Caco-2 cell monolayer integrity and effect of probiotic *Escherichia coli* Nissle 1917 components

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Abstract

OBJECTIVES: Different probiotic strains used in clinical trials have shown prophylactic properties in different inflammatory diseases of the gastrointestinal tract. This study was aimed to investigate the influence of *Escherichia coli* strain Nissle 1917 (EcN) components on the integrity of the Caco-2 cell monolayer (human adenocarcinoma cell line).

METHODS: The effect of supernatant of EcN suspension and lipopolysaccharide (LPS) isolated from EcN (in concentrations from 0.001 to 1 000 μg/ml) on paracellular transport of ^{14}C-mannitol marker through epithelial cell monolayer was estimated.

RESULTS: Both LPS and EcN supernatant exerted almost the same effect; whereas no effect was shown using high concentrations (100 and 1 000 μg/ml), low concentrations (0.001, 0.1 and 1 μg/ml) significantly decreased permeability of ^{14}C-mannitol. Concentration (0.001 μg/ml) decreased ^{14}C-mannitol permeability approximately about 20% (LPS) and 30% (EcN supernatant).

To elucidate the observed changes in monolayer permeability ("tighter monolayer") induced by concentrations of LPS or supernatant, media able to open epithelial intercellular junctions were used. The effects of Ca^{2+}-free transport medium and of medium containing 5, 10, 20, 50, and 100% of Ca^{2+} on the ^{14}C-mannitol transport in the presence of the lowest (0.001 μg/ml) and high (100 μg/ml) concentrations of LPS were studied. Using Ca^{2+}-free medium both concentrations of LPS significantly decreased apparent permeability coefficient (Papp) of ^{14}C-mannitol indicating that changes of ^{14}C-mannitol permeability are independent of dimensions of paracellular spaces.

CONCLUSION: The decrease of ^{14}C-mannitol permeability caused by EcN LPS indicates the ability of components of probiotic EcN strain to restore disrupted epithelial barrier.

ORIGINAL ARTICLE

Abbreviations:

ECACC - European Collection of Cell Cultures
HBSS - Hanks´ balanced salt solution
P_{app} - apparent permeability coefficient (cm/s)
LPS - bacterial lipopolysaccharide
EcN - probiotic *Escherichia coli* strain Nissle 1917 O6:K5:H1

INTRODUCTION

Probiotics are defined as live microorganisms which when administered in adequate amount confer a healthy benefit on the host. The mechanisms by which probiotics exert beneficial effects include immmunomodulatory effects and enhancement of intestinal barrier function. It is widely accepted that tight junctions (components of epithelial junction complexes) are altered during infections by attaching and effacing pathogens (Britton & Versalovic 2008; Guttman *et al.* 2006 a,b). Increase of intestinal permeability resulting from loss of tight junction formation and disruption of epitehalial barrier function could lead to translocation of commensal bacteria or their components. This effect may contribute to pathological consequences and development of inflammatory gastrointestinal and systemic diseases (Tlaskalová-Hogenová *et al.* 2004). The role of increased epithelial permeability in various diseases has been described *in vitro* and *in vivo* (Madsen *et al.* 2001; Welcker *et al.* 2004; Guttman *et al.* 2006 a, b). It was suggested that prevention and treatment with probiotic bacteria may prevent or reverse increased permeability of the intestinal epithelium (Schultz *et al.* 2004).

Commensal-derived probiotic bacteria have been implicated as therapy for a range of digestive symptoms or diseases such as diarrhoea, inflammatory bowel disease, bacterial gastritis, amelioration of the side effects of antibiotic therapy, and prevention of atopic disease (Schultz *et al.* 2004, Kokešová *et al.* 2006, Britton & Versalovic 2008). Non-pathogenic *Escherichia coli* strain Nissle 1917 O6:K5:H1 (EcN) has evolved into one of the best characterized probiotics, and its therapeutic efficacy and safety have been proven (Schultz *et al.* 2004, Ukena *et al.* 2007). However, the exact mechanisms by which EcN mediates its effects are not fully understood.

Before studying the interaction of probiotics with transepithelial transport of xenobiotics, this prework-study was done to evaluate the effect of probiotics on the integrity of the Caco-2 cell monolayer (an *in vitro* model of human intestinal absorption) because intestinal epithelium has been proved to be rate-limiting for the absorption of orally administered drug. The permeability is one factor determining oral absorption and bioavailability of drugs. Due to the risk of live bacteria multiplications in the cell culture medium followed by its effect on medium pH and nutrient availability (Gratz *et al.* 2007), the EcN component, the bacterial lipopolysaccharide (LPS), a glycolipid of the cell wall of gram-negative bacteria, was used (Böcker *et al.* 2003).

To differentiate the potential effects caused by LPS alone or by other factors released by EcN suspension into supernatant in the present study we compared the effects of LPS with the effects of EcN cell free supernatant on epithelial monolayer integrity.

MATERIAL AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (Praha, Czech Republic), materials for cell culture were obtained from PAA Laboratories (BioTech, Praha, Czech Republic) or Gibco Invitrogen (KRD, Praha, Czech Republic) and ^{14}C-mannitol (100 µCi/ml) from Moravek Radiochemical and Biochemicals (MGP Zlín, Czech Republic). Lipopolysaccharide (LPS) isolated by phenol extraction from the *Escherichia coli* Nissle 1917 O6:K5:H1 and EcN-supernatant were prepared at the Institute of Microbiology, Czech Academy of Sciences.

Caco-2 cells

Caco-2 cells (ECACC) were cultured in a standard manner (Bourdet and Thakker 2006) at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity. For transport studies, Caco-2 cells (passages 68–79) were seeded onto the Transwell inserts at the density of 2.5×10^5 cells/cm^2 and grown to late confluence (21–24 days).

Cell viability assay

Cell viability was determined by Trypan blue exclusion method (Smetanova *et al.* 2009). 24 h after seeding Caco-2 cells onto Petri dishes (2×10^5 cells/cm^2), LPS 100 µg/ml and 1 000 µg/ml or transport medium (control) was added and incubated for next 2 h. Then the cells were released and incubated with 0.4% Trypan blue for 5 min and counted by using microscopy.

Monolayer integrity assessment

Before the start of the experiment, the monolayer integrity was checked by 500 µmol/l phenol red permeability/1 h (Smetanova *et al.* 2008). During the transport studies, the integrity was confirmed simultaneously by ^{14}C-mannitol (0.5 µCi/ml) permeability (Artursson *et al.* 1996).

Effect of LPS or EcN supernatant on ^{14}C-mannitol transport

^{14}C-mannitol transport (marker of paracellular transport and indicator of monolayer integrity) was studied. The prepared Caco-2 cell inserts were rinsed twice and equilibrated with prewarm HBSS at 37 °C for 30 min before the transport studies.^{14}C-mannitol alone or ^{14}C-mannitol plus LPS (0.001, 0.1, 1, 100, 1 000 µg/ml) or plus supernatant (0.001, 0.1, 1, 50 µg LPS/ml) were added to the apical compartment (diluted in HBSS transport medium) and the radioactive samples from

the basolateral compartment were withdrawn at 30, 60, 90 and 120 min for measurement radioactivity using liquid scintillation counter (Beckman Instruments).

LPS pretreatment

To investigate LPS effect on cell junctions, the LPS (0.001 μg/m, 100 μg/ml) were incubated for 2 hours in apical compartment. After Caco-2 cells washing the transport of ^{14}C-manitol was evaluated.

Induction of "leaky Caco-2 monolayer" and LPS effect

The effect of Ca^{2+}-free transport medium (opening cell junctions) and of medium containing 5, 10, 20, 50, and 100% of Ca^{2+} (100% = standard concentration of Ca^{2+} in HBSS transport medium) on the ^{14}C-mannitol transport were analyzed. The effects of low (0.001 μg/ml) and high (100 μg/ml) concentration of LPS on ^{14}C-mannitol transport was studied using transport medium with 100% of Ca^{2+} or Ca^{2+}-free transport medium.

Data analysis (calculation of the P_{app})

In all transport studies, the following equation was used to calculate the apparent permeability coefficient $P_{app} = (dQ/dt) \times (1/A \times C_0)$, (cm/s) (Artursson & Karlsson 1991): 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).

Statistical analysis

All values are represented as mean ± standard deviation (SD). All treatments were carried out at least in two independent experiments (Transwell inserts, n=3–17). Statistical differences were determined using Kruskal-Wallis one way analysis of variance (ANOVA) followed by Bonferroni´s (with control) multiple-comparison test as post hoc analysis or a Student´s *t*-test for group comparison of parametric data. The differences were considered significant when $p<0.05$.

RESULTS

Cell viability assay

LPS 100 μg/ml and 1 000 μg/ml did not influence Caco-2 cell viability in comparison with viability of Caco-2 cells incubated with transport medium without LPS (97.9% and 98.4% vs. 98.0% , not significant).

Effect of LPS or supernatant on ^{14}C-mannitol transport

As the results were obtained from several tested plates, the each value of P_{app}s was expressed as percent of control value of ^{14}C-mannitol of pertinent plate (the mean ^{14}C-mannitol P_{app} was 1.50 ± 0.20 cm/s, n=10). Result (Figure 1) showed that highest concentration of LPS (1 000 μg/ml) non-significantly increased the transport of ^{14}C-mannitol (by 14%), the concentrations of 100 μg/ml LPS did not change the ^{14}C-mannitol transport, while decreasing concentrations lowered permeability of ^{14}C-mannitol significantly (the lowest concentration 0.001 μg/ml approximately about 20%). Supernatant exerted almost the same effect as LPS, the lowest concentration (0.001 μg LPS/ml) decreased ^{14}C-mannitol permeability by 33%.

LPS pretreatment

No changes in P_{app} of ^{14}C-mannitol after 2-hour pre-incubation of the Caco-2 monolayer with transport medium (control group without treatment) or with LPS in a concentration of 0.001 (100.5% of control value) and 100 μg/ml (97.6% of control value) indicated no disruption of cell junctions of the Caco-2 monolayers.

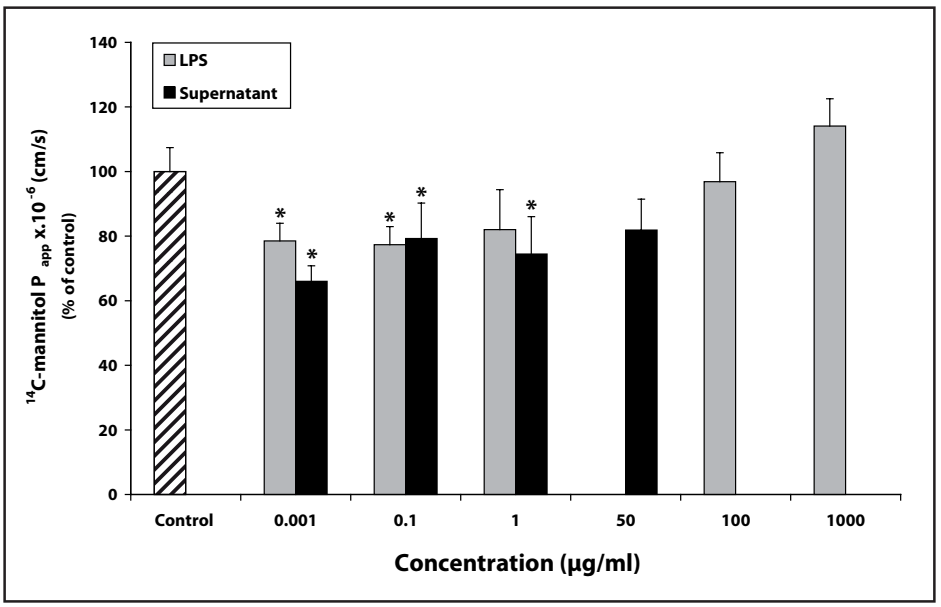

Fig. 1. Effect of various concentrations of EcN LPS or supernatant on ^{14}C-mannitol transport across Caco-2 monolayer from apical to basolateral compartment. Values are mean percentage differences ± SD of the ^{14}C-mannitol P_{app} (control group without treatment) obtained from at least two independent experiments (Transwell inserts, n=4–11), *significant differences versus control group, $p<0.05$.

Induction of "leaky Caco-2 monolayer" and LPS effect

Presence of 50, 20 and 10% of Ca^{2+} of the standard concentration of Ca^{2+} (100% Ca^{2+}) in HBSS transport medium had no effect on ^{14}C-mannitol permeability (Figure 2). However, the presence of only 5% of Ca^{2+} significantly increased ^{14}C-mannitol permeability. Ca^{2+}-free transport medium enormously increased ^{14}C-mannitol permeability; from 1.5 ± 0.20 cm/s to 18.96 ± 2.26 cm/s.

Only low concentration of LPS (0.001 µg/ml) decreased significantly transport of ^{14}C-mannitol when standard medium (100% Ca^{2+}) was used. Using Ca^{2+}-free transport medium ^{14}C-mannitol transport was significantly decreased in presence of both low (0.001 µg/ml) and high (100 µg/ml) concentration of LPS (Figure 3) suggesting protective effect of LPS.

DISCUSSION

The beneficial effect of probiotic administration acquires attention in clinical practice and for this reason it is important to know possible mechanisms of interaction of probiotics with other prescribed drugs. To focus on absorption process the effect of LPS (a key component of the outer membrane of gram-negative bacteria) or supernatant of EcN suspension on Caco-2 cell monolayer (*in vitro* model of human intestinal absorption) was studied.

There are different mechanisms by which probiotics can modulate the epithelial barrier. We concentrated on evaluation of effect of EcN components on Caco-2 monolayer integrity. The obtained results showed that LPS had no effect on Caco-2 cells viability even at the

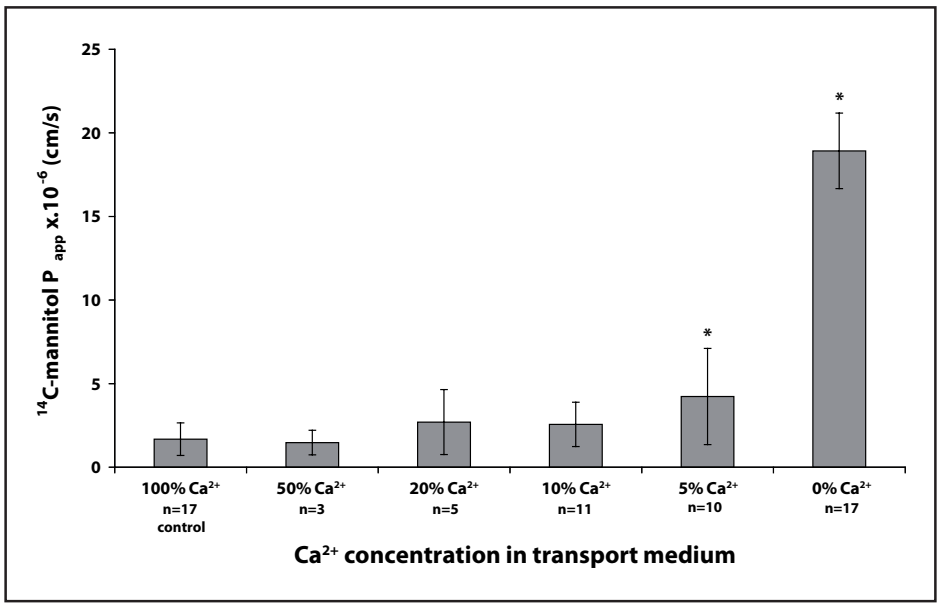

Fig. 2. Changes in permeability of ^{14}C-mannitol from apical to basolateral compartment across Caco-2 cell monolayer in presence of various concentrations of Ca^{2+} ions in transport medium. Results are reported as mean ± SD of ^{14}C-mannitol P_{app} from at least two independent experiments, *significant differences versus control group, $p<0.05$.

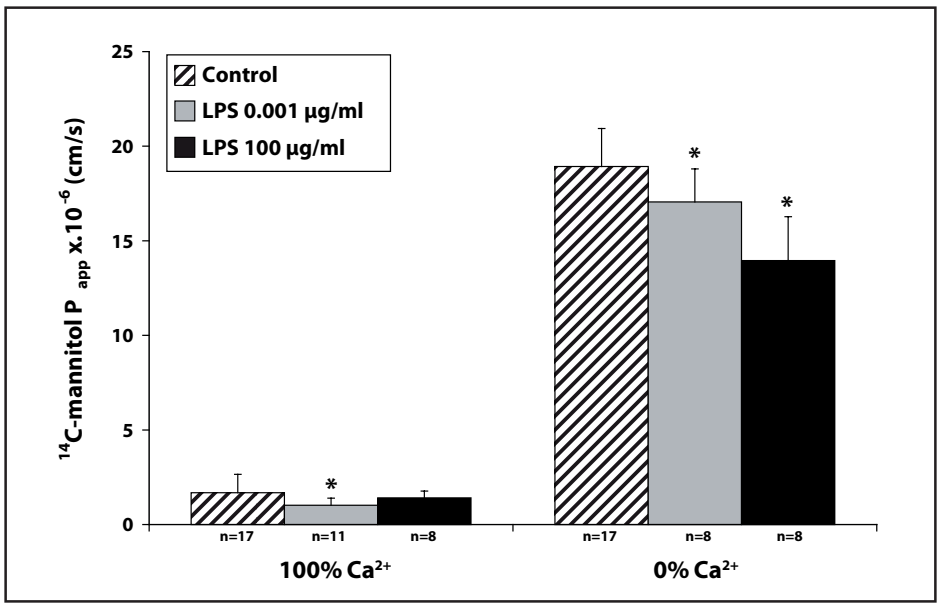

Fig. 3. ^{14}C-mannitol permeability from apical to basolateral compartment in absence or presence of Ca^{2+} ions in transport medium (control groups) and effects of EcN LPS (0.001 and 100 µg/ml) on the ^{14}C-mannitol permeability. Each column represents the mean ± SD of the ^{14}C-mannitol P_{app} from at least two independent experiments, *significant differences versus control group, $p<0.05$.

highest dose (1 000 μg/ml), which indicate non toxic effect of LPS on integrity of Caco-2 cells. Similarly, LPS in the high doses (100 and 1 000 μg/ml) exerted no effect on ^{14}C-mannitol permeability, a marker of paracellular transport, i.e. on monolayer integrity. On the other hand low doses of LPS or supernatant repeatedly decreased ^{14}C-mannitol transport. Interestingly, it seems that in contrast to EcN LPS, lipopolysaccharides from other bacterial sources increase permeability and cause mucosal damage (Fang *et al.* 2010, Hietbrink *et al.* 2009).

To elucidate the observed decreased permeability of the monolayer ("tighter monolayer") induced by lower concentrations of LPS or supernatant we attempted to analyze this effect by pretreatment of Caco-2 monolayer by LPS for 2 h. Results induced assumption that LPS had no altering effect on cell junctions because no effect on monolayer resistance measured by ^{14}C-mannitol transport was found. Then we attempted to prepare monolayers with different resistance (differently "leaky monolayer") using transport medium with different Ca^{2+} concentrations. Ca^{2+} ions are indispensable for formation of some types of cell junctions (cell-cell adhesions in adherens junctions and desmosomes are mediated by transmembrane proteins cadherins, proteins responsible for Ca^{2+}-dependent interactions -homophilic binding- of adjacent cells (Lodish 2008)). Evaluation of the dose-effect relationship (percent of Ca^{2+} in transport medium in relation to P_{app} of ^{14}C-mannitol (tightness of the monolayer) showed no significant difference of ^{14}C-manitol permeability using the transport medium with 100–10% of Ca^{2+} ions. However, there was steep increase in P_{app} of mannitol in Ca^{2+}-free medium versus P_{app} of mannitol in medium containing 5% of Ca^{2+}. Ca^{2+}-free medium increased P_{app} more than 10 times and value of SD (standard deviation) indicated uniform reaction, whereas SD of 68% in the case of 5% Ca^{2+} in transport medium (P_{app} increased only 2.5 times) signalized non-uniform answer.

As obvious "leaky monolayer" was induced only by Ca^{2+}-free medium, further research was centred on effect of probiotics in "leaky monolayer" using this Ca^{2+}-free medium. While results with the standard concentration of Ca^{2+} in transport medium showed significant decrease of ^{14}C-mannitol permeability only in the dose of 0.001 μg/ml of LPS, in the case of use of Ca^{2+}-free medium, both used doses of LPS (0.001μg/ml and 100 μg/ml) significantly decreased ^{14}C-mannitol permeability which indicated that these changes of ^{14}C-mannitol permeability were independent of actual tightness of paracellular spaces. We can state that this finding of increased monolayer resistance is in an agreement with hypothesis that probiotics are able, among others, to induce restoration of a disrupted epithelial barrier (Schultz 2004) as was described by Zyrek *et al.* (2007) using T_{84} and Caco-2 monolayers. In their work, disruption of these monolayers induced by enteropathogenic *E. coli* was restored by co-incubation with EcN for 120 min as monitored by transepithelial resistance. They suggested possible explanation that this was caused via silencing of protein kinase (whose activation is involved in dysregulation of tight junction permeability) and by redistribution of zonula occludens-2, tight junction-associated proteins, that appear to organise the transmembrane proteins and couple them to other cytoplasmic proteins and actin microfilamens (Anderson 2001). However, the participation of nitric oxide and cytokines in observed effects cannot be excluded (Zídek *et al.* 2010).

CONCLUSION

Even the highest dose of LPS (1 000 μg/ml) did not show any effect on viability of cultivated Caco-2 cells. Both, LPS and supernatant of EcN caused similar effects on ^{14}C-mannitol permeability indicating that except LPS no other factors present in supernatant are responsible for decreasing the ^{14}C-mannitol permeability. The estimated decrease of ^{14}C-mannitol permeability (independent of tightness of paracellular spaces) suggested that increased monolayer integrity was induced by probiotic components.

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