Yes to "NO" host flora symbiosis

Alexandria Nichols, Khandaker A. Ahmed and Rakesh P. Patel
(University of Alabama at Birmingham, USA)

Nitric oxide (NO) is a relatively simple molecule comprising only two atoms. Understanding how this free radical controls an array of complex biological functions provides the platform for much of the research in NO biochemistry and biology. Here, we discuss an updated perspective on how this gas is formed in the body involving a fascinating interplay between the diet, bacteria residing on the tongue, and redox reactions that are regulated by pH and local oxygen tensions. We highlight this as an area primed for novel microbe-targeted therapeutics for controlling NO production and affecting human health and disease.

The functions ascribed to nitric oxide (NO) in humans are many and diverse, including but not limited to regulating blood flow, neuronal signal transduction, innate immunity, inflammation, coagulation, cell survival and death. Like any biological signalling pathway there has to be controlled formation, selectivity in the reactions that follow and activation of a signalling cascade culminating in a cellular response. Finally, there should be mechanisms that counter this activation to allow the pathway to be slowed or turned off. NO signalling checks all these boxes with an extensive scientific literature detailing mechanisms for each of these key aspects. In the standard model of NO signalling, NO is produced by one of three nitric oxide synthase (NOS) enzymes, which catalyse the conversion of L-arginine to NO to amplify local signalling pathways. In this article, we focus on a relatively new or updated perspective on how NO formation occurs in mammals that instead, involves bacteria residing on the tongue. According to this model, these bacteria reduce internally and externally produced nitrate to nitrite, a reaction that bacteria catalyse to support bacterial metabolism. The nitrite that is produced is further metabolized by an array of mammalian (host) proteins to produce NO and mediate NO signalling in all major organs. In this paradigm, NO bioavailability is controlled not by a single host enzyme, but also by symbiotic interactions between the environment, microbes in the mouth and several host proteins.

How is nitric oxide formed? Is it all nitric oxide synthase?

It is well established that the major source of NO in mammals is controlled by NOS enzymes that catalyse the NADPH-dependent reaction of L-arginine with oxygen to form NO and L-citrulline. These enzymes are formed by the combination of two non-covalently bonded subunits with each subunit containing flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin and Fe(3+)-haem, which collectively facilitate the five-electron reduction of L-arginine to yield NO. There are three types (or isoforms) of NOSs, neuronal (NOS 1), inducible (NOS 2) and endothelial (NOS 3). These are regulated by distinct mechanisms including transcriptional, translational and post-translational processes that result in different magnitudes and temporal profiles for NO formation, ranging from low (nM) levels over long periods of time, to high (μM) levels over short time periods[1]. Another important feature of the different NOS enzymes is their compartmentalization into specific tissues, cells and subcellular locales. This allows NO production to occur in close vicinity to its targets allowing control of the activated signalling pathways. Indeed, the compartmentalized production of NO is a central feature of how this free radical modulates so many different functions.

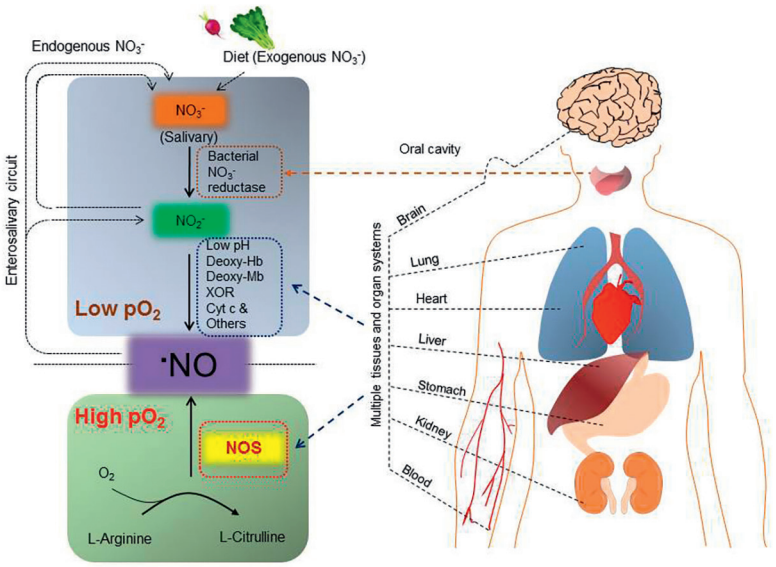
The idea that there are NOS-independent mechanisms for NO formation is not new. In plant and prokaryotic systems, the presence of nitrate- and nitrite-reductase enzymes that may generate NO is widely appreciated and known to play key roles ranging from denitrification and regulating bioenergetics to evading host immune responses. But what about mammals; are there similar reactions that reduce nitrate and nitrite to NO? In mammals, nitrate and nitrite are commonly thought of as oxidation products of NO that are inert, and often used as markers for NO formation. That said, under extreme conditions of low oxygen, and/or very low pH (e.g. in the stomach), nitrite can be reduced by one electron to NO and other NO-containing intermediates[2,3]. The concept that nitrate and nitrite may be sources of NO-signalling equivalents in mammalian systems was largely ignored for a long time because there were no known enzymatic systems equivalent to a plant or bacterial nitrate or nitrite reductase. However, around the time NOS enzymes were discovered, it was also shown that facultative anaerobic bacteria residing on the tongue express functional nitrate-reductase enzymes, and can reduce nitrate to nitrite in saliva[4]. In parallel with these observations were intriguing studies showing that nitrate from fruit and vegetables in the diet was absorbed from the upper gastrointestinal system into the blood and then concentrated into the saliva

to reach millimolar concentrations. The fact that this is a concerted process suggests biological importance and the idea emerged of an entero-salivary nitrate-reduction cycle that provides a controlled supply of nitrate to oral microbes, which use this as a substrate to generate reducing equivalents for respiration and other microbial processes[5]. While beneficial to the bacteria, the advantage to the mammalian host was less clear and limited to preventing oral infections and dental caries[4]. Systemic effects were overlooked, largely because the product was nitrite, and as indicated above, at the time of these findings, nitrite was not thought to be particularly important for NO bioavailability except under extreme biological conditions.

Nitrite-reduction mechanisms – the missing piece of the nitrate-reduction puzzle

Fast forward to today, and we now know that nitrite is not an inert anion in mammals, but in fact tightly controlled and a key player in NO homeostasis[3]. Nitrite may be reduced by a one-electron process to produce NO and other NO-containing products including S-nitrosothiols and nitrated lipids. Importantly, mechanisms controlling this biochemistry are operational at physiological nitrite concentrations. The new insight that provided the 'missing piece' of the puzzle was the understanding that metallo- and haem proteins (e.g. haemoglobin) are able to facilitate, or increase the rate of nitrite reduction via mechanisms that still involve hypoxia (low oxygen tension) and low pH. The general model is that lowering oxygen tension alters the reactivity of the metal centre with nitrite. Several candidate metalloproteins have been discussed and amongst the first and perhaps most studied is haemoglobin, which also provides a useful model to illustrate the mechanism and function[6].

Specifically, deoxyferrous haem reduces nitrite to NO. In other words, nitrite reduction to NO only occurs when haemoglobin is deoxygenated. This reaction is also faster at lower pH thereby limiting or compartmentalizing this pathway to metabolically active, high oxygen-consuming and lactate-producing tissues. This pathway has been discussed as a mechanism for how hypoxia increases local blood flow via NO formation, and couples this with oxygen delivery. The comparisons with NOS-dependent NO formation are interesting. Both are regulated by oxygen, but with NOS, oxygen is a substrate, with nitrite-reduction mechanisms, however, oxygen could be considered an inhibitor; for example, oxygen will promote oxyhaemoglobin formation, and oxyhaemoglobin oxidizes and thus depletes nitrite. Several candidate metalloproteins have been described to possess properties similar to haemoglobin that couple oxygen sensing to nitrite reduction, with specific biochemical and functional aspects differing[3]. For example, haemoglobin-dependent



Current perspective of NO-formation pathways in mammals. In the standard pathway, NO formation is catalysed by one of three nitric oxide synthases (NOS) using L-arginine as a substrate. This process is oxygen dependent and NO-signalling compartmentalization is based where the NOS is located; collectively three NOSs provide NO-generating capacity in all major organs and tissues. On the other hand is the entero-salivary circuit of NO formation whereby facultative anaerobes expressing nitrate reductase in the oral cavity, reduce salivary nitrate (NO3-) to nitrite (NO2-), nitrate being supplied from endogenous formation (via NO or nitrite oxidation) or from the diet (referred to as the entero-salivary circuit). In contrast to NOS, nitrite reduction to NO is facilitated at low oxygen and low pH, and compartmentalization of NO signalling in this paradigm is controlled by the location of an array of putative nitrite reductases (e.g. haemoglobin, myoglobin etc.)

nitrite reduction is optimal at ~27 mmHg oxygen and controls blood flow and coagulation, whereas with the related protein myoglobin, oxygen levels have to drop to below ~10 mmHg before nitrite reduction occurs with the function being regulation of mitochondrial respiration and reactive species formation in cardiac muscle cells. This comparison also illustrates how compartmentalized NO formation can occur in this paradigm. So while NOSs are the 'professional' NO-generating proteins, whose primary function is to catalyse NO formation, proteins that mediate nitrite reduction could be considered as the 'back-ups' that ensure NO signalling is maintained when oxygen levels drop and NOS pathways become inactivated. In this model, metalloproteins that possess nitrite-reduction reactivity are protein catalysts that speed-up nitrite reduction and provide regulation of this process.

Role of the entero-salivary system: why mouthwash could be bad for you

Returning to the entero-salivary system discussed earlier; nitrate is concentrated into the saliva where it is reduced to nitrite by tongue bacterial nitrate reductases. Nitrite is then swallowed. In the stomach, some of the

nitrite undergoes acidic disproportionation to form NO and nitroso-species that have local effects in controlling gastric-mucosal functions, but some is also absorbed into the blood where it supports NO signalling in all major organs via reactions with an array of metalloproteins. The functionality of this model has been demonstrated in many physiological and therapeutic contexts especially where low oxygen (i.e. tissue ischaemia) and low pH are biological features[7]. The linkage between nitrate, nitrite and NO may also partly explain the health benefits of consuming diets rich in fruits and vegetables, the latter providing more than 80% of the nitrate exposure to humans[8]. Conversely, lack of nitrate-derived NO may contribute to increased susceptibility to cardiovascular disease in communities with a low intake of fruits and vegetables. The preponderance of evidence for this cycle in humans comes from experiments showing that basal blood pressure, platelet coagulation and exercise performance are modulated by the presence of nitrate in the diet and oral nitrate-reductase activity. The basal effects referred to were gleaned by showing that oral microbe depletion using antiseptic mouthwash, increases basal blood pressure in healthy individuals[9]. This effect is worth pointing out because it demonstrates the potential for the entero-salivary system to play a key role in NO homeostasis in the presence of a functioning NOS-dependent NO-signalling system. There are several excellent reviews and articles which discuss nitrate to nitrite to NO mechanisms, biological effects and therapeutic potential. In the remainder of this article, we highlight areas of this pathway that are less understood.

Oral microbes in the spotlight

Bacteria expressing nitrate reductase enzymes (NAR) are pivotal for the entero-salivary system. However, our current knowledge regarding the specific bacteria expressing this activity, which classes of NAR are involved, how they are regulated and whether this changes in any pathophysiologic setting remains poor. This is likely to alter with the application of high throughput and sensitive analyses using 16S RNA sequencing and metagenomics, together with the ever-increasing appreciation of the importance of our microbiome in health and disease. Bacterial species that have been identified as containing genes for nitrate reductase include Staphylococcus sp. (S. aureus, S. epidermidis, S. bovis), Streptococcus sp. (S. mittis), Corynebacterium sp., Rothia sp., Actinomyces sp. Prevotella sp., Fusobacterium sp. and Veillonella sp., with the latter considered to be the largest contributor to nitrate reduction[10]. Knowledge is limited regarding their regulation however.

Most bacteria in the oral cavity are facultative anaerobes living in the crypts of the tongue, but why they predominantly occupy the posterior tongue remains unclear. Many of the nitrate-reductase expressing bacteria also express nitrite reductase enzymes that catalyse denitrification i.e. reduce nitrite to NO, N_2O and N_2. Furthermore, some bacteria may reduce nitrite to ammonia via dissimilatory nitrate-reduction pathways. Conceptually, this sets up an interesting balance where the fate of nitrite generated in the oral cavity is dependent on competing biochemical and physical processes related to saliva volume, swallowing of saliva, nitrite reduction to N_2 or ammonia. Can this balance be modulated? Does it change in different pathophysiologic scenarios related to oral health and diseases associated with altered NO bioavailability? Can it be targeted therapeutically to improve NO bioavailability? Moreover, little is known about how nitrate is concentrated into the saliva. These are all questions for which we have little current insight. That said, emerging evidence and ideas suggest that indeed the oral nitrate-reducing microbiota may be regulated.

As proposed recently[11], diversion from nitrite formation to ammonia formation may exacerbate chronic kidney and heart disease and link oral microbial dysbiosis, loss of NO and chronic inflammation. Moreover, oral microbes are spatially proximal to exposure to reactive and toxic substances present in cigarette smoke and other inhaled environmental pollutants. Such stresses are strongly associated with a host of cardiopulmonary inflammatory diseases characterized by loss of NO bioavailability. Whether inhaled toxicants negatively affect the oral nitrate-reducing microbiome has significant implications for environmental toxicology mechanisms and requires testing. Finally, emerging studies show that oral bactericidal solutions, like chlorohexidine, or the presence of nitrate in the diet shift the oral microbial community. Assuming nitrate reduction to nitrite and then to NO is concomitantly increased or decreased, such data reflect the dynamic nature of this pathway and suggest that indeed, it can be modulated to affect systemic NO bioavailability[8].

Prospects

The entero-salivary nitrate-reduction pathway and NOS-dependent NO-formation processes operate, and are regulated by, distinct and complementary mechanisms. Evidence demonstrating that the entero-salivary system is competent to mediate NO-dependent processes underscores the potential for controlling this pathway in the management of diseases associated with decreased NO bioavailability. Changes in lifestyle such as improved oral health and increased dietary nitrate may be relatively simple approaches to treat common but difficult diseases

including hypertension, atherosclerosis and diabetes. This understanding also raises intriguing possibilities regarding the use of oral probiotics and the notion of being able to repopulate the 'good' bacteria or restoring natural conditions from a microbial imbalance. That said, much still needs to be learned about this fascinating interplay of the diet, bacteria in the oral cavity and nitrite-reduction pathways. ■

Glossary:

Entero-salivary Nitrate Reduction: the system of nitrate reduction to nitrite catalysed by nitrate reductases present in bacteria residing on the tongue. Nitrite is swallowed and absorbed into the blood stream, where some is reduced to nitric oxide, and some oxidized to nitrate, which accumulates in the saliva and again provides substrate for oral nitrate reductases.

Microbial dysbiosis: development of an imbalance in the microbiota compared to the normal condition.

Nitric oxide bioavailability: the ability for NO to stimulate a signal transduction pathway depends on the concentration, or amount of NO. The amount of NO is determined by how much is formed versus how much NO is consumed by other pathways that do not lead to physiologic signalling. The term NO bioavailability reflects this balance and the amount of NO available to stimulate physiologic soluble guanylyl cyclase signalling.

Alexandria L. Nichols is a senior undergraduate majoring in Molecular Biology at the University of Alabama at Birmingham. She belongs to the Science and Technology Honors College at UAB and has been working in the Department of Pathology in Dr Rakesh Patel's lab on the development of a method for measuring and characterizing oral nitrate reductase activity. Email: aln835@uab.edu

Khandaker Ahtesham Ahmed received his PhD from Kumamoto University, Japan in 2011. He is currently pursuing his postdoctoral training at the Department of Pathology, UAB. His research interest focuses on the regulation of nitric oxide (NO) bioavailability by oral nitrate-reducing bacteria and its impact on acute and chronic lung injury models. Email: kahmed@uabmc.edu

Rakesh Patel received his PhD from the University of Essex, UK in 1996. He moved to pursue postdoctoral studies at UAB, where he is currently a Professor in the Department of Pathology and Director of the Center for Free Radical Biology. His research interests have centred on understanding the role of nitric oxide and other oxidative/nitrosative intermediates in modulating acute and chronic inflammatory diseases. Email: rakeshpatel@uabmc.edu.

References

1. Griffith, O.W. and Stuehr, D.J. (1995) Nitric oxide synthases: properties and catalytic mechanism. Annu. Rev. Physiol. **57**, 707–736
2. Lundberg, J.O., Gladwin, M.T., Ahluwalia A., et al. (2009) Nitrate and nitrite in biology, nutrition and therapeutics. Nat. Chem. Biol. **5**, 865–869
3. van Faassen, E.E., Bahrami, S., Feelisch, M., et al., (2009) Nitrite as regulator of hypoxic signaling in mammalian physiology. Med. Res. Rev. **29**, 683–741
4. Lundberg, J.O., Weitzberg, E., Cole, J.A. and Benjamin, N. (2004) Nitrate, bacteria and human health. Nat. Rev. Microbiol. 2, 593–602
5. Lundberg, J.O., Weitzberg, E. and Gladwin, M.T. (2008) The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat. Rev. Drug. Discov. **7**, 156–167
6. Gladwin, M.T., Raat, N.J., Shiva, S., et al., (2006) Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. Am. J. Physiol. Heart Circ. Physiol. **291**, H2026–2035
7. Kapil, V., Weitzberg, E., Lundberg, J.O. and Ahluwalia, A. (2014) Clinical evidence demonstrating the utility of inorganic nitrate in cardiovascular health. Nitric Oxide **38**, 45–57
8. Bryan, N.S. and Ivy, J.L. (2015) Inorganic nitrite and nitrate: evidence to support consideration as dietary nutrients. Nutr. Res. **35**, 643–654
9. Kapil, V., Haydar, S.M., Pearl, V., Lundberg, J.O., Weitzberg, E. and Ahluwalia, A. (2013) Physiological role for nitrate-reducing oral bacteria in blood pressure control. Free Radic. Biol. Med. **55**, 93–100
10. Hezel, M.P. and Weitzberg, E. (2015) The oral microbiome and nitric oxide homoeostasis. Oral Dis. **21**, 7–1
11. Briskey, D., Tucker, P.S., Johnson, D.W. and Coombes, J.S. (2016) Microbiota and the nitrogen cycle: implications in the development and progression of CVD and CKD. Nitric Oxide **57**, 64–70

Further Reading

- Bryan, N.S., Fernandez, B.O., Bauer, S.M., et al., (2005) Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. Nat. Chem. Biol. **1**, 290–297
- Coelho, C. and Romao, M.J. (2015) Structural and mechanistic insights on nitrate reductases. Protein Sci. **24**, 1901–1911
- Qin, L., Liu, X., Sun, Q., et al., (2012) (SLC17A5) functions as a nitrate transporter in the plasma membrane. Proc. Natl. Acad. Sci. USA **109**, 13434–13439
- Vitturi, D.A. and Patel, R.P. (2011) Current perspectives and challenges in understanding the role of nitrite as an integral player in nitric oxide biology and therapy. Free Radic. Biol. Med. **51**, 805–812