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NITRIC OXIDE IN THE GASTROINTESTINAL TRACT: ROLE OF BACTERIA

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ABSTRACT

Nitric oxide is produced by numerous cell types along the GI tract where it serves to regulate a variety of physiological processes including gut motility, secretions, mucosal blood flow and immunity. Classically, NO is produced from L-arginine and molecular oxygen by specific enzymes- the NO synthases, but more recently a fundamentally different pathway for NO generation was described. This involves stepwise reduction of the higher nitrogen oxides nitrate and nitrite to form NO. In this process commensal bacterial in the GI tract play a key role. Dietary nitrate (mainly provided for by vegetables) accumulates in saliva and the oral microflora reduces this nitrate to nitrite. Nitrite then enters the stomach where it is reduced to NO by the acid. A picture is now emerging suggesting an important role of entero-salivary circulation of nitrate and serial reduction to NO in regulation of gastric function. Intriguingly, the nitrite that survives gastric passage is absorbed and can later recycle to NO in blood and tissues via several enzymatic as well as non-enzymatic pathways. Such systemic NO generation is likely involved in regulation of cardiovascular function and tissue homeostasis, especially in response to ischaemia and hypoxia.

INTRODUCTION

NO is generated in our bodies from the oxidation of L-arginine and this reaction is catalysed by specific enzymes, the NO synthases. A tremendous amount of data generated over the past two decades show that NO regulates vital physiological as well as pathophysiological processes ranging from vasoregulation, neuromodulation and regulation of platelet function to host defence and immunity (*Ignarro*, 2002). Here, a previously unrecognized and fundamentally different pathway for the generation of NO in humans is discussed. This pathway is NO synthase-independent and utilizes nitrate and nitrite as substrates. Interestingly, while the classical NO synthase pathway is oxygen dependent and dysfunctional during ischaemia/hypoxia, the nitrate-nitrite-NO axis is instead greatly enhanced during these conditions. Two components are central in the generation of NO from nitrate and nitrite: The commensal bacteria and the diet.

SOURCES AND ENTEROSALIVARY CIRCULATION OF NITRATE

The main dietary source of nitrate (NO_3^-) is vegetables, which account for 60-80% of the daily nitrate intake in people on a typical western diet (*Lundberg* and *Weitzberg*, 2005). Nitrite (NO_2^-) is also found in some foodstuff. For example, it is used as a food additive in meat to prevent botulism and to enhance its appearance. The main source of endogenous nitrate in mammals is the L-arginine-NO pathway, which is constitutively active in numerous cell types throughout the body. NO is produced from the amino acid L-arginine and molecular oxygen by NO synthases (NOSs). Although in simple aqueous systems NO is oxidized to nitrite, in mammals NO predominantly reacts with oxidized haemoglobin and other compounds to form nitrate.

After ingestion, nitrate is rapidly and effectively absorbed proximally from the gastrointestinal tract into the bloodstream, where it mixes with endogenously synthesized nitrate. Peak plasma concentrations are seen within 60 minutes of nitrate ingestion and the half-life of nitrate in plasma is about 5 hours. For as-yet-unknown reasons, the concentrations of nitrate excreted in saliva are exceptionally high; up to 25% of plasma nitrate is actively taken up by the salivary glands and secreted with saliva, and the resulting salivary nitrate concentrations are at least 10 times higher than the concentrations in plasma (*Spiegelhalder* et al., 1976).

In the oral cavity salivary nitrate is rapidly reduced to nitrite by facultative anaerobic bacteria residing mainly at the dorsal part of the tongue. The levels of nitrite in fasting saliva are typically 50-150 μM but rise dramatically (to 1-2- mM) following ingestion of nitrate rich food (*Spiegelhalder* et al., 1976).

INTRAGASTRIC NO GENERATION AND ITS PHYSIOLOGICAL ROLE

In 1994 two groups independently discovered that great amounts of NO are being generated constantly in the stomach lumen (*Lundberg* et al., 1994; *Benjamin* et al., 1994). It was immediately realised that this NO was not a result of NO synthase activity but rather a non-enzymatic reduction of nitrite present in swallowed saliva. Nitrite is protonated in the acidic stomach to form nitrous acid (reaction 1), which then decomposes to a variety of nitrogen oxides including NO. Clear evidence of this pathway for NO formation came from human studies in which pre-treatment with the proton pump inhibitor omeprazole (thereby increasing gastric pH) abolished gastric NO (*Lundberg* et al., 1994). Additional *in vitro* experiments with different concentrations of nitrite in acid, or mixing of saliva and gastric juice, confirmed this (*Lundberg* et al., 1994).

$$\mathbf{NO_2^-} + H^+ \leftrightarrow HNO_2 \qquad (1)$$

$$2HNO_2 \leftrightarrow N_2O_3 + H_2O$$

$$N_2O_3 \leftrightarrow \mathbf{NO} + NO_2$$

The concentrations of NO in the stomach lumen (20 - 400 ppm.) are several orders of magnitude higher than those that are required for vasodilatation. As NO is known to easily travel across biological membranes and as NO-donating drugs are gastroprotective, it has been proposed that nitrite-derived NO, acting from the luminal side, could be involved in the regulation of

gastric mucosal blood flow (*Lundberg* et al., 1994). Several recent studies from different laboratories support this idea. *Bjorne* and colleagues (2004) studied gastric mucosal blood flow and mucus secretion in a rat *in vivo* model after local application of human saliva to the gastric mucosa. Mucosal blood flow and mucus secretion were increased after luminal application of nitrite-rich saliva, whereas saliva from a fasting individual had no effect. These effects were associated with the generation of NO and *S*-nitrosothiols. In addition, pre-treatment with an inhibitor of guanylyl cyclase markedly inhibited nitrite-mediated effects on blood flow. This indicates that the observed effects were mediated by NO.

Several other recent animal studies indicate that dietary nitrate has gastroprotective activity through the generation of NO in the stomach. We recently pre-treated rats with nitrate in the drinking water for one week and then challenged them with diclofenak; a non-steroidal anti-inflammatory drug (NSAID) known to produce gastric ulcers in animals and humans. Interestingly, the nitrate pre-treated animals were dose-dependently protected against the ulcerogenic effects of this drug (*Jansson* et al., 2007). *Miyoshi* et al. (2003) examined the effects of oral nitrate supplementation on stress-induced gastric injury in rats. Pre-treatment with inorganic nitrate was strongly protective and the effects were paralleled by intragastric generation of NO. Interestingly, NO generation and the protective effects of dietary nitrate were abolished when the oral microflora was removed by topical antibiotic treatment before the experiment.

In addition to the effects of NO on the gastric mucosa, the extremely high luminal levels of NO in the stomach also seems to be involved in the defence against swallowed pathogens *Benjamin* et al., 1994).

Taken together, these studies clearly indicate that dietary nitrate has important gastroprotective effects. The crucial step in the bioactivation of inorganic nitrate is the reduction to nitrite, which is carried out by the oral microflora.

NO GENERATION BY GUT COMMENSAL BACTERIA

The intragastric formation of NO from nitrite in saliva is non-enzymatic and a result of acid-dependent reduction of nitrite. We recently examined if NO could be generated also in lower parts of the GI tract where pH levels are much higher. Interestingly, when human faeces were incubated anaerobically in the presence of nitrate or nitrite considerable amounts of NO were produced (*Sobko* et al., 2004). In parallel *in vivo* experiments we could detect significant NO levels throughout the GI tract in conventional rats but not in germfree animals, thereby confirming the need for bacteria in this process. In addition, caecal and small intestinal NO levels increase in the rat after supplementing the diet with nitrate for one week (*Sobko* et al., 2005). When studying isolated strains of bacteria *in vitro* we found that bifidobacteria and lactobacilli generated large amounts of NO in the presence of nitrite while NO production from *E. coli* and *C. difficile* were negligible. In fact, further experiment showed that *E. coli* and *S. aureus* effectively consumed NO (*Sobko* et al., 2006). Clearly, at this stage we can say that NO is being produced by bacteria in the lower GI tract and its level will depend on the balance

between production and consumption by different bacteria. We also know that levels can be increased by increasing the dietary intake of substrate (nitrate). However, the physiological significance of this locally generated NO remains to be elucidated. In the stomach luminal NO clearly affects the host mucosa as discussed above but one should keep in mind that the levels in the stomach are orders of magnitude higher than those normally found in lower parts of the GI tract.

NITRITE REDUCTION TO NO IN THE SYSTEMIC CIRCULATION AND TISSUES

The generation of NO from nitrite occurs spontaneously in highly acidic or reducing environments. Interestingly, such non-enzymatic generation of NO can also occur systemically. In ischaemic tissues in which the pH value is decreased, NO is formed from nitrite by similar mechanisms (*Weitzberg* and *Lundberg*, 1998; *Lundberg* et al., 2004; *Gladwin* et al., 2005; *Zweier* et al. 1995). In addition, recent research indicates that nitrite can be converted to NO by several other pathways, which involve mammalian enzymes or proteins (*Weitzberg* and *Lundberg*, 1998; *Lundberg* et al., 2004; *Gladwin* et al., 2005; *Zweier* et al. 1995). It has now been shown that physiological concentrations of nitrite can dilate blood vessels through conversion to NO (*Cosby* et al., 2003). With this new knowledge, nitrite might be considered an important vascular storage pool of NO. Interestingly, it was recently found that the levels of nitrite in plasma increase 4-5-fold after ingestion of inorganic nitrate (*Lundberg* and *Govoni*, 2004). This increase was abolished if the test subject avoided swallowing after the nitrate intake, thereby illustrating its salivary origin. By extrapolation, this could in fact indicate that the commensal oral flora contributes not only to the local regulation of gastric function, as discussed above, but also to systemic NO-mediated effects, such as the regulation of vascular tone, platelet function and leukocyte adhesion. In strong support of this idea we recently noted a reduction in systemic blood pressure in healthy normotensive volunteers after a 3 day dietary supplementation with nitrate (sodium nitrate) in an amount corresponding to a daily intake of 100-300g of a nitrate rich vegetable such as spinach or lettuce (*Larsen* et al., 2006). This asks the intriguing question whether the high nitrate content in vegetables contributes to their beneficial effects on the cardiovascular system. Emerging data from numerous laboratories now also suggest that direct administration of nitrite can be therapeutically useful e.g. in treatment of ischaemia reperfusion injury (*Weitzberg* and *Lundberg*, 1998; *Gladwin* et al., 2005; *Zweier* et al. 1995). As an example, *Duranski* and colleagues (2005) pre-treated mice with extremely low doses of nitrite and then subjected them to cardiac ischaemia. Remarkably, nitrite treatment reduced their myocardial infarction by up to 70%. Interestingly, the dose that afforded the greatest protection is equivalent to what is achieved by ingestion of no more than 100 g of spinach or lettuce.

CONCLUSION

Nitrate is generally considered a water pollutant and an undesirable fertilizer residue in the food chain. Research in the 1970s indicated that, by reducing nitrate to nitrite, commensal bacteria might be involved in the pathogenesis of gastric cancers and other malignancies, as nitrite can enhance the generation of carcinogenic *N*-nitrosamines. More recent studies indicate that the bacterial and host metabolism of nitrate to nitrite can lead to formation of nitric oxide (NO) which could have beneficial roles not only locally in the stomach but also systemically. There is now accumulating evidence that nitrate-reducing commensals have a true symbiotic role in mammals and facilitate a previously unrecognized but potentially important aspect of the nitrogen cycle. This could lead to a paradigm shift in the view of dietary nitrate in relation to human health. It may be that the high nitrate content of vegetables explains some of their well known beneficial effects on health including a decreased risk of cardiovascular disorders.

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