

Short communication

Birth-related increase in intracolonic hydrogen gas and nitric oxide as indicator of host–microbial interactions

Background: Bacterial colonization of the intestine early in life might have implications for allergy development. We studied early host–bacterial interactions in the gut by simultaneous measurements of hydrogen gas (H₂) and faecal short chain fatty acid pattern (SCFAs), i.e. bacterial products, as well as of nitric oxide (NO), a marker of mucosal immune activation.

Methods: A novel minimally invasive technique was used for repeated measurements of luminal colonic H₂ and NO in 32 healthy newborn infants delivered vaginally or by Caesarean section. Luminal gas was sampled and analysed at five occasions: immediately after birth, day 1, days 3–5, 1 and 5–6 months after birth.

Results: Colonic H₂, NO and faecal SCFAs were undetectable at birth. The H₂ and SCFAs appeared within 24 h and continued to increase during the 6 months follow-up. Nitric oxide remained very low until 3–5 days after birth at which time it markedly increased. In some apparently healthy infants NO transiently reached levels similar to those seen in adults with inflammatory bowel disease.

Conclusion: Intracolonic measurements of H₂ and NO may be useful to monitor the developmental colonization process as well as mucosal responses.

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Background

Early interactions between intestinal bacteria and the host might modulate the adaptive immunity and play an important role for later development of allergy (1). Colonization of the human intestine begins at birth and is determined by a number of environmental variables, e.g. microflora of the birth canal and mode of delivery (2, 3).

One way to study host–bacterial interaction in the gut would be to simultaneously measure bacterial metabolites as well as mucosal products in the gastrointestinal tract. Hydrogen gas (H₂) and short chain fatty acids (SCFAs) are generated solely by intraluminal bacteria (4–6).

A novel approach to study inflammatory reactions in the gut is to measure luminal levels of nitric oxide (NO). The NO production is induced in various cell types during inflammatory reactions (7). In adults, intestinal formation of NO is normally low, but during inflammation, the levels of this gas are greatly increased (8, 9). We envisaged that simultaneous determinations of intraluminal H₂, NO and faecal SCFA in newborn infants could offer a possibility to monitor both the emerging bacterial colonization (H₂ and SCFA) and innate host defence responses (NO) in the gastrointestinal tract. We developed a minimally invasive sampling technique for measurements

of H₂ and NO in neonates. Intestinal gases and faecal SCFA were repeatedly collected, from birth up till 6 months of age.

Material and methods

Subjects

We studied 32 (16 boys + 16 girls) newborn infants (gestational age 38 ± 1, 6 weeks), birth weight 3456 ± 388 g (mean ± SD) delivered either vaginally (VD; *n* = 16) or by elective Caesarean section (CS; *n* = 16) before labour and rupture of foetal membranes. Exclusion criteria were asphyxia at birth, malformations or lack of informed parental consent. All infants were breast-fed on demand from the first hours of life and subsequent feeding practices were monitored throughout the study period (Table 1). Three infants developed transient hyperbilirubinaemia.

The study protocol, reviewed and approved by the local ethics committee, included measurements of intestinal gas at five consecutive postnatal ages: (i) 0–2 h, (ii) 24 h, (iii) 3–5 days, (iv) 30 days and (v) 5–6 months after birth. Two infants missed one measurement each.

An allergy questionnaire was used to interview both parents about a possible history of atopic or allergic diseases in one or several first-degree family members, the infant's living environment and allergic symptoms. During the follow-up period, all infants exhibited normal growth and development, no infant was hospitalized or showed signs of food intolerance. Subject characteristics are presented in Table 1.

Table 1. Infant characteristics in relation to mode of delivery

	Caesarean section (n = 16)	Vaginal delivery (n = 16)
Family history of atopy	9 (56)	7 (44)
Parental smoking	3 (19)	0 (0)
Pets at home	5 (31)	7 (44)
Feeding (first month)		
Exclusive breast feeding	4 (25)	12 (75)
Formula	1 (6)	0 (0)
Mixed	11 (69)	4 (25)
Dermatitis	3 (19)	2 (12)
Infections (first 6 months)		
URTI	8 (50)	8 (50)
LRTI with wheezing	4 (25)	1 (6)
Gastroenteritis	0 (0)	0 (0)
Antibiotic therapy	4 (25)	4 (25)

URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection.
Values expressed in parentheses are percentage.

Colonic gas measurements

Colonic gas samples were collected using a tonometric balloon technique (10). An all-silicone catheter (Sherwood Medical, Tullamore, Ireland) equipped with an inflatable balloon tip was inserted 10 cm into the sigmoid colon via rectum. The balloon was inflated with 5 ml NO-free air and left in the intestine for 5 min. After the incubation period, the gas was aspirated and immediately injected into a chemiluminescence NO analyser (Aerocrine AB, Stockholm, Sweden). While leaving the catheter in place, the same sampling procedure was repeated to measure H₂ gas, which was analysed by an electrochemical sensor device (Bedfont Ltd, Rochester, UK).

Short chain fatty acids

Faecal samples were collected when available in conjunction with the gas measurements and frozen at -20°C for further analysis of SCFAs. A gas liquid chromatography method (11) was used for quantification of total and specific SCFA content. The concentration of different acids was expressed as mmol/kg faeces.

Statistics

Statistical analysis and graph plotting were performed with GRAPHPAD (GraphPad Software, San Diego, CA, USA). Because of skewed distributions, H₂ and NO data are presented as median

(range), whereas SCFA-values are given as mean (SD). To analyse any possible influence of delivery mode and postnatal ages, the H₂ and NO concentrations were normalized by log-transformation and compared using Newman-Keuls multiple comparison test and repeated measures ANOVA with post-test for linear trend.

Results

The measuring procedure was well-tolerated in all infants and there were no complications. At birth, colonic H₂ and NO levels were below detection level in all newborn infants. With time a significant increase was observed (Table 2). The SCFAs showed a similar age-related pattern.

Hydrogen gas

Hydrogen gas levels were below 1 ppm at birth, irrespective of mode of delivery. There was a significantly earlier and higher H₂ increase in VD infants when compared with the CS group; already at 24 h of postnatal age, VD infants showed a colonic H₂ concentration of 47 (7–417) ppm, while only two of 16 infants in the CS group had values above the detection level (*P* < 0.05). At 3–5 days of age, H₂ had appeared in 26 of 32 infants and it had increased to an average of 220 (1–1500) in the CS group and to 429 (1–7000) ppm in the VD group, the difference was no longer statistically significant. At 30 and 180 days the H₂ levels were similar in both groups and amounted to 821 (8–15 000) and 612 (1–6000) ppm respectively (Fig. 1A, Table 2).

Between 24 h and 3–5 days intestinal H₂ increased in all except one infant. This infant had received intravenous broad-spectrum antibiotic therapy for transient tachypnea at the age of 2 days. Prior to antibiotic therapy at 24 h of postnatal age the H₂ level was 308 ppm. During therapy it dropped to 12 ppm.

Nitric oxide

Intraluminal NO levels were below or close to the detection limit at birth, and remained low at 24 h of

Table 2. Levels of colonic nitric oxide (NO), hydrogen gas (H₂) and faecal short chain fatty acids (SCFA) in infants delivered vaginally or by Caesarean section

	Postnatal age				
	0–2 h	24 h	3–5 days	1 month	5–6 months
NO (ppb)					
Vaginal delivery	8.5 (7.5–17)	11 (6.5–79)	75 (0–7113)	61 (18–721)	110 (14–1590)
Caesarean section	11 (5–31)	12 (5–21)	19 (0–3098)	63 (18–1458)	350 (15–1900)
H ₂ (ppm)					
Vaginal delivery	<1	47 (1–417)*	429 (1–7000)	373 (8–7000)	523 (19–6000)
Caesarean section	<1	<1	220 (1–1500)	1778 (46–15000)	813 (1–5846)
SCFA (mmol/kg)					
Vaginal delivery	5.4 ± 0.6	12 ± 2	44 ± 11	65 ± 10	136 ± 20
Caesarean section	4.1 ± 0.1	8.4 ± 1	21 ± 4	49 ± 10	144 ± 20

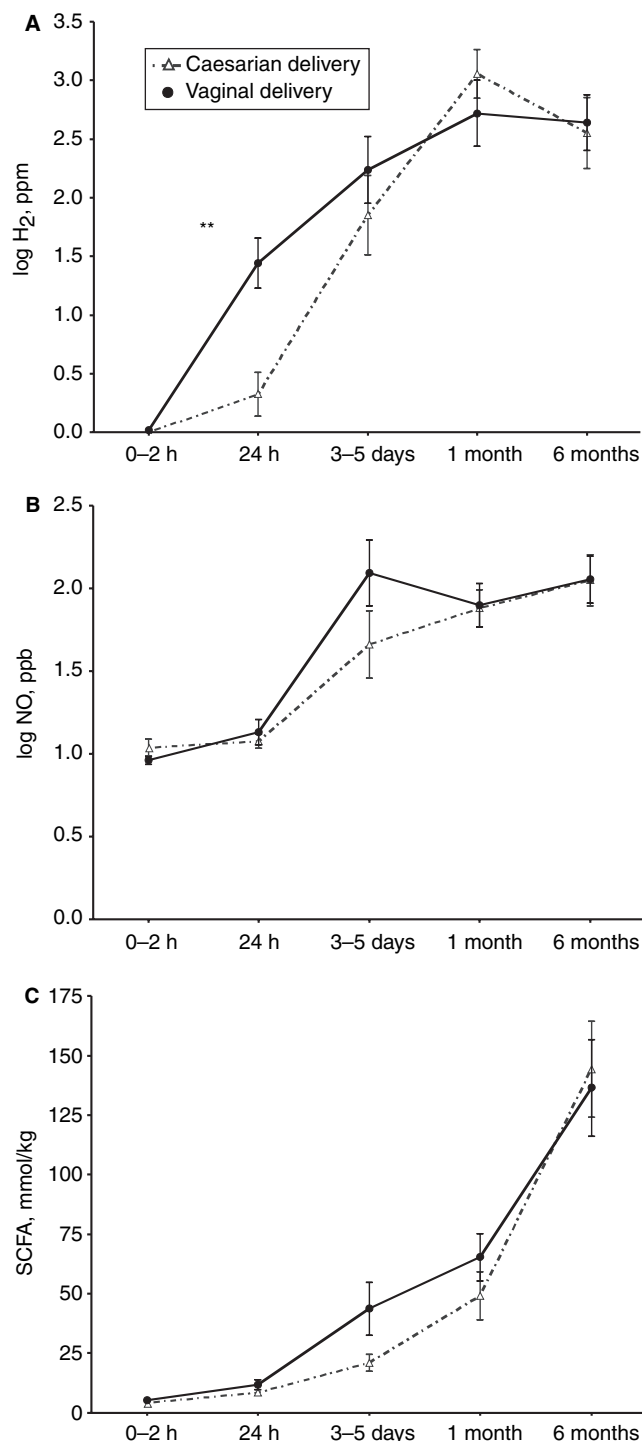


Figure 1. (A) The log-concentration of hydrogen gas (H₂) in distal colon of infants during the first 6 months after birth ($n = 16$ for each group, symbols denote mean $\pm$ SEM, ** indicates $P < 0.01$ caesarian vs. vaginal delivery). (B) The log-concentration of nitric oxide gas (NO) in distal colon of infants during the first 6 months after birth ($n = 16$ for each group, symbols denote mean $\pm$ SEM). (C) Total content of short chain fatty acids (SCFAs) in faeces of infants during the first 6 months after birth ($n = 16$ for each group, symbols denote mean $\pm$ SEM, * $P < 0.05$ for differences between groups).

postnatal age (Fig. 1B). At 3–5 days of age, NO levels had increased to 52 (0–7113) ppb. At 30 and 180 days colonic NO levels were 62 (18–1458) and 110 (14–1900) ppb respectively. The kinetics and magnitude of the increase in NO over time were not significantly influenced by the mode of delivery. However, the median NO level at 3–5 days after birth was three to four times higher in VD infants when compared with the CS group ($P = 0.14$).

In seven infants, all apparently healthy, NO occasionally exceeded 1000 ppb, i.e. levels similar to those seen in older subjects with bowel inflammation (12). In five of these infants in whom subsequent measurements were scheduled, the NO peaks were proven to be transient. Six of these infants had been fed with formula based on cow's milk.

Faecal short chain fatty acids

The progression of SCFAs for both delivery mode groups is shown in Fig. 1C. The total amount of faecal SCFAs increased gradually from zero at birth to a mean of 138 (20) mmol/kg at 6 months of age ($P < 0.05$). There was no statistical difference in SCFAs between infants delivered VD or by CS.

The main SCFA at birth was acetic acid. Although all SCFAs increased with postnatal age, the relative contribution of acetic acid to the total SCFAs decreased from 99 to 82% at 6 months of age. Levels of butyric, caproic and propionic acids increased respectively to 3, 1 and 15% during the first 6 months of life.

Taking age-related variations into account there were no correlations between H₂, NO and SCFAs.

Discussion

We show here that the intestinal gases H₂ and NO can be measured directly in the colon of newborn infants. The procedure was safe, rapid and well-tolerated, which allowed repeated measurements. Using the gas sampling method several interesting observations were made. First, there was an early rise in intestinal H₂ and it emerged faster after VD delivery than after CS. Secondly, within days after birth significant increments in intestinal NO were observed. Thirdly, in some apparently healthy infants, NO levels were comparable with those found in gut inflammation. Taken together, these findings suggest a vivid activation of intestinal immune functions when the gut becomes colonized with bacteria for the very first time. Besides mode of delivery, our observations further indicate that feeding practices as well as antibiotic treatment may have considerable influence on the concentration of the measured parameters.

Hydrogen gas

This is the first report of H₂ gas measurements directly in the human gastrointestinal tract. The sampling was

performed directly *in vivo* in the natural anaerobic environment and no further handlings were required such as those involved with faecal sampling. This method permits multiple measurements at predefined time-points independent of bowel emptying.

The absence of intestinal H₂ at birth was expected since the foetus lives in a sterile environment. Immediately after birth H₂ levels increased dramatically, reflecting the postnatal establishment of a microflora in the gastrointestinal tract. The CS infants showed a 1–2 days delay in emergence of H₂ compared with the VD group. This corresponds with other studies reporting later bacterial colonization in infants delivered by CS (13, 14). In our study, the difference related to delivery mode was no longer apparent after the first week of life. After a peak concentration at 30 days after birth, H₂ levels reached a steady-state or even declined somewhat at 6 months of age. This development is in agreement with studies in which H₂ was measured in faeces (15).

With one exception all infants increased in H₂ between 24 h and 3–5 days. This infant received intravenous broad-spectrum antibiotic therapy for suspected but later unproven systemic infection at the age of 2 days. Interestingly the H₂ level dropped almost below the detection level, probably as a direct consequence of an immense reduction of intestinal bacteria (16).

Short chain fatty acids

Apart from H₂ we also measured SCFAs as a product of bacterial metabolism. The total amount of SCFAs and the proportion of different fatty acids in the faeces changed considerably after birth. The SCFAs might interplay with the mucosa in different ways. Butyrate, for example, may be a major energy source for the colonic epithelial cells and also regulate cell differentiation (15). Such roles may change in relation to postnatal age. In accordance, we found that total levels of SCFAs and especially those of butyrate were very low at birth and increased over time.

Nitric oxide

Although the source of luminal NO in the gastrointestinal tract of newborn infants is not known, the kinetics and time course of intestinal NO suggest an association to bacterial colonization. Thus, NO was very low in the sterile intestine at the time of birth and increased in parallel with H₂ and SCFA, two specific bacterial products. Bacteria can contribute to NO generation in several ways. They could trigger NO production from NOS in the intestinal mucosa. Indeed, several bacterial products can induce expression of inducible NOS (iNOS) and this isoform has been found in colon epithelial cells (17). Apart from bacteria, components of breast-milk

could also contribute to intestinal NO generation. Breast-milk contains a multitude of compounds that may promote the development of neonatal immune competence, e.g. the soluble protein CD14, and these could trigger generation of NO from the NOS in the mucosa (18). In addition, some bacteria can produce NO from nitrite/nitrate. In this pathway, nitrite can be reduced to NO either nonenzymatically in an acidic/reducing environment or via bacterial nitrate/nitrite reductases (19).

Interestingly, in four apparently healthy neonates rectal NO concentrations were very high on days 3–5, in fact, similar to what is seen in patients with active ulcerative colitis (8, 20). Provided that this NO is derived from the intestinal mucosa, it indicates a strong activation of the immune system. Regardless of the source of this NO it is not unlikely that it can have effects on mucosal cells as well as on luminal bacteria. Thus, NO affects cell functions in numerous ways including regulation of immune responses, intestinal water and electrolyte transport (21) and motility (22). The NO also plays a role in regulation of the Th1/Th2 balance, indicating a potential for NO to contribute to the development of atopy and other allergic diseases, including bronchial asthma (23).

Earlier studies suggest that measurements of intestinal NO could be used to detect inflammatory reactions in the gut (7, 8) including those associated with adverse reactions to food. In our study, six of the seven infants with NO levels exceeding 1000 ppb had been fed formula based on cow's milk. Whether this NO increase is caused by the formula diet can be presently only speculated upon.

A central question in general NO biology is whether the increased production seen during inflammatory reactions is beneficial or harmful, but despite the extensive research this issue is still unsettled. Likewise, we cannot tell if the high transient NO peaks observed in this study are of any pathological significance. Larger groups and longer study periods are needed to investigate the relation between high intestinal NO and allergy development later in life.

In conclusion, we have developed a minimally invasive, rapid and pain-free method to directly measure luminal intestinal gases in newborn infants. This method may be a useful tool to explore the relationship between the initial bacterial colonization process, early intestinal immune activation and later development of allergy.

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