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Review

Nitrite in nitric oxide biology: Cause or consequence? A systems-based review

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Abstract

All life requires nitrogen compounds. Nitrite is such a compound that is naturally occurring in nature and biology. Over the years, the pharmacological stance on nitrite has undergone a surprising metamorphosis, from a vilified substance that generates carcinogenic nitrosamines in the stomach to a life-saving drug that liberates a protective agent (nitric oxide or NO) during hypoxic events. Nitrite has been investigated as a vasodilator in mammals for over 125 years and is a known by-product of organic nitrate metabolism. There has been a recent rediscovery of some of the vasodilator actions of nitrite in physiology along with novel discoveries which render nitrite a fundamental molecule in biology. Until recently nitrite was thought to be an inert oxidative breakdown product of endogenous NO synthesis but the past few years have focused on the reduction of nitrite back to NO in the circulation as a possible mechanism for hypoxic vasodilatation. Nitrite has evolved into an endogenous signaling molecule and regulator of gene expression that may not only serve as a diagnostic marker but also find its role as a potential therapeutic agent of cardiovascular disease. These data therefore warrant a reevaluation on the fate and metabolism of nitrite in biological systems. This review serves to encompass the history and recent evolution of nitrite, the compartment-specific metabolism of nitrite and its role in plasma as a biomarker for disease, the role of nitrite as a potential regulator of NO homeostasis, and the future of nitrite-based research.

Contents

Introduction

The long-held belief that nitrite is only an inert metabolite of the nitric oxide (NO) pathway is no longer valid. Nitrite plays an important role as a biomarker of endogenous nitric oxide synthase (NOS) activity [1,2]. Its relevance in this context

Abbreviations: BH_4, tetrahydrobiopterin; CaM, calmodulin; EDRF, endothelium-derived relaxing factor; I/R, ischemia/reperfusion; NDMA, *N*-nitrosodimethylamine; NO, nitric oxide; NOS, nitric oxide synthase; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; RNNOs, *N*-nitrosamines; SOD, superoxide dismutase.

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becomes apparent by the critical roles that NO plays in physiology and homeostasis and a growing recognition of the diverse maladies whose etiology involves NO insufficiency. Recently it has been shown that nitrite also plays a fundamental role in cell signaling and pathology whereby disturbances in steady-state nitrite concentrations may have profound consequences [3]. A systems biology approach to physiology in experimental animals has revealed that the steady-state concentration of nitrite in the circulation may not accurately reflect the concentrations in tissues either under basal conditions or with interventions [3–5]. If nitrite is an important molecule in maintaining cellular homeostasis, there must be a mechanism in place to keep its systemic concentration roughly the same in the face of varying levels of dietary intake of nitrite/nitrate and changes in endothelial NOS activity secondary to fluctuations in blood flow.

Other than nitrite-reflecting activity of the endogenous NO system, nothing is known about how nitrite itself affects NOS-derived NO. Due to the growing popularity of nitrite-based therapeutics, it is critical to understand if and how nitrite affects the NO system. Administration of nitrite may offer new therapeutic approaches in the management of myocardial infarction, stroke, pulmonary hypertension, or a host of other maladies characterized by ischemia. However, this requires an understanding of nitrite metabolism in each tissue and how this is reflected in the plasma for use as a biomarker. Diagnosis of human disease relies almost primarily on biomarkers in the blood since tissue samples are not readily available from patients. Despite the recent increased interest and research on nitrite, there are many unanswered questions. In the following review, the metabolism of nitrite in blood and tissues will be addressed with the hope of bridging the gap between basic science nitrite research and clinical medicine with an outlook of what role nitrite may play in maintaining NO nitroso-redox homeostasis.

History of nitrite

Inorganic nitrite and nitrate have been in use for as long as 5000 years in the preservation of food. However, it was not realized until the 19th century that the mechanism underlying food preservation was actually the conversion of nitrate to nitrite by bacteria [6]. This provided the rationale for the use of nitrite rather than nitrate in the meat preservation industry in the early 1900s. Nitrite in meat greatly delays the development of botulinum toxin, develops cured meat flavor and color, retards development of rancidity during storage, inhibits development of warmed-over flavor, and preserves flavors of spice and smoke [6]. Ascorbic acid or dehydroascorbate is also a common meat preservative. It was demonstrated very early on that the chemical basis for the nice red color and appearance of cured meat was the reaction of nitrite with oxyhemoglobin to form *S*-nitrosohemoglobin [7]. It was later realized that free sulfhydryl groups were necessary for this effect [8]. The process was shown to involve intermediate formation of *S*-nitrosothiols to ultimately produce nitrosylmyoglobin [9]. In the early 20th century meat preservation regulations in the United States for the first time allowed nitrite to be used as a curative salt. According to the Meat Inspections Regulations, the maximum amount of nitrite that can be used for the curing process is one ounce per 100 pounds of meat (dry cured) or ¼ ounce per 100 pounds chopped meat and/or meat by-product [10]. Nitrite has since become a common dietary nutrient in those who consume cured meats.

In the 1970s, there became a major public health concern regarding nitrite, when there was indication of endogenous formation of *N*-nitrosamines from nitrite and nitrate and its relevance to human cancer. The first report in the 1950s on the hepatocarcinogenic effects of *N*-nitrosodimethylamine (NDMA) [11], and the suggestion that low molecular weight *N*-nitrosamines (RNNOs) can be formed following nitrosation of various amines [12] ignited an enormous interest in *N*-nitrosamines and their association with cancer. Direct proof that such nitrosation reactions can occur was provided by Ender et al. [13] who identified NDMA in nitrite preserved fish, and by Sander and Seif [14] who demonstrated the in vivo formation of a nitrosamine in the acidic conditions of the human stomach. Because of the potent carcinogenicity of some low molecular weight *N*-nitrosamines, considerable effort was made to determine the levels of nitrite and nitrate in the external and internal human environment, and to assess exposure in order to correlate it with human cancer at specific sites [15]. Since the early 1980s there have been numerous reports on the association of *N*-nitrosamines and human cancers [15,16] but a causative link between nitrite or nitrate exposure and cancer is still missing [17].

Public awareness was also brought to industrial settings and exposure of workers to nitrite. Not only is nitrite used as a color fixative and preservation in meats and fish but it is also used in manufacturing diazo dyes, in nitroso compounds, in the textile industry, in photography, and in the manufacture of rubber chemicals. Nitrite is also a common clinical and laboratory chemical that is used as a vasodilator [18], bronchodilator [19], intestinal relaxant [20], and even as an antidote for cyanide poisoning [21]. Considering its widespread use there have been many toxicological studies on acute and chronic exposure to nitrite. The fatal dose of nitrite is in the range of 22–23 mg/kg body weight (from *USFDA Generally Recognized as Safe Food Ingredient: Nitrates and Nitrites (Including Nitrosamines*, by Battele-Columbus Laboratories and Department of Commence, Springfield, VA, 1972). Lower doses of either nitrite or nitrate have caused acute methemoglobinemia, particularly in infants where a high nitrite or nitrate intake has been associated with "blue baby syndrome" caused by methemoglobinemia [22–24]. These negative connotations of nitrite and nitrate have led the government to regulate and restrict the levels in food and drinking water.

In the late 1970s, despite all the fear and paranoia surrounding nitrite exposure, our appreciation and understanding of nitrite took a drastic turn. Studies on nitrogen balance in humans and analyses of fecal and ileostomy samples indicated that nitrite and nitrate are formed de novo in the intestine. These early findings by Tannenbaum et al. [25] significantly altered our conceptions of human exposure to exogenous nitrite and

nitrate and represented the original observations that would eventually lead to the discovery of the L-arginine:NO pathway. Prior to these studies it was thought that steady-state levels of nitrite and nitrate originated solely from the diet and from nitrogen fixing enteric bacteria.

Sources of nitrite

We now know and understand that endogenous sources of nitrite in mammals are derived from: (1) oxidation of endogenous NO, (2) reduction of salivary nitrate by commensal bacteria in the mouth and gastrointestinal tract, (3) nutritional sources such as meat, vegetables, and drinking water. The steady-state concentrations of nitrite in the body are tightly regulated and vary depending on each tissue or compartment and relative NOS activity [5,26]. However, there is usually more nitrite concentrated in the tissues than in the circulation [5]. NO is synthesized by mammalian cells from L-arginine through a complex oxidation reaction catalyzed by the flavo-hemoprotein NO synthase [27]. The endogenous production of NO plays an important role in vascular homeostasis, neurotransmission, and host defense mechanisms [28]. In plasma, steady-state concentrations of nitrite are conserved across various mammalian species, including humans in the range of 150–600 nM [1]. Apart from plasma, nitrite can also be transported within red blood cells [29]. The net concentration in plasma is a result of its formation and consumption. It has been shown that up to 70–90% of plasma nitrite is derived from eNOS activity in fasted humans and other mammals [1]. Humans, unlike prokaryotes, are thought to lack the enzymatic machinery to reduce nitrate back to nitrite. However, due to the commensal bacteria that reside within the human body it has been demonstrated that these bacteria can reduce nitrate, thereby supplying an alternative source of nitrite [30–33]. Plasma nitrite increases after ingestion of large amounts of nitrate. This increase is entirely due to enterosalivary circulation of nitrate (as much as 25% is actively taken up by the salivary glands) and reduction to nitrite by commensal bacteria in the mouth [34]. Therefore, dietary and enzymatic sources of nitrate are now a potentially large source of nitrite in the human body. Nitrate is rapidly absorbed in the small intestines and readily distributed throughout the body [35]. This nitrate pathway to NO has been shown to help reduce gastrointestinal tract infection and increase mucous barrier thickness and gastric blood flow [35–40]. The concentrations of nitrate in drinking water are usually <10 mg/L in the absence of bacterial contamination [41]. Vegetables, especially beets, celery, and leafy vegetables like lettuce and spinach are rich in nitrates [35,42,43]. Other vegetables contain nitrate at lower concentrations, but because they are consumed in greater quantity, they may contribute more nitrate and thus nitrite from the diet. For the average population, most nitrate exposure (86%) comes from vegetables, whereas the primary contributors to nitrite intake are cured meats (39%), baked goods and cereals (34%), and vegetables (16%). The National Research Council report *The Health Effects of Nitrate, Nitrite, and N-Nitroso Compounds* (NRC, 1981) reported estimates of nitrite and nitrate intake based on food consumption tables and report that the average total nitrite and nitrate intake in the U.S. was 0.77 and 76 mg, respectively, per day.

In plasma, nitrite remains stable for several hours [44,45]. In whole blood, however, NO and nitrite are rapidly oxidized to nitrate [46]. The half-life of nitrite in human blood is about 110 s [44,45,47] and nitrate has a circulating half-life of 5–8 h [46,48]. Tissue nitrite and nitrate, on the other hand, both have in vivo half-lives of tens of minutes [3]. Nitrite and nitrate are excreted through the kidneys. Nitrate is excreted in the urine as such or after conversion to urea [49]. Clearance of nitrate from blood to urine approximates 20 ml/min in adults [50], indicating considerable renal tubular reabsorption of this ion. There is little detectable nitrite or nitrate in feces [51]. There is some loss of nitrate and nitrite in sweat, but this is not a major route of excretion [52].

Nitrite reduction to NO

Since nitrite appears to be tightly regulated as described above, then Nature must have a use for it rather than just as a decomposition product. Much of the recent attention given to nitrite is through its ability to be reduced to NO during ischemic or hypoxic events. Indeed, NO production from nitrite has been described in infarcted heart tissue [53]. Nitrite reductase activity in mammalian tissues has been linked to the mitochondrial electron transport system [54–58], protonation [19,53], deoxyhemoglobin [19,59], and xanthine oxidase [60–62]. Mitochondrial nitrite reduction has been shown to occur by ubiquinol [56,63] and cytochrome *c* oxidase [64] with subsequent binding of the NO produced to the cytochrome bc1 site of complex III or complex IV, resulting in oxygen-dependent reversible inhibition of mitochondrial respiration [65]. The acidic reduction of nitrite requires protonation and a one-electron reduction. The relatively low p*K*a of nitrite (3.34) [66] limits this activity in physiology but it can occur in the stomach or during ischemic events when tissue pH falls. The recently described function of hemoglobin as an enzymatic nitrite reductase converts nitrite to NO, maximally effective at 50% oxygenation [67]. This characteristic has been suggested to link nitrite reduction to hemoglobin allostery [67,68] and may be responsible for oxygen sensing and hypoxic vasodilation. Xanthine oxidase is a flavoprotein enzyme that is distributed in various mammalian tissues [69] and plays an important role in both physiology and pathophysiology. In addition to its known ability to reduce molecular oxygen to superoxide (O_2^-) [70], at low oxygen tensions and low pH xanthine oxidase can also reduce nitrite to NO at the molybdenum site of the enzyme [71–74]. Oxygen acts as a strong competitive inhibitor of nitrite reduction by xanthine oxidase [60]. Furthermore conditions require abundant superoxide dismutase (SOD) to scavenge the superoxide simultaneously generated by xanthine oxidase which would otherwise rapidly react with any NO generated. As a result, a prominent role in physiological nitrite reduction by xanthine oxidase is still a matter of debate. Since all four described pathways have been shown to be able to reduce nitrite but require different conditions and substrates for optimal nitrite

reduction, it is likely that all pathways may become relevant but at different oxygen tensions, substrate availabilities, and perhaps even compartment-specific needs.

Both nitrite and nitrate have been shown to be reduced ultimately back to NO by commensal bacteria [75] and bacteria in the urogenital tract [76]. These pathways have been extensively reviewed elsewhere [77] in the vascular compartment but extend to all organ systems. These processes are all independent of NOS and are unaffected by specific NOS inhibitors. NO is also produced in the mouth of both rat [78] and humans [79], and in human sweat [52]. In these instances, commensal bacteria may play a role in NO synthesis from nitrite, and the NO that is produced may serve as a defense mechanism against pathogens.

More recently, nitrite reduction has been implicated in hypoxic vasodilation in the circulation [80]. The use of nitrite as a vasodilator is not a novelty. As early as 1880, nitrite was described in terms of its vasodilatory abilities [18] and much later, Furchgott and Bhadrakom in 1953 used acidified sodium nitrite to relax precontracted aortic strips [81]. Both studies used supraphysiological concentrations of nitrite. However, recent studies have rediscovered the vasodilatory effect of nitrite on forearm and systemic blood flow after nitrite infusion. Cosby et al. [59] suggested that nitrite is a large intravascular storage pool for NO and that nitrite bioactivation to NO could dilate regions with tissue oxygen debt in the human circulation. However, an earlier study by Lauer et al. reported that nitrite lacked intrinsic vasodilatory properties [2]. This discrepancy is likely due to kinetics and duration of infusion. Two independent groups have recently demonstrated the cytoprotective effects of nitrite in ischemia–reperfusion injury [62,82]. Duranski et al. attribute nitrite's protective effects to the reduction of nitrite to NO by the reductase activity of hemoglobin. However, the study by Webb et al. using an isolated heart setup was in the absence of blood, clearly demonstrating that the myocardial tissue itself can metabolize nitrite without the need for hemoglobin and instead implicate xanthine oxidase as the source of nitrite-derived NO. Ischemia leads to hypoxia due to a decrease or stop in blood supply and thus oxygen tension and tissue pH will decrease providing more optimal conditions for nitrite reduction back to NO either through acidification/protonation or through enzymatic reduction.

While nitrite has been shown to be protective in ischemia/reperfusion (I/R) injury in the heart and liver, it was recently revealed that nitrite provides no protection in renal I/R injury [83]. This clearly demonstrates tissue-specific metabolism of nitrite. Careful comparison on the nitrite-reducing efficacy of tissues from 7 major organ systems clearly illustrates that tissues, particularly heart, liver, and skeletal muscle, have a much higher and efficient nitrite reductase activity than red blood cells under anoxic conditions. There is a significant correlation in brain, heart, liver, and lung between anoxic nitrite reductase activity and tissue mitochondrial membrane surface area but not in the kidney (Bryan, under review). For whatever reason, the renal mitochondria do not reduce nitrite as effectively as other tissues and thus may provide an explanation for the lack of protection by nitrite in the kidney.

The central role of nitrite as a reservoir of NO clearly extends beyond the circulation and is primarily utilized by the tissues, demonstrating the disparity between what occurs in the circulation and what occurs in tissues [3]. In addition to modulating vascular tone during hypoxia, nitrite may be involved in the regulation of processes coupled to the mitochondria [84,85] by nitrite reduction to NO [56,57]. NO reversibly inhibits the activity of mitochondrial cytochrome *c* oxidase (i.e., complex IV of the mitochondrial electron transport chain) by competing with O_2 binding at the cytochrome's heme [86,87]. This well-recognized interplay between NO and O_2 through mitochondrial cytochrome *c* oxidase and the overall physiology of mitochondria at the interface between tissue O_2 consumption and energy production would appear to position this organelle to serve as an intrinsic tissue sensor of cellular O_2 demand distinct from the way in which the RBC, which does not contain mitochondria, might coordinate and transduce O_2-dependent processes [88]. Under hypoxic conditions whereby mitochondrial electron carriers would tend to remain reduced, isolated mitochondria produce NO when supplied with NO_2^- and respiratory substrate [54,89], with the suggested loci of this "NO_2^- reductase" activity being the ubiquinone cycle of complex II [56] or complex IV (cytochrome *c* oxidase) [64]. There appears to be diverse and differentiated pathways for nitrite reduction which vary from cytoplasmic (XO) in location to organelle specific (mitochondria). The purpose of such may extend well beyond just hypoxic vasodilation.

Nitrite may serve several roles during ischemia or hypoxia. It may first act to reversibly nitrosate critical thiols to prevent irreversible oxidation during reperfusion and thus maintain function. Secondly, a portion of the nitrite may be reduced to NO which may then diffuse to smooth muscle and dilate vessels in order to bring blood flow in line with oxygen demand within the deprived tissue beds. Furthermore nitrite reduction to NO may act to inhibit mitochondrial respiration to regulate energy production or extend oxygen gradients within tissues [84]. One possible mechanism is via NO's interaction with cytochrome *c* where it inhibits electron transport at complex IV of the mitochondrial electron transport chain [87]. This decreases the respiration rate and consumption of oxygen to allow the cell to essentially slow down and withstand the oxidative burst and signals that are associated with reperfusion injury. This would suggest a critical and fundamental role for nitrite in maintaining homeostasis and blood flow regulation throughout the entire physiological oxygen gradient, including complete anoxia.

Nitrite signaling

The evidence cited above has led scientists in the field to appreciate the potential importance of nitrite in pathophysiology. While there is growing evidence that nitrite serves as an important molecule during hypoxia or ischemia, a physiological role for nitrite had yet to be established until very recently. Nitrite was reported to be a signaling molecule and regulator of protein expression. Administration of nitrite in vivo leads to an acute accumulation of cGMP in some tissues, inhibition of P450 enzyme activity, and a change in the expression of two

archetypical proteins 24 h later. It was further demonstrated that a linear association existed between tissue nitrite and RSNO concentrations, suggesting that physiological concentrations of nitrite sufficiently account for basal levels of heme nitrosylation and *S*-nitrosation [3]. *S*-Nitrosation is known to be involved in cell signaling pathways [90]. Thus, nitrite itself, through the formation of RSNOs, can act as a biological signaling molecule and regulate protein expression under physiological conditions. While it is possible that free NO is formed from nitrite reduction in particular cellular compartments, the evidence presented by Bryan et al. [3] includes the fact that *S*-nitrosation and nitrosylation by nitrite proceeds unchanged in the presence of the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) in vivo, and in the presence of oxyhemoglobin and carboxy-PTIO in vitro. Although we were not able to affect *S*-nitrosation by nitrite with NO scavengers or oxygen, other scenarios have recently been described by Rikfind and colleagues [91] whereby nitrite reduction by deoxyheme is coupled to the formation of a thiyl radical and subsequent RSNO formation via an intramolecular shuffle of an NO intermediate. Similarly Stamler and colleagues describe RSNO formation by nitrite's reaction with deoxy-heme proteins, which produces heme-Fe(II)NO and then proceed to form RSNOs through the intramolecular oxidative transfer from the heme to β-93 cysteine thiol [92]. In both cases, the NO (or an elusive NO intermediate) produced by such reactions would likely be inaccessible for scavenging or even detection which makes proof of these proposed mechanisms difficult. Both of these alternative pathways are described using only hemoglobin as a model protein. Whether this may occur in other heme proteins is still unknown. Despite the lack of a unified mechanism on nitrite-mediated *S*-nitrosation, it is clearly a viable pathway demonstrated now by several independent laboratories.

The study by Duranski et al. also describe nitrite-based signaling by reduction of near-physiological levels of nitrite, albeit through the classical NO-dependent signaling pathways and not through nitrite itself [82]. Nitrite-mediated signaling (NO-independent) stems from the fact that there is a lack of inhibition of platelet aggregability in response to various doses of nitrite, the lack of effect of oxygen on nitrite-mediated nitrosation in vitro, and the linear relationship of *S*-nitrosation to nitrite addition [3]. Furthermore the change in protein expression which occurs 24 h post nitrite administration is at a time when blood and tissue nitrite concentrations have returned to their steady-state concentrations and in some tissues actually little or no nitrite remaining [3]. These downstream effects as a result of a bolus nitrite administration further support the role of nitrite as a signaling molecule whereby transient changes in blood and tissue concentrations affect gene/protein expression hours later. The ability of nitrite to signal without NO would require a novel chemical pathway, and indeed one appears to be in place which requires a cooperative action between thiols and hemes as has been previously described [3].

These results demonstrate a fundamental and critical role for nitrite in cellular signaling. Up to now, nitrosylation reactions, including those leading to stimulation of sGC and inhibition of mitochondrial respiration [86,87], have been attributed exclusively to reactions between a heme metal and free NO. In light of the most recent in vivo results [3], this view may have to be reconsidered. Thus, via direct formation of nitroso and nitrosyl products, endogenous or exogenous nitrite may account for some of the biochemistry and pharmacology previously attributed to NO. The definitive role of nitrite-mediated nitrosation in vivo is unclear at the present time but may serve a role other than preserving NO bioactivity through RSNO formation as in SNO-Hb. Nitrite, through steady-state equilibrium with RSNOs, may act in vivo to form a reversible, transient modifications, i.e., nitrosation on critical thiols to protect from irreversible oxidative damage during oxidative stress.

Disease diagnostics, prevention, and therapeutics

Endothelial dysfunction is a hallmark of a number of diseases including atherosclerosis and has been attributed to impaired NO bioactivity and enhanced formation of oxygen-derived free radicals [93]. Given that endothelial dysfunction is at least in part reversible [94], early detection is of important diagnostic and prognostic value provided an accurate biomarker exists. Identifications of such alterations in NO bioavailability may help in targeting asymptomatic individuals who are at risk for cardiovascular diseases and would likely benefit from preventive measures. Therefore, establishing a biomarker to assess NO bioavailability in the form of a blood test is highly desirable. Studies have shown that stimulation of eNOS results in an acute change in plasma nitrite concentration in the human forearm vasculature [2]. Further studies revealed that 70 to 90% of the circulating plasma nitrite is derived from eNOS activity [1,95]. Therefore plasma nitrite seems ideally suited as a biomarker for diseases associated with NO insufficiency.

Beside its role in diagnostics, it has recently been assumed that nitrite may play an important role in the therapy of vascular diseases. Diseases that have in common an association with reduced tissue oxygen, pH, and impaired regional blood flow are those which may be treated with nitrite. In vitro observations in the heart and liver showed that nitrite prevents ischemia–reperfusion injury [62,82]. Moreover, inhalation of nitrite selectively dilates the pulmonary circulation under hypoxic conditions in vivo in sheep [19]. Experiments in primates revealed a beneficial effect of long-term application of nitrite on cerebral vasospasm [96]. Topical application of nitrite improves skin infections and ulcerations [97]. Furthermore, in the stomach, nitrite-derived NO seems to play an important role in host defense [78,98] and in regulation of gastric mucosal integrity [39]. All of these studies together along with the observation that nitrite can act as a marker of NOS activity [1] opened a new avenue for the diagnostic and therapeutic application of nitrite.

A reduced NO availability is a hallmark of a number of cardiovascular disorders. Hyperlipidemia, arterial hypertension, diabetes, smoking, and aging are major risk factors for the manifestation of cardiovascular events [99]. A recent report by Kleinbongard et al. [100] demonstrated that plasma nitrite levels

progressively decrease with increasing cardiovascular risk load. Risk factors considered include age, hypertension, smoking, and hypercholesterolemia. Although a correlation exists in the plasma, it is not known whether the situation is mirrored in the heart. Since nitrite acts as a protective molecule during ischemic events, these data raise the intriguing possibility that the underlying problem with these patients is their diminished nitrite levels. Therefore establishing disease states with decreased NO or nitrite availability may then open the door for increasing their nitrite availability by nitrite-based therapeutics, perhaps in conjunction with L-arginine supplementation which may provide benefit and provide an alternate route to increase their NO availability as well as conferring protection from I/R injury. The paramount question then becomes do these correlations from plasma nitrite translate to what is occurring in tissues at risk?

Do plasma nitrite concentrations accurately reflect tissue concentrations?

It was recently demonstrated that tissues handle nitrite much differently than blood, either in plasma or red cells [3]. Nitrite performs nitrosative chemistry in the heart and liver under normoxic conditions while it is quickly and completely oxidized to nitrate in blood. Until recently it was thought that the majority of endogenous nitrite derives from the NO pathway. While this may hold true in the circulation of fasted subjects, our own investigations have revealed that the tissue nitrite can be affected by dietary changes [3]. Understanding dietary nitrite and nitrate consumption and metabolism therefore becomes very prudent. Most of the data presented above only represented what is occurring in plasma but in the past extrapolated to what may occur elsewhere, perhaps naively. A systems-based approach to biology and physiology has demonstrated a great variety between what is occurring in tissue and what is reflected in blood in terms of nitrite metabolism [3]. Nitrite alone, in a single compartment, then may not be enough to reflect specific changes in NO or nitrite availability in select tissues. The differences in plasma and tissue nitrite and nitroso concentrations first revealed itself in our studies with NO donors. The dynamics of GTN or other NO donor-derived nitrite and nitrate in blood does not reflect the nitrite and/or nitros(yl)ation patterns in the circulation or in tissues [4]. Although there was an increase in circulating nitrite after GTN administration, the magnitude and quality of the response was much different than in tissues. Similar observations were made with sodium nitrite administration [3]. While there was little nitrosative chemistry occurring in the plasma, most tissues experienced nitrosative modifications several magnitudes higher than what occurred in the plasma [3]. This was further confirmed in experiments with systemic inflammation as a result of LPS administration in the rat (unpublished observation). However, kinetics and mode of administration become an important consideration when correlating plasma with tissue nitrite metabolism. In all cases examined where nitrite was not changed acutely, i.e., those measured basally and under the low-nitrite/nitrate diet, the levels of tissue nitroso/nitrosyl modifications seemed remarkably resilient to changes in tissue nitrite concentrations. They remained unchanged in the face of depleted tissue nitrite on a low NOx diet [3]. Nitrite added acutely via ip injection readily increased tissue nitroso/nitrosyl levels in a dose-dependent manner that was correlated with an increase in tissue nitrite concentrations at very early time points. Nitrite given in the drinking water, rather than bolus ip, where there is gradual uptake and transport, only modestly increased tissue nitroso/nitrosyl levels (unpublished data). This apparent discord between kinetics and mode of administration suggests involvement of a regulatory mechanism able to maintain constant basal nitroso/nitrosyl levels over the physiological range of nitrite concentrations (0.2–2 μM), provided nitrite influx/production levels change slowly. Nitrite, given ip, has been shown to appear first in the blood at high concentrations and is delivered to the tissues down a concentration gradient [3]. Nitrite concentrations in both compartments (plasma vs tissues) appear tightly correlated immediately following ip injection but appear totally dissociated under the effects of a low nitrite/nitrate diet. The former case suggests that in acute doses the levels of nitrite in most tissues are dictated by passive transport of this anion across membranes due to the high concentration gradient from plasma to tissues. In the latter case, the steadiness of nitrite levels in plasma in the face of its virtual disappearance in tissues strongly argues for an active mechanism that attempts to maintain basal values within the circulation, perhaps as a reservoir for tissues to draw upon in times of need. Part of this active regulatory mechanism may involve stimulation of NOS activity in blood cells or in the endothelium and an increase in nitrite conversion into nitrate, nitroso, and nitrosyl products in the tissues. Clearly this shows a complex and regulated mechanism of nitrite homeostasis in tissues but more research is needed to understand the uptake and regulation in tissues and how this may eventually be reflected in plasma as a clinical biomarker.

NOS and nitrite: A concert in NO homeostasis

Nitric oxide synthase enzymes produce ·NO by catalyzing a five-electron oxidation of a guanidino nitrogen of L-arginine (L-Arg). Oxidation of L-Arg to L-citrulline occurs via two successive monooxygenation reactions producing *N*hydroxy L-arginine as an intermediate. Two moles of O_2 and 1.5 mol of NADPH are consumed per mole of ·NO formed [101]. NOS enzymes are the only enzymes known to simultaneously require five bound cofactors/prosthetic groups: FAD, FMN, heme, tetrahydrobiopterin (BH_4), and Ca^{2+}-calmodulin (CaM). All NOS isozymes are catalytically self-sufficient provided all required substrates and cofactors are available. CaM binding to nNOS has been shown to regulate catalytic activity by triggering electron flux from FMN to heme, thereby coupling the oxygenase and reductase domains. CaM also facilitates NADPH dependent reduction of cytochrome *c* and ferricyanide in BH_4 and heme-depleted nNOS. If any of the cofactors become limiting, then NO production from NOS shuts down, and in many cases NOS then produces superoxide instead. This is indeed a very complex and coordinated effort to

enzymatically produce NO which normally proceeds very efficiently. However, in disease characterized by oxidative stress where cofactors become oxidized, NOS uncoupling, or conditions of hypoxia where oxygen is limiting, this process can no longer maintain NO production. Therefore there has to be an alternate route to NO production. It is highly unlikely that Nature devised such a sophisticated mechanism of NO production as a sole source of a critical molecule. Does nitrite then act as a backup system to the NOS system? Part of this may occur through nitrite reduction during low oxygen availability.

Nitrite reduction to NO can occur in a much simpler mechanism. The one-electron reduction of nitrite can occur by ferrous heme proteins (or any redox active metal) and an electron donor through the following reaction:

$$NO_2^- + Fe^{(II)} + H^+ \leftrightarrow NO + Fe^{(III)} + OH^-.$$

This is the same biologically active NO as that produced by NOS just instead of using L-arg as the substrate, nitrite is used. This is a relatively inefficient process. Therefore for this to occur, the tissues or biological compartment must have a sufficient pool of nitrite stored. Since plasma nitrite is a direct measure of NOS activity, a compromised NOS system can also affect downstream nitrite production and metabolism. This can perhaps exacerbate any condition associated with decreased NO bioavailability. Nitrite supplementation may therefore act as a protective measure to compensate for insufficient NOS activity under conditions of hypoxia or in critically ill patients unable to swallow [102]. It is very likely that exogenous nitrite contributes to whole-body NO production and homeostasis. Considerable published support for this theory derives from the following facts: NO produced from nitrite in the upper intestine is up to 10,000 times the concentrations that occur in tissues from enzymatic synthesis [103], nitrite can act as a circulating NO donor [104], and nitrite can itself perform many actions previously attributable to NO [105] without the intermediacy of NO [3].

It is possible that an interaction between nitrite and NOS exists in which the former acts as a negative feedback inhibitor of endogenous NO production. This explains why blood pressure actually increases after application of low doses of nitrite [3,18]. The persistent increase in platelet aggregability upon nitrite administration [3] suggests that platelet NOS remains inhibited at all dose levels of exogenous nitrite but blood pressure drops, perhaps due to (an NO-independent) nitrosylation of soluble guanylyl cyclase and concurrent relaxation of aortic vascular tissue. In the opposite scenario, i.e., when nitrite intake is decreased by dietary means, a dysinhibition and maybe even a compensatory stimulation of NOS activity is observed in an attempt to make up for the missing nitrite, keeping systemic levels (i.e., the concentration in plasma) the same. These assumptions are supported by currently ongoing experiments in eNOS knockout mice (Bryan and Feelisch, observations). Clearly, much more must be learned before an informed judgment can be made as to the physiological role of individual regulatory pathways that are modulated or triggered by nitrite. Nevertheless, one clear picture emerging from these results is that changes in nitrite availability into either direction may have a direct effect on endogenous NO production and elicit measurable changes in the activity and expression of multiple proteins and enzyme products.

Although it may be argued that nitrite is simply a storage pool that can be reduced to NO under appropriate conditions, we consider this the unlikely role for nitrite in physiology, i.e., normoxic and neutral pH, conditions under which nitrite is stable and tightly regulated. Alternatively, nitrite acts as an important molecule in its own right but which has regulatory effects on the NO pathway. It may be that both hypoxic reduction of nitrite to NO and normoxic metabolism of nitrite represent an advantageous oxygen sensing system which is a vestige of denitrifying microorganisms that existed long before the advent of aerobic respiration and the emergence of an NO synthase system. The fact that both systems still exist today highlights the importance of nitrite in all cellular processes throughout the entire physiological oxygen gradient [105].

Outlook

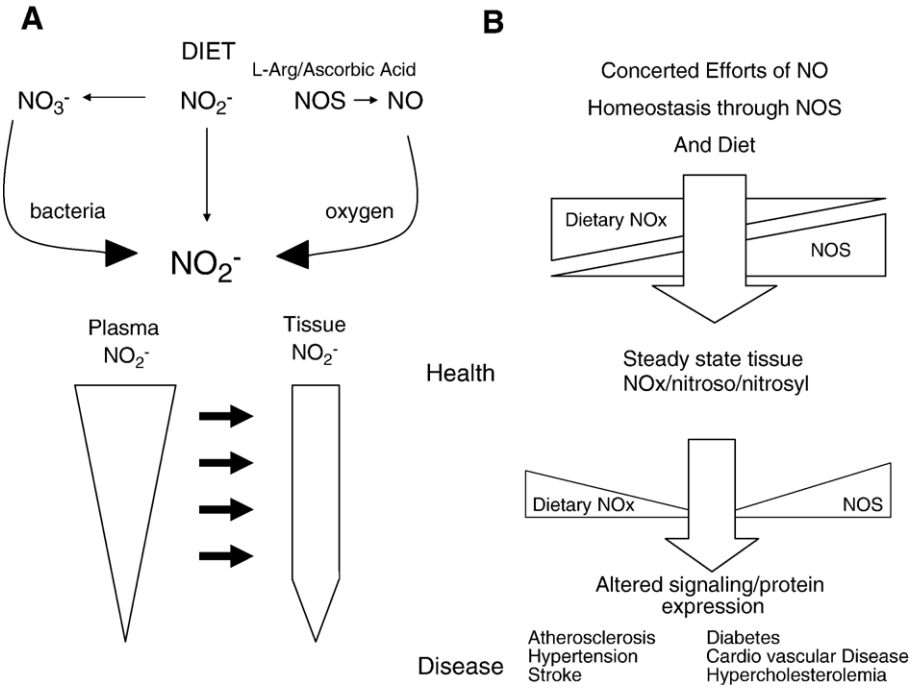
What once was a simple inert oxidation product overlooked in terms of a regulatory molecule in physiology, nitrite is now the subject of intense investigations in physiology, pathophysiology, and therapeutics [105]. There is now growing evidence in our laboratory of an intimate relationship between endogenous production of nitrite from NO synthase and exogenous nitrite from the diet. Similar to the semiessential nature of L-arginine for optimal NOS activity, nitrite may emerge as an essential nutrient. Changes in nitrite availability into either direction may have a direct effect on endogenous NO production and elicit measurable changes in the activity and expression of multiple proteins and enzyme products. These findings suggest the possibility of nitrite or nitrate supplementation which may act as preventative medicines for cardiovascular diseases or any disease characterized by an insufficiency in NO or conditions whereby enzymatic NO production from NOS is insufficient. If our current understanding is true, then an optimal diet may then consist of a sufficient supply of nitrite and nitrate for health and disease prevention. Under normal healthy conditions of a high NOx (vegetarian) diet and active NOS system, sufficient nitrite exists from both pathways. This is reflected in an increased circulating nitrite concentration in the plasma. Tissue nitrite and nitroso/nitrosyl concentrations initially increase with plasma concentrations but very quickly return to baseline concentrations and remain constant likely until end-stage disease. During periods of fasting or low nitrite intake, NOS then has to become more active to maintain NO production as well as a sufficient pool of nitrite, provided ample L-arginine and other cofactors are available. Under conditions when NOS is insufficient (endothelial dysfunction) due to depletion of L-arginine or oxidation of cofactors combined with a poor diet low in NOx, neither system can maintain homeostasis and disease ensues. This scenario is

depicted in Scheme 1B. Although there is some disparity between plasma and tissue nitrite, it is possible that the tissues rely on their own production and metabolism and only draw upon the plasma reservoir in times of distress such as oxidative stress. Therefore nitrite becomes an important regulatory molecule in physiology and may serve as a potential rescue molecule under conditions of insufficient NO as a result of impaired NOS activity.

The discovery of NO as endothelium-derived relaxing factor (EDRF) and its signaling in the cardiovascular system led to the Nobel Prize in Physiology or Medicine in 1998. Research over the past 25 years has taught us that nitrite and nitrate are metabolic end products of NO production and thus markers of local NO formation. It becomes apparent how the bioactivity of nitrite went unrecognized because NOS inhibition studies inhibit NO production and thus inhibit nitrite production and NO donors are actually quite good nitrite donors. Although EDRF was discovered to be NO, NO is much more than EDRF. Early research focused strictly on the vascular relaxation effects of NO and nitrite is known to be a poor vasodilator via cGMP pathway [106]. In terms of its vasomotor effects, nitrite was dismissed as a biologically active molecule. There is now a growing appreciation that NO does much more than dilate vessels so now may be an ideal time to reinvestigate nitrite in terms of other NO signaling pathways, such as preconditioning, gene expression, and tissue remodeling, as well as regulation of mitochondrial respiration and energy production. Nitrite may provide an explanation for many of the dichotomous actions previously attributed to NO if nitrite and NO are divergent signaling molecules.

Nitrite became a research interest in the 1970 when it was discovered that nitrite in industry and in foods might increase the incidence of cancer by the formation of *N*-nitrosamines. The paradigm on nitrite immediately developed into one in which nitrite must be regulated and nitrite in any form was detrimental to health. Could it be that the stringent regulations on nitrite/nitrate in drinking water and in foods contribute to the contemporary diseases of today due to inadequate nitrite or nitrate in the diet and NOS activity unable to supply enough nitrite to maintain cellular homeostasis?

While the most recent attention to nitrite has been in the circulation and its role in vascular control, a system biology approach to physiology has now confirmed an essential role for nitrite across all major organ systems of mammals. Nitrite appears to be a homeostatic redox and oxygen-sensitive molecule, whereby under normoxic conditions can perform unique roles in maintaining protein nitrosation and under hypoxic conditions reduce back to NO when NOS activity is insufficient. Although the past 10 years have brought about an enormous research effort on the biological role of nitrite, it is with hope this review highlights the major finding over the past several years and reveals the importance of investigating many different biological compartments to understand a physiological pathway rather than focusing on a single compartment.



Scheme 1. (A) Plasma nitrite is derived from dietary nitrite and nitrate and also from oxidation of endogenous nitric oxide. Tissues maintain a tight steady-state concentration of nitrite despite wide fluctuations in plasma nitrite. Therefore plasma nitrite may not always accurately reflect tissue concentrations. Plasma can act as a reservoir for tissue nitrite to draw upon in times of need as long as there is a sufficient buffer of plasma nitrite available. (B, upper) There is a concerted effort through dietary NOx and NOS to maintain both nitrite and NO bioavailability and thus steady-state tissue nitroso/nitrosyl. One may compensate for the other provided both pathways are active with ample substrate. (Lower) When dietary intake of NOx is limited, combined with a dysfunctional NOS, there is no source of nitrite or NO and as a result there is altered signaling and protein expression which may lead to the progression of disease.

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