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Original Contribution

Generation of NO by probiotic bacteria in the gastrointestinal tract

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Abstract

Probiotic bacteria elicit a number of beneficial effects in the gut but the mechanisms for these health promoting effects are not entirely understood. Recent in vitro data suggest that lactobacilli can utilise nitrate and nitrite to generate nitric oxide, a gas with immunomodulating and antibacterial properties. Here we further characterised intestinal NO generation by bacteria. In rats, dietary supplementation with lactobacilli and nitrate resulted in a 3–8 fold NO increase in the small intestine and caecum, but not in colon. Caecal NO levels correlated to nitrite concentration in luminal contents. In neonates, colonic NO levels correlated to the nitrite content of breast milk and faeces. Lactobacilli and bifidobacteria isolated from the stools of two neonates, generated NO from nitrite in vitro, whereas *S. aureus* and *E. coli* rapidly consumed NO. We here show that commensal bacteria can be a significant source of NO in the gut in addition to the mucosal NO production. Intestinal NO generation can be stimulated by dietary supplementation with substrate and lactobacilli. The generation of NO by some probiotic bacteria can be counteracted by rapid NO consumption by other strains. Future studies will clarify the biological role of the bacteria-derived intestinal NO in health and disease.

Keywords: Nitrite; Nitrate; Allergy; Colitis; Lactobacilli; Newborn infants

Introduction

It has been suggested that dietary supplementation with live microorganisms, known as probiotics (e.g. lactobacilli and bifidobacteria), may be beneficial for the host. Acting either themselves or by influencing the host flora, probiotics may enhance various defence mechanisms, modulate innate and adaptive immunity, eliminate toxins, carcinogens and pathogens, release antioxidants and stimulate gastrointestinal motility [1]. The molecular mechanisms governing these activities are currently the subject of intense investigation. It seems clear that both agents secreted by metabolically active viable probiotics (bactericions, short-chain fatty acids and peptides) and components from nonviable bacteria (DNA, protein constituents) can mediate protective pathways, but for many of these effects the exact mechanism of action still remains to be elucidated.

Nitric oxide (NO) is a free radical gas involved in numerous physiological and pathophysiological events in the gastrointestinal tract. Endogenous NO generated from L-arginine by NO synthases (NOS) serves to regulate mucosal blood flow, mucus generation, water and electrolyte transport, motility and host defence responses. In addition, NO synthesis is upregulated during inflammatory conditions [2,3]. We and others have been studying an alternative NOS-independent route for NO generation in the gut that involves commensal bacteria [4–7]. Oral commensal bacteria reduce salivary nitrate to nitrite which then spontaneously decomposes to NO and other nitrogen oxides in the acidic stomach [8,9]. In a recent study, we found that isolated strains of lactic acid producing bacteria generated NO in vitro when nitrite was added to the growth medium [10]. Again, NO generation was a result of pH reduction, this time caused by the bacteria themselves via formation of lactic acid. All together, this has led to the speculation that NO generated locally in the intestinal lumen can mediate some of the beneficial effects of probiotic bacteria [7,10,11].

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At birth, intestinal NO concentrations are very low (<10 parts per billion, ppb) in humans [12]. Once the amniotic membranes are ruptured, bacterial colonization of the neonatal intestinal tract begins. In association with exposure to increasing numbers of gut bacteria, intestinal NO in healthy newborn infants increase rapidly to, in some cases, very high (>1000 ppb) levels [12]. The origins and physiological role of this neonatal peak in intestinal NO are unclear.

In the process of revealing the biological significance of NO formed in the GI tract it will be important to understand how the generation is controlled. The aim of the present study was to investigate if commensal bacteria can generate NO in vivo in the gut and to further explore the importance of substrate (nitrate/nitrite) availability. We first studied if dietary supplementation with a live lactobacilli strain and inorganic nitrate would generate NO in rats. In addition, we measured colonic NO during the first days of life in newborn infants; here the aim was to study the relation between the NO levels in the colon and the amounts of nitrate and nitrite in ingested breast milk. Finally, we also investigated in vitro generation and consumption of NO by different strains isolated from newborns.

Material and methods

Animal experiments

Adult male Wistar rats ($n=36$, weight 325±15 g) were divided into four groups: control group, (Control), rats fed with *Lactobacillus rhamnosus* (LGG, ATCC 53103; Valio Ltd., Helsinki, Finland) for 7 days, rats fed with nitrate (NO_3^-), and rats fed LGG together with nitrate (LGG+ NO_3^-). Either sodium nitrate ($NaNO_3$, 0.1 mmol/kg/day) or the same amount of NaCl (Control) was given in distilled drinking water. LGG was anaerobically cultered in MRS medium (de Man, Rogosa and Sharp, Merck, Darmstadt, Germany) at 37°C for 24 hours. Then 1 ml (10^9 CFU/ml) of the culture was given orally to each animal and the remaining contents (10 ml) were spread on the fur and bedding material. The animals were kept at standard conditions ($t=21\pm2$°C, 12 h light/12 h darkness). They received a rodent diet, (TD 99366 chow for rats, Harlan, USA) found to be very low in nitrate.

At the day of the NO measurements, 120 mg/kg of sodium pentobarbital anaesthetic was administered intra-peritoneally, followed by laparotomy. Luminal NO gas measurements were performed as described [13,14]. Briefly, using a 5 ml syringe with a thin needle, NO-free air (<3 ppb) was directly inflated into the caecum, stomach, small intestine and colon. The NO-free air was obtained by sampling room air (NO<10 ppb) via charcoal filter. After incubating the air for 15 sec in the different compartments the intestinal gas was aspirated and immediately injected into a chemiluminescence analyser (Aerocrine AB, Stockholm, Sweden) and the peak NO concentration was measured. The instrument's detection limit for NO was 1 ppb. Calibration of the instrument was performed with cylinder gas (10 ppm NO in nitrogen; AGA AB, Lidingö, Sweden).

Samples of intestinal contents from the small intestine, caecum and colon were tested for nitrate and nitrite concentrations: 0.5 mg wet weight was diluted in 0.5 ml sterile 0.9% NaCl and centrifuged to separate faecal material from a clear supernatant. The supernatant was stored at −20°C and later analysed with chemiluminescence after reductive cleavage and subsequent determination of the NO released into the gas phase as described in detail recently [15]. The detection limit for nitrate/nitrite in this assay is <10 nM. At the end of the experiments the rats where killed with an overdose of sodium pentobarbital given intra-peritoneally.

Studies in newborn infants

A total number of 34 healthy, newborn infants (14 girls/20 boys), delivered either vaginally ($n=24$) or by elective Caesarean section ($n=10$), were included in this study (gestational age 38±2 w, birth weight 3718±510 g, mean±SD values). Exclusion criteria were maternal antibiotic therapy during the last month of pregnancy, preterm birth, asphyxia at birth, signs or symptoms of neonatal disease, malformations or lack of informed parental consent.

The study protocol included a single measurement of intestinal NO at 3-6 days after birth. Colonic gas samples were collected using a tonometric balloon technique described recently [12]. Briefly, an all-silicone catheter (Sherwood Medical, Tullamore, Ireland) equipped with an inflatable balloon tip was inserted 8-10 cm into the sigmoid colon via rectum. The balloon was inflated with 5 ml NO-free air and left to equilibrate in the intestine for 5 min. After the incubation period, the gas was aspirated and immediately injected into the chemiluminescence NO analyser determined and expressed in parts per billion (ppb).

During the investigation faeces was collected after spontaneous defecation and treated with aseptic precautions. To analyse nitrite and nitrate, 0.5 g wet weight was diluted in 0.5 ml sterile 0.9% NaCl and centrifuged (20, 000 rpm for 2 h) to separate faecal material from a clear supernatant. Supernatant was stored at −20°C for later analysis of nitrate and nitrite.

All infants were breastfed and 5 ml of the breast milk was collected and frozen at −20°C for later analysis of nitrate and nitrite concentrations. The sample of breast milk was taken at the day of NO measurements.

In vitro experiments with bacteria

Using fresh faeces from two of the healthy neonates, simple colonies with typical appearance were isolated and identified by conventional bacteriologic methods as *Lactobacilli* sp, *E. coli*, *Bifidobacterium* sp and S*taphylococcus aureus*. For lactobacilli and bifidobacteria no further species identification was performed. Bacteria were inoculated anaerobically at 37°C for 24–48 hours, on different agars supplemented with 0,1 mM $NaNO_2$ as described recently [10]. Briefly, 100 μl (about 10^9 CFU/ml) of the precultured inoculates was put on either lactobacilli agar AOAC (pH=6.8, Difco, USA) for lactobacilli and bifidobacteria. Alternatively, we used ISO-sensitest agar plates, (pH=7.0, Oxoid, Basingstoke, England), to culture *E. coli* and *S. aureus*. After inoculation, the plates were inserted