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INVITED MEDICAL REVIEW

The oral microbiome and nitric oxide homoeostasis

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The tiny radical nitric oxide (NO) participates in a vast number of physiological functions including vasodilation, nerve transmission, host defence and cellular energetics. Classically produced by a family of specific enzymes, NO synthases (NOSs), NO signals via reactions with other radicals or transition metals. An alternative pathway for the generation of NO is the nitrate–nitrite–NO pathway in which the inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-) are reduced to NO and other reactive nitrogen intermediates. Nitrate and nitrite are oxidation products from NOS-dependent NO generation but also constituents in our diet, mainly in leafy green vegetables. Irrespective of origin, active uptake of circulating nitrate in the salivary glands, excretion in saliva and subsequent reduction to nitrite by oral commensal bacteria are all necessary steps for further NO generation. This central role of the oral cavity in regulating NO generation from nitrate presents a new and intriguing aspect of the human microbiome in health and disease. In this review, we present recent advances in our understanding of the nitrate–nitrite–NO pathway and specifically highlight the importance of the oral cavity as a hub for its function.

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Introduction

The discovery in the late 1980s that a tiny gaseous molecule like nitric oxide (NO) was an endogenous signalling substance radically changed our view on biological function (Moncada and Higgs, 1993). Now 30 years later, a vast knowledge has accumulated on the role of NO in health and disease, and it is evident that this radical is involved in a vast spectrum of human physiology including vasodilation, endothelial function, anti-aggregation of platelets, nerve transmission, host defence, metabolism and mitochondrial function (Lundberg *et al*, 2008). The specific enzymes [NO synthases (NOSs)] responsible for converting the amino acid L-arginine and molecular oxygen to NO and L-citrulline have been thoroughly characterized as have the signalling characteristics of NO (Forstermann and Sessa, 2012). In spite of this vast knowledge, few new treatment options or drugs have reached the clinical setting. Inhaled NO is used in infants with pulmonary distress (Kinsella *et al*, 2006), and phosphodiesterase inhibitors, for example sildenafil, work by preventing the degradation of cyclic GMP, a classical downstream target of NO (Goldstein *et al*, 1998).

Research that started in the mid-1990s has revealed an alternative pathway for the generation of NO that is independent of NOS (Benjamin *et al*, 1994; Lundberg *et al*, 1994). The inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-) can be reduced to bioactive NO and other reactive nitrogen intermediates. These anions are oxidation products of endogenous, NOS-dependent NO generation and are also a part of our diet, especially in green leafy vegetables, which are very high in nitrate. Systemic nitrate and nitrite in blood and tissues are now considered a pool for potential conversion to NO, which can be enhanced by intake of nitrate-rich food (Weitzberg and Lundberg, 2013).

An intriguing aspect of this nitrate–nitrite–NO pathway is the obligatory role of oral commensal bacteria. Nitrate is actively taken up by the salivary glands and concentrated in saliva (Spiegelhalder *et al*, 1976). Facultative anaerobic bacteria on the surface of the tongue efficiently reduce nitrate to nitrite, a process poorly performed by mammalian cells. These bacteria use nitrate and nitrite as final electron acceptors in their respiration and at the same time help the host with the first step in converting nitrate to NO. Salivary nitrite is then swallowed and reaches the stomach and intestinal tract where it is efficiently taken up. Once nitrite reaches the systemic circulation, there are several enzymatic and non-enzymatic pathways for its reduction to NO and other reactive nitrogen intermediates (Lundberg *et al*, 2008). The nitrate–nitrite–NO pathway is an alternative system for the generation of NO, supporting and complementing canonical NOS-dependent NO generation, especially in situations where the latter is malfunctioning. Novel therapeutic and nutritional interventions

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based on this pathway are now being explored as well as the basic physiological role of this system.

The oral cavity is central in the nitrate–nitrite–NO pathway, and this review will present recent advances in this field of research with special focus on how nitrate is handled by our salivary glands and the oral commensal bacteria.

Classical nitric oxide generation and signalling

Endogenous NO generation was first identified in the vascular endothelium where it acts as a paracrine vasodilator (Furchgott and Zawadzki, 1980; Ignarro *et al*, 1987). The importance of the discovery of NO and associated signalling pathways led to the Nobel Prize in Physiology or Medicine in 1998. The complex oxidation reaction of L-arginine catalysed by NOSs requires several cofactors such as haem, tetrahydrobiopterin and flavin reductases as well as the presence of molecular oxygen which makes these enzymes more or less malfunctioning during hypoxia (Griffith and Stuehr, 1995). In addition, lack of substrate or the cofactor tetrahydrobiopterin can lead to NOS uncoupling where the enzyme switches from NO production to superoxide generation (Xia *et al*, 1996; List *et al*, 1997). Three NOS isoforms have been described: neuronal NOS (nNOS, NOS 1), endothelial NOS (eNOS, NOS 3) and inducible NOS (iNOS, NOS 2; Moncada and Higgs, 1993). eNOS was initially discovered in endothelial cells and is important in modulating vascular tone and upholding endothelial integrity (Sessa *et al*, 1992). nNOS was initially identified in the brain and is involved in central and peripheral neuronal signalling (Bredt *et al*, 1991). Homodimers of eNOS and nNOS are constitutively expressed in various tissues and require the presence of calcium and calmodulin to be activated. iNOS is an inducible form of NOS, and expression is stimulated by bacterial products and various cytokines (Xie *et al*, 1992). This form does not require calcium to be active as long as it is bound to calmodulin. iNOS is found in macrophages and is involved in fighting off bacteria, virus and tumour cells (Fang, 2004). While the different NOS enzymes received their names from the areas where they were first identified, their expression is in many different cell types. In the circulation, haemoglobin rapidly scavenges NO resulting in a very short half-life (seconds) under biological circumstances. The reaction with haemoglobin oxidizes NO to nitrate, which together with nitrite (NOx) have been extensively used as surrogate markers of NO production in biological samples.

As mentioned above, there are myriad effects of NO as a result of its unique structure with an unpaired electron, its lipophilic nature and its ability to diffuse to nearby targets, acting in both an autocrine and paracrine fashion. Many of its signalling functions, including vasodilation, are transmitted by the activation of soluble guanylyl cyclase leading to the production of cGMP (Hobbs and Ignarro, 1996; Figure 1). Other ways that NO can signal are via modification of proteins and fatty acids through nitrosation (–NO; Stamler *et al*, 2001) and nitration ($-NO_2$). In addition, NO regulates mitochondrial function by direct binding to and inhibition of cytochrome C oxidase

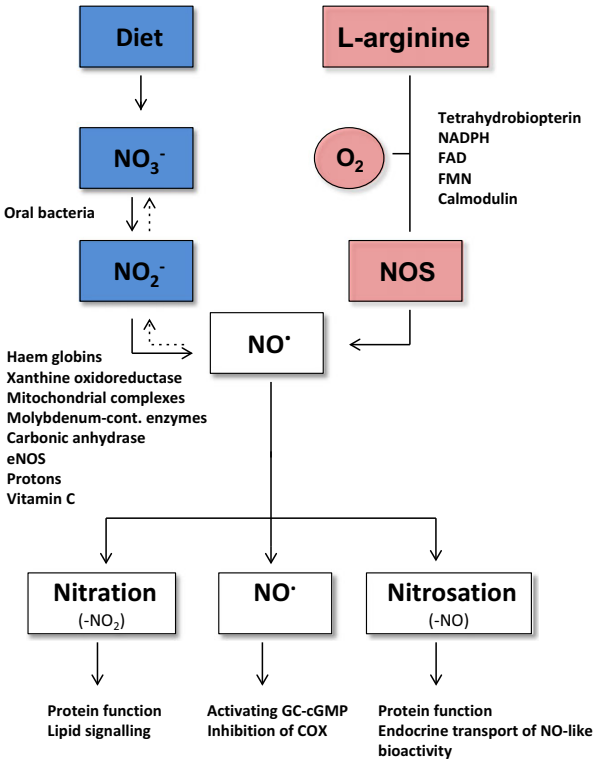

Figure 1 Generation of nitric oxide by nitric oxide synthase and the nitrate–nitrite–NO pathway. Nitric oxide synthases (NOS) catalyse the oxidation of the amino acid L-arginine in the presence of molecular oxygen and several cofactors. In biological fluids, NO is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) (dashed arrows). Endogenously generated nitrate and nitrate from the diet can be reduced to nitrite by oral commensal bacteria. Nitrite is absorbed systemically, and there are several enzymatic and non-enzymatic pathways for further reduction to NO and other reactive nitrogen intermediates. These nitrogen oxides can signal via nitration ($-NO_2$), direct NO signalling and nitrosation (–NO). NADPH, Nicotinamide adenine dinucleotide phosphate-oxidase, FAD, Flavin adenine dinucleotide, FMN, flavin mononucleotide, COX, cytochrome C oxidase

(Cleeter *et al*, 1994). Finally, high levels of NO such as those produced by iNOS in activated macrophages can be directly toxic which is used in killing of bacteria and tumour cells (Lundberg *et al*, 2004).

Role of nitric oxide in oral physiology

Nitric oxide synthase isoforms are found in the oral cavity of many mammals but with some species differences. In humans, nNOS was identified as localizing to the salivary gland parenchyma, ducts and blood vessels (Ceccatelli *et al*, 1994). nNOS localization was also described in nerve fibres around the submandibular and labial salivary gland acini, ducts and blood vessels (Looms *et al*, 2000), while the parotid and sublingual gland had limited staining (Soinila *et al*, 2006). Rats seem to have similar nNOS staining as humans but with a stronger intensity (Soinila *et al*, 2006). eNOS is localized to the glandular vascular endothelium and the luminal epithelium of the salivary ducts (Bentz *et al*, 1998; Soinila *et al*, 2006). iNOS has been identified in the salivary ducts of normal tissue

(Brennan *et al*, 2000). Another group identified slight ductal iNOS staining in the salivary glands (Soinila *et al*, 2006).

In general, the role of NO produced by eNOS and nNOS is for the regulation of oral blood flow as recently reviewed by Toda *et al* (2012). eNOS is activated through shear stress, bradykinin and insulin, while nNOS is important in non-adrenergic non-cholinergic transmission upon parasympathetic activation. The stimulation of perivascular nerves and increased blood flow by nicotine in the oral cavity is NOS-dependent (Toda *et al*, 1997).

Nitric oxide signalling may also play a physiological role in salivary gland secretion because nNOS is expressed in postganglionic parasympathetic neurons surrounding acini of salivary glands in many species (Modin *et al*, 1994). NOS is also present within the acinar cells (Looms *et al*, 2002).

Nitric oxide and other reactive nitrogen intermediates probably play a role in the oral non-specific immune system against microorganisms, bacteria and protozoa. Oral neutrophils generate NO and superoxide (O_2^-) leading to a complex chemistry of nitrogen intermediates involved in host defence (Takahama *et al*, 2008; Figure 2). Moreover, the high concentrations of nitrate and nitrite in normal saliva contribute to local generation of nitrogen intermediates with potentially protective effects including anti-bacterial properties and increased mucosal blood flow and oral mucus production. However, these aspects of salivary nitrate and nitrite have not yet been investigated. In contrast, salivary nitrate and nitrite have been implicated in oral and gastric carcinogenesis, but there is still a great deal of controversy within this field of research (Korde Choudhari *et al*, 2012). In general, NO is considered to be a 'double-edged sword' with respect to cancer where some aspects of NO signalling can promote tumour growth, while other aspects have anti-tumour effects (Burke *et al*, 2013).

The nitrate–nitrite–NO pathway

Dietary nitrate and nitrite

Besides being oxidation end products of NOS-derived NO, nitrate and nitrite are also ingested in our diet and drinking water. Much of ingested nitrate comes from green leafy vegetables having the highest nitrate content (EFSA, 2008). The vegetables with the highest nitrate content include beetroot, spinach, rocket, kale, celery, fennel and lettuces (Weitzberg and Lundberg, 2013). Daily ingested nitrate is on average 2–2.5 times that produced endogenously (Granli *et al*, 1989). Nitrate and nitrite are also found in meat products, where they are used in inhibiting microbial growth and preserving colour (Skibsted, 2011). Cured meats are the main source of dietary nitrite and have been postulated as initiating adverse health effects of nitrite in humans (Sindelar and Milkowski, 2012).

Nitrate is also found in water. In the mid-20th century, there were a number of case studies of infants with methaemoglobinemia (blue-baby syndrome) attributed to bacte-

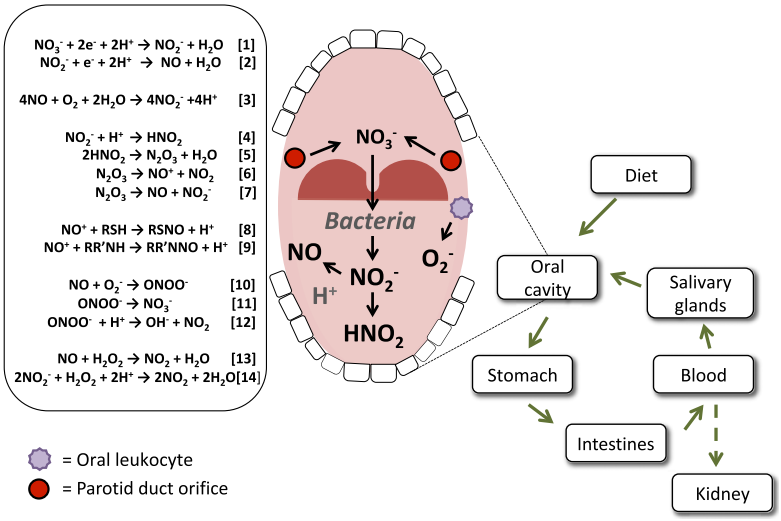

Figure 2 Enterosalivary circulation of nitrate and oral nitrogen oxide chemistry. *Enterosalivary circulation of nitrate* (*lower right*): Nitrate in our diet is efficiently absorbed in the gastrointestinal tract. From the blood stream, it mixes with endogenously generated nitrate from NOSs, some is taken into tissue, or excreted via the kidney, but 25% is actively taken up in the salivary glands and released with saliva into the oral cavity. In the oral cavity, bacteria reduce nitrate to nitrite which is swallowed along with the remaining nitrate. The nitrate re-enters the enterosalivary loop, and nitrite is subjected to further reduction to NO and other nitrogen oxides in blood and tissues. *Oral nitrogen oxide chemistry* (*middle and left*): The oral cavity is a complex milieu with bacteria, leucocytes, proteins, ions and other molecules having potential reactions with nitrate (NO_3^-), nitrite (NO_2^-) and nitric oxide (NO). Nitrate and nitrite can be reduced by commensal bacteria [reactions 1, 2]. NO can be oxidized to nitrite in the presence of oxygen [3]. Nitrite in an acidic environment can be protonated to nitrous acid (HNO_2) that can form intermediate dinitrogen trioxide (N_2O_3) and decomposes into NO and nitrogen dioxide (NO_2) or nitrosonium ion (NO^+) and nitrite [4–7]. The reaction of nitrite in an acidic environment is potentiated by reducing agents such as thiocyanate, ascorbate or urate. The nitrosonium ion can react with either reduced thiols (RSH) or secondary amines (RR'NH) to form nitrosothiols (RSNO) or N-nitrosamines (RR'NNO) [8, 9] Nitric oxide can also react with superoxide (O_2^-), a product of leucocytes to form peroxynitrite ($ONOO^-$) [10]. Depending on the environment, peroxynitrite can isomerize into nitrate [11] or in an acidic environment a hydroxyl radical (OH^-) and nitrogen dioxide [12]. NO and nitrite and can also react with hydrogen peroxide (H_2O_2) from bacteria and leucocytes to form nitrogen dioxide [13, 14]. This is not a comprehensive list of reactions but describes the various pressures that can affect nitrogen oxide components in the oral cavity

rially contaminated high-nitrate well water (Fawns and Aldridge, 1954). This increased occurrence was coupled to the growing application of nitrates in crop fertilization, leaking into the ground and contaminating well water. However, later research and re-examination of historical cases show a more complex aetiology with gastrointestinal infection and inflammation as being the cause in many cases (Avery, 1999) The raised susceptibility of infants to methaemoglobinemia is due to the fact that foetal haemoglobin may oxidize more easily than adult haemoglobin and infants have lower levels of methaemoglobin reductase.

The existing view on nitrate in our diet is one of concern due to its suggested carcinogenic effects. Acceptable daily intake levels are postulated by international authorities, and nitrate levels are controlled in food and drinking water (EFSA, 2008). However, in the recommendations from both the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority, it is concluded that nitrate intake from the diet is not associated with increased cancer risk (Speijers and van den Brandt, 2003; EFSA, 2008). Conversely, approximately 80% of our nitrate intake comes from vegetables, which are strongly associated with a decreased risk of cancer and cardiovascular disease (Hung *et al*, 2004).

Enterosalivary nitrate circulation

The body has an innate ability to circulate and preserve nitrate (Figure 2). Ingested inorganic nitrate is very efficiently absorbed into the circulation from the intestinal tract. After nitrate intake plasma levels rapidly increase (within 15 min) and stay elevated for a long time (half-life 5–6 h; Lundberg and Weitzberg, 2013). A major part (70–75%) of the ingested nitrate is excreted by the kidneys, but considerable salvage takes place through selective reabsorption of nitrate in the renal distal tubule (Packer *et al*, 1989) and by biliary (Fritsch *et al*, 1985) and salivary recirculation (Spiegelhalder *et al*, 1976; Granli *et al*, 1989).

Salivary recirculation of nitrate has been known for a long time, but the physiological importance of this active process has not been understood until recently. Approximately 25% of the circulating nitrate is actively taken up by the salivary glands and concentrated in the saliva (Spiegelhalder *et al*, 1976). There is an almost linear relationship between the amounts of nitrate ingested and amounts of nitrate found in saliva (Eisenbrand *et al*, 1980). The mechanism of nitrate concentration into the salivary gland has not been understood, but a competition with iodine, percholate and thiocyanate for uptake and concentration in saliva was early established (Edwards *et al*, 1954). Recently Qin *et al* (2012) suggested that the plasma membrane protein sialin mediates nitrate influx into the salivary gland by acting as an electrogenic NO_3^-/H^+ transporter in salivary acinar cells. They could show that knockdown of sialin expression reduced nitrate transport and that fibroblasts from humans with mutations in the sialin gene have a lower transport of nitrate compared with controls. This is a potentially important finding because uptake of nitrate and excretion into the saliva is a *sine qua non* for a functional nitrate–nitrite–NO pathway.

The parotid gland is the main contributor to salivary nitrate with its saliva containing approximately three times more nitrate compared with mixed whole saliva (Granli *et al*, 1989). Salivation caused by chewing and in response to taste increases the flow of saliva and decreases the salivary nitrate concentration, although the overall salivary nitrate output is increased (Granli *et al*, 1989). The concentration of nitrate found in saliva is influenced by multiple factors, and there is a great inter-individual variation, which could be dependent on the salivary flow rate, differences in the oral microflora, but also on different sampling techniques and methodologies for nitrate analysis (Hart and Walters, 1983; Granli *et al*, 1989; Takahama *et al*, 2008). In addition, there seems to be a diurnal variation with higher concentrations during night time (Mirvish *et al*, 2000).

Fasting levels of salivary nitrate are in the 100–500 μM range, which is already approximately 10 times higher than in plasma but after a nitrate load levels can increase 20- to 40-fold and reach 5–8 mM (Lundberg and Govoni, 2004). Due to the long half-life of circulating nitrate, salivary nitrate levels remain elevated many hours after a nitrate-containing meal (Klein *et al*, 1978; Pannala *et al*, 2003). A substantial part of the salivary nitrate is efficiently reduced by oral bacteria as described below, while the majority re-enters the gastrointestinal tract and cycles into the enterosalivary loop again. The nitrate ion in itself is inert and needs to be reduced to nitrite in order to exert any biological functions. Here, the oral microbiome plays a pivotal role.

Bacterial nitrate reduction

There are more than 300 different bacterial species residing in the human oral cavity of which a variety of facultative anaerobic bacteria with effective nitrate reductases are harboured in the crypts in the posterior part of the tongue (Duncan *et al*, 1995). These bacteria are able to respire on nitrate when oxygen availability is limited (Li *et al*, 1997). Having ample access to substrate, these bacteria constitutively and very efficiently reduce nitrate to nitrite leading to salivary nitrite concentrations during fasting conditions in the same range as nitrate. As fasting plasma nitrite levels are normally in the 50–100 nM range, there is a 100- to 1000-fold higher concentration in saliva and this difference increases markedly after nitrate intake (Lundberg and Govoni, 2004). Many bacterial species have nitrate and nitrite reductases, but some have been especially implicated in the reduction of nitrate to nitrite including *Streptococcus salivarius*, *S. mitis*, *S. bovis*, *Veionella* spp., *Staphyloccocus aureus* and *S. epidermidis*, *Nocordia* spp., *Corynebacterium* sp. (Li *et al*, 1997; Palmerini *et al*, 2003). Many of these bacteria also have additional reductases that enable denitrification of nitrate all the way to nitrogen gas (N_2) via formation of NO and N_2O (Zetterquist *et al*, 1999; Schreiber *et al*, 2010). Interestingly, this denitrification pathway has been found in dental plaques, and the generation of NO has interesting aspects in relation to the pathophysiology of periodontitis and caries (Schreiber *et al*, 2010).

The nitrite produced in the oral cavity is relative to the nitrate concentrations and also follows the temporal

increases in salivary nitrate (Zetterquist *et al*, 1999; Lundberg and Govoni, 2004). The reducing activity in the oral cavity seems to be consistent before and after a nitrate load (Walters and Smith, 1981). Current data are scarce on how the oral microbiome relates to the nitrate–nitrite–NO pathway, and the biological effects of dietary nitrate and more work are certainly needed.

Even if some nitrate reductase activity has been reported by mammalian cells (Jansson *et al*, 2008), nitrate reduction by the oral commensal bacteria is absolutely central in order for nitrate to be converted to nitrite, thereby enabling biological effects. Some bacteria residing more distally in the gastrointestinal tract have the same intrinsic capacity (Sobko *et al*, 2005), but they seem not be important in this context. This is probably due to the efficient uptake of nitrate and nitrite proximally in the GI-tract, which minimizes exposure of these anions to the bacteria residing in more distal parts of the gut. The experimental evidence for this assumption is provided by several studies where the biological effects of dietary nitrate are completely abolished by the use of an oral anti-bacterial mouthwash (Shapiro *et al*, 1991; Govoni *et al*, 2008; Petersson *et al*, 2009; Hendgen-Cotta *et al*, 2012; Kapil *et al*, 2013). It is worth mentioning here that the use of an anti-bacterial triclosan-containing toothpaste does not seem to affect nitrate reduction, probably due to not reaching the bacteria involved (Bondonno *et al*, 2012).

Significant amounts of nitrite-containing saliva are continuously swallowed (approximately 1-l per day) and in the acidic gastric environment nitrite is rapidly protonated yielding several bioactive nitrogen oxides as mentioned above (Figure 2; Lundberg *et al*, 1994). The possible physiological importance of these reactions will be discussed below. However, most of the nitrite enters the lower GI-tract unchanged where it is rapidly absorbed and reaches the systemic circulation. After nitrate ingestion, an increase in plasma levels of nitrite can be observed already after 15–30 min reaching peak levels after 60–90 min (Lundberg and Govoni, 2004). Nitrite itself has a relatively short plasma half-life of 20–30 min, but when generated from ingested nitrate, the enterosalivary circulation of nitrate will generate new nitrite for a more prolonged time.

In summary, facultative anaerobic bacteria residing in the low-oxygen environment of the crypts of the tongue use nitrate and nitrite as final electron acceptors in their respiration. By doing so, they help the mammalian host to convert the inert nitrate anion to nitrite that can then be further used systemically to generate NO and other bioactive nitrogen oxides. Without this symbiotic relationship, the nitrate–nitrite–NO pathway would not be functional, and in the next section, we will describe different pathways for systemic generation of NO from nitrite.

Systemic nitrite reduction

Once nitrite reaches the systemic circulation, there are multiple endogenous enzymatic and non-enzymatic pathways in blood and tissues for its reduction to NO and other reactive nitrogen intermediates (Figure 2; Gladwin *et al*, 2005). Common denominators for all these pathways are that nitrite reduction is enhanced by hypoxia and low pH (van Faassen *et al*, 2009). This presents a striking difference compared with the canonical NOS-dependent generation of NO, which is oxygen dependent.

Haemoglobin and myoglobin are important nitrite reducers. In the oxygenated state, they act as scavengers of NO, thereby partly regulating the biological half-life and action of NO, but in the deoxygenated state, they have been identified to reduce nitrite most efficiently (Doyle *et al*, 1981; Cosby *et al*, 2003). This nitrite reduction is dependent on the haemoglobin conformation and is most active at 50% oxygen saturation levels (Huang *et al*, 2005). This dependency on oxygen saturation to produce NO has been suggested as a mechanism for hypoxia regulated vasodilation (Cosby *et al*, 2003; Maher *et al*, 2008). Similarly deoxygenated myoglobin is an important intracellular nitrite reductase which by comparison is more potent than haemoglobin (Shiva *et al*, 2007a). Myoglobin in the presence of nitrite has a protective effect in myocardial ischaemia–reperfusion injury, which is lost in myoglobin null mice (Hendgen-Cotta *et al*, 2008). Other haemoglobins such as neuroglobin and cytoglobin have also been shown to reduce nitrite under *in vitro* conditions (Tiso *et al*, 2011; Li *et al*, 2012).

Mammalian molybdenum-containing enzymes have structures similar to bacterial and plant nitrate- and nitrite-reducing enzymes and have been identified for the ability to catalyse the reduction in nitrite (Figure 2). In humans, this includes xanthine oxidoreductase, aldehyde oxidase and sulphite oxidase. Xanthine oxidoreductase is mainly involved with purine catabolism and producing hydrogen peroxide and superoxide, but has been shown to reduce nitrite under low oxygen levels (Li *et al*, 2001; Webb *et al*, 2004). Xanthine oxidoreductase has also been identified as a nitrate reductase and as such can potentially reduce nitrate and nitrite to NO (Millar *et al*, 1998; Jansson *et al*, 2008). Aldehyde oxidase is a cytosolic protein that can reduce nitrite to NO under physiological anaerobic and aerobic conditions (Li *et al*, 2008). Sulphite oxidase has been presented to reduce nitrite similarly (Wang *et al*, 2011). The activity of these enzymes as nitrite reductases is influenced by the presence of oxygen and the redox state of the metal centres associated with the enzymes.

The mitochondrial complexes III and IV have also been identified to reduce nitrite to NO, but the importance of these reactions for mitochondrial function is still controversial (Shiva, 2010; Larsen *et al*, 2012). In addition, carbonic anhydrase (Aamand *et al*, 2009) and even eNOS (Vanin *et al*, 2007) have been suggested to reduce nitrite to NO.

Taken together, there are several pathways for nitrite reduction to bioactive NO in the circulation and tissues that are differentially regulated by oxygen availability and redox status and are to some extent tissue specific. In the next section, we will discuss recent findings on the role of the nitrate–nitrite–NO system in health and disease.

The nitrate–nitrite–NO pathway in health and disease

Gastric integrity and host defence

Swallowed salivary nitrite is rapidly protonated in the acidic gastric milieu resulting in the formation of NO and

other nitrogen intermediates including HNO_2, N_2O_3 and NO_2 (Benjamin *et al*, 1994; Lundberg *et al*, 1994; Lundberg and Weitzberg, 2013). Some of this chemistry underlies the long withstanding view of nitrite causing cancer through the formation of N-nitrosamines, a versatile class of carcinogens. Indeed, N-nitrosamines can be formed from nitrite in the stomach, but there is no solid evidence that nitrate ingestion, even with the conversion to nitrite in the oral cavity, is associated with a higher risk of cancer (EFSA, 2008).

In contrast, recent data suggest a protective role of salivary nitrite in the stomach (Lundberg and Weitzberg, 2013). Petersson and colleagues showed that dietary nitrate in rats increased mucosal blood flow and mucus production, two major protective mechanisms in the stomach. This was accompanied by reduced mucosal leakage after administration of aggressors (Petersson *et al*, 2007). In addition, Björne *et al* (2004) found that human saliva collected after high nitrate intake increased blood flow and mucus production when placed onto the gastric mucosa of rats. In addition, dietary nitrate protects against gastric ulcers induced by a non-steroidal anti-inflammatory drug (NSAID) in the rat (Jansson *et al*, 2007).

Salivary nitrite in the acidic milieu of the stomach has potent anti-bacterial effects. Benjamin *et al* (1994) elegantly showed potent anti-bacterial effects of nitrite on enteropathogens such as *Salmonella*, *Shigella*, *Yersinia* and *Escherichia coli* grown under acidic conditions. Similar effects were found by Björne and colleagues who could show that bacterial growth of *E. coli* and *Candida albicans* was inhibited by a mixture of human gastric juice and human saliva rich in nitrite compared with saliva low in nitrite. These findings were paralleled by a high production of NO and nitroso/nitrosyl species from nitrate-rich saliva (Björne *et al*, 2006).

In aggregate, these data suggest that salivary nitrite could be a part of a first-line defence to protect the stomach against aggressors and bacteria. In addition, when swallowed nitrite-containing saliva reaches the acidic gastric environment, a variety of nitrogen species including NO are formed that may have beneficial effects not only in the stomach but also systemically (Bonacci *et al*, 2012; Lundberg and Weitzberg, 2013).

Cardiovascular function

As inorganic nitrate can be serially reduced to NO in the body, it was reasonable to investigate whether dietary nitrate could have NO-like effects such as vasodilatation and lowering of blood pressure. In 2006, Larsen *et al* could show that dietary sodium nitrate, in amounts comparable to a high vegetable intake, reduced blood pressure in healthy volunteers. These findings were confirmed by a series of studies using beetroot juice, a natural source of nitrate. Beetroot juice dose dependently and acutely lowers diastolic and systolic blood pressure in healthy subjects as well as in patients with hypertension (Webb *et al*, 2008; Kapil *et al*, 2010; Ghosh *et al*, 2013). In addition, nitrate in human studies also improves vascular endothelial function (Heiss *et al*, 2012), inhibits platelet aggregation (Webb *et al*, 2008) and promotes the release of circulating angiogenic cells from the bone marrow (Heiss *et al*, 2012; Figure 3). In some studies, a placebo beetroot juice has been used, in which inorganic nitrate was selectively eliminated, and this placebo juice had no effect (Lansley *et al*, 2011b). This strongly indicates that inorganic nitrate is the responsible ingredient behind the observed effects by beetroot juice. The central role of the oral microbiome in these studies is evident because disruption by either an anti-bacterial mouthwash or spitting abolishes the effects (Webb *et al*, 2008). Taken together, these findings suggest protective effects of dietary nitrate in the cardiovascular system and that the well-established beneficial effects on cardiovascular function by diets rich in vegetables (Appel *et al*, 1997) may be related to the nitrate content. Interestingly, green leafy vegetables, which are especially high in nitrate, often stand out in epidemiological studies as

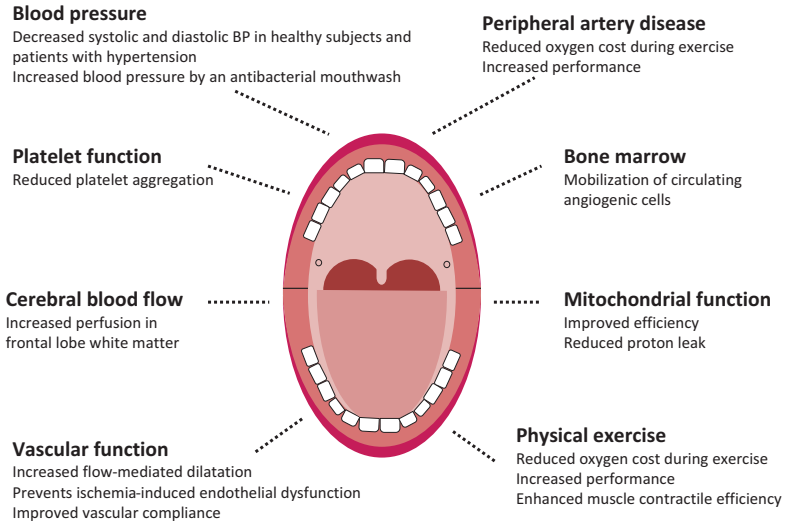

Figure 3 Human studies with dietary nitrate. Physiological and therapeutic effects of inorganic nitrate in humans. The centrally located oral cavity highlights the importance of the oral microbiome in converting nitrate to nitrite.

particularly protective against cardiovascular disease (Hung *et al*, 2004) and type 2 diabetes (Carter *et al*, 2010; Cooper *et al*, 2012).

Other effects of nitrate and nitrite

In animal studies, dietary nitrate or nitrite protect against ischaemia–reperfusion injury in the heart (Duranski *et al*, 2005), liver (Duranski *et al*, 2005), brain (Jung *et al*, 2006), kidney (Carlstrom *et al*, 2011) and skeletal muscle (Kumar *et al*, 2008). The exact mechanism(s) has not yet been pinpointed and probably varies in different organs, but a reversible inhibition of complex I in the mitochondrial respiratory chain, leading to reduced generation of oxygen radicals as well as less apoptosis, has been suggested (Shiva *et al*, 2007b).

Several human studies now show that dietary nitrate reduces oxygen cost and improves performance during exercise (Larsen *et al*, 2007; Bailey *et al*, 2009; Lansley *et al*, 2011a). For a recent meta analysis on the ergogenic effects of dietary nitrate, the reader is referred to the study by Hoon *et al* (2013). There are several plausible mechanisms underlying these effects, and our group has shown that dietary nitrate improves skeletal muscle mitochondrial efficiency by down-regulation of two proteins involved in mitochondrial uncoupling (uncoupling protein 3 and adenine nucleotide translocator; Larsen *et al*, 2011). Other suggested mechanisms are increased skeletal muscle intracellular Ca^{2+} release and contractility (Hernandez *et al*, 2012) and improved skeletal muscle blood flow (Ferguson *et al*, 2013). With respect to the pluripotency of NO, several different mechanisms could very likely work in parallel.

For a more extensive overview on additional effects of inorganic nitrate and nitrite, the reader is referred to recent review articles (Lundberg *et al*, 2008; Kevil and Lefer, 2012; Weitzberg and Lundberg, 2013).

Physiological role of endogenous nitrate

The obligatory role of oral bacteria for conversion of nitrate to nitrite has been shown in several experimental settings. In humans, the blood pressure-lowering effects of dietary nitrate mentioned earlier are completely abolished with an anti-bacterial mouthwash (Lansley *et al*, 2011b). Spitting as another means of disrupting the enterosalivary circulation of nitrate abrogates the effects of beetroot juice on blood pressure in humans (Webb *et al*, 2008). In intubated patients in the Intensive Care Unit who have reduced salivary production and reduced capacity to swallow saliva, stomach levels of NO are almost abolished (Björne *et al*, 2005; Weitzberg *et al*, 2010). Moreover, in germfree animals, salivary nitrate reduction is absent (Sobko *et al*, 2004). Most of these studies have shown that disruption of the oral microflora abolishes effects of exogenous nitrate, but what about the role of endogenous nitrate derived from oxidation of NOS-derived NO? Is this formerly considered inert end product of endogenous NO-generation recyclable and does it have any physiological function?

This question was addressed in a recent study by Kapil *et al* (2013) in healthy subjects. By limiting exogenous sources of nitrate from the diet combined with daily treatments with an anti-bacterial mouthwash, they found markedly reduced conversion of salivary nitrate to nitrite in conjunction with a decrease in circulating plasma nitrite. These effects were accompanied by a significant increase in systolic and diastolic blood pressure that correlated strongly to the reduction in plasma nitrite. This study nicely shows that recycling of endogenously produced nitrate by oral bacteria has a physiological role in modulating blood pressure and therefore suggests that oral bacteria are involved in regulating cardiovascular function. Another interesting aspect of this study still to be proven is that chronic use of an anti-bacterial mouthwash in a population may lead to an overall elevation in blood pressure, which is highly coupled to the incidence of stroke. Based on these preliminary findings, further studies on the chronic use of mouthwash in relation to cardiovascular disease would be extremely interesting, especially because other oral bacteria causing periodontitis have been associated with increased risk of cardiovascular disease (Kim and Amar, 2006). There might be a fine balance between protecting commensal nitrate-reducing bacteria and treating bacteria causing periodontitis, and the net effect on the risk of cardiovascular disease is unknown. Even if there might be an overlap between the bacterial species responsible for reducing salivary nitrate and the ones involved in periodontitis, the difference in anatomical location might be of importance.

Summary

The inorganic anion nitrate, until recently considered only harmful to man, is now becoming appreciated as the largest pool of potential NO bioactivity in the body. Via the nitrate–nitrite–NO pathway, NO and other nitrogen intermediates are generated. The therapeutic potential of nitrate and nitrite is currently being investigated in several cardiovascular diseases such as hypertension, pulmonary hypertension, myocardial ischaemia and peripheral artery disease. Moreover, the ergogenic effects of dietary nitrate are explored and were quickly adopted by athletes in the sports world. From a nutritional viewpoint, the possibility that inorganic nitrate might contribute to the well-established health effects of vegetables is gaining increasing interest. A role of endogenously generated nitrate in physiological regulation of cardiovascular function was very recently suggested, and data show that the amount of nitrate generated by NOSs is sufficient to participate in physiological regulation of blood pressure.

The obligatory role of the oral microflora for the nitrate–nitrite–NO pathway is yet another example of the importance of the human microbiome. This symbiotic relationship is important for systemic NO homoeostasis and modulation of cardiovascular and metabolic function. Further studies should be directed at exploring the uptake mechanisms and excretion of nitrate in the salivary glands and the importance of inter-individual variance in the oral microflora, including possible gender differences. Moreover, the consequences of the widespread use of anti-bacterial mouthwashes among the population ought to be investigated from a cardiovascular perspective. Clearly, these are important areas of research where scientific interest in oral physiology could be of great importance for allopathic medicine.

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Author contributions

EW produced the outline. EW and MPH shared in the writing, editing and figure design.

Conflict of interest

EW is listed on patents related to the therapeutic use of inorganic nitrate and nitrite.

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