DOI 10.1007/s10517-016-3166-z
Bulletin of Experimental Biology and Medicine, Vol. 160, No. 3, January, 2016

MICROBIOLOGY AND IMMUNOLOGY

Role of Nitric Oxide Produced by Lactobacilli in Relaxation of Intestinal Smooth Muscles

D. R. Yarullina, R. O. Mikheeva*, G. I. Sabirullina*, P. V. Zelenikhin, O. N. Ilinskaya, and G. F. Sitdikova*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 160, No. 9, pp. 328-331, September, 2015
Original article submitted July 31, 2014

Application of NO-producing lactobacilli to a rat jejunum segment induced muscle relaxation that was potentiated after activation of bacterial NO production with NO synthase substrate L-arginine. Similar changes in the intestinal contractile activity were observed in response to exogenous NO formed by sodium nitroprusside. These results indicated the involvement of NO synthesized by probiotic lactobacilli in the regulation of the intestinal motor function.

Key Words: *Lactobacillus plantarum; nitric oxide; spontaneous contractile activity; smooth musculature; rat jejunum*

NO is involved in many important physiological functions of the gastrointestinal system (GIT): it maintains vascular tone, participates in immune reactions and nerve pulse transmission, and supports GIT motor function by causing intestinal smooth muscle relaxation. This effect of NO is realized via cGMP-dependent or non-cGMP-dependent mechanisms directly in the smooth muscle cells or via inhibition of release of stimulatory neurotransmitters, such as acetylcholine or substance P [13]. The sources of NO in GIT are NO synthases (NOS) and enteric microflora that produces NO during nitrate- and nitrite reduction and, by reducing pH, creates conditions for chemical formation of NO from nitrites in acid medium [11,12,14]. NO produced by bacteria easily passes through membranes into tissues, where it exhibits its functional activity. In the intestine of *Caenorhabditis elegans*, NO synthesized by *Bacillus subtilis* regulates gene expression in cells and modulates worm lifespan and stress resistance [6]. However, the role of bacterial NO in GIT deserves further studies.

Department of Microbiology, *Department of Human and Animal Physiology, Kazan (Volga Region) Federal University, Kazan, Russia. ***Address for correspondence:*** kasfes@gmail.com. D. R. Yarullina

Lactobacilli are components of normal human enteric microflora; for this reason, they are widely used in modern probiotics and dairy foodstuffs [5]. Some of lactobacilli (*Lactobacillus plantarum*, *L. fermentum*, and *L. farciminis*) produce NO [4,7,8,15]; this fact seems to be partly responsible for the positive effect of probiotics [7,11,15].

We studied the effects of *Lactobacillus plantarum* 8P-A3 on contractile activity of rat intestine by evaluating the role of NO produced by symbiotic enteric microorganisms in GIT motor function and substantiating additional effects of probiotics based on NO-producing lactobacilli.

MATERIALS AND METHODS

The study was carried out in accordance with International Regulations on Animal Experiments and was approved by local ethic committee. Spontaneous contractile activity (SCA) was measured in rat jejunum preparations on a Biopac Systems Inc. instrument [1]. The animal was anesthetized with 5% isoflurane (Abbott Laboratories); an 8-mm long jejunum segment was fixed vertically in a 20-ml cuvette. The lower

end was tightly fixed to the block, while the upper end was connected to tensometric pickup (TSD 125C, Biopac Systems Inc.). The preparation was washed in Krebs medium (in mM): 121.0 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, and 8.0 $C_6H_{12}O_6$ (pH 7.2-7.4) at 37°C, aerated with a mixture of 95% O_2 and 5% CO_2. The preparation was equilibrated for 60-90 min after fixation in order to attain stable stretching; if stable spontaneous contractile responses were not attained, the preparations were excluded from the study. Each experimental series was carried out on 2-4 strips from 6 animals.

NO synthase substrate L-arginine (100 and 1000 μM), NO synthase blocker L-NAME (200 μM), NO donor sodium nitroprusside (SN; 10-1000 μM; Sigma-Aldrich), and *L. plantarum* 8P-A3 suspension (10^7-10^8 cell/ml; Lactobacterin dry; Perm Biomed Factory [4]) served as intestinal SCA effectors. The effectors were added (200 μl) into the cuvette with jejunum segment in the above concentrations. Suspension of *L. plantarum* 8P-A3 cells was prepared from bacteria cultured in MRS by the microanaerophilic method for 24 h and washed from the medium 3 times by centrifugation in isotonic NaCl. Viability of bacteria was evaluated by propidium iodide staining (2.5 μg/ml; Sigma-Aldrich) with subsequent flow cytofluorometry on a BD FACS-Canto II instrument. Bacterial biosynthesis of NO was evaluated by staining with 1,2-diaminoantraquinone sulfate (DAA, 50 μg/ml, Molecular Probes; 30 min incubation at 37°C) with subsequent microscopy of bacteria washed from stain in a Leica DM 6000B fluorescent microscope [3]. The concentration of NO in SN solutions was estimated by calibration curve plotted with the use of fresh solutions of $NaNO_2$ (Sigma-Aldrich) in concentrations of 0-100 μM. Nitrites, NO oxidation products under aerobic conditions, were measured spectrophotometrically (λ=540 nm) using Griess reagent (Sigma-Aldrich).

SCA was recorded with a DA100C amplifier, subsequent analysis of preparation contractions force (in grams) was carried out using AcqKnowledge 4.1 software. The significance of differences was evaluated by Student's *t* test.

RESULTS

We studied the effects of probiotic *L. plantarum* 8P-A3 lactobacilli on rat intestinal SCA. These microorganisms formed NO, which was shown by the results of staining with DAA (fluorescent indicator of NO; Fig. 1, *a*). Despite high permeability of cell membranes for NO, fluorescence was observed exclusively in bacteria, which indicated the bacterial origin of NO.

Incubation of the jejunum with lactobacillus suspension led to reduction of SCA; relaxation increased with increasing in the concentration of microorganisms (Fig. 1, *e*). Addition of lactobacillus suspension (10^7 cell/ml) reduced the amplitude of jejunum segment contractions by 6.8% from the initial level; suspension with a higher concentration of cells (10^8 cell/ml) caused more marked reduction of contraction amplitude – by 13.4% (n=6, p<0.05).

Application of lactobacillus suspension containing NOS substrate L-arginine (100 μM) reduced contractions by 23.1% (n=6, p<0.05; Fig. 1, *c*, *d*, *e*). We have previously demonstrated that arginine stimulated NO synthesis in *L. plantarum* 8P-A3 [3], and hence, we attributed relaxation of the preparation to higher level of NO. The amplitude of contractions in this variant decreased more markedly than in response to L-arginine alone (Fig. 1, *e*), and hence, muscle relaxation observed in this experiment was not due to L-arginine induction of the intestinal NOS.

The relaxing effect of lactobacilli on rat jejunum coincided with the effect of SN. The strength of spontaneous contractions of the jejunum decreased in response to SN in proportion to the concentrations of NO released from SN (Fig. 1, *f*). The level of NO in the experimental system in response to addition of 100 μM SN reached 0.63 μM, which was paralleled by reduction of the amplitude of contractions by 19.4% in comparison with the values before SN addition (n=6, p<0.05), which was comparable with the effect of lactobacillus suspension under conditions of L-arginine activation of NO synthesis (amplitude reduction by 23.1%; Fig. 1, *e*).

Micromolar concentrations of NO are normally toxic for bacteria [2]; however, we have shown by fluorescent staining with propidium iodide that the level of viable cells in lactobacillus population with the basal level of NO synthesis and in bacteria with L-arginine induced NO synthesis is higher than 95% (Fig. 1, *b*). Hence, the forming NO is not toxic for lactobacilli.

Hence, NO found in lactobacillus cells stimulates relaxation of rat jejunum segment, this effect similar to SN and is amplified in response to NOS substrate L-arginine, which fact suggests regarding *L. plantarum* 8P-A3 bacteria as an exogenous NO donor in the intestine.

NO, causing smooth muscle relaxation in GIT, is synthesized by endogenous systems: neuronal (nNOS) and endothelial (eNOS) NOS [13]. Functional activity of NO synthesized by NOS in the preparation can be evaluated by an increase in the amplitude of intestinal contractions in response to addition of NOS inhibitor L-NAME (200 μM) and by smooth muscle cell relaxation in response to L-arginine application (100 μM) (Fig. 1, *e*). The reduction of smooth muscle SCA under the effect of lactobacilli can be due to iNOS induction by the bacteria, which was demonstrated in porcine

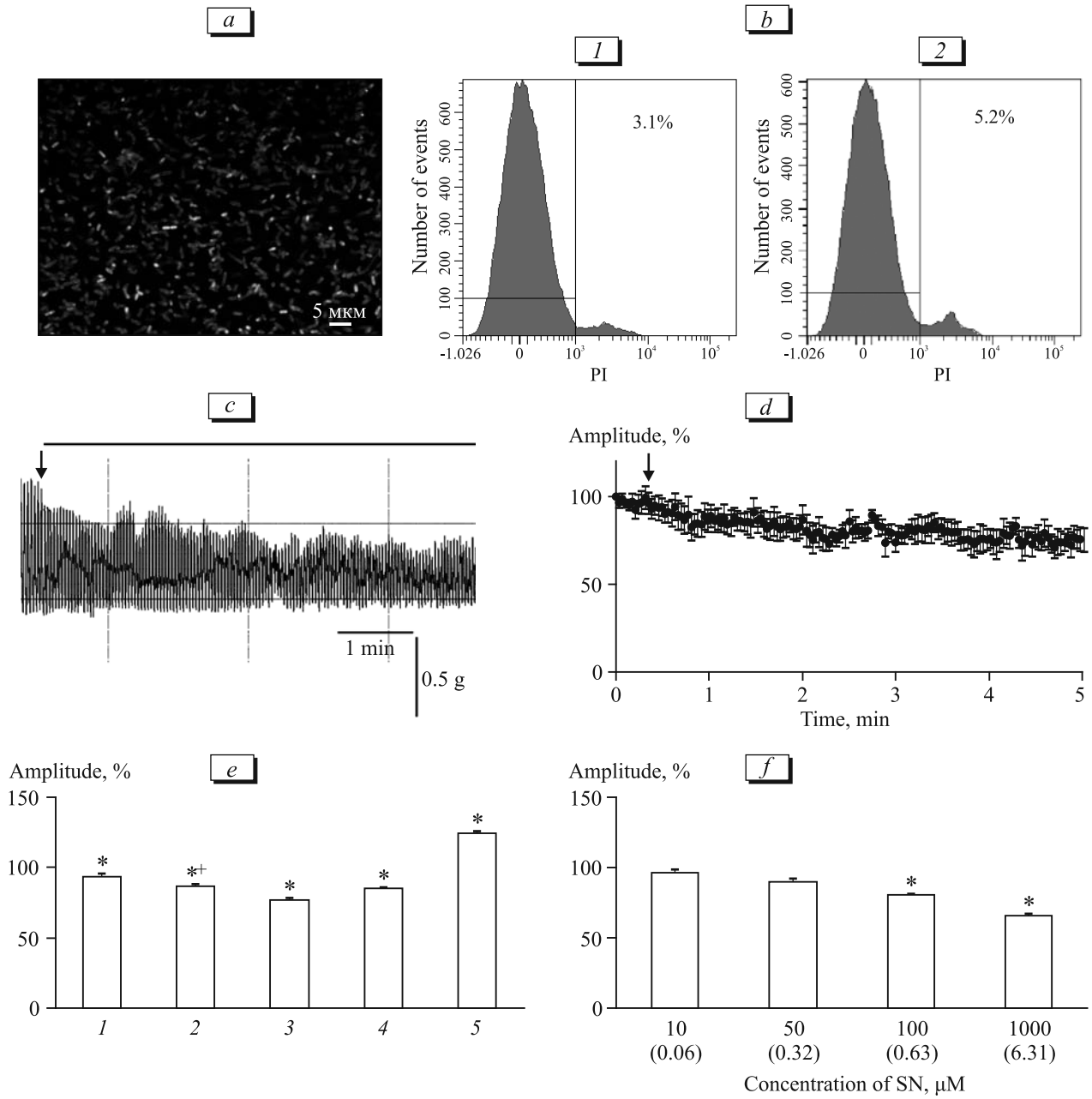

Fig. 1. Effects of NO producing *Lactobacillus plantarum* 8P-A3 on the rat jejunum SCA. *a*) Detection of NO in *L. plantarum* 8P-A3 by DAA staining and fluorescent microscopy; *b*) viability of *L. plantarum* 8P-A3 cells. Bacteria resuspended in isotonic NaCl without (*1*) and with 100 μM L-arginine (*2*); *c*) mechanogram; *d*) time course of contractions of rat jejunum segment in response to application of *L. plantarum* 8P-A3 suspension (10^8 cell/ml) with 100 μM L-arginine (arrow). Initial force of the preparation contractions is taken for 100%; *e*) effects of *L. plantarum* 8P-A3, L-arginine, and L-NAME on rat jejunum SCA: *1*) *L. plantarum* 8P-A3, 10^7/ml; *2*) *L. plantarum* 8P-A3, 10^8/ml; *3*) *L. plantarum* 8P-A3, 10^8/ml+L-arginine, 100 μM; *4*) L-arginine, 100 μM; *5*) L-NAME, 200 μM; *f*) SN effects on rat jejunum SCA. Concentrations of NO (μM) released from SN are shown in parentheses. $p<0.05$ in comparison with the corresponding parameter *before exposure (100%), $^+$in the presence of *L. plantarum* 8P-A3, 10^8/ml+L-arginine, 100 μM.

alveolar macrophage culture [9]. However, iNOS induction takes several hours [10], while the muscle tone changed within just 3-5 min in response to application of bacteria. It seems that the increase of NO level in the intestine under the effect of lactobacilli in our experiment was not due to iNOS activation by the bacteria, but to bacterial synthesis of NO.

The increase of NO concentration in the intestine as a result of lactobacillus induction of iNOS is traditionally regarded as a factor of probiotic activity of the bacteria, stimulating the immune function of GIT [7,11,15]. Our results confirm the positive effects of NO formed due to enteric microflora, but we for the first time study the probiotic bacteria as an exogenous

source of NO, promoting relaxation of the intestinal smooth musculature.

The study was carried out within the framework of Program of Improving Competitiveness of the Kazan (Volga Region) Federal University with support from the Russian Foundation for Basic Research (grants Nos. 12-04-012261 and 14-04-31661_mol_a).

REFERENCES

1. M. U. Shafigullin, R. A. Zefirov, G. I. Sabirullina, *et al.*, *Bull. Exp. Biol. Med.*, **157**, No. 3, 302-306 (2014).
2. D. R. Yarullina, L. V. Vakatova, A. V. Krivoruchko, *et al.*, *Mikrobiologiya*, **82**, No. 4, 417-421 (2013).
3. D. R. Yarullina and O. N. Ilinskaya, *Mikrobiologiya*, **76**, No. 4, 570-572 (2007).
4. D. R. Yarullina, O. N. Ilinskaya, A. V. Aganov, *et al.*, *Mikrobiologiya*, **75**, No. 6, 731-736 (2006).
5. G. Giraffa, N. Chanishvili, and Y. Widyastuti, *Res. Microbiol.*, **161**, No. 6, 480-487 (2010).
6. I. Gusarov, L. Gautier, O. Smolentseva, *et al.*, *Cell*, **152**, No. 4, 818-830 (2013).
7. F. Lamine, J. Fioramonti, L. Bueno, *et al.*, *Scand. J. Gastroenterol.*, **39**, No. 1, 37-45 (2004).
8. H. Morita, H. Yoshikawa, R. Sakata, *et al.*, *J. Bacteriol.*, **179**, No. 24, 7812-7815 (1997).
9. N. Pipenbaher, P. L. Moeller, J. Dolinsek, *et al.*, *Int. Dairy J.*, **19**, No. 3, 166-171 (2009).
10. K. Sade, D. Schwartz, Y. Wolman, *et al.*, *J. Lab. Clin. Med.*, **134**, No. 5, 471-477 (1999).
11. T. Sobko, L. Huang, T. Midtvedt, *et al.*, *Free Radic. Biol. Med.*, **41**, No. 6, 985-991 (2006).
12. T. Sobko, C. L. Reinders, E. Jansson, *et al.*, *Nitric Oxide*, **13**, No. 4, 272-278 (2005).
13. N. Toda and A. G. Herman, *Pharmacol. Rev.*, **57**, No. 3, 315-338 (2005).
14. J. Vermeiren, T. Van de Wiele, W. Verstraete, *et al.*, *J. Biomed. Biotechnol.*, doi:10.1155/2009/284718 (2009).
15. J. Xu and W. Verstraete, *Appl. Microbiol. Biotechnol.*, **56**, Nos. 3-4, 504-507 (2001).