The emerging biology of the nitrite anion

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Nitrite has now been proposed to play an important physiological role in signaling, blood flow regulation and hypoxic nitric oxide homeostasis. A recent two-day symposium at the US National Institutes of Health highlighted recent advances in the understanding of nitrite biochemistry, physiology and therapeutics.

Over the last five years, a growing body of evidence suggests that the nitrite anion (NO_2^-), present in abundance in blood and tissues, may represent the largest intravascular and tissue storage form of nitric oxide (NO). During physiological and pathological hypoxia, nitrite is converted to NO via reactions with hemoglobin, myoglobin, xanthine oxidoreductase and heme- and thiol-containing enzymes, and by acidic reduction. These different reactions provide for graded nitrite reduction to NO along the entire physiological and pathological oxygen and pH spectrum (**Fig. 1**). Nitrite is now being recognized as a critical 'hypoxic buffer', potentially contributing to the regulation of hypoxic vasodilatation and hypoxic mitochondrial respiration, and to the modulation of ischemia-reperfusion tissue injury and infarction. Additionally, a previously unknown function for hemoglobin as an enzymatic nitrite reductase has now been identified that converts nitrite to NO, an effect that is maximal at about 50% oxygen saturation. This newly understood role of hemoglobin as a nitrite reductase, as well as other pathways for nitrite reduction to NO, are relevant in the context of signaling, blood flow regulation, oxygen sensing and nitrite-based therapeutics. These mechanistic discoveries have now led to further identification of potential roles for nitrite in the treatment of a variety of diseases characterized by ischemia and hemodynamic dysregulation, including neonatal pulmonary hypertension, subarachnoid hemorrhage associated vasospasm, ischemia-reperfusion injury to heart and liver, hypertension, and sickle cell disease.

On September 8th and 9th, 2005, a two-day symposium was held at the US National Institutes of Health (NIH) in Bethesda, Maryland, to address recent advances in our understanding of the role of nitrite in physiology, pathophysiology and therapeutics. The topics covered a wide breadth, including mechanisms of nitrite formation, transport, and enzymatic reduction to NO; nitrite as a substrate for nitration, nitrosation and nitrosylation reactions; the existence of a symbiotic 'nitrogen cycle' in humans; and the emerging role for nitrite in blood flow homeostasis, hypoxic vasodilatation, and therapeutics.

Historical perspective: the 'rediscovery' of nitrite

Until quite recently, our understanding of the impact of inorganic nitrogen oxides on humans was confined to their well-known actions as environmental pollutants. However, knowledge of their beneficial and/or physiological effects has a long and rich history (**Box 1**). By far, the most ancient example is the use of nitrate salts to cure foods, which not only imparts a pleasing color to meats but also is a very effective agent against the bacterium that causes botulism. However, as Anthony Butler (St. Andrews University) pointed out, documents dating to around 800 AD suggest that nitrite and nitrate were used by the Chinese medicinally to relieve "acute heart pains, and cold in the hands and feet". The 20th century witnessed important insights into the physiological effects of nitrite in particular. With the discovery of mammalian NO synthase enzymes in the late 1980s, nitrite was largely considered to be only an end product of NO metabolism. In the past few years, evidence has been mounting that shows nitrite *per se* may have important physiological and pathophysiological functions.

Chemical biology of the nitrite anion

In simple aqueous systems, the major product of the oxidative decomposition of NO is nitrite, whereas *in vivo* NO can react with oxygenated heme proteins (such as hemoglobin and myoglobin) to form nitrate. Despite the presence of heme proteins in blood and tissue that should preferentially oxidize NO to nitrate, significant quantities of nitrite form, suggesting the presence of 'NO oxidase' metal complexes in plasma and tissue. These were discussed by Sruti Shiva (National Heart, Lung, and Blood Institute (NHLBI), NIH). Except for the red blood cell, little is known about the cellular uptake and metabolism of nitrite, although surprisingly high levels have been reported for vascular tissue[1], supporting the notion that nitrite may represent a storage form of NO. Once nitrite reaches the intracellular space, its fate depends on enzymatic activities (reductases or oxidases), the prevailing level of oxygenation and likely the redox state. Under physiological conditions, with oxygen concentrations ranging from

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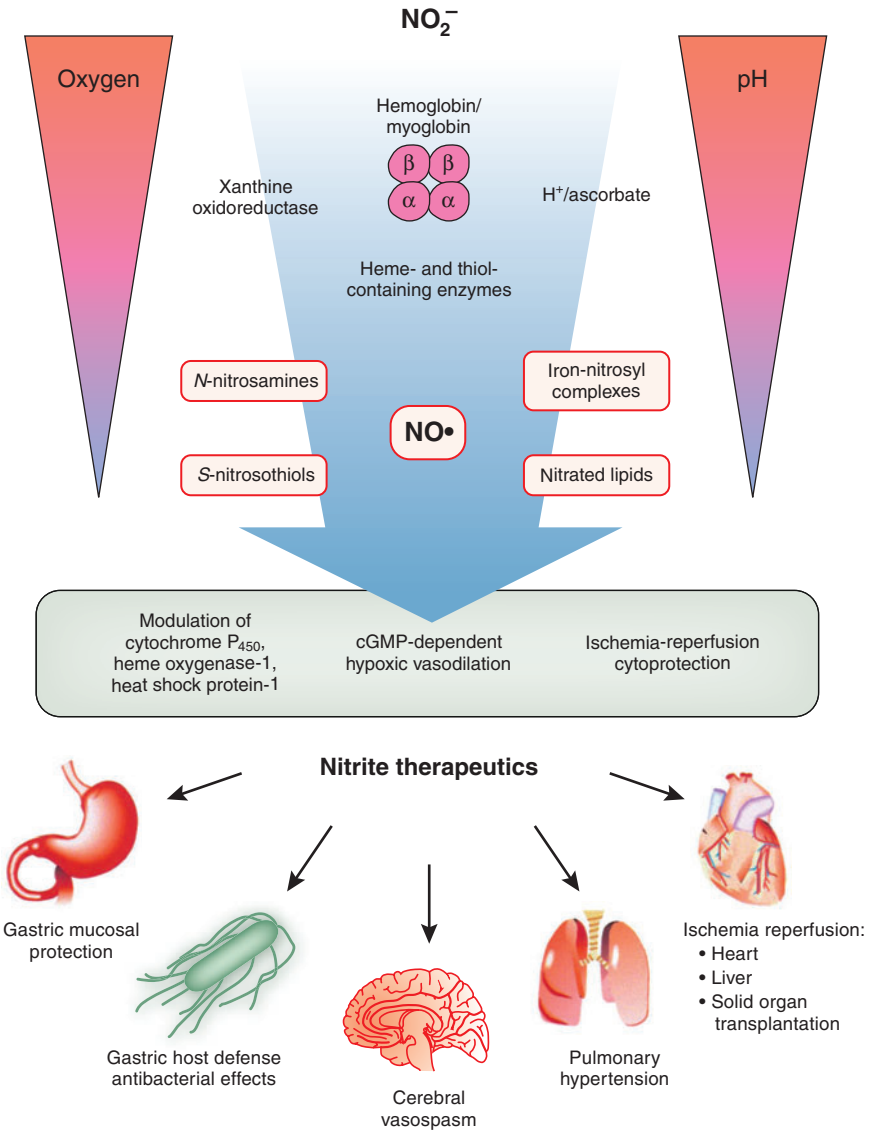

Figure 1 Nitrite chemistry, physiology and therapeutics. Nitrite is reduced to NO and potentially nitros(yl)ates and nitrates proteins and lipids along the physiological oxygen and pH gradient, ultimately modulating important signal transduction pathways, physiological functions and disease.

micro- to low nanomolar, depending on vascular bed and cellular compartment, nitrite can be rapidly transformed into nitroso and nitrosyl species[2,3]. Under ischemic conditions, as in episodes of prolonged hypoxia or anoxia, considerable amounts of nitrite are consumed to form nitroso and nitrosyl species and NO (refs. 3–7), leading to the concept of a 'hypoxic buffer' that provides NO in an oxygen-independent manner (as opposed to NO synthase, which requires oxygen as a cofactor). Thus, nitrite offers a unique redox chemistry that can generate either radical, NO or NO_2, depending on whether it undergoes reduction or oxidation[8]. Stanley Hazen (the Cleveland Clinic) outlined how, during inflammation, nitrite also serves as a substrate of peroxidases, giving rise to formation of nitrated species[9,10], including nitrotyrosine and nitrated unsaturated fatty acids. As discussed by Bruce Freeman (University of Pittsburgh), basal human plasma levels of bioactive nitrated oleic and linoleic acids exceed 1 μM in concentration, thus representing a large pool of 'stored' NO, in addition to nitrite. During cycling of the erythrocyte between normoxia and hypoxia in the presence of nitrite, an even greater extent of fatty acid nitration was noted, raising the possibility that fatty acid nitro derivatives are transducing some of the actions of nitrite. This is a realistic possibility, as nitroalkenes can undergo decay to yield NO and also mediate receptor-dependent signal transduction, via action as high-affinity peroxisome proliferator-activated receptor (PPAR) ligands[11,12].

Nitrite as a signaling molecule

Bryan *et al.* recently reported that nitrite is a signaling molecule in its own right, even under physiological conditions (that is, in the absence of ischemia). Specifically, they demonstrated that nitrite increases cyclic GMP (cGMP) formation, inhibits cytochrome P_{450} activity and affects the expression of two archetypical proteins, heat shock protein 70 and heme oxygenase-1. These effects were observed in a number of tissues *in vivo* and at nitrite concentrations far lower than those required to decrease blood pressure or oxidize hemoglobin[3]. Most surprisingly, a number of these actions of nitrite did not seem to be mediated by NO, but rather were consistent with *S*/*N*-nitroso and iron-nitrosyl-heme species formed from nitrite via mechanisms involving a thiol- and heme-containing enzyme system. In the context of these discussions, Martin Feelisch (Boston University) hypothesized that the present cell signaling functions of nitrite represent a vestige of early metabolic processes that originated from denitrifying microorganisms long before the advent of aerobic respiration and the emergence of an NO synthase enzyme system. The finding that both systems (nitrite reductase and NO synthase) still coexist today suggests that the nitrite pathway must serve a critical function that is distinct from that of NO (**Fig. 2**). Thus, the tissue-protective actions of nitrite may not be limited to ischemic events but may operate, by means of different mechanisms, under physiological conditions as well.

Mechanisms of nitrite reduction to NO

Considering the apparent facile chemical transformations of nitrite into NO and nitros(yl)ated proteins *in vivo*, an improved understanding of mechanisms of bioactivation is required. This is of particular interest to therapeutics, considering the fact that nitrite is a product of the metabolism of nitroglycerin and other organic nitrates. A number of pathways have been explored, including nonenzymatic pathways, such as acidic reduction (disproportionation)[4,13,14], and enzymatic pathways, including reduction by xanthine oxidoreductase[15,16] and deoxygenated hemoglobin and myoglobin[17]. The acidic reduction of nitrite requires protonation and a one-electron reduction. The relatively low pK_a of nitrite of 3.4 limits this activity to ischemic tissue or the stomach mucosa (the latter pathway discussed below). Jay Zweier (Ohio State University) discussed the contribution of this pathway in the

ischemic heart as evidenced by the anaerobic formation of iron-nitrosylated myoglobin during ischemia in the Langendorf model and *in vivo* as measured by EPR imaging of blood iron-nitrosyl-hemoglobin formation in mouse models of anoxic arrest.

Although xanthine oxidase is known to reduce molecular oxygen to superoxide (O_2^-), at low oxygen tensions and pH values, this enzyme can also reduce nitrite to NO at the molybdenum site of the enzyme, with xanthine, NADH or aldehyde substrates providing the reducing equivalents. A role for this enzyme in physiological nitrite reduction was the subject of considerable debate, as this reaction requires low oxygen tensions and abundant superoxide dismutase to scavenge O_2^-, which will otherwise react rapidly with NO, forming peroxynitrite, NO_2 and/or N_2O_3. In modeling physiological conditions, Margaret Tarpey (University of Pittsburgh) demonstrated that minimal NO forms from nitrite by this pathway in the absence of superoxide dismutase. However, inhibition of xanthine oxidase in heart homogenates during anoxia inhibits approximately 50% of NO generation from nitrite[5]. How much xanthine oxidoreductase contributes to nitrite reduction, versus peroxynitrite production, during pathological ischemia or during graded reductions in oxygen during physiological stress, such as exercise, clearly requires further study. An additional mechanism of nitrite reduction by mitochondria was proposed by Hans Nohl (University of Vienna), with the demonstration that respiring mitochondria reduce nitrite to NO by ubiquinol that binds to the cytochrome bc1 site of complex III, resulting in measurable reductions in respiratory rate at nitrite concentrations of 50 μM[18].

One major focus of the meeting was the reaction of nitrite with hemoglobin, myoglobin and heme systems (inspired by recent discoveries that hemoglobin possesses a nitrite reductase enzymatic activity[2,19]) and the vasodilator activity of nitrite under physiological conditions, associated with the rapid *in vivo* formation of iron-nitrosyl-hemoglobin[2]. These physiological observations are consistent with a reaction between nitrite and deoxyhemoglobin to form NO characterized by Doyle and colleagues in 1981 (ref. 20):

$$NO_2^- + HbFe^{II}\ (\text{deoxyhemoglobin}) + H^+ \rightarrow NO\ (\text{nitric oxide}) + HbFe^{III} + OH^-$$

Much of the formed NO is then captured as iron-nitrosyl-hemoglobin ($HbFe^{II}$-NO) on vicinal hemes, which may serve as a 'dosimeter' of NO production in blood:

$$NO + HbFe^{II} \rightarrow HbFe^{II}\text{-NO}$$

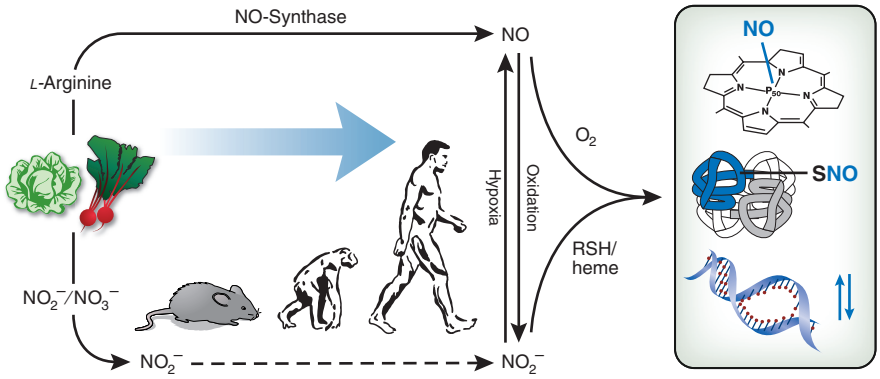

Figure 2 Origin and interconversion of nitric oxide (NO) and nitrite (NO_2^-) and their role in cell signaling and gene expression. Nitrite is the major oxidative decomposition product of NO, but it also represents a major storage form that is bioactivated and/or recycled to form NO under hypoxic conditions. The system is fueled by the dietary intake of precursor molecules. Both pathways have been conserved throughout evolution, coexisting in present-day mammals both to regulate the function and activity of multiple proteins by S-nitrosation and heme nitrosylation, and to modulate reactions with other biological molecules.

Recent studies require a reappraisal of this apparently simple reaction. Rather than a simple reaction between nitrite and deoxyheme, the Hogg, Kim-Shapiro and Gladwin groups (Medical College of Wisconsin, Wake Forest University, and NHLBI, respectively) have shown that the reaction is in fact under allosteric control[17,21]. During the anaerobic reaction of nitrite with deoxyhemoglobin (T-state tetrameric conformation) the products of this reaction, iron-nitrosyl-hemoglobin and methemoglobin, both stabilize the R-state and drive a T-to-R allosteric transition in the hemoglobin quaternary structure. This lowers the heme redox potential (making the heme a better electron donor), which effectively increases the bimolecular rate constant for the reaction between nitrite and the remaining deoxyhemes (on R-state tetramer). This anaerobic reaction between nitrite and deoxyhemoglobin represents a novel chemical reaction process, termed 'allosteric autocatalysis', where the reaction of one nitrite molecule with one heme decreases the redox potential of vicinal hemes on either the same tetramer or neighboring tetramers, which in turn reduce nitrite more rapidly and generate more R-state tetramers. Interestingly, this reaction can be considered the reverse of reductive nitrosylation, as reviewed by Peter Ford (University of California at Santa Barbara), in which methemoglobin is reduced by NO to form iron-nitrosyl-hemoglobin and nitrite ($Fe^{III} + 2NO + e^- \rightarrow Fe^{II}\text{-}NO + NO^+$)[22]. The rate of this latter reaction increases as the heme redox potential increases (that is, the heme is more readily reduced) and is catalyzed by nitrite.

As described by Mark Gladwin (NHLBI, NIH), under physiological conditions, oxygen, rather than NO-heme and metheme, dictate the allosteric state of hemoglobin. Under these conditions the rate of nitrite reduction is maximal at an ideal balance between the most available deoxyhemes necessary for nitrite binding (maximal in T-state) and low redox potential of the heme necessary for electron transfer from heme to nitrite (maximal in R-state). This balance is maximum at the P_{50} of hemoglobin, when hemoglobin is approximately 50% saturated with oxygen (**Fig. 3**). This chemistry appears ideally suited for hypoxic oxygen sensing and NO generation, as the hemoglobin P_{50} 'set point' is chemically and thermally responsive to tissue metabolism and allosteric effectors. Consistent with this thesis, Eric Feigl (University of Seattle, Washington), presented evidence that nitrite consumption across the canine coronary circulation was directly proportional to myocardial oxygen consumption; this correlation is necessary for a putative feedback vasodilator, although it is certainly not sufficient to prove that nitrite is responsible for this physiological activity. Although these studies support a potential physiological role for hemoglobin, myoglobin, neuroglobin and other heme proteins as hypoxia-dependent nitrite reductases, more work is required to fully characterize this biochemical oxygen sensor-nitrite reductase paradigm.

Examination of the molecular mechanism of bacterial nitrite reductases (NIR) may provide clues as to how hemoglobin reduces nitrite. Grant Mauk (University of British Columbia) reviewed the two classes of bacterial nitrite reductases: the copper-containing enzymes and the multiheme-containing enzymes with two histidine residues at the active site by the d_1 heme[23,24]. Some analogy may be proposed

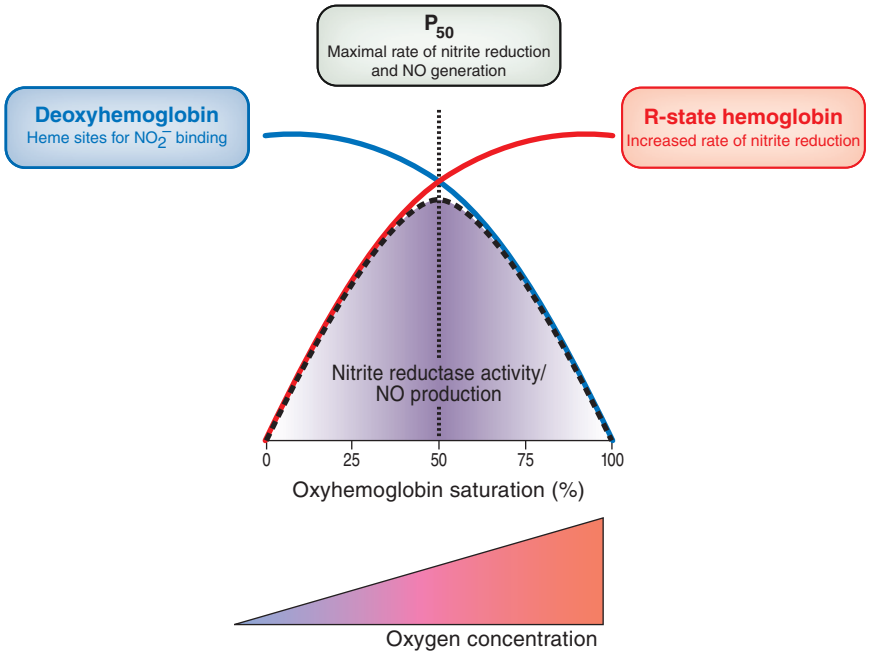

Figure 3 Nitrite reductase activity of hemoglobin. The rate of nitrite reduction is maximal at the balance point between the highest amount of available deoxyhemes, which are necessary for nitrite binding (maximal in T-state), and the lowest redox potential of the heme, which is necessary for electron transfer from heme to nitrite (maximal in R-state). This balance occurs at the P_{50} of hemoglobin, when hemoglobin is approximately 50% saturated with oxygen.

between these enzymes and hemoglobin: For example, the copper nitrite reductase coordinates nitrite at the type 2 Cu site via proton donation from an aspartate residue, followed by a 12.5 Å electron transfer across a cysteine-histidine bridge from the type 1 Cu to the type 2 Cu site. Hemoglobin is known to coordinate water in the heme pocket via proton donation from the distal histidine, and electron transfer between the heme iron and the 13 Å distant β-93 cysteine (which can bind Cu) of hemoglobin has been described[25,26], suggesting analogous activity. In hemoglobin, coordination of nitrous acid (which is consistent with the pH dependence of the kinetics of the reaction[17,20]) may be analogous to nitrite binding at the active site of the multiheme and copper nitrite reductases. This mechanism might also explain allosteric control of the hemoglobin nitrite reductase activity reported recently[17,21]; the R-state conformation would be better positioned for the histidine to form a hydrogen bond with the heme-bound nitrous acid. Consistent with these mechanisms, Joseph Rifkind (National Institute on Aging, NIH) reviewed data suggesting that the heme pocket of hemoglobin traps an intermediate that can be converted to NO under reductive conditions but is not scavenged and does not exhibit an iron-nitrosyl EPR spectrum[19]. He proposed an electronic delocalization between Fe^{II}-NO^+, a cysteine thiyl radical and Fe^{III}-NO, which may stabilize nitrite as an intermediate state for NO or *S*-nitrosothiol release. As emphasized by Mauk of the University of British Columbia, coupled proton and electron transfer reactions are currently of considerable interest, and the source of electrons and possible involvement of the β-93 cysteine in hemoglobin-mediated nitrite reduction deserves further exploration.

Nitrite as an index of NO synthase activity

Owing to the rapid metabolism of nitrite in blood (largely forming nitrate, NO_3^-) and the difficulties in its analytical determination, the published literature on the levels of nitrite in mammalian blood have been inconsistent, and thus the value of plasma nitrite as a general marker of constitutive endothelial NO synthase (eNOS) activity *in vivo* has been unclear. With the advent of more reproducible and sensitive methodologies, coupled with a better understanding of nitrite biochemistry, Malte Kelm (Heinrich-Heine University) presented data showing that plasma nitrite levels are conserved across various mammalian species, including humans, in the range of 150–600 nM[27]. This accounts for ~40% of the entire nitrite pool in blood; red blood cells carry the residual proportion. As discussed by Alan Schechter (National Institute of Diabetes and Digestive and Kidney Diseases, NIH), levels of nitrite in human blood have been found to be at the lower end of this range, and erythrocytes thus represent a major storage pool of nitrite in blood, the levels of which are modulated by oxygen tension and eNOS activation[28]. Frank Jensen (Syddansk University, Denmark) reviewed the mechanisms of nitrite uptake in fish and mammals and described how the concentration of nitrite within the red cell seems to be under oxygen-dependent control, with increased uptake rates during red cell deoxygenation; this flux likely occurs via Cl^- channels and protonation (Donnan equilibrium), but a role for the anion exchange protein (band 3) cannot be excluded[29].

Approximately 80–90% of basal plasma nitrite is derived from eNOS-derived NO, suggesting that nitrite could be used as a biomarker to evaluate endothelial function[27]. Consistent with this proposal, studies assessing blood flow in the human forearm showed that plasma nitrite mirrors acute changes in eNOS activity after stimulation with a variety of eNOS-dependent vasodilators[30]. Kelm also reviewed data suggesting that plasma nitrite levels reflect endothelial dysfunction in patients suffering from cardiovascular disease. In a cohort of 351 blood donors, plasma nitrite levels decreased with increasing number of cardiovascular risk factors. In another subset of patients with endothelial dysfunction, plasma nitrite levels correlated significantly with the degree of (NO-dependent) flow-mediated vasodilatation and inversely with intima-media thickness. These studies suggest that plasma and red cell nitrite levels are reliably measurable in humans using new analytical techniques and that low levels reflect endothelial dysfunction and correlate with cardiovascular risk factors.

Role of the red cell in the transduction of NO-dependent activity

Although a physiological role for nitrite in blood flow regulation had been deemed unlikely based on the low potency of nitrite in classical aortic ring preparations (EC_{50} of 100 μM nitrite)[31], Gladwin and colleagues observed artery-to-vein gradients of nitrite in the human circulation and increased consumption of nitrite during exercise stress[32]. As reviewed by Richard Cannon (NHLBI, NIH), during NO gas inhalation in humans, a peripheral vasodilatation was measurable during pharmacological inhibition of NO synthase activity, suggesting endocrine transport of NO bioactivity in blood[33]. Because this vasodilation was associated with increases in blood nitrite, further human studies were performed

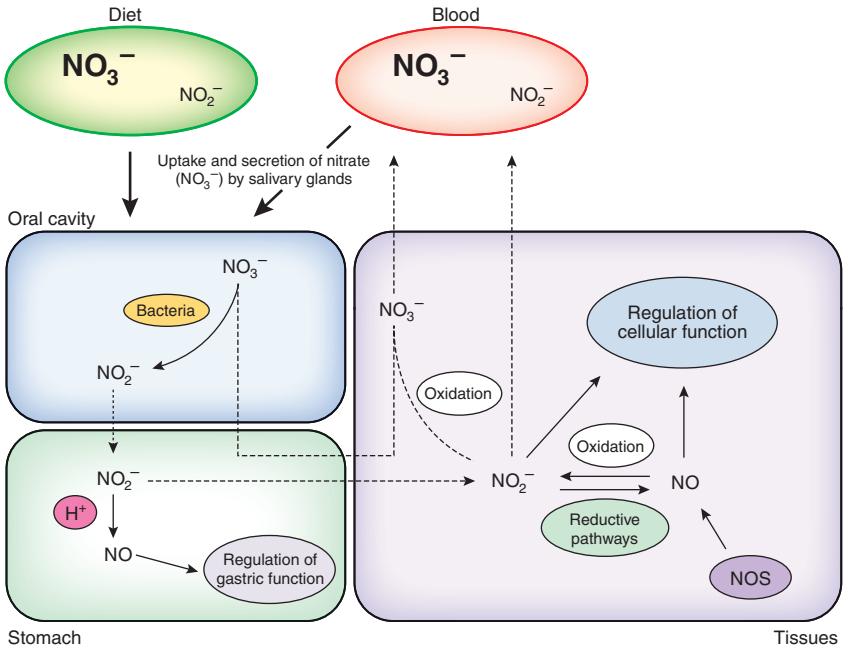

Figure 4 The human nitrogen cycle. Dietary nitrate is rapidly absorbed into the bloodstream, where it mixes with endogenous nitrate from the NOS/NO pathway. A large portion of nitrate is taken up by the salivary glands, secreted with saliva and reduced to nitrite by symbiotic bacteria in the oral cavity. Salivary-derived nitrite is further reduced to NO and other biologically active nitrogen oxides in the acidic stomach. Remaining nitrite is rapidly absorbed and accumulates in tissues, where it serves to regulate cellular functions via reduction to NO or possibly by direct reactions with protein and lipids. NO and nitrite are ultimately oxidized to nitrate, which again enters the enterosalivary circulation or is excreted in urine.

evaluating the vasodilatory activity of nitrite infusions and intravascular formation of NO and NO-modified proteins[2]. Surprisingly, nitrite rapidly vasodilated the human forearm circulation at near-physiological nitrite concentrations (2.5 μM) and was converted to iron-nitrosyl-hemoglobin in direct proportion to the degree of physiological hemoglobin deoxygenation. Consistent with a relatively high vasodilatory potency for nitrite *in vivo*, Andrew Arai (NHLBI, NIH) presented data showing that 8 μM nitrite decreases systolic blood pressure by more than 17 mm Hg in anesthetized dogs.

Rakesh Patel (University of Alabama at Birmingham) reviewed recent studies suggesting that nitrite, red cells (or hemoglobin) and hypoxia are required for *in vitro* hypoxic vasodilatation of rat aortic rings. Indeed, in the presence of hypoxia and erythrocytes (conditions not tested in historical aortic ring bioassay studies[31]), nitrite vasodilated aortic rings at physiological concentrations of 100–500 nM (ref. 2). Kinetic deoxygenation studies suggest that this vasodilation occurs as hemoglobin unloads oxygen to 50% saturation, and that this vasodilation is mediated by a maximal nitrite reductase activity of hemoglobin allosterically linked to its intrinsic P_{50} (ref. 34). Coupled with data from the Kelm group showing that nitrite concentrations are lower and may predict vascular dysfunction in cardiovascular disease, the mechanisms outlined here support a bioactive function for nitrite as a hypoxia-activated pool of NO bioactivity.

A major focus of discussion and controversy surrounded the potential mechanisms for NO escape from the red blood cell. Daniel Kim-Shapiro (Wake Forest University) and Jack Lancaster (University of Alabama at Birmingham) reviewed the kinetic constraints on this escape: NO produced after nitrite reduction would react at nearly diffusion-limited rates with vicinal oxy- or deoxyhemes, effectively inactivating or trapping the NO, respectively. Despite these biophysical limitations, Patel presented data demonstrating NO gas production from the reaction of nitrite with red cells and NO-dependent aortic ring vasodilation and formation of cGMP, indicating NO-dependent signal transduction[34]. Accordingly, Christian Hunter (Loma Linda University) presented data from studies in the anesthetized sheep showing that infusions of nitrite produced vasodilation associated with simultaneous increases in exhaled NO gas, and Jensen reviewed data showing NO formation (measured by amperometric electrode) after addition of nitrite to blood in a fish heart perfusion model.

Gladwin discussed potential solutions to this paradox including (i) the formation of chemical intermediates such as N_2O_3, HNO_2^-, NO_2^{-2}, $NO_2\bullet$ and $ONOO^-$ (formed from NO from nitrite reduction and superoxide from hemoglobin autooxidation, both maximal around P_{50}), (ii) formation of low–molecular weight *S*-nitrosothiols in the heme pocket, and (iii) facilitated NO release from the red cell nitrite reductase 'metabolon'. According to the latter hypothesis, the assembly of proteins within the lipid-rich red cell membrane raft, including AE1/band 3 (which binds deoxyhemoglobin and methemoglobin and could transport nitrite), carbonic anhydrase (which generates protons), Rh and aquaporin channels (potentially transporting NO or intermediates) and mixed hybrids of deoxyhemoglobin, methemoglobin and carboxyhemoglobin (providing R-state nitrite reductase activity), could catalytically amplify nitrite reduction and potentially facilitate NO or intermediate export. The investigation of mechanisms responsible for the export of NO bioactivity from the red cell was identified as a major challenge for the field.

Nitrite therapeutics and cytoprotection

Several therapeutic applications for nitrite were presented, all targeting diseases with reduced tissue oxygen and pH associated with critical regional decreases in blood flow. For example, as reviewed by Hunter, inhaled nitrite was shown to selectively vasodilate the pulmonary circulation in a hypoxic sheep model, with hypoxic potentiation of NO gas formation, iron-nitrosyl-hemoglobin formation and vasodilation[35]. Ryszard Pluta (National Institute of Neurological Disorders and Stroke, NIH) presented data on two-week infusions of nitrite in a primate model of postaneurysmal hemorrhage-induced middle cerebral artery vasospasm that resulted in stable plasma nitrite levels, clinically insignificant methemoglobinemia, increased levels of cerebral spinal fluid nitrite and *S*-nitrosothiols, and complete inhibition of cerebral vasospasm[36]. Nigel Benjamin (Universities of Exeter and Plymouth) reviewed the application of topical acidified nitrite as an NO donor for the treatment of skin infections and ulcerations. Multiple groups presented data indicating that low doses of nitrite prevent ischemia-reperfusion cellular infarction in the Langendorf heart preparation[5], in the mouse liver and heart[6] and in dogs. These effects were observed at extremely low doses of nitrite with concentrations approaching physiological levels (as low as 1.2 nmol doses, producing

200 nM levels in blood; peak effects observed at 10 μM). Mechanisms proposed for this effect included xanthine oxidoreductase dependent nitrite reduction to NO[5], deoxyhemoglobin- and myoglobin-mediated nitrite reduction to NO[6], a direct effect on mitochondrial susceptibility to anoxia, and transcriptional induction of the mitochondrial biogenesis program (data presented by Shiva of NHLBI).

The human nitrogen cycle: symbiosis and host defense

Second only to the NO synthases, the diet represents the major source of nitrite/nitrate in mammals[37]. Nitrite formation occurs mainly via reduction of dietary nitrate, which is abundant in many foods, such as vegetables and drinking water. After absorption in the small intestine, about 25% of circulating nitrate is actively taken up by the salivary glands and concentrated in saliva. Commensal bacteria in the oral cavity then reduce nitrate to nitrite[37] and, when swallowed, this nitrite reacts with the acidic gastric juice, thereby yielding a variety of reactive nitrogen oxides including NO (**Fig. 4**)[14,37].

There has been concern about the effects of dietary nitrate and nitrite on human health because nitrite can promote the generation of potentially carcinogenic nitrosamines[38]. However, any link between nitrate or nitrite and cancer in humans remains unclear[37]. Moreover, recent studies suggest important gastroprotective effects of the NO produced locally by acidification of salivary-derived nitrite. These include potent antibacterial effects[14] and enhancement of mucosal blood flow and mucus generation (**Fig. 4**)[39].

Nitrite as an essential nutrient?

An exciting possibility exists that dietary nitrate and nitrite have systemic NO-like effects, and a study by Lundberg & Govoni supports this notion[40]. They showed that plasma nitrite increases greatly after ingestion of inorganic nitrate. This increase is entirely due to entero-salivary circulation of nitrate and reduction to nitrite by oral bacteria. The total body load of nitrite after ingestion of a nitrate-rich meal (for example, 100 g of spinach) would amount to >1 μmol kg^{-1}, which is intriguing, as potent cardioprotective effects of nitrite have been observed in the same dose interval (0.1–2 μmol kg^{-1})[6]. Interestingly, Feelisch presented data suggesting that the majority of nitrite in tissues in fact originates from the exogenous intake of nitrite and nitrate and not from endogenous eNOS-dependent NO formation. Removal of nitrate and nitrite from the diet in these studies resulted in decreased tissue levels of nitrite and a concomitant downregulation of the signaling pathways modulated by this anion. Interestingly, although tissue levels varied greatly with nitrate and nitrite intake, the plasma levels of nitrite changed only slightly, suggesting 'crosstalk' between NO- and nitrite-regulating pathways in blood.

Taken together, these findings illustrate the pivotal role of diet and symbiotic nitrate-reducing bacteria in regulating basal systemic levels of nitrite and other bioactive nitroso/nitrosyl compounds. This new knowledge could have a profound impact on our view of the role of diet and commensal bacteria in the regulation of normal physiological processes and in relation to cardiovascular disease. Most provocatively, these studies suggest that the cardioprotective effects of leafy green vegetables could derive from nitrite, in addition to the often cited antioxidant effects of these food groups.

Conclusions and future research directions

We have attempted to summarize the breadth of information covered in this meeting on nitrite biochemistry, physiology and therapeutics, which we believe represents the

BOX 1 HISTORY OF NITRITE AND NITRIC OXIDE

- **5000 years ago:** 'wall saltpeter' or 'nitre' ($Ca(NO_3^-)_2$) used in cave communities near the Dead Sea to preserve food[41]
- **Ancient Greeks, Romans, Phoenicians:** salt used extensively for food preservation[41]
- **Eighth century:** China, Dunhuang scrolls: first written description of use of nitrate/nitrite for a cardiovascular disorder
- **1817–1822:** Kerner in Germany: identifies omission of nitrate from sausage salt as common feature of 'sausage poisoning' or 'botulism' (from Latin 'botulus' or sausage)[41]
- **1865:** Hermann: describes of reaction of nitric oxide with hemoglobin[42]
- **1867:** Brunton: discovers amyl nitrite as a treatment for angina, first nitrovasodilator[43]
- **Late 1800s:** addition of saltpeter for development of 'cured color'[41]
- **1897:** Van Ermengen: cause of botulism is a neurotoxin produced by a bacterium, which he identified and named *Bacillus botulinus*[41]
- **1899:** Lehmann, Kisskalt: nitrite rather than nitrate confers red color to meats[41]
- **1901:** Haldane: on basis of experiments with blood and hemoglobin, he proposes that the reaction of hemoglobin with nitric oxide derived from nitrite is the chemical basis for cured meat color[44]
- **1916:** Mitchell: humans produce nitrate[45]
- **1925:** Haldane: description of NO-hemoglobin in blood of a septic patient[46]
- **1928:** Lewis and Moran: suggest nitrite has antimicrobial effects[41]
- **1937:** Brooks: reaction of nitrite with deoxyhemoglobin[47]
- **1953:** Furchgott: nitrite vasodilates aortic strips (although unphysiological concentrations)[31]
- **1970s:** Definitive evidence for the relative contribution of nitrite in controlling *C. botulinum* in foods[41]
- **1977:** Murad: NO activates guanylate cyclase[48]
- **1981:** Tannenbaum: mammals synthesize nitrate[49,50]
- **1981:** Doyle: reaction of nitrite with deoxyhemoglobin[20]
- **1986:** Furchgott, Ignarro: independently propose that endothelium-derived relaxing factor (EDRF) is NO (refs. 52,53)
- **1987:** Feelisch and Noack: differing potencies of nitrovasodilators correspond with ability to liberate NO[51]
- **1987:** Ignarro, Moncada: EDRF and NO are indistinguishable based on key chemical properties[54,55]
- **1987:** Hibbs: macrophages produce nitrite, nitrate from L-arginine; are inhibited by L-N^G-monomethyl arginine[56]
- **1988:** Garthwaite: neuronal cells produce EDRF (NO; ref. 57)
- **1988:** Marletta: macrophage nitrite, nitrate derived from NO (ref. 58)

convergence of information around a new scientific field. Future research must now address fundamental questions highlighted in these sessions:

- The contribution of NO-dependent and NO-independent signaling in cellular processes regulated by nitrite.
- The mechanisms of cytoprotection afforded by nitrite after ischemia-reperfusion, and the role of endogenous nitrite and diet in modulating these events.
- The role of myoglobin and other heme proteins, xanthine oxidoreductase, and other enzyme systems in the 'physiological' reduction of nitrite to NO in different tissues at different pH or oxygen gradients.
- The potential role for the nitrite-hemoglobin reaction in regulating vascular homeostasis, signaling and hypoxic vasodilation, and the study of potential intermediates in these reactions and mechanisms of NO export from the red cell.

The solution to these problems should open the door to novel therapeutics, lead to the potential consideration of nitrite as an 'essential nutrient' and engage future researchers in the study of nitrite biochemical physiology and pathology.

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