drug interactions mediated by probiotics and about changes in efficacy of simultaneously administered drugs. Only paper describing survival of the EcN in gastrointestinal tract given in combination with oral 5-ASA to healthy volunteers was published (Joeres-Nguyen-Xuan et al. 2010). Authors of this study found no significant effect of 5-ASA on the survival of EcN in healthy volunteers. In contrast to this findings Swidsinski et al. (2007) described a significant reduction of amenability, adherence, and concentrations of mucosal bacteria in the 5-ASA-treated patients with IBD. As drug´s fate in the intestinal barrier depends among others on absorption and first pass metabolism, we focused on the study of the effect of EcN bacterial lipopolysaccharide (LPS), a glycolipid of the cell wall of gram-negative bacteria, and EcN bacteria-free supernatant on the 5-ASA intestinal transport (i.e. 5-ASA permeability, transepithelial transport mechanisms of 5-ASA and of its intracellularly formed metabolite N-acetyl-5-aminosalicylic acid) using the in vitro model of the human intestinal barrier, Caco-2 cells monolayers.

Materials and Methods

Materials

Mesalazine (5-ASA) substance was obtained from PRO. MED.CS, Praha a.s., Czech Republic. Phenol red, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 2-morpholinoethanesulfonic acid (MES), and scintillation liquid (Universal LSC cocktail) were purchased from Sigma-Aldrich (Czech Republic), propionic anhydride (99%) and potassium dihydrogenphosphate (KH₂PO₄) and methanol (HPLC grade) were from Merck (Germany). Sodium hydrogenphosphate dodecahydrate (Na₂HPO₄·12 H₂O) were obtained from Penta (Czech Republic), sodium hydroxide p.a. from Fluka (Switzerland) and hydrochloric acid (35%) from Lach-Ner (Czech Republic). The standards (N-propionyl-5-ASA, N-acetyl-5-ASA, internal standard N-acetyl-4-ASA) used in HPLC determination, were synthesized in our laboratories. Dulbecco´s modified Eagle´s medium with high glucose (DMEM), Hanks´ balanced salt solution (HBSS) with
Ca\textsuperscript{2+}, Dulbecco’s phosphate buffered salt solution (DPBS), Trypsin-EDTA (1:250), antibiotic-antimycotic solution were obtained from PAA Laboratories (BioTech, Praha, Czech Republic). Fetal bovine serum (FBS) was purchased from Gibco Invitrogen (KRD, Praha, Czech Republic) and \textsuperscript{14}C mannitol (100 µCi/ml) from Moravák Radiochemical and Biochemicals (MGP Zlín, Czech Republic).

Isolation of EcN lipopolysaccharide (EcN LPS)

EcN LPS was isolated by the phenol-water extraction from the \textit{Escherichia coli} Nissle 1917 O6:K5:H1 (Zídek et al. 2010).

Preparation of EcN supernatant

EcN supernatant was obtained after the third washing of EcN suspension in RPMI-1640 medium (Zídek et al. 2010). Both EcN LPS and EcN supernatant were prepared at the Institute of Microbiology, Academy of Sciences of the Czech Republic.

Cell culture

The Caco-2 cell line was purchased from the European collection of cell culture (Sigma-Aldrich, Czech Republic) and used between passages 71 and 79. The cells were routinely grown (Bourdet and Thakker 2006, Štětinová et al. 2009) in plastic tissue culture flasks (75 cm\textsuperscript{2} growth area, TPP AG, Switzerland) in DMEM containing 25 mmol/l glucose and supplemented with 4 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg amphotericin. Cell line was cultured at 37°C in an atmosphere of 5% CO\textsubscript{2} and 90% relative humidity, and passaged at about 90% confluence, using 0.05% trypsin (1:250) – 0.02% EDTA in calcium-free and magnesium-free phosphate-buffered saline (DPBS). The medium was changed three times a week.

For 5-ASA transport experiments, the cells were seeded onto polycarbonate filter cell culture chamber inserts (dimensions Ø 13 × 11 mm, area available for growth 0.5 cm\textsuperscript{2}, pore diameter 0.4 µm) of tissue culture plates containing 24 wells (TPP AG, Switzerland) at the density of 2.5 × 10\textsuperscript{5} cells/cm\textsuperscript{2}. Caco-2 cells were used for transport experiments 21–25 days post-seeding (Štětinova et al. 2009). Before experiments, the integrity of the monolayer was checked by 500 µl from the basolateral compartment were withdrawn at 30, 60, 90 and 120 min for HPLC analysis and the same volume of the prewarmed transport medium was added to the basolateral compartment. During withdrawing samples the 24-transwell plate was placed on a heated plate (37°C). Between sampling, the cells were kept in an incubator at 37°C and 5% CO\textsubscript{2}.

All used solutions and transport media were sterile filtered just before the experiments. All experiments were carried out under sink conditions so that the concentrations of the drug in the receiver compartment would not exceed 10% of the applied dose in the donor side and under iso-pH conditions (pH 7.4 in both sides).

HPLC analysis of 5-ASA

The quantification of 5-ASA was performed by high performance liquid chromatography (HPLC) with gradient elution. A modified method of Nobilis et al. (2006) was used. Withdrawn samples of 5-ASA in the transport medium were analyzed for 5-ASA and \textit{N}-acetyl-5-aminosalicylic acid (N-Ac-5-ASA). Chromatographic separation was performed on the analytical column N-acetyl-5-aminosalicylic acid n LichroCART\textsuperscript{®} 250 × 4 mm packed with Purospher RP-18e 5 µm and precolumn LiChroCART\textsuperscript{®} 4-4 with the same stationary phase (Merck, Darmstadt, Germany). The mobile phase A consisted of an acetonitrile – UHQ water (8:2, v/v), the mobile phase B contained acetonitrile-0.01M Na\textsubscript{2}HPO\textsubscript{4} buffer (pH 3) in the ratio 15:85 (v/v). The flow-rate was 1 ml/min and elution of 5-ASA and N-Ac-5-ASA was monitored at a fluorescence excitation wavelength of 300 nm and an emission wavelength of 406 nm, the total run was 21 min for each sample. The quantitative determination was based upon the integration of fluorescence peak area.

Transport studies

Transport experiments were performed with transport medium (Ingels and Augustijns 2003) – Hanks’ balanced salt solution (HBSS) buffered with 25 mmol/l HEPES (pH 7.4). The prepared Caco-2 cell inserts were rinsed twice with prewarm HBSS and equilibrated with HBSS at 37°C for 30 min before the transport experiments (Walle and Walle 1998).

The transport experiments from apical to basolateral side (AP-BL) were initiated by replacing the transport medium with 5-ASA alone (300 µmol/l) or in combination with either EcN LPS (100 µg/ml) or EcN supernatant (50 µg/ml) in the transport medium (500 µl) on the apical side. The samples of 500 µl from the basolateral compartment were withdrawn at 30, 60, 90 and 120 min for HPLC analysis and the same volume of the prewarmed transport medium was added to the basolateral compartment. During withdrawing samples the 24-transwell plate was placed on a heated plate (37°C). Between sampling, the cells were kept in an incubator at 37°C and 5% CO\textsubscript{2}.

Similarly, for BL-AP (basolateral to apical) transport, the drug (5-ASA alone or in combination) was added to the basolateral compartment (650 µl) and the volume of 300 µl was withdrawn from the receiver side in the same time intervals.

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Data analysis

Calculation of $P_{\text{app}}$ (cm/s) of the total 5-ASA (5-ASA + N-Ac-5-ASA)

$P_{\text{app}}$ was calculated according to the equation: $P_{\text{app}} = (dQ/dt) \times (1/(A \times C_0))$, where $dQ/dt$ is the permeability rate, the amount of drug appearing in the receiver compartment in function of time (nmol/s), $C_0$ is the initial concentration in the donor chamber (nmol/ml), and $A$ is the surface area of the monolayer ($cm^2$) (Artursson and Karlsson 1991).

Evaluation of the bidirectional flux

Transport in both directions across monolayer enables to calculate an efflux ratio (Ungell and Karlsson 2004):

$$P_{\text{app efflux ratio}} = P_{\text{app BL-AP}} / P_{\text{app AP-BL}}$$

Cumulative transport of the total 5-ASA

Calculated total cumulative amount of 5-ASA (5-ASA + N-Ac-5-ASA; nmol) estimated in received chamber were plotted versus time.

Percent transport

Percent transport was calculated for each time interval as the ratio of the cumulative concentration of the total 5-ASA (5-ASA + N-Ac-5-ASA) or 5-ASA and N-Ac-5-ASA, separately, in the receiver chamber to the concentration in the donor chamber × 100.

Results

Calculation of $P_{\text{app}}$ of the total 5-ASA (5-ASA + N-Ac-5-ASA)

Effect of EcN LPS

The $P_{\text{app}}$ of 5-ASA showed that the secretory flux (BL-AP) was significantly greater than absorptive flux (AP-BL). The calculated $P_{\text{app efflux ratio}}$ was 1.68. EcN LPS slightly lowered (nonsignificantly) $P_{\text{app}}$s of 5-ASA in both directions, the efflux ratio in the presence of EcN LPS was 1.96 (Fig. 1A).

Effect of EcN supernatant

Similarly as in the case of EcN LPS, the asymmetric transport of 5-ASA was not influenced by EcN supernatant; EcN supernatant slightly (nonsignificantly) increased the $P_{\text{app}}$ values in both directions (Fig. 1B) and the $P_{\text{app efflux ratio}}$s for 5-ASA transport and for transport of 5-ASA in combination with EcN supernatant were 1.86 and 1.87, respectively.

Figure 1. Bidirectional transport (AP-BL and BL-AP) of the total 5-ASA across Caco-2 monolayer in the absence or in the presence of EcN LPS (A) or EcN supernatant (B). Data represent the mean ± SD of the permeability coefficient ($P_{\text{app}}$), ($n = 4–6$), * significant differences between AP-BL and BL-AP directions ($p < 0.05$).
**Cumulative transport of the total 5-ASA (5-ASA + N-Ac-5-ASA)**

**Effect of EcN LPS**

The cumulative BL-AP transport of 5-ASA (nmol) and 5-ASA in combination with EcN LPS seems to be linear up to 2 h. The cumulative AP-BL transport of 5-ASA and 5-ASA in combination with EcN LPS were linear up to 90 min and the amount of 5-ASA and 5-ASA in combination with EcN transported in the last time interval (Fig. 2A) was lower than the amount transported in each previous interval.

**Effect of EcN supernatant**

The cumulative bidirectional transport of 5-ASA (nmol) and 5-ASA with EcN supernatant were roughly linear up to 120 min, except the BL-AP transport of 5-ASA with EcN supernatant that demonstrated higher transport in the last time interval (Fig. 2B).

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 2.** Cumulative bidirectional transport of the total 5-ASA across Caco-2 monolayer (nmol) and the percent cumulative bidirectional transport at 120 min in the absence and in the presence of EcN LPS (A) or EcN supernatant (B). Data represent the mean ± SD (n = 4–6), * significant differences between AP-BL and BL-AP directions (p < 0.05).
Percent transport of the total 5-ASA (5-ASA + N-Ac-5-ASA)

The percent transport of the total 5-ASA and 5-ASA in combination with EcN LPS or EcN supernatant did not exceed 1%.

Effect of EcN LPS

The percent cumulative transport estimated at each time interval showed that the percent transport was greater in BL-AP direction than in AP-BL for both 5-ASA and 5-ASA with EcN LPS (only values at 120 min are presented, Fig. 2A). EcN LPS slightly lowered transport of 5-ASA in both directions.

Effect of EcN supernatant

The percent cumulative BL-AP transport of 5-ASA was higher in comparison with AP-BL direction. As for effect of EcN supernatant (Fig. 2B), data from the last time interval (120 min) showed slightly increased percent transport of 5-ASA in both directions.

Percent transport of parent 5-ASA and intracellularly formed metabolite N-Ac-5-ASA

Effect of EcN LPS

The bidirectional transport of 5-ASA expressed as (i) percentage cumulative amount of parent drug (5-ASA) and as (ii) percentage cumulative amount of its main metabolite (N-Ac-5-ASA) estimated in receiver sides and the effect of EcN LPS is shown in Fig. 3. The AP-BL transport of the produced N-Ac-5-ASA was minimal in comparison with metabolite flux in BL-AP direction at each time interval (data not shown). At 120 min the absorptive metabolite flux was significantly lower (at mean 6.6-times). The EcN LPS slightly (nonsignificantly) decreased the percent transport of the parent 5-ASA and its metabolite in each time interval in both directions (data not shown). At 120 min, the percent transport of the parent drug was decreased 1.3-times in AP-BL direction and was almost the same in BL-AP direction. The percent transport of N-Ac-5-ASA was lowered 1.2-times in AP-BL direction and was the same in BL-AP direction in comparison with 5-ASA transport (Fig. 3).

Effect of EcN supernatant

Up to 90 min the transport of 5-ASA (parent drug) and its metabolite were almost the same after coadministration with EcN supernatant (data not shown). Only at 120 min (Fig. 4) nonsignificantly higher transport of parent drug in BL-AP direction (1.36-times) and transport of metabolite in AP-BL direction (1.5-times) was observed after coadministration of EcN supernatant in comparison with values reached after administration of 5-ASA alone.

Discussion

With the increasing interest in probiotics exerting preventive or therapeutic effects in several experimental models and clinical studies, their interactions with medications cannot be excluded even though interactions with medications or other supplements are not known so far (Kligler and Cohrs 2008).

Figure 3. Effect of EcN LPS on 5-ASA (300 µmol/l) percent cumulative transport (5-ASA and intracellularly formed N-ac-5-ASA, separately) at 120 min. Data represent the mean ± SD (n = 4–6). * significant differences between AP-BL and BL-AP directions (p < 0.05).
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As recent knowledge indicates efficacy of probiotics in IBD, we focused on the interaction of EcN components with 5-ASA (effective agent for the treatment of IBD). To evaluate the transport of 5-ASA alone or in combination with EcN LPS 100 µg/ml or EcN supernatant 50 µg/ml, the Caco-2 permeability model was used as an in vitro tool to predict absorption in man (Ungell and Karlsson 2004). In many respects Caco-2 cells are functionally similar to the human small intestinal enterocyte, despite the fact that they originate from a human colorectal carcinoma (Artursson and Tavelin 2004). Evaluation of the drug intestinal permeability is essential as the rate and extent of drug absorption and metabolic enzymes activity are the most important factors determining total drug bioavailability.

We have described earlier (Štětinová et al. 2011) that the transport of 5-ASA is asymmetric in Caco-2 cells, 5-ASA BL-AP transport being higher than AP-BL direction. In the present study we have shown that the transport of 5-ASA was unchanged by EcN LPS or EcN supernatant. At given conditions EcN LPS slightly lowered 5-ASA transport in both directions, whereas EcN supernatant slightly increased the bidirectional transport of 5-ASA (cumulative transport (nmol), percent transport at 120 min and P app values of 5-ASA). However, these changes were not significant and seem to be accidental caused only by biological fluctuation. These insignificant changes in 5-ASA transport induced by EcN LPS or EcN supernatant are in accordance with the fact that neither the high concentration of LPS (1000 µg/ml) have significant effect on the viability of Caco-2 cells nor the concentrations of EcN LPS (1000, 100 µg/ml) and EcN supernatant (50 µg/ml) revealed effect on the tightness of cell junctions, as it has been recently estimated by testing the integrity of Caco-2 monolayer using 14C mannitol as marker of paracellular transport across Caco-2 monolayer (Štětinová et al. 2010). Thus, we can conclude that neither EcN LPS nor EcN supernatant has effect on the 5-ASA transport.

It is believed that asymmetric transport of 5-ASA at low concentration (preferentially transported in the basolateral to apical direction) indicates presence of carrier-mediated saturable transepithelial transport. Our results showed in concordance with Zhou et al. (1999a) that this asymmetric transport of total 5-ASA is due to a strictly secretory transport of N-Ac-5-ASA. Several authors investigated which efflux pumps on the apical membrane are involved in N-Ac-5-ASA efflux. Xin et al. (2006) demonstrated that 5-ASA is not a substrate of P-glycoprotein, Yoshimura et al. (2009) deduced that this metabolite is pumped out by an MRP-like transporter. Futhermore, it has been shown recently that intracellular accumulation of 5-ASA is mediated by members of the OATP uptake transporter family (OATP1B1, OATP1B3, and OATP2B1), which are expressed in small and large intestine (Köning et al. 2011) and also in Caco-2 cells (Englund et al. 2006, Seithel et al. 2006, Maubon et al. 2007). According to published data (Köning et al. 2011) 5-ASA OATP-mediated uptake may be modified by genetic factors and by comedication. Showing no changes in transport of intracellularly formed main metabolite N-Ac-5-ASA it can be indirectly supposed that neither EcN LPS nor EcN supernatant had effect on the N-acetyltransferase 1 activity and on the metabolite transport. Both these factors has to be kept in view even though the direct effects of probiotics on transporters function and on their expression are not extensively elucidated; e.g., Saksena et al. (2010) cited stimulation effect of probiotics

Figure 4. Effect of EcN supernatant on 5-ASA (300 µmol/l) percent cumulative transport (5-ASA and intracellularly formed N-ac-5-ASA, separately) at 120 min. Data represent the mean ± SD (n = 4–6). * significant differences between AP-BL and BL-AP directions (p < 0.05).
(Lactobacilli or their soluble factors) on P-glycoprotein expression in Caco-2 cells and in vivo in MDR1-deficient mice. It can be generally supposed that the fate of intracellularly formed metabolite depends on the current state of both actions - inhibition versus stimulation of transporters expression and N-acetylttransferase 1 activity.

These transport experiments indirectly showed no factors or mechanisms (e.g., effect on transporters, on enzyme activity) which would be able to magnify the 5-ASA effect and evoke synergistic effect of concomitant administration of 5-ASA plus EcN LPS or EcN supernatant. On the other hand, neither EcN LPS nor EcN supernatant has harmful effect on 5-ASA transport in vitro conditions.

In summary, higher secretory than absorptive flux of the total 5-ASA (parent drug plus metabolite) and preferential secretory flux of intracellularly produced 5-ASA metabolite (N-Ac-5-ASA) were not significantly changed by concomitant administration of EcN LPS or EcN supernatant.

Acknowledgements The authors wish to thank Ms Hana Machova for her skilful technical assistance. The study was supported by the research projects GAČR No. 305/08/0535 and No. P304/11/1252.

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Received: September 24, 2012
Final version accepted: February 11, 2013