

Sulfide- and nitrite-dependent nitric oxide production in the intestinal tract

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Summary

In the gut ecosystem, nitric oxide (NO) has been described to have damaging effects on the energy metabolism of colonocytes. Described mechanisms of NO production are microbial reduction of nitrate via nitrite to NO and conversion of L-arginine by NO synthase. The aim of this study was to investigate whether dietary compounds can stimulate the production of NO by representative cultures of the human intestinal microbiota and whether this correlates to other processes in the intestinal tract. We have found that the addition of a reduced sulfur compound, i.e. cysteine, contributed to NO formation. This increase was ascribed to higher sulfide concentrations generated from cysteine that in turn promoted the chemical conversion of nitrite to NO. The NO release from nitrite was of the order of 4% at most. Overall, it was shown that two independent biological processes contribute to the chemical formation of NO in the intestinal tract: (i) the production of sulfide by fermentation of sulfur containing amino acids or reduction of sulfate by sulfate reducing bacteria, and (ii) the reduction of nitrate to nitrite. Our results indicate that dietary thiol compounds in combination with nitrate may contribute to colonocytes damaging processes by promoting NO formation.

Introduction

Nitric oxide (NO) is one of the smallest and simplest biologically active molecules in the human body. As one of

the most widespread signalling molecules in mammals, NO is a major player in controlling nearly every cellular and organ function in the body (Ignarro, 2000). Particularly in the colon, NO plays an important role in the first defence of the host by exerting antimicrobial effects on the invading pathogens. NO is produced by the conversion of L-arginine by an inducible isoform of NO synthase (iNOS) which is expressed by inflammatory cells, such as activated macrophages. iNOS has been described to be upregulated in the colon of patients with inflammatory bowel diseases (IBD) (Boughton-Smith *et al.*, 1993; Middleton *et al.*, 1993; Rachmilewitz *et al.*, 1995). Moreover, dysbiosis in the NO metabolism has been linked to ulcerative colitis (UC), one of the main forms of IBD (Roediger, 2008). Co-action of NO and sulfide has even been suggested to be involved in the initiation of UC, although this has not been confirmed (Roediger and Babidge, 2000).

Besides the production of NO by epithelial cells, microbial and chemical processes have been described with NO as intermediate or end-product. First, some Gram-positive bacteria, i.e. *Staphylococcus aureus*, *Bacillus anthracis* and *Bacillus subtilis*, producing NO from arginine by a bacterial NOS, have been shown to produce NO as a cytoprotective against oxidative stress and antibiotics (Gusarov and Nudler, 2005; Shatalin *et al.*, 2008; Gusarov *et al.*, 2009). Second, NO is produced as an intermediate in the denitrification, a four-step reduction process of nitrate over nitrite, NO and N₂O to N₂ gas. A broad range of denitrifying bacteria, mainly Gram-negative, have been described and the genes and enzymes involved in the process have been thoroughly investigated (Zumft, 1997). Denitrification has been suggested to have a minor role in the gastrointestinal tract (Allison and Macfarlane, 1988). Thirdly, conditions in the intestinal tract have been described to stimulate dissimilatory nitrate reduction to ammonium (DNRA), the reduction of nitrate/nitrite to ammonium. The functional gene of DNRA, *nrfA*, has been identified in *Bacteroides* species (Mohan *et al.*, 2004) and gamma-, delta- and epsilon-subclasses of the *Proteobacteria* (Smith *et al.*, 2007) and even sulfate reducing bacteria (SRB) (Mitchell *et al.*, 1986; Seitz and Cypionka, 1986; Dannenberg *et al.*, 1992; Pereira *et al.*, 1996). *Escherichia coli*, capable of DNRA but not denitrification has been shown to generate substantial amounts of NO (Ji and Hollocher, 1989).

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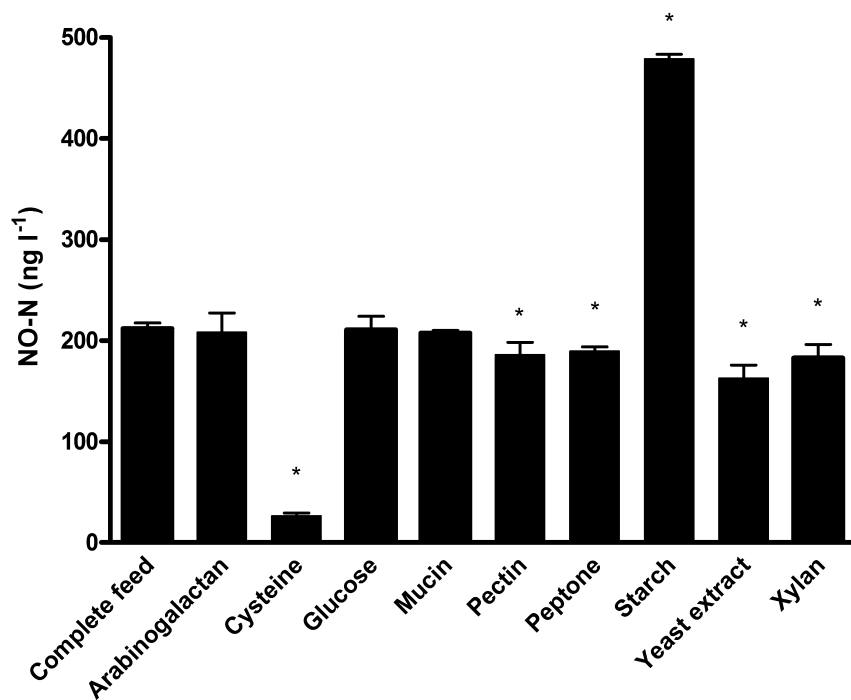


Fig. 1. NO-N (ng l⁻¹) production by the human intestinal microbiota incubated in feed medium for 24 h of which all compounds were excluded separately, and the complete feed medium. The mean \pm SD is shown. The asterisk indicates significant differences compared to the control feed medium ($P < 0.05$).

Moreover, DNRA has been shown to be the most prevalent nitrate reduction process in the colon and NO was suggested as intermediate or side-product during this process (Vermeiren *et al.*, 2009). Finally, non-enzymatic NO production from nitrite at a pH below 5.5 has been described and is considered to occur in the stomach, on the surface of the skin, in the ischaemic heart, and in infected nitrite-containing urine (Weitzberg and Lundberg, 1998).

Nitrate has previously been shown to be positively correlated to concentrations of NO produced by gut microbial species (Sobko *et al.*, 2005; Vermeiren *et al.*, 2009). In this paper, *in vitro* experiments inoculated with intestinal microbial communities were performed in order to identify the influence of dietary compounds on the formation of NO. Finally, it was investigated which microbial or chemical processes occurring in the gut environment, can be correlated to the formation of NO.

Results and discussion

Nitric oxide production by the human intestinal microbial community

NO concentrations produced by a representative human intestinal microbial community in feed medium were measured after 24 h of batch incubation. The community produced 211.9 ± 5.6 ng NO-N l⁻¹ when incubated anaerobically in the feed medium (Fig. 1). The initial concentration of nitrate present in the feed medium was 0.12 mg NO₃-N l⁻¹. In healthy persons, it is calculated that levels in the order of a few mg NO₃-N l⁻¹ reach the large

intestine (Xu, 2001) and nitrate concentrations in faeces were shown to be around 0.15 mg NO₃-N l⁻¹ (Reinders *et al.*, 2007). Therefore, the nitrate concentrations of the feed medium are representative for the intestinal environment.

To further investigate which compounds, besides the trace amounts of nitrate, were necessary to induce NO production, all compounds were excluded one by one from the feed medium and the NO production was measured after 24 h of incubation. Most remarkably, when cysteine or starch were excluded, NO concentrations reached significantly lower levels, i.e. 26.7 ± 2.7 ng NO-N l⁻¹ (factor 8 decrease) or significantly higher levels, i.e. 479.1 ± 4.4 ng NO-N l⁻¹ (factor 2 increase) respectively (Fig. 1). Still significant decreases were obtained when pectin (factor 1.1), peptone (factor 1.1), yeast extract (factor 1.3) or xylan (factor 1.2) were excluded from the feed medium (Fig. 1). The concentrations of NO produced by the abiotic controls of the different media were below 0.5 ng NO-N l⁻¹. To our knowledge, no literature is available on the direct influence of cysteine, starch or any of these compounds on microbial NO production. As the results were most remarkable for cysteine, this compound was chosen for further investigation elucidating the NO formation mechanism.

Positive correlation between cysteine and NO production

Cysteine was added to the feed medium at two different concentrations to study the concentration-dependent

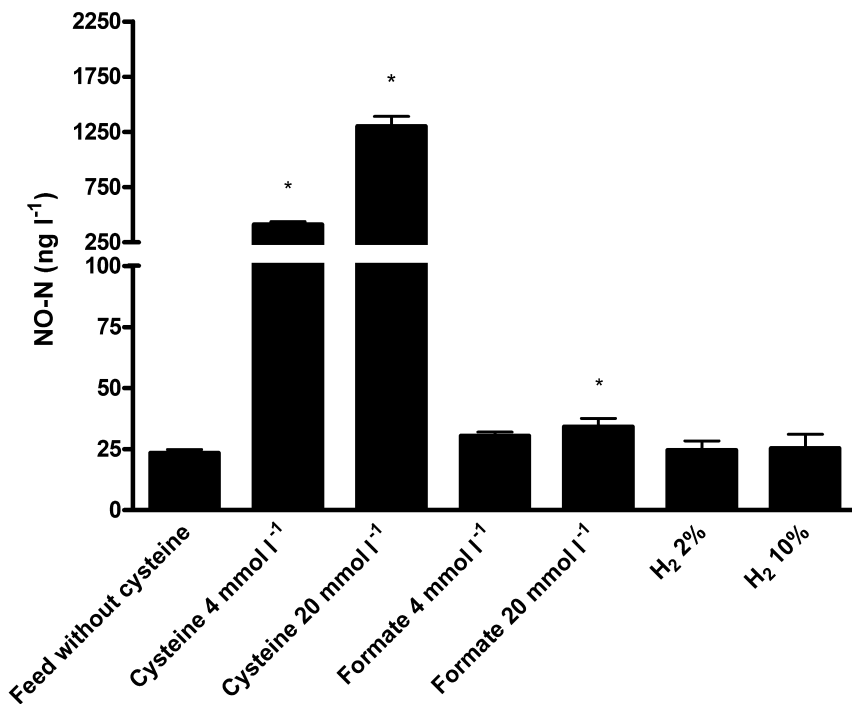


Fig. 2. NO-N (ng l⁻¹) production by the human intestinal microbiota incubated for 24 h in feed medium without cysteine (control), supplemented with cysteine (4 or 20 mmol l⁻¹), formate (4 or 20 mmol l⁻¹) or H₂ gas (2 % or 10 % in headspace). The mean ± SD is shown. The asterisk indicates significant differences compared to the feed without cysteine ($P < 0.05$).

effect of cysteine on the NO production (Fig. 2). Feed medium without cysteine was considered as control and reached a NO concentration of 23.5 ± 1.4 ng NO-N l⁻¹. Cysteine added at 4 mmol l⁻¹ and 20 mmol l⁻¹ increased the production of NO significantly to concentrations of 411.8 ± 26.5 ng NO-N l⁻¹ (factor 18 increase) and 1303.8 ± 85.4 ng NO-N l⁻¹ (factor 56 increase) respectively. In the abiotic controls, NO concentrations of 2.0 and 8.0 ng NO-N l⁻¹ were measured for 4 mmol l⁻¹ and 20 mmol l⁻¹ cysteine respectively. A positive linear correlation between cysteine and NO was found ($r = 0.99$). Three possible explanations for this correlation were suggested, i.e. redox potential, sulfide production or DNRA.

The redox reducing potential of cysteine

As cysteine is frequently used during anaerobic incubations for its redox reducing potential, this characteristic might have changed the growth conditions for the microbiota and concomitant NO production. Cysteine has been shown to influence the growth and hydrogen production of *Enterobacterium bacterium*. In the latter study, L-cysteine concentrations above 4 mmol l⁻¹ showed a marked decrease in the redox status, hydrogen production and biomass growth (Chen *et al.*, 2010). To investigate whether the reducing power of cysteine was responsible for the increased NO concentrations in our study, the redox potentials were measured after 24 h of incubation. The redox potential of the inoculated feed medium was around -174 ± 3 mV, while addition of 4 mmol l⁻¹ or

20 mmol l⁻¹ cysteine decreased this value to -205 ± 9 mV ($P = 0.17$) and -223 ± 4 mV ($P < 0.05$) respectively. Yet, the redox potential of both cysteine concentrations were not significantly different from each other ($P = 0.41$). To conclude, a five times increase in the cysteine concentration resulted in a 20 times increase in the NO concentration. Thus, the redox reducing potential of cysteine was not considered to cause the significant increases in NO production.

Increased NO production by reduced sulfur compounds

Second, as bacterial degradation of cysteine to H₂S has been described (Forsberg, 1980) and H₂S has been shown to be an electron donor for DNRA (Eisenmann *et al.*, 1995), a comparison was made between the NO and sulfide produced when equimolar concentrations of cysteine or Na₂S were added to the feed medium. The feed medium without cysteine produced 72.7 ± 6.4 ng NO-N l⁻¹ and these concentrations increased significantly to 730.2 ± 30.3 ng NO-N l⁻¹ (factor 10 increase) and 2832.5 ± 181.7 ng NO-N l⁻¹ (factor 39 increase) for 4 and 20 mmol l⁻¹ cysteine respectively. It was shown that Na₂S stimulated the NO production in a similar way as cysteine. Na₂S addition resulted in significantly higher NO concentrations of 684.0 ± 52.8 ng NO-N l⁻¹ (factor 10 increase) and 2881.4 ± 99.2 ng NO-N l⁻¹ (factor 40 increase) for 4 and 20 mmol l⁻¹ respectively (Fig. 3A). The NO concentrations measured in feed with cysteine or Na₂S added at 4 mmol l⁻¹ or 20 mmol l⁻¹ respectively, were not

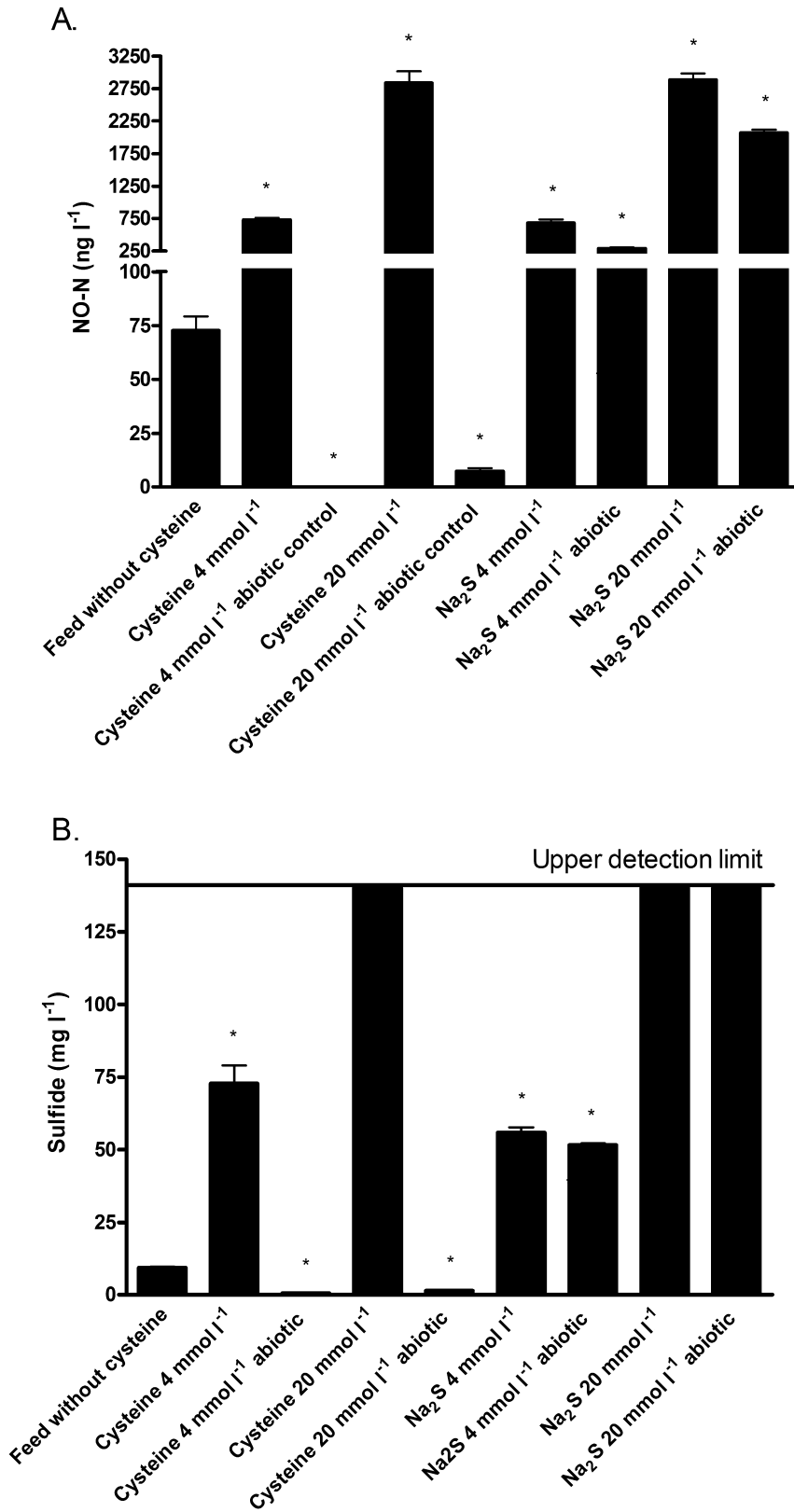


Fig. 3. (A) NO-N (ng l⁻¹) and (B) sulfide (mg l⁻¹) production in feed medium with or without cysteine or Na₂S supplemented (4 or 20 mmol l⁻¹) inoculated or abiotic after 24 h of incubation. The mean ± SD is shown. The asterisk indicates significant differences compared to the feed without cysteine (*P* < 0.05).

significantly different ($P = 1.00$ for both concentrations). Sulfide concentrations after 24 h in feed without cysteine reached concentrations around $9.3 \pm 0.5 \text{ mg l}^{-1}$. Addition of 4 mmol l^{-1} cysteine or Na_2S increased this sulfide concentration significantly to $72.8 \pm 6.2 \text{ mg l}^{-1}$ and $55.9 \pm 1.9 \text{ mg l}^{-1}$ respectively. Higher concentrations resulted in sulfide concentrations above the upper detection limit (Fig. 3B). In the abiotic controls, NO concentrations of 0.08 ± 0.04 and $7.4 \pm 1.5 \text{ ng NO-N l}^{-1}$ were measured for 4 mmol l^{-1} and 20 mmol l^{-1} cysteine respectively. Remarkably, in the abiotic controls of Na_2S , NO concentrations of $288.8 \pm 13.5 \text{ ng NO-N l}^{-1}$ (factor 4 increase, $P < 0.05$) and $2070.2 \pm 48.5 \text{ ng NO-N l}^{-1}$ (factor 29 increase, $P < 0.05$) were measured for 4 mmol l^{-1} and 20 mmol l^{-1} Na_2S (Fig. 3A). These abiotic controls were however still significantly lower than their biotic counterpart ($P < 0.05$) indicating that NO formation resulted from a combination of chemical and biological processes.

Small increase in NO production by stimulation of DNRA

Thirdly, DNRA is believed to be the most important microbial process for the removal of nitrate in the intestinal tract (Allison and Macfarlane, 1988; Vermeiren *et al.*, 2009). H_2S has been shown to be an electron donor for DNRA (Eisenmann *et al.*, 1995), and we have found that Na_2S stimulated the formation of NO. Therefore, the influence of two alternative DNRA stimulating electron donors, i.e. formate and H_2 (Simon, 2002) on NO formation was further investigated. The addition of 4 and 20 mmol l^{-1} formate slightly increased the NO concentrations to $30.4 \pm 1.5 \text{ ng NO-N l}^{-1}$ (factor 1.3 increase) and $34.2 \pm 3.4 \text{ ng NO-N l}^{-1}$ (factor 1.5 increase, $P < 0.05$) respectively (Fig. 2). H_2 gas added to the headspace to final concentrations of 2% and 10% did not show an increase in the production of NO (Fig. 2). The NO concentrations of all abiotic controls were below $0.6 \text{ ng NO-N l}^{-1}$. Stimulation of DNRA by formate increased the NO concentrations significantly; however, the impact of formate was very small compared to the impact cysteine had on the NO production. Thus, stimulation of DNRA was not considered as one of the main driving forces for NO formation under intestinal conditions.

Abiotic NO production from nitrite, but not nitrate or hydroxylamine

Considering our previous results, the abiotic controls of Na_2S showed remarkably high NO concentrations. To identify the specific N-compound from which NO is formed abiotically, solutions of nitrate, nitrite and hydroxylamine (5 mg N l^{-1}) were incubated abiotically for 24 h at 37°C with or without $20 \text{ mmol Na}_2\text{S l}^{-1}$. Nitrate and hydroxy-

lamine did not produce NO at either condition, while concentrations of $13 \text{ ng NO-N l}^{-1}$ were measured for nitrite after 24 h. Remarkably, these concentrations increased significantly with a factor 1000 when Na_2S was added (data not shown).

Release of NO from nitrite is pH-dependent

As non-enzymatic NO release from nitrite at a pH below 5.5 has been described (Weitzberg and Lundberg, 1998), the influence of pH on the chemical production of NO from nitrite with or without Na_2S was studied. As expected, NO concentrations around $5100 \text{ ng NO-N l}^{-1}$, $15 \text{ ng NO-N l}^{-1}$ and 8 ng NO-N l^{-1} were measured for pH 5, 7 and 9 respectively, confirming previous reports on chemical decomposition of nitrite at a pH < 5.5 . The NO released was only a minor fraction of the nitrite added, i.e. 1‰ at pH 5. When Na_2S was added, concentrations around $19000 \text{ ng NO-N l}^{-1}$, $12700 \text{ ng NO-N l}^{-1}$ and $50 \text{ ng NO-N l}^{-1}$ were measured for pH 5, 7 and 9 respectively (Fig. 4A). Under these conditions, 3.8‰, 2.5‰ and 0.01‰ of nitrite was converted to NO at pH 5, 7 and 9 respectively. Although literature is limited concerning this process, Roediger and Babidge (2000) have suggested a similar process. They described an increased release of NO, with a factor 3 to 4, from the NO donor nitrosoglutathione (GSNO) when adding Na_2S . Moreover, our results corroborate a recent publication describing the production of NO from nitrite induced by H_2S (Grossi, 2009). The latter author describes a NO release from the NO donor sodium nitroprusside (SNP) or nitrite mediated by $\text{HS}^-/\text{H}_2\text{S}$, particularly at a pH lower than 7. An increased NO formation with a decreasing pH can be explained by the fact that only the HNO_2 form of nitrite can be reduced to NO.

To confirm chemical NO formation in the feed medium, an additional experiment was performed incubating the feed medium abiotically without cysteine at pH 5, 7 and 9 for 24 h with or without 20 mmol l^{-1} Na_2S . Without Na_2S added to the feed medium, no NO was formed (Fig. 4B). With Na_2S , NO was formed but the concentrations were around five times lower compared to NO produced in the nitrite solution described above. This can be explained by the very low concentrations of nitrite in the feed which are below 0.05 mg l^{-1} . A negative correlation between NO and the pH was seen, similarly as for the nitrite solution (Fig. 4B).

NO production is regulated by dietary compounds and microbial processes

Taking these findings together, a combination of biological and chemical reactions lead to the production of NO by the human intestinal microbiota (Fig. 5). As such, dietary compounds may influence the NO production significantly.

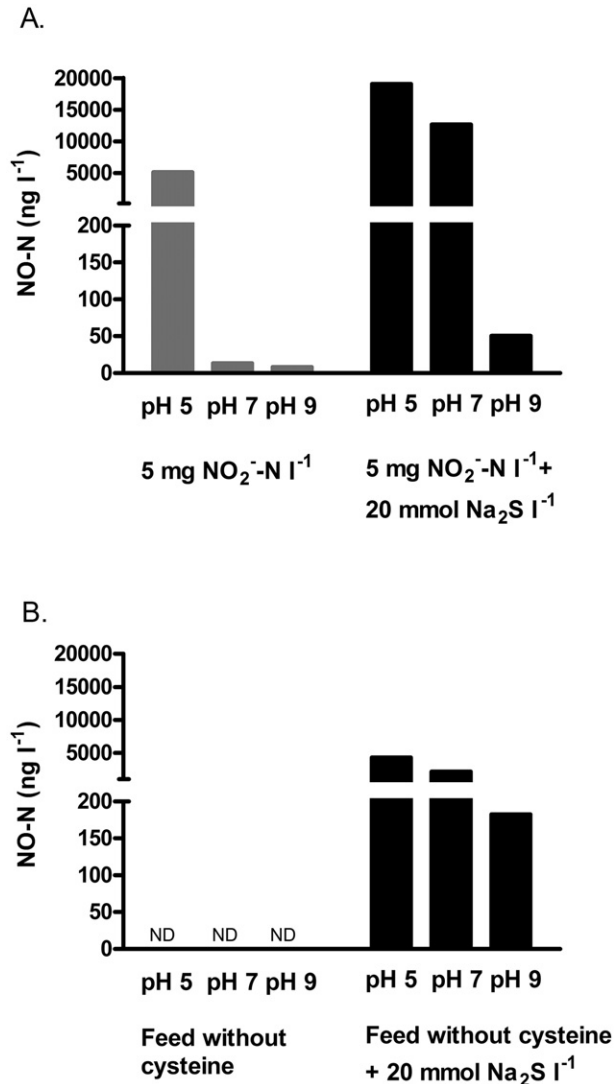


Fig. 4. Abiotic NO-N (ng l⁻¹) production from (A) 5 mg NO₂⁻-N l⁻¹ solution and (B) feed without cysteine incubated with or without 20 mmol Na₂S l⁻¹ at pH 5, 7 or 9 for 24 h at 37°C. ND indicates that the values were below the detection limit.

Nitrate in the lower parts of the gastrointestinal tract originates from dietary products, like leaf vegetables [accounting for 60 to 80% of the total dietary nitrate intake (Ysart *et al.*, 1999)] and from endogenously synthesized nitrate, which mainly comes from the L-arginine–NO pathway (Leaf *et al.*, 1989). Nitrate is reduced to nitrite by the bacteria in the oral cavity or is mainly absorbed in the small intestine but levels of a few mg can reach the large intestine (Xu, 2001). Nitrite is used as a food additive for example in meat to prevent botulism and to stabilize the colour (Weiss *et al.*, 2010), but concentrations in faecal samples are very low, i.e. around 70 µg N l⁻¹. Considering the low levels of NO (in the range of ng l⁻¹ to µg l⁻¹), direct chemical reduction from nitrite cannot be excluded. Yet, many species residing in the intestinal tract have the

ability to reduce nitrate to nitrite, thereby creating a major source of nitrite (Zumft, 1997).

The second microbial process necessary to reduce nitrite to NO is the production of H₂S which can be partly related to the reduction of sulfate by SRB. Sources of inorganic sulfate include bread, dried fruits, nuts, fermented beverages, garlic and brassica vegetable (Florin *et al.*, 1991). Second, dietary protein, especially meat, has been shown to be an important substrate of H₂S. Sulfur amino acids, such as cysteine and methionine, rather than sulfate have been suggested to be a more readily utilizable sulfur source (Florin *et al.*, 1991; Levine *et al.*, 1998; Magee *et al.*, 2000). H₂S has been shown to reach concentrations of up to 3 to 4 mmol l⁻¹ in healthy individuals (Florin *et al.*, 1991; Magee *et al.*, 2000).

To come back to the results of the first experiment, exclusion of starch from the feed medium increased the concentrations of NO significantly. We suggest that due to a lack of starch, the fermentation of sulfur-containing amino acids is increased leading to higher H₂S and concomitant NO concentrations. Indeed, when measuring the sulfide concentrations after 24 h, feed medium without starch showed significantly higher sulfide concentrations (factor 1.2) than the feed with cysteine (data not shown).

Due to the fact that inocula were used from different SHIME runs, we found variability in the concentrations of NO produced during the batch experiments. This inter-individual variation can be ascribed to variability in the microbial production of nitrite and sulfide. Also Sobko and colleagues (2005) have described large individual variation in the NO production between the faecal samples.

Physiological consequences for colonocytes

Co-action of NO and sulfide has been described to be more damaging for colonocytes than either of the compounds (Roediger and Babidge, 2000). NO impairs the energy metabolism of colonocytes by reducing the acyl-CoA production, necessary for the generation of energy

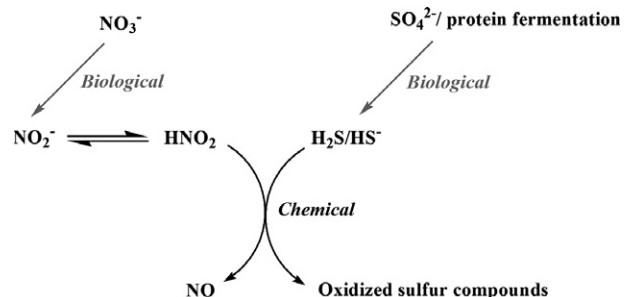


Fig. 5. Hypothesis on the production of NO in the intestinal tract. The end-products of two biological processes, i.e. the reduction of nitrate to nitrite and the reduction of sulfur compounds to H₂S, are necessary for the chemical production of NO.

from butyrate. Co-incubation with sulfide showed an increased generation of NO from the NO donor GSNO which further reduced CoA-metabolism in the colonocytes (Roediger and Babidge, 2000). In our study, we have shown that the production of NO can be stimulated directly by H₂S and indirectly by cysteine. To what extent H₂S or thiol compounds indirectly contribute to the NO induced damage to colonocytes, should be further investigated. This may relate to the genotoxicity of sulfide, which is mediated by free radicals (Attene-Ramos *et al.*, 2007). In respect to our findings, further studies may investigate whether sulfide genotoxicity may indeed be related to the formation of NO radicals from nitrite in the medium.

Conclusions

To conclude, this study has shown that the combination of two biological and one chemical process contributes to the reduction of nitrate to NO in the intestinal tract. Dietary compounds and the microbial community composition determine the conditions in the colon and hence the chemical production of NO. NO is formed by the reduction of nitrite with H₂S, both products of the microbial metabolism in the intestinal tract. Only small fractions of NO are released from nitrite, yet these amounts may impact the metabolism of colonocytes (Roediger and Babidge, 2000). The significance of the chemical release of NO from nitrite, mediated by H₂S and negatively correlated to the pH, might be underestimated in several biological processes.

Experimental procedures

Growth media

Unless stated otherwise, all products were ordered from Sigma Aldrich (Bornem, Belgium). Feed medium was composed of arabinogalactan (1 g l⁻¹), pectin (2 g l⁻¹), xylan (1 g l⁻¹), potato starch (3 g l⁻¹; Anco, Roeselare, Belgium), glucose (0.4 g l⁻¹), yeast extract (3 g l⁻¹; Oxoid, Aalst, Belgium), special peptone (1 g l⁻¹; Oxoid, Aalst, Belgium) and mucin from porcine stomach type II (4 g l⁻¹). L-cysteine (0.5 g l⁻¹) was added to scavenge-dissolved oxygen and to lower the initial redox potential. Feed medium is based on a diet frequently used during *in vitro* incubation experiments of the gastrointestinal microbiota (Molly *et al.*, 1993; Possemiers *et al.*, 2004; Van de Wiele *et al.*, 2004; Grootaert *et al.*, 2009). Analyses of the feed medium indicated that very low concentrations of nitrate (0.115 ± 0.009 mg NO₃-N l⁻¹) and nitrite (0.053 ± 0.004 mg NO₂-N l⁻¹) were present.

Inocula

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) is a dynamic *in vitro* model of the human gastrointes-

tinal tract. It is composed of five double-jacketed vessels, simulating the stomach, small intestine and the three colon regions (ascending, transverse and descending colon). The first two compartments are a fill-and-draw setup and represent the stomach and small intestine. Peristaltic pumps add a defined amount of feed medium (140 ml, three times per day) and pancreatic and bile liquid (60 ml, three times per day), respectively, to the stomach and duodenum compartments and empty the respective compartments after specified intervals. The last three compartments are continuously stirred reactors with constant volume and pH control, specific for each compartment. Isolation of the bacterial inoculum, retention time, pH, temperature settings and reactor feed composition were described previously (Molly *et al.*, 1993; Possemiers *et al.*, 2004). For the batch experiments, samples from the colon ascendens were chosen as inocula as in this vessel the highest levels of NO were measured previously (Vermeiren *et al.*, 2009). Three different SHIME runs, inoculated with faecal samples from three healthy individuals (male; 24, 25 and 27 years) who had no history of antibiotic intake 6 months prior to the study, were used as inocula for the batch experiments. Samples were taken at least 2 weeks after the stabilization period. SHIME samples were preferred over faecal samples because of the stability of the microbial community composition in this continuous system.

Batch experiments

Anaerobic incubations were performed in penicillin flasks with a total volume of 120 ml. The bottles were filled with 42.5 ml feed medium and 7.5 ml of a 1 M phosphate buffer (88 g K₂HPO₄ l⁻¹ and 68 g KH₂PO₄ l⁻¹). The pH was set between 6.8 and 7.0 using a 1 mol l⁻¹ NaOH solution at the beginning of the incubation and decreased maximum one unit during the 24 h of incubation (data not shown). To obtain anaerobic conditions the bottles were closed with butyl rubber stops and flushed with Argon during 15 cycles of 2 min each at 800 mbar overpressure and 900 mbar underpressure (De Weirdt *et al.*, 2010). Before starting the incubations, bottles were put on atmospheric pressure. To perform the experiments with a representative microbial community of the intestinal tract, 1 ml of SHIME suspension from the colon ascendens was used as inoculum. The penicillin flasks were incubated at 37°C for 24 h while shaking at 100 r.p.m.

To investigate chemical NO production, a nitrite solution of 0.3 mM NO₂-N l⁻¹ or feed medium without buffer was incubated sterile at pH 5, 7 or 9 with or without 20 mM Na₂S for 24 h at 37°C while shaking at 100 r.p.m.

Electron donors

Formate, Na₂S, cysteine and H₂ were separately added to the feed medium from which cysteine was excluded in two different concentrations. Cysteine, Na₂S and formate were added to final equimolar concentrations of 4 and 20 mmol l⁻¹. This corresponds to final concentrations of 0.5 and 2.5 g l⁻¹ for cysteine, 0.31 and 1.56 g l⁻¹ for Na₂S, and 0.27 and 1.36 g l⁻¹ for sodium formate respectively. A defined volume of the headspace of the penicillin flasks was replaced with 100% (v/v) H₂ gas to final concentrations of 2% and 10% H₂.

Nitrogen compound analysis

NO₃⁻, NO₂⁻ and NO concentrations were measured as described previously (Vermeiren *et al.*, 2009). In short, NO₃⁻ and NO₂⁻ concentrations were measured by first reducing NO₃⁻ to NO₂⁻ by a copper-cadmium reductor coil at pH of 8.0. NO₂⁻ concentrations were determined colorimetrically by an imidazole buffered reaction with N-1-naphthylethylenediamine. The NO₃⁻ concentration was quantified by subtraction of the concentrations of NO₂⁻ from the concentration of (NO₃⁻ + NO₂⁻) (Hauck, 1982). The samples were centrifuged at 4000 g for 3 min, filtered using 0.45 µM membrane filters (Millipore, NYSE:MIL, USA) and diluted in milli-Q water before analysis. In short, NO measurements were based on the principle of chemiluminescence using Eco Physics CLD 77 AM (Eco Physics AG, Duernten, Switzerland) with a detection limit of 1 ppbv. A standard curve prepared with NO standard gas (9.8 ± 0.05 ppmv) diluted in air.

Sulfide analysis

Sulfide concentrations of the samples were measured using standard sulfide cuvette tests (range 0.1–2.0 mg l⁻¹) (Hach Lange, Mechelen, Belgium). This method accounted for all dissolved sulfide species (H₂S, HS⁻ and S₂⁻).

Calculations

All headspace NO measurements were subsequently converted to concentrations present in the feed medium. The NO concentrations measured in the gas phase (ppbv) were converted to molar concentrations using the ideal gas law $pV = nRT$ with $T = 294.65$ K. Taking into account $[NO]_g / [NO]_{aq} \cong 20$ at equilibrium, the NO concentrations in the gas phase were converted to the concentrations in the liquid phase and expressed as ng NO-N l⁻¹.

Statistical analysis

All experiments were performed in triplicate. SPSS version 16.0 (SPSS, Chicago, USA) was used to carry out the statistical analyses. Normality of the data and homogeneity of variances was assessed using the Kolmogorov–Smirnov and the Levene test respectively. Comparison of normally distributed data was performed with ANOVA or Dunnett T3; when ANOVA indicated significant differences, means were compared using the Bonferroni comparison test. Comparison of means of not normally distributed data was evaluated with the non-parametric Kruskal–Wallis test; when Kruskal–Wallis indicated significant differences, means were compared with the Behrens–Fisher comparison test using the npmc package of R software (<http://www.r-project.org/>) (Munzel and Hothorn, 2001). A *P*-value of less than 0.05 was considered significant.

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