Gastrointestinal nitric oxide generation in germ-free and conventional rats

Tanja Sobko,1,2 Claudia Reinders,2 Elisabeth Norin,1,3 Tore Midttvedt,3 Lars E. Gustafsson,1,2 and Jon O. Lundberg2
1Centre for Allergy Research, 2Department of Physiology and Pharmacology, 3Microbiology and Tumor Biology Centre, Karolinska Institutet, S-17177 Stockholm, Sweden

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Sobko, Tanja, Claudia Reinders, Elisabeth Norin, Tore Midttvedt, Lars E. Gustafsson, and Jon O. Lundberg. Gastrointestinal nitric oxide generation in germ-free and conventional rats. Am J Physiol Gastrointest Liver Physiol 287: G993–G997, 2004.—Nitric oxide (NO) is a central mediator of various physiological events in the gastrointestinal tract. The influence of the intestinal microflora for NO production in the gut is unknown. Bacteria could contribute to this production either by stimulating the mucosa to produce NO, or they could generate NO themselves. Using germ-free and conventional rats, we measured gaseous directly in the gastrointestinal tract and from the luminal contents using a chemiluminescence technique. Mucosal NO production was studied by using an NO synthase (NOS) inhibitor, and to evaluate microbial contribution to the NO generation, nitrate was given to the animals. In conventional rats, luminal NO differed profoundly along the gastrointestinal tract with the greatest concentrations in the stomach (>4,000 parts per billion (ppb)) and cecum (~200 ppb) and lower concentrations in the small intestine and colon (~20 ppb). Cecal NO correlated with the levels in incubated luminal contents. NOS inhibition lowered NO levels in the colon, without affecting NO in the stomach and in the cecum. Gastric NO increased greatly after a nitrate load, proving it to be a substrate for NO generation. In germ-free rats, NO was low (~30 ppb) throughout the gastrointestinal tract and absent in the incubated luminal contents. NO also remained low after a nitrate load. Our results demonstrate a pivotal role of the intestinal microflora in gastrointestinal NO generation. Distinctly compartmentalized qualitative and quantitative NO levels in conventional and germ-free rats reflect complex host microbial cross talks, possibly making NO a regulator of the intestinal eco system.

intestine microflora; nitrate; nitrite; N⁴-nitro-L-arginine methyl ester; colitis

NITRIC OXIDE (NO) exhibits a variety of biological actions in the gut and is involved in regulation of regional blood flow, gut motility, and secretory and immunological functions (4, 24, 27). In higher concentrations, e.g., when produced in white blood cells, NO and related products are toxic to bacteria and virus and possibly also to host cells (3, 19, 33).

Increased local NO production occurs in patients with different inflammatory conditions, such as inflammatory bowel disease (20). This NO can be measured in gas samples taken from the inflamed gut e.g., during colonoscopy (19) or via a rectal sampling method (14). Whether this greatly increased intestinal NO exacerbates or inhibits the injury or solely is a marker of inflammation is still unclear.

NO is produced in the mucosa from the amino acid L-arginine by different NOSs. Site and onset of NO production from the individual NOS isoforms seem to play different roles in inflammatory processes (2). Bacteria can also contribute to NO production. Bacterial products, e.g., LPS, are powerful inducers of inducible NO synthase (iNOS) in many cell types, including white blood cells and epithelial cells (16, 25). The gene expression of iNOS is mediated by certain transcription factors of which NF-kB is thought to be central (26). In addition, some commensal bacteria, e.g., Escherichia coli, can generate NO by themselves; however, this pathway does not involve NOSs and L-arginine. Instead, these facultative anaerobic microorganisms generate NO by enzymatic reduction of nitrate and nitrite (5, 11). In the stomach, NO is generated in large quantities through acid-dependent nonenzymatic reduction of salivary nitrite (33). Bacteria contribute also to this process by first reducing ingested and salivary nitrite to nitrite, a process that cannot be catalyzed by mammalian cells (7). Taken together, it seems clear that NO has an important role in gastrointestinal physiology and pathophysiology and that several fundamentally different pathways for NO generation in the gut may exist.

The overall involvement of bacteria in the production of NO in the gastrointestinal tract, which might be critical in determining its overall impact, has not yet been studied. Characterizing the cross talk between bacteria and the host during normal noninflammatory conditions will contribute to the understanding of the shift between pathogenic and nonpathogenic settings in the bacteria in gastrointestinal tract. To further study the interplay between the host and bacteria in the intestinal NO production, we have developed techniques for intraluminal gas measurements, using germ-free (GF) and conventional (Conv) rats. The influence of intestinal mucosal NO synthesis was studied by using an NOS inhibitor, whereas bacterial contribution was further characterized by feeding the rats with inorganic nitrate.

MATERIALS AND METHODS

Animals. The study protocol was reviewed and approved by the Local Ethics Committee for Animal Experiments at the Karolinska Institutet. Adult GF male and female Agus rats (10) were used (n = 20, weight 325 ± 15 g) and compared with Conv Agus rats (n = 11, weight 315 ± 10 g). The GF animals were inbred for ~90 generations at the Laboratory of Medical Microbial Ecology and they were kept in lightweight stainless-steel isolators (13). They received autoclaved rodent diet R36 (Lactamin, Södertälje, Sweden). The GF status was checked weekly by culturing fecal samples, both aerobically and anaerobically, by incubating feces at 20 and 37°C for up to 4 wk (1). In addition to Agus rats, in separate experiments, we also used adult Conv male Wistar rats (n = 17, weight 265 ± 3 g), kept in standard conditions (21 ± 2°C, 12:12-h light-dark, humidity 55 ± 10%). All animals were given the above-mentioned diet and water ad libitum.

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although, due to the experimental design, Wistar rats fasted before the nitrate load and the \(l\)-NAME studies for 8–12 h.

The study was performed in three subsets: 1) NO gas was measured in the gastrointestinal tract of GF rats and the levels were compared with those found in Conv rats of the same strain and age, 2) NO gas was measured in the gastrointestinal tract of Conv Wistar rats after treatment with a NOS inhibitor, and 3) NO gas measurements were made after a nitrate load in GF and Conv Wistar rats.

**Luminal NO gas measurements.** A total number of 110 samples of gastrointestinal gas was collected from the Agus GF rats \((n = 17)\) and Conv rats \((n = 11)\), with the use of a 5-ml syringe with a thin needle. Anesthesia was performed by administration of 120 mg/kg ip pento-barbital sodium, followed by laparotomy. Different volumes of NO-free air \( [<3 \text{ parts per billion (ppb)} \] \) was directly inflated into four different luminal compartments of the gastrointestinal tract: the stomach \((4 \text{ ml})\), 5 cm of a midsection of the small intestine \((2.5 \text{ ml})\), the cecum \((5 \text{ ml})\), and 4 cm of the distal colon \((3 \text{ ml})\). External clamps were used to prevent the air from passing into neighboring compartments. The air was incubated for 15 s in the above-mentioned compartments, and thereafter, the intestinal gas was aspirated and immediately injected into a rapid-response chemiluminescence analyzer \((\text{Aerocline, Stockholm, Sweden})\) to determine the peak NO concentration. Samples with a volume <5 ml were diluted to 5 ml before measurements to ensure full recovery of the NO signal. The ambient NO was <10 ppb in all experiments, and by passing this air through a charcoal filter, NO was decreased to <3 ppb. The instrument’s detection limit for NO was 1 ppb. Calibration of the instrument was performed with cylinder gas \((10 \text{ ppm NO in nitrogen; AGA, Lidingö, Sweden})\).

**Effect of sodium nitrate and measurements of NO and nitrate from luminal contents.** To further study bacterial contribution to the intestinal NO production, a nitrate load was given to eight Conv Wistar rats. Either sodium nitrate \((0.1 \text{ mmol/kg Na NO}_3, n = 5)\) or the same amount of NaCl \((\text{control, } n = 3)\) was given in 1 ml of distilled water through a gastric gavage to fasting rats, and intestinal NO was measured 60 min later as described in Luminal NO gas measurements. Sodium nitrate \((0.1 \text{ mmol/kg})\) was also given to three Agus GF rats followed by NO measurements.

To measure the NO release from luminal contents of these rats, 3 g of intestinal contents from the cecum and distal colon were sampled and placed in closed 50-ml containers. After aerobic incubation for 60 min at 37°C, headspace gas was aspirated and immediately injected into the chemiluminescence analyzer to determine NO concentrations.

For nitrate measurements, 0.4 g of the cecal or colon content was diluted to a concentration of 100 mg/ml in distilled water and centrifuged at 37°C for 5 min. The supernatant was removed, and nitrate levels were determined by using a commercially available kit \((\text{Cayman Chemical, Ann Arbor, MI})\).

**Effect of \(l\)-NAME inhibitor.** To further study the contribution of mucosal NOS to luminal NO, we used the NOS inhibitor, \(N^\text{G}-\text{nitro-l-arginine methyl ester (\(l\)-NAME)}. Either \(l\)-NAME \((100 \text{ mg/kg, } n = 5)\) or water \((\text{control, } n = 4)\) was given to the fasting Conv Wistar rats via gastric gavage. Intraluminal NO gas was measured 2 h later as described in Luminal NO gas measurements.

**Statistics.** Statistical analysis and graph plotting were performed with Prism 4.0 \((\text{GraphPad Software, San Diego, CA})\). NO values are given as means ± SE. When the number of rats in the group was <10, NO values were given as median and range. Statistical difference between groups was calculated by Mann-Whitney U-test. A \(P\) value ≤0.05 was considered significant.

**RESULTS**

Gastrointestinal NO in Conv and GF animals. Luminal intestinal NO levels differed profoundly along the gastrointestinal tract of Agus Conv rats, with by far the greatest concentrations in the stomach \((4,236 \pm 698 \text{ ppb})\) and lower levels in the small intestine \((17 \pm 3 \text{ ppb})\), cecum \((205 \pm 49 \text{ ppb})\), and colon \((20 \pm 2 \text{ ppb})\).

In contrast, in GF rats, luminal NO was very low throughout the gastrointestinal tract. Thus in the stomach, the concentration of NO was 31 ± 6 ppb, in small intestine was 10 ± 2 ppb, in cecum was 9 ± 1 ppb, and in colon was 13 ± 3 ppb \((\text{Fig. 1})\). NO levels were significantly higher in all parts of the gastrointestinal tract in Conv rats compared with GF rats.

**Effect of sodium nitrate and measurements of NO and nitrate from luminal contents.** In these experiments, control rats \((\text{Conv Wistar rats})\) treated with NaCl had NO levels of ≤55 ppb in the stomach and ≤51 ppb in the colon. Rats treated with nitrate had higher levels in the stomach \((1.560 \text{ (range, 1.112–4.005) ppb})\), but the levels did not differ in the colon \((15–48) \text{ ppb}) \((\text{Fig. 2})\). In nitrate-treated GF animals NO levels were ≤9 ppb in the stomach, and ≤3 ppb in the colon.

After incubating the luminal contents from the rats treated with NaCl \((n = 3)\), we found NO levels of 200–400 ppb from cecal contents and 50–100 ppb from colon contents. In the rats treated with NaNO\(_3\), levels of NO in feces were 179 \((\text{range, 46–282}) \text{ ppb in the cecum and 41} \text{ (range, 31–143) ppb in the colon}) \((\text{Fig. 3})\). In GF animals, we found levels close to the detection limit, measured in cecum \(<3 \text{ ppb})\) and the colon \(<1 \text{ ppb})\). NO levels released from the contents of cecum correlated with the intraluminal NO levels found in vivo \((r = 0.72)\).

Cecal levels of nitrate \((25 \pm 3 \mu M)\) were significantly higher compared with the levels found in the colon contents \((15 \pm 1 \mu M)\) \((P < 0.05)\).

**\(l\)-NAME in Conv rats.** In rats treated with \(l\)-NAME, the NO levels in the stomach were 42 \((\text{range, 12–68}) \text{ ppb}) compared with the levels of 24 \((\text{range, 12–41}) \text{ ppb}) \text{ in control rats treated with NaCl. NO levels in the small intestine after \(l\)-NAME treatment were 11 \((\text{range, 7–24}) \text{ ppb})\text{ compared with 14 \text{ (range, 7–44}) ppb in the control rats}; in the cecum 95 \((51–103) \text{ ppb}) \text{ vs. control 200 \text{ (range, 46–416)} \text{ ppb and in the colon 7 \text{ (range, 6–9}) ppb vs. control 17 \text{ (range, 15–19}) ppb.) The groups did not differ in their NO concentrations in any parts of the gastrointestinal tract except in colon where they were significantly lower in \(l\)-NAME treated animals \((P < 0.02)\) \((\text{Fig. 4})\).

**DISCUSSION**

The major finding of this study is that bacteria play a pivotal role in normal NO production in the gastrointestinal tract. The most compelling evidence for this is that NO levels are much lower in GF animals along the entire gastrointestinal tract compared with the Conv controls. In addition, we also showed that luminal NO levels differ profoundly in different compartments of the gastrointestinal tract in Conv rats.

In principle, there are two ways in which bacteria could contribute to the intestinal NO production, although it is difficult to pinpoint the strains responsible for this contribution and the exact pathway by which this occurs. The first pathway might be that bacteria stimulate cells in the mucosa to produce NO from NOS, and the second alternative is that bacteria produce NO themselves (35). Judging from the results of this study, both mechanisms are likely, but they seem to operate at different locations. The high NO levels found in the stomach and in the cecum of Conv rats were not affected by \(l\)-NAME, which suggests predominantly NOS independent pathways for...
NO generation at these sites. Also, when incubated in closed containers, NO was released directly from luminal cecal contents of Conv rats but not from GF rats, and this NO release correlated with the levels found in the cecum in vivo.

In the colon, however, NO levels were lower in L-NAME treated animals, which indicates a larger contribution from an NOS in the mucosa in that particular compartment of the GI tract. In addition, NO levels differed only slightly between GF and Conv rats in the small intestine and in the colon, thereby further supporting a mucosal origin of NO at these locations.

Basal NO levels were considerably lower in the small intestine and the colon compared with the stomach and the cecum. Difference in bacterial contribution to NO levels in various parts of the intestinal tract may depend on subtle differences among the number and strains of bacteria in different compartments.

Fig. 1. Intraluminal nitric oxide (NO) in different parts of gastrointestinal tract of germ-free (GF) and conventional (Conv) Agus rats. Stomach and cecum (***P < 0.001), small intestine (*P < 0.01), and colon (*P < 0.01) vs. corresponding values in GF animals. ppb, Parts per billion.

Fig. 2. Intraluminal NO in the stomach and colon of Conv Wistar rats after administration of nitrate (0.1 mmol/kg) n = 5 rats, or saline (Control), n = 3 rats.

Fig. 3. NO released from the contents of cecum and colon of GF Agus and Conv Wistar rats incubated in closed containers for 60 min at 37°C.
different compartments of the GI tract, the amount of NO2 and NO3 available, local redox conditions, pH, and the status of the intestine (e.g., diarrhea, constipation, etc.). The reason for the much higher NO levels found in the gastric lumen compared with the cecum is probably due to substrate availability as well as the mechanism of NO generation. In the stomach, much substrate (nitrite) is delivered continuously via saliva rich in nitrite, and NO generation occurs through rapid pH-dependent nonenzymatic reduction of nitrite in gastric juice (3, 4). Interestingly, we have observed a difference in the basal gastric NO levels in Conv rats during the nitrate load and L-NAME gavage experiments. This might be explained by the fact that these rats had fasted 8–12 h before these experiments, which led to a decreased salivary production and increased gastric pH, conditions that greatly reduce stomach NO generation (22). The pH in the fasting rats is known to be high, pH 7, due to the anatomic absence of the gallbladder and the retrograde influx of bile into the rat stomach, which we also confirmed by measuring pH levels in some of our experiments (data not shown).

The finding of very high gastric NO levels in nonfasting rats are in accordance with earlier studies in humans (22). The fact that gastric NO is uniformly low in GF rats firmly establishes that bacteria are essential in this production. Gastric pH, being an important factor for NO generation, is also described to be significantly higher in the GF rats (32). The posterior part of the rat tongue is heavily colonized by facultative anaerobic bacteria and is the source of nitrite formation (8). Several facts imply that salivary-derived NO is important for the integrity of the gastric mucosa and that locally produced NO might have protective effects on the host (4, 6, 18, 21, 31), creating a first-line regulation of the eco system in the upper gastrointestinal tract.

In the cecum, nitrate/nitrite is probably reduced to NO entirely via bacterial enzymes (nitrate and nitrite reductases), and substrate availability is likely due to the direct nitrate secretion from the blood. Unlike humans, rats can secrete nitrate directly into the lower intestinal tract (34). Substrate availability may also explain why cecal NO was higher than colon NO levels. Indeed, the nitrate measured in the luminal contents was higher in cecum than in colon.

It is likely that also in the cecum, locally produced NO could have biological effects, e.g., in regulation of superficial blood flow or mucosal secretions, because a role of NO in mucosal secretion has been found in other tissues (28). Gaseous NO, when inhaled, has been shown to dilate the pulmonary vasculature already at concentrations <100 ppb. In this study, we found mean NO levels in the cecum of ~200 ppb after having diluted luminal gases with 5 ml of air. The actual levels in the cecum are most likely considerably higher.

NO may also be involved in regulation of immunological functions in the gut (9). Interestingly, a recent study (17) indicates that NO produced by intestinal bacteria can modulate the inflammatory response in the colon during experimental colitis. Luminal NO, released from Lactobacillus farciminis given orally, attenuated inflammation in a colitis model and this effect was mimicked by intracolonic administration of an NO donor. Taken together, this implies that NO produced by luminal bacteria can affect the intestinal mucosa both in health and disease.

After it has become possible to measure NO in the gaseous form, increasing interest has developed in studying NO, both as a mediator (30) and inhibitor of inflammation (12), as well as an inflammatory marker (20). There are still questions about how much of the measured NO in the gastrointestinal tract comes from the mucosa and how much is produced or consumed by bacteria (29). If NO gas can be transported from one GI compartment to another, it could affect the NO measurements performed locally. Our results show that this is not probably the case. Thus NO levels measured in the stomach

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**Fig. 4.** Effect of Nω-nitro-L-arginine methyl ester (NO inhibitor; n = 5) on NO production in the stomach and colon (*P < 0.01) of Conv Wistar rats vs. corresponding values in controls (n = 4). ns, Not significant.

**Fig. 5.** NO levels measured at different parts of the gastrointestinal tract of Conv nonfasting rat.
differed greatly from the neighboring small intestine (Fig. 5). Furthermore, the effect of mucosal NOS inhibition in the colon also showed that much of measured luminal NO in this compartment was possibly of local mucosal origin.

Although a similar measuring method has been used before in humans and large animal models (15, 23), to our knowledge, this is the first time luminal NO gas has been measured directly in the gastrointestinal tract of the rat. This could be a valuable tool to directly measure intestinal NO gas e.g., in animal models of intestinal inflammation.

In conclusion, luminal concentrations of NO vary considerably in different compartments of the gastrointestinal tract and are critically dependent on the presence of bacteria. Regardless of its origin, luminal NO may have both protective and regulatory functions, and NO in gaseous form may be used as a marker for a local status of the intestine.

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GRANTS

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