PROBIOTIC BACTERIA ESCHERICHIA COLI STRAIN NISSLE 1917 ATTENUATES ACUTE GASTRIC LESIONS INDUCED BY STRESS

P.C. KONTUREK1, Z. SLIWOWSKF, J. KOZIEL2, A. PTAK-BELOWSKA2, G. BURNAT1, T. BRZOZOWSKF, S.J. KONTUREK2

1Department of Internal Medicine, Thuringia-Clinic Saalfeld/Saale, Teaching Hospital University Jena, Germany; 2Department of Physiology, Jagiellonian University Medical College, Poland; 3Department of Microbiology, Faculty of Biochemistry, Biophysics, and Biotechnology, Jagiellonian University, Krakow, Poland

INTRODUCTION

Living microorganisms that enter the gastrointestinal tract (GIT) in an active state and exert a positive influence on the host tissues are called probiotics (1). Recent evidence has suggested the potential therapeutic role of probiotics in the prevention or treatment of GIT diseases (2). A particularly interesting strain is Escherichia coli Nissle 1917, which was isolated by A. Nissle in 1917 from the feces of a soldier, who in contrast to all of his comrades, did not develop enterocolitis during the war on the Balkan peninsula, was highly contaminated by enteropathogens at that time. For that reason, the strain is known by the name Escherichia coli Nissle 1917 (EcN). At present EcN is contained in a probiotic drug called Mutaflor (3). Efficacy of EcN against inflammatory cytokines, enhancement of gut barrier function, antagonistic activity against pathogenic microorganisms in the GIT, immunomodulatory effects, support of colonocyte metabolism and modulatory effect on gut motility (12).

EcN was investigated mainly in the lower GIT, showing a strong anti-inflammatory effect in patients with ulcerative colitis and in animal models for experimental colitis, while the effects of this probiotic on the upper GIT, in particular, on acute gastric damage and the mechanism of its action have been little studied. However, previous studies demonstrated that some probiotics such as Lactobacillus and Bifidobacterium play a role in the stabilization of the gastric barrier function and decrease of mucosal inflammation (13, 14). Moreover, some probiotics such as Lactobacillus acidophilus have been shown to have an inhibitory effect on the growth of Helicobacter pylori (Hp) in vitro and to increase eradication rate of Hp in patients infected with this bacterium (13).

The aim of the present study was: 1) to investigate the effect of probiotic EcN on the formation of stress-induced gastric lesions and accompanying changes in gastric mucosal blood flow in rats, 2) to study the possible implications of probiotics (PG) and sensory nerves in the gastroprotective action of EcN, 3) to analyze the effect of pretreatment with EcN...
on gastric mucosal expression of cyclooxygenase-2 (COX-2), peroxisome proliferator receptor gamma (PPARγ), heat shock protein 70 (Hsp70) and pro-inflammatory cytokine IL-1β.

MATERIAL AND METHODS

Induction of stress lesions and determination of gastric mucosal blood flow

The study was performed according to the guidelines of Helsinki Declaration regarding handling of experimental animals in accordance with the permission of the Local Ethical Committees at the Jagiellonian University of Medical College and University of Jena.

Acute gastric stress lesions were induced in rats by water immersion restrain-method as described before (15). Briefly, the animals were placed in restraint cages and immersed vertically to the level of the xyphoid process in a water bath of 23°C for 3.5 hours. After 3.5 h of WRS, the rats were lightly anaesthetized with ether, the abdomen was opened and the stomach was exposed. The stomach by means of local H2-gas clearance method using an electrolytic regional blood flow meter (Biomedical Science, Model RBF-2, Osaka, Japan) as described before (15). The measurements were made in the three areas of the mucosa and the mean values of the measurements were calculated and expressed as percent changes of those recorded in the intact rats not exposed to WRS (control group) saline-treated animals. The stomach was then removed, opened along the greater curvature and placed flat to count the number of gastric lesions by two investigators, unaware of the treatment given as described in our previous studies. The stress lesions were defined as round or linear mucosal defects of at least 0.1 mm in diameter.

Animals with sensory denervation induced by capsaicin

The role of sensory afferent nerves in gastroprotective action of EcN against WRS-induced gastric lesions was determined in rats with deactivation of sensory nerves by capsaicin. For this purpose the animals were pretreated with capsaicin (Sigma Co., St. Louis, MO, USA) injected s.c. for 3 consecutive days at a dose of 25, 50 and 50 mg/kg for 2 weeks before experiments with WRS exposure as described before (16). All injections of capsaicin were performed under the anaesthesia to counteract the pain reactions and respiratory impairment associated with injection of this agent. To check the effectiveness of the capsaicin denervation, a drop of 0.1 mg/ml solution of capsaicin was instilled into the eye of each rat and the protective movements were counted as described previously (17). Rats with or without capsaicin-denervation received vehicle or EcN (104 CFU) 1 h prior to exposure to 3.5 h of WRS. At the end of WRS, animals were anesthetized and the GBF and the number of gastric lesions were measured in a similar manner as mentioned above.

Involvement of nitric oxide (NO) and prostaglandins E2 (PGE2) in the gastroprotection induced by EcN

In these studies two series (A and B) of experiments were carried out. In series A of experiments, the involvement of NO in gastroprotection by EcN was tested by employing Nω-nitro-L-arginine (L-NNA) and L-arginine (both purchased from Sigma, St. Louis, USA), an inhibitor of nitric oxide synthase (NOS) injected i.p. at a dose 20 mg/kg. The following groups consisting of 6 rats each were used: 1) vehicle (saline i.p.) followed 60 min later by WRS, 2) EcN (104 CFU i.g.) followed 60 min later by WRS, 3) L-NNA (20 mg/kg i.p.) followed 15 min later by vehicle and then 60 min later by WRS, 4) L-NNA (20 mg/kg i.p.) followed 15 min later by EcN (104 CFU i.g.) and then 60 min later by WRS and 5) L-arginine (200 mg/kg i.g.) followed 15 min later by L-NNA (20 mg/kg i.p.) and by EcN (104 CFU i.g.) and then finally 60 min later by 3.5 h of WRS.

Series B was used to investigate the involvement of PG in the gastroprotection induced by EcN. For this purpose following groups, each consisting of 6 rats, were used: 1) vehicle (saline i.p.) followed 60 min later by WRS, 2) EcN (104-106 CFU/ml i.g.) followed 60 min later by WRS, 3) indomethacin (5 mg/kg i.p.) followed 15 min later by vehicle and then 60 min later by WRS, 4) indomethacin (5 mg/kg i.p.) followed 15 min later by EcN (104 CFU i.g.) and then 60 min later by WRS.

Determination of the mRNA expression for IL-1β and ghrelin

The expression of IL-1β and ghrelin was determined by RT-PCR in the gastric mucosa of intact rats or following exposure to WRS with or without pre-treatment with EcN applied in graded concentrations in terms of colony forming units per ml (104-106 CFU/ml) either alone or in combination with indomethacin (5 mg/kg i.p.). Samples of the gastric oxyntic mucosa were scraped off on ice using glass slide and then immediately snap frozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated from the gastric oxyntic mucosa using a rapid guanidinium isothiocyanate/phenol chloroform single step extraction kit from Stratagene (Stratagene GmbH, Heidelberg, Germany). Following precipitation, the RNA was resuspended in RNase-free TE buffer and the concentration was estimated by absorbance at 260 nm wave length. Samples were frozen at -80°C until analysis. First strand cDNA was synthesized from total cellular RNA (5 µg) using 200 U Stratata Script TM reverse transcriptase (Stratagene GmbH, Heidelberg, Germany) and oligo (dT) primers (Stratagene GmbH, Heidelberg, Germany). After the reverse transcription, the transcriptase activity was destroyed by heating, and the cDNA was stored at -20°C until PCR. A 543-bp fragment of IL-1β and 394 bp segs of ghrelin were amplified from single-stranded DNA by PCR using specific oligonucleotide primers to IL-1β and ghrelin. The IL-1β sense primer was 5' GCT ACC TAT GTC TTG CCC GT3' and antisense primer was 5'GAC CAT TGC TGT TTC CTA GG 3'. The ghrelin sense primer was 5'TTG AGC CCA CAG CAC CAG AAA 3' and antisense primer was 5'AGT TGC AGA GGA GCC GCC AGA AGC T3'. The primers for IL-1β, ghrelin and β-actin were synthesized by Biometra (Gottingen, Germany) as described previously (17, 18). Concomitantly, amplification of control rat β-actin (Clon Tech, Palo Alto, CA, USA) (764 bp) was performed on the same samples to verify RNA integrity.

DNA amplification was carried out under the following conditions; denaturation at 94°C for 1 min, annealing at 60°C for 45 s, and extension at 72°C for 45 s. The number of amplification cycles was 30 for IL-1β and 31 for ghrelin. Each PCR-product (8 µL) was electrophoresed on 1.5% agarose gel stained with ethidium bromide, and then visualized under UV light. Location of predicted PCR product was confirmed by using a 100-bp ladder (Gibco BRL/Life Technologies, Eggenstein, Germany) as standard marker. The intensity of bands was quantified in a semiquantitative manner using densitometry (LKB Ultrascan, Pharmacia, Sweden). The mRNA signals for IL-1β and ghrelin were standardized against the β-actin mRNA signal for each sample and results were expressed as IL-1β mRNA/or ghrelin/β-actin mRNA ratio.

Determination of the gastric mucosal protein expression for COX-2, PPARγ and HSP70 by Western blot

For Western blot analysis, proteins were extracted from the same gastric mucosa samples as mentioned above.
Approximately 10 µg of total protein extracts was loaded on SDS polyacrylamide gels and run at 40 mA, followed by transfer onto nitrocellulose membrane (Protran, Schleicher and Schuell, Germany) by electrophoretic solution. Solution of 3% bovine serum albumin (BSA - Sigma Aldrich, Germany) in TBS/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris-HCl, pH 7.4, 0.1% Tween-20) was used to block filters for at least 1 h at room temperature. Specific primary antibody against COX-2 (rabbit polyclonal, dilution 1:500; Santa Cruz), PPARγ (rabbit polyclonal, dilution 1:300; Santa Cruz), HSP70 (rabbit polyclonal, dilution 1:200; StressGene) or β-actin (mouse monoclonal, dilution 1:3000; Sigma Aldrich, Germany) was added to the membrane, followed by an anti-rabbit-IgG or anti-mouse-IgG HRP-horseradish peroxidase conjugated secondary antibody (dilution 1:40000 or 1:20000) dissolved in 1% non-fat milk in TBS-Tween-20 buffer. Incubation of primary antibody was followed by 3 washes with TBS-Tween-20 buffer for 5 min. Incubation of the secondary antibody was followed by 6 washes for 5 min. Immunocomplexes were detected by the SuperSignal West Pico Chemiluminescent Kit (Pierce, USA). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Wiesbaden, Germany).

For NFκB-p65 protein expression analysis, nuclear extracts were performed according to the method described by Suzuki et al. (19). The expression of NFκB-p65 was determined by Western blot using rabbit polyclonal antibody (C-20, 1:500, Santa Cruz, USA). Total isolated proteins were measured by BCA method according to the manufacturer’s recommendation (Sigma Aldrich, Germany). One hundred µg of proteins were loaded on SDS-polyacrylamide gel. Remaining part of the Western blot analysis was performed as described above.

Statistical analysis

Results are expressed as means±S.E.M. from 6 rats per group. Statistical significance of the difference was determined using analysis of variance (one-way ANOVA). Further statistical analysis for post hoc comparisons was carried out using Bonferroni/Duncan test or Student’s t-test when appropriate. Differences with p<0.05 were considered as significant.

RESULTS

Effect of cyclooxygenase (COX) and NOS inhibitors on EcN-induced gastroprotection against WRS induced gastric damage and the alterations in GBF

Exposure of rats to WRS was associated with the induction of acute gastric mucosal lesions and a significant decrease in GBF. Pretreatment with EcN (10^10 CFU i.g.) significantly and dose-dependently reduced gastric lesions induced by WRS. The gastroprotective effect of EcN was accompanied by a significant increase in gastric mucosal blood flow. The i.p. administration of indomethacin at a dose 5 mg/kg, which is known to inhibit the generation of PGE2 by about 80%, aggravated the WRS-induced gastric lesions and significantly decreased GBF as compared to respective values in control animals treated with vehicle. Pretreatment with indomethacin reversed almost completely the protective effect of EcN against WRS-induced gastric lesions and the accompanying increase in GBF (Fig. 1).

The administration of L-NNA, an inhibitor of NOS, significantly aggravated the number of gastric mucosal lesions induced by WRS and accompanying fall in GBF. The pretreatment with L-NNA almost completely abolished the protective effect of EcN against WRS-induced lesions and the increase in GBF caused by this probiotic. However, co-treatment with L-arginine (200 mg/kg i.g.) added to EcN restored the gastroprotection and the accompanying increase in GBF induced by EcN in L-NNA-treated animals (Fig. 2).

Effect of deactivation of sensory nerves by capsaicin on EcN-induced gastroprotection against WRS induced gastric damage and the alterations in GBF

As shown in Fig. 3, the deactivation of sensory nerves by pretreatment with neurotoxic dose of capsaicin, carried out on separate group of animals, increased the area of WRS-induced gastric lesions and produced a significantly greater fall in GBF as compared to vehicle-pretreated rats with intact sensory nerves exposed to WRS. In capsaicin-denervated animals, the protective

![Fig. 1. Effect of EcN applied in graded concentrations (10^1-10^9 CFU) without or with pretreatment with indomethacin (5 mg/kg i.p.) on the mean number of acute stress lesions and the accompanying changes in gastric blood flow. Results are means±SEM of 6-8 rats. Asterisk indicates a significant change as compared to vehicle treated group. Cross indicates a significant change as compared to respective values without indomethacin pretreatment.](image-url)
activity of EcN applied in a standard dose of 10⁴ CFU and accompanying rise in the GBF, were significantly reduced as compared with those in rats with intact sensory nerves. Co-administration of calcitonine gene related peptide (CGRP) with EcN in rats with capsaicin denervation restored the protective and hyperemic effects of EcN against WRS-induced gastric lesions.

**Gastric mucosal expression of mRNA for IL-1β and ghrelin**

As shown in Fig. 4, mRNA for IL-1β was not detected in the intact gastric mucosa. In vehicle-treated rats exposed to WRS, the expression of mRNA for IL-1β was significantly increased as compared to that observed in intact rats not exposed to stress alone. Pretreatment with EcN at increasing doses, starting from 10¹ to 10⁸ CFU/ml applied prior to the exposure to WRS, dose-dependently decreased the signal of mRNA for IL-1β below that detected in gastric mucosa exposed to WRS alone. The ratio of IL-1β mRNA over β-actin mRNA confirmed that IL-1β mRNA was increased in rats exposed to WRS, but it was downregulated when EcN was applied prior to WRS. The addition of indomethacin (5 mg/kg i.p.) to EcN (10⁴ CFU/ml) prior exposure to WRS completely abolished the decrease in expression of IL-1β induced by EcN and the ratio of IL-1β to β-actin reached even higher value than that observed in rats exposed to WRS alone.
Ghrelin mRNA was detected in intact rat gastric mucosa (Fig. 5). Exposure to WRS resulted in upregulation of the mRNA for ghrelin. Pretreatment with EcN at increasing doses (10^4–10^8 CFU/ml) applied prior to the exposure to WRS, dose-dependently increased the mRNA expression for ghrelin above that detected in gastric mucosa exposed to WRS alone. The ratio of ghrelin mRNA over β-actin mRNA confirmed that ghrelin mRNA was increased in rats exposed to WRS, but it was further upregulated by EcN, especially when this probiotic was administered in concentration of 10^6 CFU/ml. The co-treatment with indomethacin (5 mg/kg i.p.) and EcN (10^6 CFU/ml) prior exposure to WRS was associated with a significant decrease in the expression of ghrelin towards the level observed in intact rats not exposed to WRS (Fig. 5).

**Gastric mucosal protein expression of PPARγ, COX-2 and HSP70**

As shown in Fig. 6, the expression of COX-2 and HSP70 was negligible in the intact gastric mucosa. In rats exposed to 3.5 h WRS, the expression of COX-2 and HSP-70 significantly increased when compared to that observed in control animals without exposure to WRS. The reduction in the signal intensity for COX-2 expression and a significant increase in HSP70 expression were observed in rats pretreated with EcN. In contrast, a significant up-regulation of COX-2 and down-regulation of HSP70 protein expression were recorded in rats co-treated with indomethacin and EcN. PPARγ was detected as a relatively weak signal in the intact gastric mucosa. The exposure to WRS caused a significant up-regulation of protein for PPARγ. In rats co-treated with indomethacin and EcN, the PPARγ expression remained at the increased level but not significantly different from that in EcN-treated rats. The pretreatment with EcN prior to WRS exposure was accompanied by a decrease in PPARγ expression.

**Effect of EcN pretreatment on NFκB-p65 expression in nuclear extracts by Western blot**

In intact gastric mucosa no expression for NFκB-p65 was detectable, indicating a low activation of this transcription factor in the gastric mucosa. In contrast, in rats exposed to WRS, a significant and strong upregulation of NFκB-p65 was detected in gastric mucosa. In rats exposed to EcN prior to WRS exposure as well as those co-treated with indomethacin and EcN before
Acute and life-threatening gastrointestinal bleedings in patients at risk of stress are often associated with mucosal lesions induced by stress. Candida strain (22, 23, 24). All these observations are of importance as they indicate that probiotics may be beneficial in the prophylactic treatment and protection against acute gastric mucosal lesions.

The mechanism of gastroprotective activity of EcN has not been fully explained and as shown in present report, it may involve numerous important mediators. The fact that L-NAME, the blocker of NOS almost completely attenuated the gastroprotective effect of EcN against stress-induced gastric lesions, while exogenous L-arginine, the substrate of NOS, restored the effect, indicates that nitric oxide (NO) could be considered as an important mediator of this gastroprotection afforded by EcN. Therefore, NO seems to play a central role in the mechanism of gastric mucosal defense. Moreover, endogenous NO contributes also to the healing of acute gastric mucosal injury by mediating the mucosal hyperaemic response (25).

In the present study, we observed for the first time a significant increase in ghrelin mRNA expression in gastric mucosa of rats treated with EcN prior to exposure to WRS. This new aspect of the action of EcN deserves further studies to clarify the involvement of ghrelin in the gastroprotection by this probiotic. Ghrelin has been recently shown to exert a marked protective action against acute stress-induced gastric mucosal injury and to accelerate the ulcer healing (26, 27).

Herein, we provide evidence that gastroprotective actions of this compound are mediated, at least in part, by PG. The EcN-induced protection against WRS-induced gastric lesions was almost completely abolished by a potent COX-inhibitor such as indomethacin. Interestingly, in this study, the expression of COX-2 protein in the gastric mucosa pretreated with EcN, tended to decrease when compared to that observed in rats exposed to WRS only. The possible explanation for this finding is that the probiotic EcN strain causes the significant down-regulation of proinflammatory cytokine IL-1β, as documented in our study by determination of IL-1β mRNA expression. We found that EcN dose-dependently inhibited expression of mRNA for IL-1β, a cytokine that is well known to stimulate COX-2 expression and its activity. Therefore, our study suggests that the protective effects by EcN may be partly mediated by PG because COX-2 was actually downregulated by EcN, whereas indomethacin, a potent inhibitor of COX activity, markedly attenuated the protection and accompanying hyperemia caused by EcN.

The mechanism of gastroprotective activity of EcN appears to be also dependent on the functional activity of sensory nerves releasing CGRP. Our finding that functional ablation of sensory afferent nerves by capsaicin attenuated EcN-induced protection and accompanying hyperemia supports this notion. In addition, co-treatment with exogenous CGRP in order to replace the deficit of this peptide in capsaicin-treated animals restored the protective efficacy of EcN. This is in keeping with original observation that sensory nerves play a crucial role in the gastric mucosal protection (28).

The present study confirms our previous results showing a significant up-regulation of HSP70 in gastric mucosa exposed to stress. HSP70 is regarded as a major heat shock protein involved in the protection of gastric mucosa against different forms of acute mucosal injury (29, 30). The induction of HSP70 by geranylgeranylandalactone has a potential in prevention of acute gastric mucosal injury in rats (31). In view of previous evidence suggesting the protective activity of HSP70 we found a significant up-regulation of HSP70 by EcN in the rat gastric mucosa. It is of interest that this effect was abolished by co-treatment with indomethacin suggesting a mediatory function of PG in the upregulation of HSP70 by EcN. Although it is a first report on induction of HSP70 by EcN in the rat gastric mucosa, previous studies implicated probiotics in the activation of heat shock protein in rat gastric mucosa exposed to stress.

**DISCUSSION**

Our present study demonstrates for the first time that probiotic strain *E. coli Nissle* (EcN) 1917 dose-dependently attenuates the acute gastric lesions provoked by the exposure to stress. This remains in keeping with previous studies showing that probiotics exhibit the protective activity not only in the lower portion of GIT such as colon, but also protect gastric mucosa against acute mucosal lesions. Our present finding is in keeping with the study of Materia et al. (20) who reported for the first time that milk is an effective protector against stress-induced gastric ulcers in rats. Moreover, Uchida et al. (21) demonstrated recently that yogurt containing *Lactobacillus gasseri* attenuated the formation of acute experimental gastric ulcers and this gastroprotective action was attenuated by indomethacin, a potent inhibitor of PG biosynthesis in the gastric mucosa. Our own studies demonstrated that some probiotics, besides inhibiting the development of acute gastric mucosal lesions, as shown in this study, can also accelerate the process of ulcer healing and ameliorate the augmentation of ulcerative colitis induced by *Candida* strain (22, 23, 24).

All these observations are of significant practical importance because the acute gastric mucosal lesions induced by stress may be responsible for the acute and life-threatening gastrointestinal bleedings in patients at

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**Fig. 6**. Representative Western blot analysis of protein expression of β-actin (house-keeping protein, internal control), HSP70, COX-2, PPARγ and NFκB-p65 (in nuclear extracts) in the intact gastric mucosa (lane 1), in rat gastric mucosa after stress exposure (lane 2), rats exposed to stress and pretreated with EcN (10^8 CFU) with (lane 3) or without application of indomethacin (lane 4).

Exposure to WRS, the expression of NFκB-p65 protein remained at the increased level (Fig. 6).
The gastroprotective activity of EcN could be also attributed to the modulation of PPARs expression which is known to trigger cell survival pathways via stimulation of release of gastrin (32). Although gastrin was not determined in the present study, we observed a significant up-regulation of PPARs at protein level in gastric mucosa exposed to stress. The pretreatment with EcN was accompanied by the decrease in PPARs expression, which rather militates against the major protective action of PPAR in response to EcN and may reflect the attenuation of inflammatory reaction in the gastric mucosa by EcN. This is supported by the fact that indomethacin, which by itself aggravates stress-induced lesions, greatly reduced the protective and hyperaemic effects of EcN against stress-induced lesions and caused further increase in PPAR protein expression.

In summary, this study demonstrates that the pretreatment with probiotic strain of E. coli Nissle attenuated the acute gastric mucosal lesions induced by stress through the anti-inflammatory actions, induction of protective factors such as ghrelin and HSP70 synthesis in the gastric mucosa and the enhancement of gastric microcirculation. Endogenous PG, NO and neuropeptides released from sensory nerves such as CGRP are also involved in the gastroprotective activities of EcN. Further studies are needed to clarify whether the gastroprotective potential of EcN and other probiotic bacteria exists also in human stomach and whether the prevention of stress-induced lesions can be also observed in human stomach.

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Author’s address: Prof. Dr. Peter C. Konturek, Department of Internal Medicine, Thuryngia-Clinic Saalfeld, Teaching Hospital University Jena, Rainweg 61, 03671 Saalfeld/Saale, Germany; E-mail: pkonturek@thueringen-kliniken.de