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Inorganic Nitrite Therapy: Historical perspective and future directions

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Abstract

Over the past several years, investigators studying nitric oxide (NO) biology and metabolism have come to learn that the one electron oxidation product of NO, nitrite anion, serves as a unique player in modulating tissue NO bioavailability. Numerous studies have examined how this oxidized metabolite of NO can act as a salvage pathway for maintaining NO equivalents through multiple reduction mechanisms in permissive tissue environments. Moreover, it is now clear that nitrite anion production and distribution throughout the body can act in an endocrine manner to augment NO bioavailability that is important for physiological and pathological processes. These discoveries have led to renewed hope and efforts for an effective NO based therapeutic agent through the unique action of sodium nitrite as an NO pro-drug. More recent studies also indicate that sodium nitrate may also increase plasma nitrite levels via the enterosalivary circulatory system resulting in nitrate reduction to nitrite by microorganisms found within the oral cavity. In this review, we discuss the importance of nitrite anion in several disease models along with an appraisal of sodium nitrite therapy in the clinic, potential caveats of such clinical uses, and future possibilities of nitrite based therapies.

Keywords

nitric oxide; ischemia; cardiovascular disease; inflammation; clinical trials

Introduction

‘Back door’ pathways and avenues are essential ingredients for success in nearly all aspects of biology, this idea is also true for nitric oxide biology through the discovery of nitrite anion reduction back to NO as an important salvage pathway to maintain NO bioavailability.

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Conflicts of Interest

C.G.K. holds intellectual property interest in sodium nitrite, and C.G.K & T.G. have commercial interests in TheraVasc.

Much debate has been given to the role of nitrite anion in various aspects of NO biology including physiological functions such as vasodilation. However, there is now wide agreement and abundant experimental evidence that nitrite anion represents an important reservoir of NO bioequivalents to augment tissue NO levels during pathophysiological states where NO synthesis from enzymatic pathways may be unavailable. Previous reviews have discussed several principles of nitrite chemistry, however in this review we address the pathophysiological and translational implications of sodium nitrite therapy for various disease conditions.

Historical account of Nitrite

Recent years have seen a tremendous step forward toward the discovery that a broad range of compounds can fulfill one or more of the roles of true NO (e.g. nitrosothiols, nitroxyl anion, etc) [1–2]. However, nitrite anion has emerged as a reversible metabolite of NO that facilitates endogenous signaling responses differentially affecting gene expression [3, 4], serves as a diagnostic marker of cardiovascular health, and acts as a potential therapeutic agent for several disease states. It is important to note that though the metabolic connections between NO and nitrite has been known for many years [5,6] and the role of nitrite was confined to the belief that it was simply an inert metabolic by-product of NO.

The origin of the use of nitrite dates back at least to 850 B.C., where salt containing nitrates were used in the meat curing process. Although, the effect of nitrates were not completely understood until early in the 20th century, it is clear that for thousands of years nitrite/nitrate played an important role in meat curing. Research shows that pure salt does not impart flavor and color to preserved meat, but that these characteristics come from sodium or potassium nitrate as an additive of the salt [7]. The medicinal use of nitrates is also evident from documents dating around 800 AD demonstrating that potassium nitrate was used by the Chinese medicinally to relieve “acute heart pains, and cold in the hands and feet” [8]. However, only in the past several years has mounting evidence demonstrated that nitrite and nitrate play important physiological and pathophysiological roles.

NO Generation and metabolism

It is well known that endothelium-derived nitric oxide (NO) plays an important role in vascular homeostasis and cardiac function based on seminal studies leading to the Nobel Prize in Physiology or Medicine in 1998 [9, 10]. NO generation is classically accomplished through the activity of a family of enzymes termed nitric oxide synthases (NOS). There are three isoforms which were initially named based on their location of expression and function which include: NOS1-neuronal nitric oxide synthase (nNOS), NOS2-inducible nitric oxide synthase (iNOS), and NOS3-endothelial nitric oxide synthase (eNOS) [11, 12]. All three of these isoforms progressively oxidize and reduce L-arginine to L-citrulline resulting in NO production [13]. Importantly, NOS enzymes require several cofactors to facilitate this reaction, which has lead to a detailed understanding of complex mechanisms (e.g. post-translational modifications, substrate and cofactor availability) controlling NO synthesis in multiple tissues under different physiological conditions [14, 15]. The biological activity of NO is broad and the molecule interacts with several molecular targets including proteins,

nucleic acids, lipids, carbohydrates, and other free radicals or can be acutely terminated by oxidation to nitrite and subsequently nitrate (figure 1).

The signaling action of gaseous NO in the vasculature is short-lived as a result of its rapid reaction with oxyhemoglobin. However studies conducted in this area over the last few years have unraveled mechanisms by which NO bioactivity in blood and in tissue have been preserved in the form of nitrite, nitrate, S-nitrosothiols (RSNO), N-nitrosamines (RNNO), nitrosylhemoglobin (NOHb), and S-nitrosohemoglobin (SNOHb) [16–21]. Recent studies show the potential role of nitrite reduction back to NO under appropriate pathophysiological conditions to generate many nitroso species or that nitrite itself may mediate intracellular nitrosation reactions (e.g. RSNO formation) [22, 23]. Due to the identification of this unique NO salvage pathway specifically under conditions where NO is insufficiently produced or poorly bioavailable, numerous studies have been performed to investigate the utility of nitrite anion for therapeutic applications.

Relation between Nitrite and NO

NO released from the endothelium serves as a potent biological second messenger that regulates important functions of the cardiovascular system. Gaseous NO freely diffuses through the endothelial cell into the underlying smooth muscle cells to promote vasorelaxation [24], and can also affect cells passing through the blood stream, such as platelets and leukocytes to control inflammation [25–28]. However NO is known to be an unstable gaseous molecule often being metabolized before it reaches eventual target cells. How then is NO able to mediate an array of biological effects (e.g. vasoregulator, anti-inflammatory, anti-thrombosis)?

Although NO is known to protect tissues from ischemic injury, the ability of nitric oxide synthases to generate NO during ischemia is compromised because of the requirement for oxygen, BH4, and other co-factors which may be limiting during tissue ischemia [29–31]. However, conditions during tissue ischemia-reperfusion or chronic ischemia are optimal for the reduction of nitrite to NO, where multiple reduction mechanisms may be invoked (e.g. deoxyhemoglobin/myoglobin, xanthine oxidoreductase, acidosis, and others). NO is a well-characterized regulator of mitochondrial function that inhibits cytochrome c oxidase, decreases mitochondrial ROS formation, and limits apoptotic cytochrome c release [32–36]. Accumulating data demonstrates that NO modulation of mitochondrial function during ischemia and reperfusion strongly influences ischemic tolerance during preconditioning leading to cytoprotection after nonlethal ischemia-activated cell-survival pathways [37, 38]. It has also been reported that nitrite mediated NO formation dynamically modulates mitochondrial function to resist cellular injury and prevent apoptosis allowing cells to recover from ischemic insult [39]. Moreover, nitrite administration can precondition liver and heart tissue against subsequent ischemic injury by protecting against mitochondrial dysfunction, enhancing tissue reperfusion respiration and energetics, and limiting ROS-mediated cellular dysfunction [40]. These findings suggest that nitrite reduction to NO may serve as a critical mechanism to maintain NO reservoirs during pathophysiological states to minimize tissue dysfunction or injury.

Sources of Nitrite

Endogenous sources of nitrite

There are 2 primary sources of nitrite anion in mammalian systems that involve endogenous and exogenous points of origin. Endogenous nitrite generation primarily involves two pathways; 1) one electron oxidation of endogenously produced NO to nitrite, and 2) reduction of salivary nitrate to nitrite by commensal bacteria and reabsorption back into the organism.

1. Oxidation of endogenous NO—The main source of endogenous nitrite in mammals is produced through the L-arginine-NO pathway, which is constitutively active in multiple cell types throughout the body [41]. It has been reported that up to 70–90% of plasma nitrite is derived from eNOS activity in humans and rodents [17]. Importantly, during inflammatory reactions or infection involving increased iNOS expression large amounts of NO are produced resulting in significant increases in plasma nitrate and nitrite [42–44]. NO is readily oxidized to nitrite and subsequently nitrate in an oxygen dependent manner; thus, the physiological concentrations of nitrite vary between tissues reflecting the degree of NOS activity as well as oxidation pathways of NO [20, 45]. Usual concentrations of plasma nitrite range from 200–600 nM in many mammals including humans [17]. Conversely, nitrite is more concentrated within cells and tissues (low μM) compared to plasma [20, 46, 47].

2. Reduction of salivary nitrate by commensal bacteria—Unlike prokaryotes, humans lack defined enzymatic machinery to efficiently reduce nitrate to nitrite although it has been reported that mammalian tissues can reduce nitrate on a less efficient scale [48]. As such, nitrate dependent nitrite production is predominantly generated from the enterosalivary circulatory system delivery of nitrate to the oral cavity where it is reduced by commensal bacteria nitrate reductase activity present in the digestive system (Figure 2) [49–53]. A report by Duncan et al has shown in rats that the reduction of salivary nitrate to nitrite occurs at a discrete posterior area of the tongue that is colonized by nitrate-reducing bacteria [54]. This has also been confirmed in pigs, where high concentrations of these nitrate-reducing bacteria are present on the posterior portion of the tongue [55]. The contribution of the commensal pathway is significant as decreasing bacterial load using antiseptic mouthwash or antibiotics can significantly diminish plasma nitrite levels [56]. Although nitrite ingestion can occur as a food additive (i.e. preservative), it is mainly ingested as a product of the enterosalivary cycling of nitrate from plasma to saliva, reduction of nitrate to nitrite in the mouth, and reingestion. It has been reported that about 7% of ingested nitrite comes from food whereas 93% from nitrate in saliva as illustrated by a report demonstrating that continuous spitting can diminish nitrate recirculation into nitrite after consumption of a nitrate load [57].

Exogenous sources of nitrite

1. Nutritional sources—Dietary intake of nitrate and to a lesser degree nitrite found in meats (through preservatives), certain vegetables, and water provide important exogenous sources of nitrite [50]. Ingestion of foods rich in nitrates results in elevated plasma levels of both nitrate and nitrite and may help explain the health benefits of certain diets (e.g.

Mediterranean) [58]. It is important to appreciate that not all foods contain similar amounts of nitrate. For example, vegetables, such as beets, celery, and green leafy vegetables are rich in nitrate, whereas others such as broccoli, cauliflower, spinach and root vegetables contain lower amounts of nitrate [59–62].

2. Environmental sources—Apart from food sources, nitrite (NO_2^-) is also found in environmental toxicants such as cigarette smoke and car exhaust [63]. These and other environmental pollutants contain volatile nitrogen oxides that are converted to nitrate or nitrite in the body act as a source of nitrite. Nitrate intake from sources other than food was estimated to be around 35–44 mg/person/day [64]. Intake of nitrite from environmental sources also occurs through contaminated ground water, commonly due to fertilizers, livestock and human excreta, which pose a potential hazard to health [59, 65].

Nitrite chemical reactions and homeostasis

NO is a gaseous free radical with a relatively short physiological half-life. As such, oxidative and nitrosative products of NO reactions can serve as storage pools for bioavailable NO throughout the body [66]. NO readily reacts with molecular oxygen through a single electron oxidation to HNO. Further oxidation of this species results in the formation of nitrite (NO_2^-) with the major pathway for NO metabolism being oxidation to nitrite and nitrate under normal physiological conditions [67]. The oxidation reaction of NO by molecular oxygen leading to the formation of nitrite (NO_2-) is depicted below (reactions (1), (2) and (3)):

$$2NO+O_2 \rightarrow 2NO_2 \quad (1)$$

$$2NO+2NO_2 \rightarrow 2N_2O_3 \quad (2)$$

$$2N_2O_3+2H_2O \rightarrow 4NO_2^-+4H^+ \quad (3)$$

In aqueous solution, NO_2 may also decompose to give equal amounts of nitrite and nitrate (reactions (4) and (5)).

$$2NO_2 \rightarrow N_2O_4 \quad (4)$$

$$N_2O_4+H_2O \rightarrow NO_2^-+NO_3^-+2\,H^+ \quad (5)$$

Earlier studies reported the half-life of NO_2- in blood to be approximately 110 seconds; however, it is now clear that the half life of exogenously administered nitrite can range between 20–30 minutes thus making it a readily available buffering metabolite to maintain NO bioavailability [52, 68, 69]. Steady state levels of nitrite or nitrate do not solely depend on NO oxidation, but also on the dietary intake of nitrate, which can further elevate plasma nitrite levels upon entry into the nitrate/nitrite enterosalivary circulatory system where nitrate is subsequently reduced to nitrite and absorbed in the GI tract as discussed above.

Lastly, chemical interaction of NO and nitrite with other molecular targets also influences steady state nitrite levels within tissues [70]. A combined balance of the aforementioned factors determines overall nitrite levels in vivo.

Nitrite concentrations in plasma and in tissues correlate closely following administration, as transport of nitrite is aided by anion transporters across membranes due to the high concentration gradient from plasma to cells [71]. NOS3 dependent NO production plays an important role in regulating steady state plasma nitrite levels, while also influencing nitrate, nitroso, and nitrosyl products in tissues [72, 73]. Together, these facts demonstrate that NO/nitrite levels are regulated in a complex manner with several redundant pathways in order to maintain bioavailable NO equivalents.

Nitrite- Heme interactions

NO and nitrite are rapidly oxidized to nitrate in whole blood. The mechanisms by which NO and NO_2^- are converted to NO_3^- in vivo are not entirely clear. John Haldane showed that the reaction of nitrite with deoxyhemoglobin and deoxymyoglobin generated NO, and this NO bound to myoglobin was responsible for the bright red color of cured meat [74]. Brooks further described deoxyhemoglobin–nitrite reaction where deoxygenated ferrous hemoglobin reduces nitrite in presence of a proton, yielding NO and methemoglobin, which was reported in “The Action of Nitrite on Haemoglobin in the Absence of Oxygen” [75].

Ignarro et al reported reaction pathways defining nitrite/nitrate formation from NO auto-oxidation by oxyhemoproteins (P-Fe2+O2) such as oxyhemoglobin or oxymyoglobin [76] through the following equations:

$$2P-Fe2+O2+3NO2-+2H+ \rightarrow 2P-Fe3++3NO3-+H2O \quad (1)$$

or

$$4P-Fe2+O2+4NO2-+4H+ \rightarrow 4P-Fe3++4NO3-+O2+2H2O \quad (2)$$

This report clearly demonstrated that the dominant oxidation product of NO in aqueous solution is nitrite and that nitrate formation requires additional oxidation by heme proteins.

Deoxyhemoglobin plays a central role in the bioactivation of nitrite by reducing it to bioavailable NO under physiological and pathophysiological conditions like hypoxia. Formation of nitrite is not favored in blood or tissues, since NO reacts rapidly with heme proteins. In blood, NO reacts with oxygenated hemoglobin to yield nitrate and methemoglobin [77]. However, Cosby et al has shown that the presence of deoxygenated hemoglobin reduced nitrite to NO, which may activate soluble guanylate cyclase to mediate vasodilation [19]. It was also shown that the addition of NO to blood generates significant concentrations of plasma nitrite that involved copper oxidation, which was found to be ceruloplasmin dependent NO oxidation [78, 79]. This concept is confirmed in mice deficient for ceruloplasmin that showed lower basal concentrations of plasma nitrite and less plasma nitrite elevation upon NO administration [78].

Thus, nitrite present in blood can react either with oxyhemoglobin (oxyHb) to form nitrate (NO_3^-) and methemoglobin (metHb) or with deoxyhemoglobin (deoxyHb) to form NO, nitrosylhemoglobin (NO-Hb), and NO adducts. It is currently unknown whether NO formed from the reaction of nitrite with deoxyhemoglobin is directly exported from the red blood cell or is transformed into an NO adduct (RX-NO) to mediate nitrite induced vasodilation [18, 19, 80, 81]. The role of nitrite-heme interactions clearly requires further study to better understand specific reaction mechanisms contributing to physiological functions such as regulation of blood flow and storage of NO.

From Nitrite to NO

For quite some time, nitrite has been considered an inert oxidation product of NO [73, 82, 83]. However, now it is clear that nitrite anion is recycled back to NO in multiple ways. Initial appreciation of nitrite reduction to NO in tissues came from Zweier et al demonstrating that ischemic myocardium can produce NO from nitrite [84]. There are several alternative sources of nitrite-dependent NO generation available in biological systems. Acidic disproportionation of nitrite can lead to increased levels of NO [84–86]. Heme dependent nitrite reduction to NO can be accomplished through different globin proteins including deoxyhemoglobin, deoxymyoglobin, neuroglobin, and cytoglobin [19, 87]. Enzymatic reduction can also occur by several different proteins throughout the cell such as xanthine oxidase and proteins within the mitochondria (cytochrome c oxidase), which contribute to NO production under ischemic conditions [39, 88–90]. Commensal bacteria may also participate in nitrite and nitrate reduction and NO bioavailability in mucosal tissues such as the gastrointestinal and urogenital tracts [50, 91]. Importantly, all of these pathways serve to maintain adequate NO levels independent of typical NO generation through a L-arginine/NOS pathway. Below we discuss major pathways that mediate the conversion of nitrite/nitrate to NO under pathophysiological conditions.

Acidic reduction of nitrite to NO

Studies have shown that acidic disproportionation of nitrite occurs throughout the body [50, 84, 92]. Moreover, oral ingestion of nitrite into the acidic environment of the stomach produces NO that augments mucus secretion, antibacterial effects of gastric fluids, and ulcer healing [93–97]. Thus nitrite acidification and reduction to NO is an important mechanism of conversion in different tissue compartments.

At physiological pH (7.0–7.4), nitrite anions are fairly stable in the absence of compounds like oxyhemoglobin. Under acidic conditions (pH 3.2–3.4), nitrite converts to HNO_2, which is unstable and forms N_2O_3 that can subsequently generate free NO (reactions (1), (2) and (3)).

$$NO_2^- + H^+ \rightarrow HNO_2 \quad (1)$$

$$2HNO_2 \rightarrow N_2O_3 + H_2O \quad (2)$$

$$N_2O_3 \rightarrow NO + NO_2 \quad (3)$$

Though acidic reduction is a well known pathway for nitrite reduction to NO, the amount of NO generated in this way is dependent not only on pH and nitrite concentrations but also on the presence of other reducing agents, proximity to heme groups, thiols, proteins and oxygen concentration [98]. Work from the Zweier lab demonstrated that nitrite reduction to NO in ischemic rat hearts occurred in a NOS independent manner but was associated with tissue pH of 5.5 or below [84]. However, such extreme changes in physiological pH is not that common outside of significant tissue ischemia. A later study by Modin et al demonstrated that vascular nitrite reduction could occur under moderately acidic pH changes indicating that this pathway may indeed be important for augmenting tissue NO levels [92]. This report demonstrated that physiological levels of nitrite relaxed rat aortic rings at a pH of 6.6, which can readily be observed in tissues during ischemia [92]. Vessel relaxation was blocked by the soluble guanylyl cyclase inhibitor, ODQ, supporting the conclusion that NO was responsible for these effects. These observations indicate that moderately acidic environments are capable of reducing nitrite to NO thus maintaining bioavailability during tissue stress.

Reduction by Xanthine oxidase

Xanthine oxidase (XO) has critical implications in the physiology and pathophysiology of the cardiovascular system [99]. Xanthine oxidase is classically regarded as an important source for ROS generation during cardiovascular pathophysiology. However, XO may also catalytically reduce nitrite back to NO under several circumstances [90, 100, 101]. Xanthine oxidase is structurally related to bacterial nitrate and nitrite reductases and can reduce both nitrate and nitrite [102]. XO nitrite reduction to NO occurs at the molybdenum site with either NADH or xanthine serving as reducing substrates.

Different groups have shown that NADH can act as an electron donor to XO to catalyze nitrite reduction [90, 101]. Moreover, xanthine or hypoxanthine was found to inhibit this reaction [101]. Conversely, Gobler et al reported that xanthine might also serve as a reducing substrate to stimulate nitrite reduction [100]; however, this has been debated by conflicting reports [101, 103]. Although xanthine has efficient reducing potential of XO-catalyzed nitrite reduction, excessive xanthine could clearly influence this response. Moreover, a study by Zhang et al indicates that XO enzyme inactivation can result from XO-induced NO production involving nitrite reduction [90]. It has been reported that peroxynitrite markedly inhibits XO activity in a dose-dependent manner, thus it is possible that subsequent nitrosation of XO due to nitrite reduction to NO in the presence of superoxide may also influence XO activity [104].

Reduction by heme containing proteins

Oxygenated hemoglobin is an extremely effective NO scavenger that reacts rapidly with NO to form nitrate and methemoglobin (met-Hb) [105]. However, experiments by Cosby et al showed that intra-arterial infusion of nitrite at near-physiological levels causes vasodilation in an arterio-venous gradient manner that is influenced by R-T state conversion and oxygen

tension implicating red cell reduction of nitrite to NO [19, 106, 107]. Nagababu et al also reported that deoxyhemoglobin mediated nitrite reduction to NO via chemiluminescent techniques coupled with EPR identifying chemical intermediates of the reaction [108]. Lastly, Crawford et al further demonstrated that deoxygenated red cells were able to reduce nitrite to NO which mediated vessel ring dilation, solidifying the biological importance of deoxyhemoglobin reduction of nitrite [109]. Greater details of this reaction are now understood and the reader is referred to a recent review by Patel et al that elegantly addresses the latest understanding of hemoglobin mediated nitrite reduction [110].

Other heme containing tissue proteins have been reported to mediate nitrite reduction to NO. Recent work from several laboratories indicates that myoglobin serves as an important reductase during tissue ischemia [111, 112]. In these studies, genetic deletion of myoglobin in the heart significantly diminished the protective effect of sodium nitrite on myocardial ischemia-reperfusion [113, 114]. Moreover, recent reports also demonstrate that neuroglobin and cytoglobin may also facilitate nitrite reduction [39, 115]. Importantly, the reductase efficacy of these enzymes is directly related to oxygen tension typically requiring a large drop in tissue oxygen tension before acting as a nitrite reductase. These results again highlight that nitrite reduction back to NO serves as an alternative pathway to augment tissue NO levels.

Reduction by Mitochondrial Enzymes

NO metabolism in the mitochondria plays a key role in regulating cellular respiration, mitochondrial transmembrane potential and transmembrane pH gradient [35]. Moreover, mitochondria derived NO plays an important role as an antioxidant by reacting with potential harmful ROS. Components of the mitochondrial respiratory chain are capable of reducing nitrite to NO due to their roles as carriers during electron transport. Older in vitro studies with porcine skeletal muscle revealed that cytochrome c oxidase may catalyze nitrite reduction under anaerobic conditions [88, 116–118]. Reutov and Sorokina went on to posit that nitrite reduction may occur in mitochondria and suggested that cytochrome c oxidase (complex 4) was the enzyme most likely responsible for carrying out this reaction [119]. This was later confirmed by Kozlov's group who illustrated that cytochrome c oxidase was an important regulator of nitrite reduction to NO in the mitochondria [120, 121]. Moreover, Meyer also suggested a role for cytochrome c oxidase in the conversion of nitrite to NO in Zweier's experiments with the ischemic rat heart but this was not confirmed by the Zweier lab [84, 117]. Castello and coworkers have also shown nitrite-derived NO production by rat liver mitochondria was inhibited by cyanide and enhanced cytochrome c oxidase in a pH dependent manner [84, 117]. Other mitochondrial molecules may also participate in nitrite reduction reactions. Recent work from Basu and colleagues reported that liposomes containing cytochrome c also reduces nitrite to NO in a pH and nitrite concentration dependent manner [122]. Given the fact that nitrite reduction occurs within the mitochondria, it is reasonable to predict that cytochrome c participates in nitrite conversion to NO to confer cytoprotection. However, additional studies are needed to elucidate the pathophysiological importance of cytochrome c nitrite reduction in mitochondria and tissues.

Recent reports demonstrate that nitrite/nitrate effects on mitochondria significantly affect several physiological responses. In two studies by Larsen et al, it is clear that dietary nitrate consumption increased plasma nitrite levels and decreased muscle oxygen cost during both sub-maximal and maximal exercise [123, 124]. These studies further demonstrated that the effect of dietary nitrate did not alter heart rate, lactate accumulation, or ventilation, and correlated with an increased time to exhaustion. Likewise, work from the Jones laboratory also found that dietary nitrate administration through beetroot juice consumption decreased oxygen cost during exercise that was also associated with increased plasma nitrite levels and extended time to exhaustion [125–127]. Most recently, Larsen et al demonstrated that dietary nitrate enhancement of muscle function during exercise involved increased muscle mitochondrial oxidative phosphorylation efficiency that is associated with reduced ATP/ADP translocase expression [128]. Together, these studies demonstrate that nitrite/nitrate positively affects mitochondrial function to alter muscle metabolism, which could similarly affect mitochondrial function of other tissues.

While it has been well known that prokaryotes contain numerous nitrite/nitrate reductases, the confirmation that eukaryotic cells employ enzymatic nitrite reduction has only recently become clear [129]. Numerous other nitrite reduction mechanisms have been identified besides those discussed above including: aldehyde dehydrogenase, carbonic anhydrase, and endothelial nitric oxide synthase [130–132]. While most attention in the literature has been devoted to deoxyhemoglobin, -myoglobin, and XO as mediators of anoxic/hypoxic nitrite reduction in vivo, it is certain that additional discoveries in this area remain to be seen that will provide additional insight into the importance of nitrite metabolism to maintain NO homeostasis.

Therapeutic implications of sodium nitrite

Current therapeutic use of sodium nitrite is for the treatment of cyanide poisoning. The beneficial effect of sodium nitrite stems from the fact that cyanide has a high binding affinity for ferric iron, which is the basis of cyanide inhibition of cytochrome oxidase a_3 thus decreasing mitochondrial respiration [133]. Therapeutic administration of sodium nitrite 30 mg/ml over 5–20 minutes (~300 mg total) significantly increases metHb (i.e. ferric heme) levels (~20%) that can bind with cyanide to form cyanmethemoglobin thus competing with cytochrome oxidase a_3 [134]. However, sodium nitrite mediated metHb formation is slower than other cyanide antidotes (e.g. 4-dimethylaminophenol) and has lead to the notion that the cardiovascular effects of nitrite may also be protective against cyanide mediated increases in central venous pressure [135].

However, given the appreciation of the reciprocal relationship between nitrite and NO plus the fact that NO bioavailability is well known to contribute to numerous disease states [136, 137], therapeutic use of inorganic nitrite may be of widespread benefit. Perhaps the most appreciated relationship between NO and disease is that of the cardiovascular system. It is well accepted that decreased NO levels contribute to endothelial cell dysfunction, atherosclerotic plaque development, arteriosclerosis, vessel restenosis, peripheral vascular disease, and ischemia-reperfusion injury [14, 15, 138–140]. However, numerous other pathological conditions involving acute or chronic inflammation, neurological dysfunction,

and cancer are also significantly affected by NO. Figure 3 illustrates several pathophysiological conditions where nitrite therapy is reported to be beneficial as discussed below.

Nitrite - a marker of NOS activity

Given the importance of NO for tissue homeostasis and its role in modulating pathophysiological events, numerous studies have been reported detailing methods in which to measure NO levels [82, 141–144]. From the outset, measurement of tissue or plasma nitrite/nitrate levels was central in discerning NO production as the majority of NO produced is oxidized to nitrite/nitrate. This was first examined through various different chemical assays including the colorimetric Griess assay and others using fluorescent substrates (e.g. diaminonapthalene). Over time, it was clear that these assays were problematic for different reasons, with a lack of sensitive discrimination between respective nitroso species being a primary issue often yielding differences in measurements between labs that led to a misunderstanding of biological ‘levels’ of NO. Many of these problems were resolved upon closer chemical reaction evaluation coupled with the application of chemiluminescence techniques to sensitively measure very low amounts of nitrite, nitrate, and other nitroso species [141, 145, 146]. Today, NO chemiluminescence detection is considered the standard analytical method for measuring low concentrations of NO metabolites; however, other potentially more sensitive assay formats for nitrite and nitrate are growing in popularity such as the EICOM nitrite/nitrate HPLC.

Recent studies indicate that plasma and tissue levels of nitrite can serve as accurate indicators of NO generation. Lauer et al have shown that plasma concentrations of nitrite reflect acute changes in regional eNOS activity and NO bioavailability that are sensitive to pharmacological modulation of the L-arginine - NO pathway [147]. A separate study by Kleinbongard and colleagues reported that plasma nitrite levels reflect NOS activity in mammals [17]. Moreover, studies by the Kelm laboratory further revealed that plasma nitrite levels are inversely proportional to the number of cardiovascular risk factors and endothelial cell dysfunction [17, 80]. Together, these and other findings suggest that plasma and tissue levels of nitrite closely reflect NOS activity as well as NO metabolism that is associated with pathophysiological conditions.

Nitrite pharmacokinetics

Recent work by Hunault and colleagues provides informative pharmacokinetic and dynamic data on oral versus intravenous sodium nitrite administration in healthy subjects [69]. Upon oral consumption, inorganic nitrite absorption occurs quickly and primarily in the upper small intestinal region (duodenum and jejunem) typically reaching a peak plasma level between 15–30 minutes that may be influenced by the amount administered and the presence of food in the stomach. The circulating half-life of nitrite is brief with an average between 25–35 minutes. Conversely, peak plasma nitrate levels from sodium nitrite consumption are observed on average by 1.5 hours with a circulating half-life of 8–13 hours depending on dose and route of administration. Sodium nitrite is very well absorbed with a bioavailability of > 98% in humans and rodents. Other studies examining sodium nitrite pharmacokinetics report similar findings in rodents and non-human primates involving both intravenous as

well as oral dosing regimens [148–150]. Importantly, continuous sodium nitrite administration in non-human primates was not associated with tolerance as is seen with organic nitrates [148]. Lastly, it has also been reported that side effects such as mild methemoglobinemia (1–3%) may be observed with doses >2–3 mg/kg of sodium nitrite.

Nitrite relationship with hypoxia

Accumulating evidence demonstrates that nitrite anion acts as a physiological intermediary for several cellular responses known to involve nitric oxide. Specifically, nitrite contributes to intravascular endocrine nitric oxide (NO) transport, hypoxic vasodilation, signaling responses, and tissue cytoprotection after ischemia-reperfusion. Human and animal studies of NO gas inhalation suggest that nitrite mediates many of the systemic therapeutic effects of inhaled NO, including peripheral vasodilation and prevention of ischemia-reperfusion-mediated tissue infarction [19, 109, 148, 151, 152]. With regard to nitrite-dependent hypoxic signaling, biochemical and physiological studies suggest that hemoglobin allosteric reduction of nitrite to NO along arterial to venous vasculature contributes to physiological hypoxic vasodilation [19, 87, 153]. Importantly, expanded understanding of nitrite as a hypoxia-dependent source of NO and signaling responses suggest that nitrite itself may be an important participant in physiological vasoregulation responses to tissue hypoxia. The loss of nitrite vasodilation upon removal of the endothelial monolayer also implicated this cell compartment as an important source of nitrite reduction within the vasculature [154]. As previously mentioned, multiple nitrite reduction mechanisms are possible within the cardiovascular system. However, the respective contribution of each compartment for nitrite reduction remains poorly understood and requires additional study.

Sodium nitrite therapy for ischemic tissue disorders

Without a doubt, the surprisingly potent effects of sodium nitrite therapy during experimental hypoxia or ischemia-reperfusion injury drew significant attention to this new field during 2003–2006. During this time, several studies detailing nitrite mediated protection against various experimental ischemia-reperfusion conditions in different organs were reported [19, 83, 155–158]. Moreover, these critical papers provided key insight into the cytoprotective effects of sodium nitrite. Webb et al initially investigated the effect of different sodium nitrite concentrations on ischemic heart function using the rat langendorff heart model [89]. In this study, the authors demonstrated that sodium nitrite treatment of ischemic hearts ex vivo could generate NO upon treatment with 10–100 μM sodium nitrite. Data was also provided demonstrating this response was similar in human heart homogenates and that sodium nitrite therapy could protect against ischemia induced pump dysfunction as nitrite therapy increased rat heart left ventricular developed pressure after ischemic insult. Shortly thereafter, Duranski and colleagues reported a highly comprehensive set of data detailing a dose response range of sodium nitrite therapy during both myocardial and hepatic ischemia-reperfusion injury [156]. This study provided the initial mechanistic understanding of nitrite mediated ischemic cytoprotection in vivo. This paper proved that near physiological doses of sodium nitrite (high nanomolar) were highly cytoprotective in both myocardial and hepatic ischemia-reperfusion extending previous work from Cosby et al demonstrating that near physiological levels of nitrite anion mediated hypoxic vasodilation responses [19]. Moreover, studies in the Duranski paper were

performed in vivo with the presence of blood which provides critical reducing capacity due to deoxyhemoglobin and deoxymyoglobin thus providing an explanation as to why less nitrite was needed to confer cytoprotection compared to ex vivo organ studies [84, 89]. Lastly, Duranski et al showed that nitrite therapy protects against ischemic tissue injury through a heme oxygenase-1 pathway which further implicated the generation of nitrite dependent NO during therapy. Several other studies extended the idea that nitrite therapy is protective against ischemia-reperfusion injury in different organs including the brain [157] and kidney (depending on route of administration) [158, 159]. Moreover, cytoprotective effects were also examined using a myocardial ischemia-reperfusion model in dogs, further illustrating the therapeutic potential for this molecule [160]. Together, these papers formed the foundation for subsequent studies into mechanisms of nitrite protection against acute ischemia reperfusion injury.

The potent effect of low doses of nitrite on limiting ischemia-reperfusion infarction suggests that nitrite may modulate critical pathophysiological stress responses, particularly those involved during tissue ischemia [47, 161]. Interestingly, work from Bryan et al demonstrated that nitrite anion itself could stimulate cGC activity and other signaling mediators as treatment with the NO scavenger carboxy-PTIO was unable to prevent these effects by nitrite administration [20]. Conversely, cPTIO treatment was shown by numerous groups to attenuate nitrite mediated protection against ischemia-reperfusion injury in vivo indicating that protective mechanisms likely involve NO dependent signaling pathways and molecular responses [47, 89, 156, 162]. Consistent with this hypothesis, Shiva et al elegantly demonstrated that nitrite therapy significantly blunted mitochondrial uncoupling during in vivo ischemia reperfusion through nitrite mediated, NO dependent, inhibition of mitochondrial complex I activity [40]. Subsequent work by the same group revealed that nitrite therapy potently decreases mitochondrial respiration during ischemia thereby preventing uncoupling of electron transport that increases reactive oxygen species formation leading to cytochrome c release and apoptosis activation [4, 39, 40]. These data demonstrate the complexity associated with nitrite-mediated cytoprotection, which remains incompletely understood.

It is crucial in therapeutic approaches to consider the route of administration and the effective dose in the organ, which undoubtedly have varied effects on the system. Altering sodium nitrite time of administration and/or route of delivery was performed in ischemic models. Initial papers investigating nitrite effects on ischemia-reperfusion injury administered nitrite midway through ischemia or immediately prior to reperfusion [89, 156–158]. However, Gonzalez et al demonstrated that even after 5 minutes of reperfusion nitrite therapy conferred myocardial cytoprotection [160]. Moreover, the route of administration did not significantly alter the protective outcome of nitrite therapy as oral administration of sodium nitrite was able to attenuate myocardial ischemia-reperfusion injury [163]. However, it is not clear whether other routes or frequencies of sodium nitrite dosing similarly affect plasma and tissue nitrite levels, which confer protection against ischemic stress.

NO production of nitrite can also act in an endocrine manner to augment distal tissue function. In an elegant transgenic animal study where eNOS expression was selectively over-expressed using a cardiac myocyte specific promoter (CS-eNOS-Tg), Elrod et al

documented significant increases in nitrite, nitrate, and nitrosothiols in the heart, plasma, and liver [164]. Moreover, CS-eNOS-Tg mice displayed a significant protection against hepatic I/R injury due to the increase in hepatic NO metabolites originating from the myocardium. Experiments also showed that pharmacological scavengers PTIO and NEM inhibited the protective effects in CS-eNOS-Tg mice, indicating a role for nitrite reduction to NO and S-nitrosylation for cytoprotection [164]. These findings clearly demonstrated that NO derived nitrite from distant tissues could be transported in the blood to significantly affect remote organs.

Potential use of nitrite as an adjunctive therapeutic agent to attenuate transplantation ischemia-reperfusion injury has been reported to a limited degree. In a report by Zhan et al, the authors examined the efficacy of nitrite supplementation on cardiac allograft survival using a fully allogeneic rat heart transplantation model [165]. It was shown that supplementation of nitrite reduced allograft inflammation, apoptosis, and macrophage recruitment and mitigated donor-specific alloimmune responses to prolong allograft survival. A previous study from Lang et al demonstrated that inhaled NO therapy before liver transplanation significantly elevated tissue nitrite levels that were associated with cytoprotection [166]. Following that observation, Li and colleagues reported that nitrite administration confers protection against liver cold ischemia-reperfusion injury [167]. Together, these studies indicate that sodium nitrite may be useful in preventing transplantation ischemia-reperfusion injury; however, additional investigation in this area is certainly needed.

Chronic tissue ischemia

NO bioavailability also plays an important role modulating chronic tissue ischemia responses [168, 169]. Chronic tissue ischemia due to peripheral arterial disease (PAD) progressively leads to restricted blood flow in the extremities that can progress to critical limb ischemia (CLI). PAD is associated with a host of vascular pathophysiological complications that contribute to disease progression including hypertension, atherosclerosis, hyperlipidemia, diabetes, obesity, and stroke. Moreover, limited effective therapeutic options exist for patients with PAD or CLI thus highlighting the importance of this clinical problem.

The effects of sodium nitrite therapy have been investigated during chronic ischemia using the unilateral femoral artery permanent ligation model to elicit hind limb ischemia [47]. Our group has reported that continuous sodium nitrite administration increases ischemic hind limb blood flow after permanent femoral artery ligation. Several important conclusions were made from this study which are: 1) low dose sodium nitrite therapy quickly restored ischemic hind-limb blood flow within several days and did not affect flow in the contralateral non-ischemic limb, 2) nitrite therapy selectively induced endothelial cell proliferation and angiogenesis only in the ischemic limb, 3) nitrite mediated changes in blood flow and endothelial cell growth were blocked by the NO scavenger cPTIO, 4) nitrite therapy significantly increased ischemic tissue nitrite/nitrosothiol levels, and 5) sodium nitrate therapy was unable to mediate similar responses to nitrite therapy over the duration of study. This report was the first to demonstrate selectively targeted NO therapy in

chronically ischemic tissues that augmented therapeutic revascularization. These results were in contrast to previous reports using non-specific NO donors or growth factors that elicited off target tissue responses. Importantly, administration of a single dose of sodium nitrite after induction of ischemia did not augment hind limb reperfusion (unpublished observations) and delayed sodium nitrite therapy 5 days after ischemic induction still augments ischemic tissue reperfusion and arteriogenesis development (Pattillo in press).

Our group has also discovered that the endocrine NO/nitrite activity confers protection against chronic ischemia [170]. Various anti-platelet agents like dipyridamole have shown some clinical efficacy in managing PAD with the speculation that they may alter arteriogenesis/angiogenesis or ischemic vascular function [171, 172]. Upon closer examination, we found that dipyridamole therapy significantly increased contralateral non-ischemic limb eNOS activity and NO production that increased plasma nitrite levels to augment ischemic limb reperfusion and angiogenesis [170]. These beneficial effects were PKA and eNOS dependent as the effects of dipyridamole were absent in eNOS knockout mice. This finding together with the study by Elrod and colleagues clearly indicates the importance and therapeutic potential of manipulating the NO/nitrite endocrine system.

Recent work from Allen et al indicates that plasma nitrite levels may be an important indicator of vascular health and function in PAD patients. In the first study by the Allen lab, induction of plasma nitrite levels during supervised exercise therapy were significantly blunted in diabetic patients with PAD versus non-diabetic PAD patients or diabetics alone [173]. In a follow up study, the authors found that plasma nitrite flux predicted the time to claudication onset pain reflecting exercise performance ability [174]. Kenjale et al has also recently reported that dietary nitrate can enhance exercise performance in PAD patients thus it appears that nitrite therapy maybe clinically beneficial [175]. However, additional studies are needed to better understand the effect of nitrite for PAD therapy.

Sickle cell disease

Sickle cell disease (SCD) is an autosomal co-dominant genetic blood disorder due to a point mutation of Glu to Val in the hemoglobin β-chain resulting in fibrilar protein rearrangement that deforms the red cell into a sickle shape [176, 177]. The pathophysiology of SCD is characterized by increased oxidative stress, decreased NO bioavailability, and increased RBC adhesion to the vasculature [178, 179]. These factors contribute to defects in SCD vasoregulation that appears to be largely influenced by endothelial dysfunction and a loss of NO [180]. Moreover, numerous studies have shown that SCD patients accumulate large concentrations of cell free hemoglobin in plasma due to intravascular haemolysis, which clearly decreases NO bioavailability [181–185]. Intermittent ischemia is also prominent in these patients due to a multitude of factors including red cell lysis and adhesion, and defective NO levels all of which could be ameliorated by hydroxyurea in an NO dependent manner [178, 186]. Therefore, it is reasonable to predict that exogenous nitrite therapy may be beneficial for this disease.

Mack et al has shown that in human subjects with SCD, small doses of sodium nitrite were adequate to provide detectable changes in systemic plasma nitrite levels and augment forearm blood flow [187]. These data are encouraging and strongly suggest that sodium

nitrite could ameliorate endothelial cell dysfunction and ischemic injury in this patient population. Importantly, there were no indications of nitrite-mediated side effects like methemoglobinaemia or reduced blood pressure with the infusions of sodium nitrite.

Sodium Nitrite for Hypertension and Pulmonary Hypertension

Over a century ago, amyl nitrite and sodium nitrite were used for relieving symptoms of acute bouts of hypertension or asthma attack [188]. However, over the course of several years use of sodium nitrite fell out of favor due to the more rapid metabolic effects of organic nitrites and side effects of high dose sodium nitrite consumption. In addition to these uses, sodium nitrate was given orally to treat chronic bronchitis. It was however unclear whether the apparent effectiveness of this treatment was secondary to its conversion to nitrite causing bronchial relaxation and antibacterial effects or due to an effect of nitrate itself [189].

Recent studies in humans have rekindled an interest in nitrite/nitrate consumption for control of hypertension. An initial study by Larsen et al reported that 3 day dietary nitrite supplementation of healthy volunteers with sodium nitrate (0.1 mmol/kg/day) significantly increased plasma nitrite/nitrate levels and decreased diastolic pressure by 3.7 mm Hg [190]. Similarly, Webb et al investigated the possibility of increasing plasma nitrite levels through dietary nitrate by consumption of beetroot juice (~500 mls) that was also found to acutely lower arterial BP, augment endothelial function during ischemia, and inhibit platelet aggregation as a result of bioconversion to NO in healthy individuals [191]. Their work nicely demonstrated that increasing plasma nitrite levels resulted in a marked reduction in blood pressure levels in normotensives; however, it is not exactly clear what the effect would be in patients with various forms of hypertension. These findings suggest that dietary nitrate highlight the beneficial effects of a vegetable-rich diet and demonstrates the potential of a “natural” approach for the treatment of cardiovascular disease.

NO generation and metabolism is also known to play an important role in the development of pulmonary artery hypertension (PAH) as tissue levels of nitroso species and cGMP are depressed during PAH and phosphodiesterase 5 inhibitor (e.g. sildenafil) therapy ameliorates disease severity [192–194]. Reports from several laboratories demonstrate that sodium nitrite can stimulate hypoxic vasodilation of the pulmonary vasculature indicating that nitrite therapy might be clinically useful for PAH [87, 195, 196]. A recent study by Zuckerbraun et al reported that nebulized nitrite administered either once or three times a week significantly prevented or reversed hypoxia or monocrotaline induced pulmonary artery hypertension [197]. Interestingly, sodium nitrite therapy minimally increased plasma and lung nitrite levels but still ameliorated disease. This observation of small changes in systemic nitrite levels associated with biological benefit reaffirms a previous report showing a similar result [195]. It was also found that nitrite mediated therapy partially involved p21 cyclin-dependent kinase inhibitor as hypoxia pulmonary hypertension was still evident upon nitrite therapy in p21 deficient mice yet reduced pulmonary pressures and ventricular remodeling responses were still reduced.

Nitrite may also attenuate unsafe elevations in blood pressure during cell free hemoglobin volume resuscitation. The use of cell free hemoglobin has long been sought after for use

during trauma but is associated with significant side effects such as hypertension and mortality. In a study by Rodriguez et al it was reported that administration of bolus nitrite (30–100 nmol) at the onset of hemoglobin based oxygen carriers (HBOC-201) resuscitation prevented hypertension [198]. This report supports the idea that nitrite can be used as an adjunct therapy to prevent HBOC-dependent hypertension to alleviate associated side effects and possibly mortality.

Cerebral vasospasm

NO deficiency has been associated with cerebral vasospasm due to ruptured intracranial aneurysms through increased NOS antagonist metabolites (e.g. ADMA) [157, 199]. This clinical condition entails the development of regional ischemia within the affected area that precipitates vasoconstrictor responses compromising blood flow. A study from Pluta et al examined the effects of sodium nitrite infusion in a primate model of cerebral vasospasm due to subarachnoid hemorrhage and found that sodium nitrite therapy using a low (90 mg over 24 hours with a 45 mg daily bolus) or high (180 mg over 24 hours) dose completely prevented the development of cerebral vasospasm over a two week period as measured by cerebral arteriograms [199]. This report showed that nitrite therapy augmented vasodilation indicating that this modality might be useful for clinical cerebral vasospasm.

Cystic fibrosis and biofilm infection

Cystic fibrosis is a recessive genetic disease involving a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Patients suffer from a wide range of problems involving multiple organ systems; however, chronic infections are especially problematic. Sodium nitrite is known to have antibacterial properties (which contributes to its role as a food preservative) prompting investigation into whether it might prevent bacterial growth in a CF setting. Work by Yoon et al demonstrated that acidified sodium nitrite mediated anaerobic killing of mucoid *Pseudomonas aeruginosa* in cultured biofilms and enhanced killing against this and antibiotic resistant strains in lungs in vivo [200]. Acidified nitrite also exhibits bactericidal action against several pathogens in other tissue compartments including the skin, dental caries, and gastric mucosa [201–206]. Major et al has also reported that sodium nitrite itself displays effective killing against other cystic fibrosis microbial pathogens including *S aureus*, and *B cepacia* under anaerobic and biofilm conditions [207], suggesting that aerosolized sodium nitrite therapy might be beneficial for controlling CF associated bacterial infections. Lastly, it is possible that probiotic approaches using healthy bacteria containing high nitrate reductase activity could be useful in controlling pathogenic bacterial growth [208].

Sodium Nitrite Therapy for Inflammatory Diseases

NO has a notorious role in regulating immune responses in both acute and chronic settings [209]. Therefore, it is not surprising that nitrite may also modulate inflammatory responses under certain conditions. Septic shock is one inflammatory setting where NO production and metabolism has garnered a conflicted reputation. Several studies have implicated excessive NO production by iNOS and/or eNOS in the development of experimental sepsis and shock [210, 211]. However, other studies suggested that additional NO protection may be needed

during sepsis and shock, which was observed from NOS inhibition in animal models and septic shock patients [212, 213]. A recent study by Cauwels et al shows that exogenous sodium nitrite treatment, in sharp contrast to exacerbation by NOS inhibition, significantly attenuates hypothermia, mitochondrial damage, oxidative stress, tissue ischemia, and mortality in a TNF-α induced murine shock model [214]. Nitrite was shown to protect mitochondria against oxidative stress thus shielding organs from shock-induced damage. It was also determined that nitrite mediated protection was independent of eNOS function but was lost in sGC knockout animals. Lastly, the protective effect of nitrite was not restricted to TNF-α but also protected against LPS induce sepsis. While these findings may be at odds with previous reports, the authors suggest that nitrite administration may work in a multifaceted manner to diminish perfusion deficits while influencing immune responses.

Hypercholesterolemia

Hypercholesterolemia leads to a decrease in NO bioavailability and increased production of ROS resulting in a proinflammatory environment within the vascular system. Restoration of NO availability either by exogenous NO stimulation or NOS activation effectively attenuates vascular inflammation and restores endothelial cell function [215–218]. Couple this information with the fact that plasma nitrite levels decrease in an inversely proportional manner to cardiovascular risk factors, it is logical to predict that nitrite therapy may confer benefit during hypercholesterolemia. Thus, Stokes et al used dietary nitrite in drinking water as a means to enhance NO bioavailability in a hypercholesterolemic mouse model, by feeding C57Bl/6J mice with a high fat western diet for 3 weeks [219]. This study shows that nitrite supplementation attenuated hypercholesterolemia mediated microvascular inflammation and that nitrite therapy preserved reduced BH_4 levels during dyslipidemia highlighting an important aspect of the antioxidant effects of nitrite therapy. Moreover, nitrite administration significantly attenuated hypercholesterolemia mediated leukocyte recruitment, which plays a key role in controlling vascular inflammation. Recent work from Carlström et al reveals that long term dietary nitrate consumption can reverse metabolic parameters associated with eNOS dysfunction that are associated with the development of cardiovascular disease [72]. It was found that daily administration of 85 mg/L^{-1} in the drinking water for 8–10 weeks decreased body weight, plasma triglycerides, and visceral fat while improving glucose tolerance and fasting blood glucose levels in eNOS deficient mice. Together, these data reveal a novel role for nitrite/nitrate as a therapeutic agent to diminish microvascular inflammation and restore endothelial function associated with cardiovascular and metabolic disease.

Experimental colitis

Colitis is a chronic inflammatory disorder of the intestinal tract with excessive production of cytokines, adhesion molecules, and reactive oxygen species (ROS) [220]. There is substantial evidence for the involvement of oxidative stress and alterations in the biosynthesis of NO in the pathogenesis of colitis [221–224]. The role of nitric oxide during inflammatory bowel disease has been considered both beneficial and harmful, and its true role remains poorly understood [221–224]. Acute and chronic colitis elicited by various methods are characterized by mucosal inflammation of the large bowel, leading to increased NO production secondary to iNOS up-regulation following barrier dysfunction [86, 225,

226]. Exogenous NO donors have been used in experimental colitis models as a therapeutic intervention [227, 228]. Salas et al. showed that DETA NONOate donor significantly protected against DSS colitis in mice with a reduction in pro-inflammatory cytokine production observed in the early phase of the model [228]. Recent studies have also shown that eNOS mRNA levels decrease after induction of colitis with DSS challenge [229]. Moreover, eNOS deficient mice develop more severe DSS colitis than wild type mice indicating that eNOS plays an important role in limiting intestinal injury and inflammation most likely through pro-inflammatory cytokine production such as IL-12 and IFN-γ and preventing leukocyte recruitment [230–232].

Ohtake et al recently investigated the effects of oral nitrite therapy during DSS-induced colitis in mice [233]. They found that therapeutic intervention with a high dose (25 mM ≈ 1.73 g/L) of oral nitrite conferred significant protection against tissue histopathology. It was reported that during the development of colitis, tissue levels of nitrite dropped quickly along with a concomitant increase in colonic TNF-α expression by day three followed by increased myeloperoxidase activity, and iNOS and HO-1 expression at day 7. Oral nitrite therapy reversed the loss of colonic nitrite levels and decreased TNF-α expression, which was associated with clinical and histological improvements. These data suggest that nitrite may be able to attenuate acute inflammatory flares of colitis by preserving tissue NO bioavailability or possibly through antioxidant mechanisms.

The notion that tissue nitrite levels are involved during the development of colitis was confirmed by another study. Saijo et al examined tissue and plasma nitroso species in both the acute and chronic dextran sodium sulfate (DSS) induced rat colitis model [234]. This study found that the development of DSS colitis involves an early loss of colon tissue nitrite levels with a concomitant increase in systemic nitrite. Moreover, tissue nitrite levels were repleted within four days after DSS withdrawal that may or may not be associated with chronic inflammation. Interestingly, the effects of 5-aminosalicylic acid (5-ASA- an anti-inflammatory agent for the treatment of inflammatory bowel disease) attenuated depletion of colon tissue nitrite levels, increased systemic nitrite levels, and oxidative stress during DSS colitis. Together, these findings indicate that tissue nitrite levels somehow influence the development of experimental colitis and that nitrite therapy may alleviate some of the histopathology due to DSS administration. It is possible that this could be related to the recent understanding that DSS colitis instigates mucosal hypoxia that contributes to inflammatory histopathology [235] which could be alleviated by nitrite therapy.

Gastric ulcer and wound healing

NO is known to influence gastric mucus production and epithelial cell function [236]. As such, investigators posited that nitrite therapy may be beneficial for gastric mucus secretion and ulcer healing due to acidic disproportionation within the gastric milieu. Work from Uchida et al has clearly demonstrated that NO donors augment gastric ulcer healing [237]. Along these lines of thought, Bjorne et al demonstrated that salivary nitrite increases gastric mucosal blood flow and mucus production [93]. Moreover, Jansson et al reported that dietary nitrate protects against non-steroidal anti-inflammatory drug induced ulceration responses [238]. Moreover, NO is well known to augment wound healing responses in other

tissues through different responses including neovascularization, tissue matrix remodeling, and granulation tissue formation [239, 240]. Likewise, Isenberg et al has reported that nitrite therapy augments surgical flap wound healing responses in mice involving increased angiogenesis and tissue blood flow [241]. However, little mechanistic information exists regarding the effects of sodium nitrite on wound healing responses, which requires further investigation.

Innate and acquired immune responses

Though NO plays a role in cutaneous skin physiology [242, 243], the biological function of nitrite as an NO storage pool is not completely understood [244], nor is the mechanism by which dermal nitrite couples to systemic NO/nitrite physiology. It is interesting to note that apart from NO, nitrite concentrations in human skin are considerably higher than those in circulation [245, 246].

Recently, Garcia-Saura et al has shown that dermal nitrite administration provides a noninvasive alternate delivery route that can be extended to percutaneous systemic therapy [247]. This study reported that topical administration of 10 mg/kg^{-1} nitrite, markedly increased nitrite, nitrate, RXNO, and NO-heme species in most tissue compartments sparing the kidney [nitrite], brain [nitrate, RXNO, NO-heme], and plasma [RXNO, NO-heme], where no change of the bracketed NO metabolites occurred. However, there were notable increases in NO-related metabolites within the thymus, spleen, and peripheral lymph nodes. It was observed that a 45–60% drop in T cells occurred shortly after dermal nitrite application. NO is well known to regulate T-cell, dendritic cell, and macrophage function in various immune responses [248–250]. Moreover, dermal nitrite administration potently inhibited Th2-derived cytokine, IL-13 levels, in bronchial alveolar lavage fluid. Together, these findings indicate that topical sodium nitrite therapy effectively augments tissue compartment nitrite levels while revealing unique aspects of immune regulation.

Inflammatory responses are not solely governed by immune cell function with vascular cells serving a critically important role in regulating both acute and chronic inflammation [251]. We have recently examined the effect of nitrite or dipyridamole therapy on immune responses during chronic tissue ischemia [252]. We found that nitrite anion therapy in chronically ischemic tissue results in selective down regulation of innate immune response genes such as chemokine ligands, toll like receptors, and leukotriene and formyl peptide receptors, while increasing expression of VE cadherin. Interestingly, sodium nitrite therapy in the hind limb ischemia model strongly depressed interleukin-10 (IL-10) expression. This observation is important for two reasons as IL-10 expression has previously been shown to be anti-angiogenic in the ischemic hind limb model and it can also promote pathological angiogenesis during hypoxia by activating macrophage behavior to a pro-angiogenic phenotype in the retina [253, 254]. These data demonstrate that nitrite works to simultaneously modulate inflammatory responses while increasing vascular remodeling of ischemic tissues. This opens a novel role of nitrite derived therapy in targeting redox regulation of innate immune responses that could represent useful clinical strategies to treat vascular diseases.

Summarizing the above information, it is obvious that inorganic nitrite therapy confers substantial benefit to numerous pathophysiological conditions. However, important conclusions can be made with regard to specific aspects of inorganic nitrite therapy. First, permissive tissue and biochemical conditions (e.g. hypoxia, reductase activity, affected organs) clearly influence whether nitrite therapy manifests a favorable response. Second, the required dose of nitrite necessary for beneficial effects differs significantly amongst disorders such that high nanomolar to low micromolar nitrite levels are protective during tissue ischemia, yet high micromolar to low millimolar doses appear to be required for attenuating colitis pathology and bacterial growth associated with cystic fibrosis. This is an important distinction given the fact that high doses of sodium nitrite can be toxic thus making nitrite therapy for certain diseases largely unrealistic. Third, the duration and route of administration of inorganic nitrite clearly influences observed beneficial effects such that continuous dosing is required to alleviate chronic tissue ischemia versus single dose therapy for acute ischemia, and that topical/oral administration (skin, mucosal surfaces, or internal organs) of nitrite can differentially affect biological responses versus intravascular or intraperitoneal delivery. Thus, it is clear that design of future therapeutic trials must consider these issues when designing trial protocols.

Sodium Nitrite Therapy and Cancer

Nitrite became a major research health interest in the early 20th century when it was suggested that nitrite from industry and in foods might increase the incidence of cancer by the formation of N-nitrosamines. This belief quickly lead to the idea that nitrite in any form was detrimental to health. As such, the toxic nature of high nitrite levels was confirmed by Ringer and Murrell [255].

In 1956, Magee and Barnes reported that certain nitrosamines could cross react with nitrite and naturally occur as secondary amines in food, compounds that act as carcinogens in rodents [256]. However, the relationship between nitrite and nitrate exposure and human cancer remains equivocal. Nevertheless, medical use of nitrite has ceased for decades, except as an antidote in emergencies, with strict regulations worldwide. In light of the negative image nitrite acquired over the years, it is rather surprising that the use of nitrite as an antibacterial agent in canned food has continued.

Inhalant organic nitrites (e.g. isobutyl nitrite) have been shown to enhance tumor growth rate in mice and are epidemiologically linked to an increased risk of Kaposi’s sarcoma [257, 258]. Soderberg has previously reported that amyl nitrite inhalant exposure led to a significant increase in tumor growth rate in mice [259]. However, it is important to understand that organic inhalant nitrites are completely different than inorganic nitrite anion including separate metabolic pathways for NO formation and lack of tissue specificity. In short, inhalant organic nitrite is analogous to other organic nitrates and acts as a rapid, non-selective NO donor to multiple tissues that has been associated with tumor progression.

It is now well known that intra tumor acidosis pH is a common feature of many tumors [260, 261]. Nitrite anion is known to represent an important source of bioactive NO under low pH conditions. The characteristic of NO, by driving local vasodilation and increasing perfusion,

suggests that it may have therapeutic potential in tumors to allow transient increase in the delivery and efficacy of anti-cancer drugs. Recently Frerart et al has evaluated the effects of nitrite anion therapy on tumor blood flow, oxygenation, and response to radiotherapy [262]. It was found that nitrite selectively induce a transient increase in tumor partial O_2 pressure (pO_2), which improved the efficacy of radiotherapy. These effects of nitrite were suggested to be due to changes in O_2 consumption rather than an increase in tumor blood flow. This report highlights nitrite as a promising, safe, and inexpensive modality to augment anti-tumor strategies and sensitize tumors for radiotherapy. Further work by the Lundberg group has confirmed this observation in a bladder tumor model such that 50 μM nitrite inhibited thymidine incorporation in human T24 bladder cancer cells under acidic conditions [263]. Thus, inorganic nitrite therapy may actually be a useful adjuvant for anti-cancer therapy. Conversely, it is also possible that nitrite reduction to NO might augment tumor perfusion or angiogenesis that could positively influence cancer cell growth. Direct investigation of inorganic nitrite therapy effects on carcinogenesis in vitro and in vivo are clearly needed to provide greater understanding on how nitrite mediated therapy affects tumor progression and development.

Clinical Trials using Sodium Nitrite

There is now little doubt that clinical trials investigating the biological effects of sodium nitrite in various pathophysiological conditions are well justified and needed. The overwhelming amount of beneficial experimental data in different models of experimental disease clearly indicates that this molecule may be useful for a number of conditions. However, the number of clinical trials involving sodium nitrite therapy is relatively small reflecting that this endeavor has a long way to go before the field begins to fully appreciate the clinical utility of this molecule. Table 1 lists all of the completed trials that are registered at Clinicaltrials.gov and their associated information. However, it is important to keep in mind that this list likely underestimates the number of clinical studies that have been performed due to investigator initiated study protocols performed at individual institutions not reported to this database. Indeed, the recent study by Hunault et al examining oral sodium nitrite pharmacokinetics and dynamics along with changes in blood pressure and methemoglobin levels was not found on the clinical trials website [69]. These completed clinical trials were sponsored by the National Heart Lung and Blood Institute (NHLBI) and the National Institute of Neurological Disorders and Stroke (NINDS) to examine basic physiological and pharmacologic parameters following nitrite infusion in healthy volunteers. Various parameters that were investigated included vasoregulation responses, blood pressure, heart and lung function, methemoglobin levels, as well as other relevant parameters such as ischemic tolerance and preconditioning, physiological exercise responses, and many of the results reported in different manuscripts [264–266]. In short, these studies established biochemical and physiological parameters as well as addressing some safety concerns that provide useful baseline information for the field moving forward.

More recent trials have begun to focus on specific disease conditions in affected populations using varying approaches for nitrite therapy. Table 2 reports clinical trails underway for specific disease conditions. Many of these treatment studies involve multiple institutional sponsors and it is clear that the trials exhibit a wide range of designs with respect to

randomization, blindedness, and controls. It is very important to emphasize that clinical trial design parameters strongly influence data outcome, interpretation, and conclusions [267, 268]. Details such as inclusion/exclusion criteria, target population enrollment (i.e. statistical power), blinding, data safety monitoring, and evaluation methods must be carefully considered as numerous trials in several disciplines have suffered from inappropriate attention to these matters. Nonetheless, the nitrite field is at an exciting crossroads that will involve new understanding in both clinical and basic science aspects.

Potential Downsides?

While sodium nitrite therapy appears to hold great promise for treating several disorders, it is important to address potential complications of sodium nitrite administration. The overarching concern pertains to the purported role of inorganic nitrite/nitrate and carcinogenesis. Early studies suggested that continuous feeding of high milligram doses of sodium nitrite could facilitate the development of tumors [269–271]. However, studies also indicated that sodium nitrite does not significantly contribute to cellular transformation [272, 273]. A comprehensive study on sodium nitrite toxicity was published in May 2001 by the U.S. Department of Health and Human Services National Toxicology Program that exhaustively examined several toxicological parameters associated with long term, high dose sodium nitrite consumption [150]. To the point, no evidence of carcinogenicity was noted in male or female F344/N rats, or male B6C3F1 mice administered a range of continuous sodium nitrite doses (35–150 mg/kg) over a two year period. Equivocal evidence for carcinogenicity was noted in female B6C3F1 mice with forestomach epithelial hyperplasia at the highest doses administered (150 mg/kg). It is important to clarify that the majority of pharmacological sodium nitrite doses (μg/kg range) are a log fold less than those used in the NTP studies (mg/kg range). Lastly, this study demonstrated no genetic toxicity responses in animals. Together, these facts suggest that sodium nitrite mediated carcinogenicity is of minimal concern.

A broader question arises with respect to the effects of sodium nitrite therapy on existing cancer progression and development. Studies have addressed this concern with Veselik et al indicating that nitrite could potentially bind and activate ER-α^+ breast cancer cells thereby influencing their growth [273–275]. Conversely, it has been reported that sodium nitrite administration to tumor bearing mice does not significantly affect growth rates and that nitrite can inhibit cell division of certain tumor cell lines [263, 277]. As discussed above, sodium nitrite therapy may actually be an effective anti-tumor therapy through NO mediated radiosenitization or tumoricidal properties [278, 279]. However, additional work is needed in this arena to better understand the effects of sodium nitrite therapy on tumor development and metabolism.

Future directions: where do we go from here?

It is fair to say that the future of nitrite research is just beginning to mature. As discussed above, clinical investigation and therapeutic trials are progressing forward. However, it is critically important to remember that it only takes one poorly designed or executed trial to throw a ‘wet towel’ on the fire of progress. Thorough, well controlled, and designed clinical trials are needed to elucidate whether nitrite therapy could be as effective as claimed by

numerous investigators. Several companies have taken the charge including Hope Pharmaceuticals, AiRES Pharmaceuticals, TheraVasc, and HeartBeet.

Clinical investigation aside, much remains unknown about the effects of nitrite at the physiological and pathological levels. Additional information is clearly needed regarding mechanistic effects of nitrite on multiple aspects of physiology and disease. In particular, recent developments regarding nitrite modulation of immune responses are very intriguing and merit further investigation as it is now known that inflammatory activity influences nearly every aspect of human disease. Lastly, recent appreciation of the NO/nitrite/nitrate endocrine system as an important mechanism in which to closely regulate bioavailable NO requires further study. In conclusion, the potential of inorganic nitrite based therapeutics for restoring bioavailable NO and treating numerous disorders remains highly promising and will continue to expand our understanding of NO biology in health and disease.

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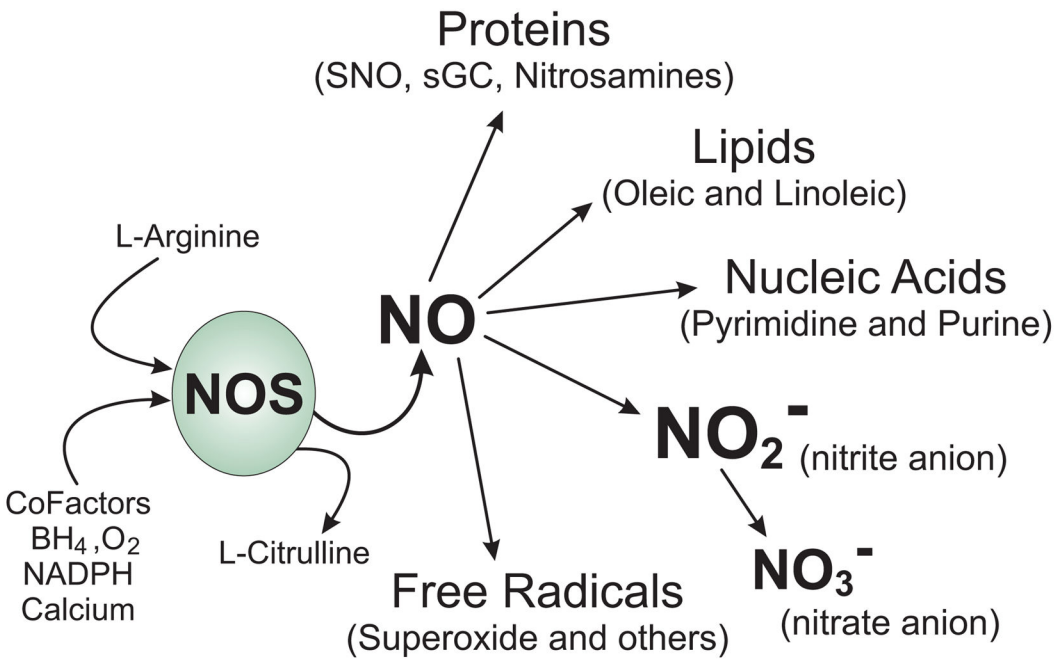

Figure 1. Nitric oxide generation and metabolism

NO is primarily generated by NOS enzymes within tissues through the conversion of L-arginine to L-citrulline. NO readily reacts with multiple molecular targets including proteins, lipids, nucleic acids, and free radicals or can be oxidized to nitrite/nitrate through oxyheme protein interactions.

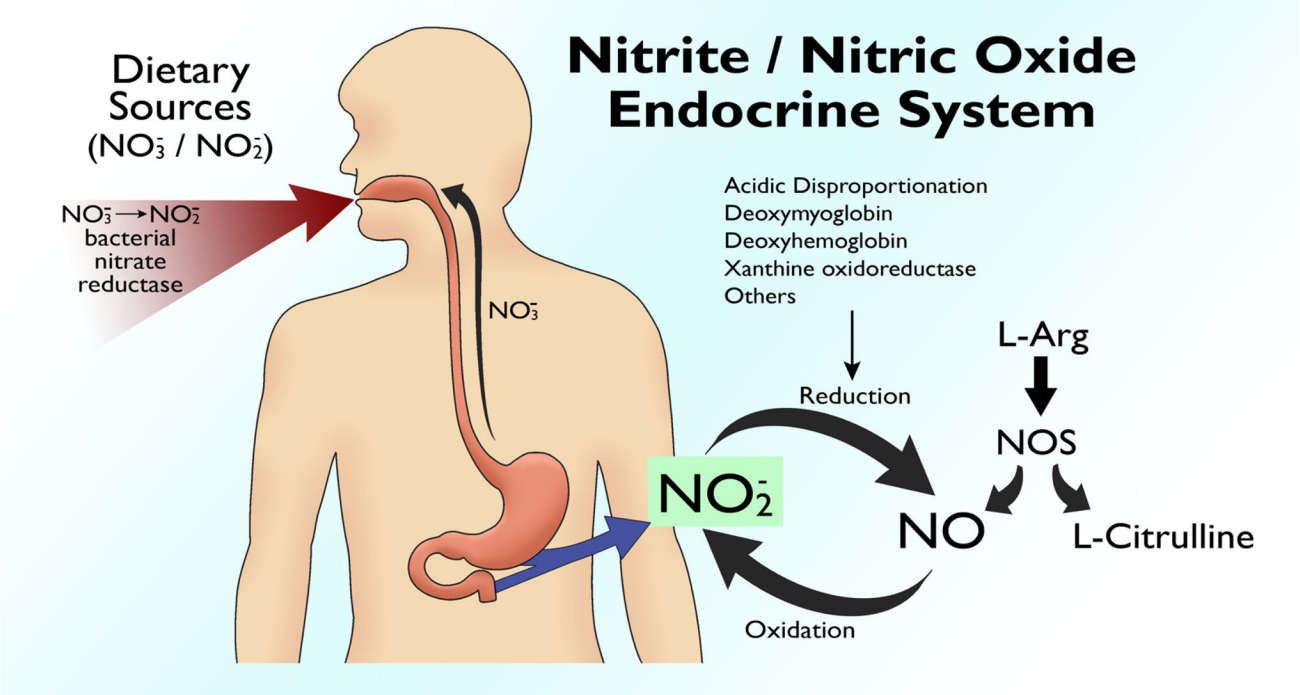

Figure 2. The nitrite/NO endocrine system

NO bioavailability can be augmented through dietary consumption of nitrate/nitrite leading to salivary nitrate secretion and reduction to nitrite by commensal bacteria. Nitrite can also be reduced to NO by various cellular mechanisms associated with decreased oxygen tension or tissue stress.

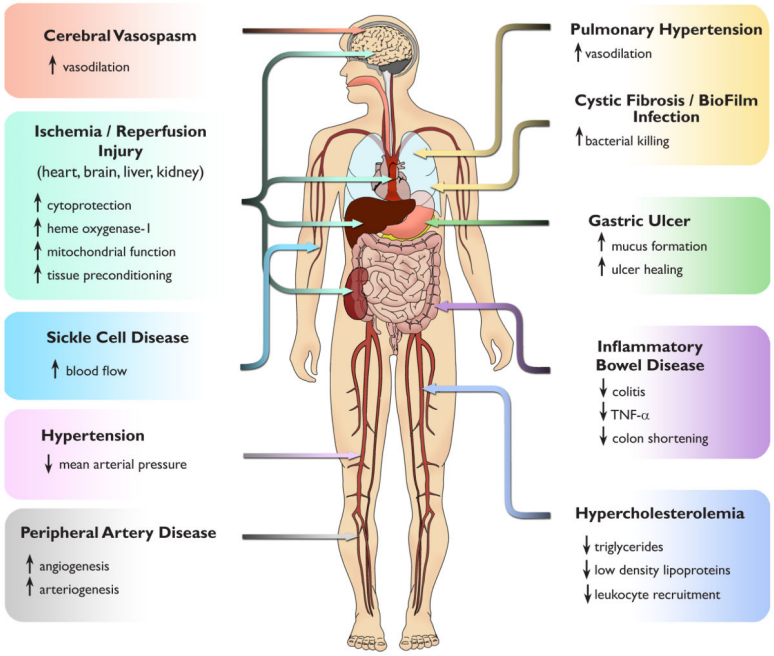

Figure 3. Therapeutic potential of inorganic nitrite

This figure illustrates the various pathophysiological conditions whereby nitrite therapy has been reported to be beneficial in either experimental or clinical conditions. Major effects of nitrite therapy are indicated for each condition.

Table 1

Completed clinical trials investigating effects of sodium nitrite

Trial #/Phase	Condition	Status	Patient #'s	Study Design	Outcome Measures	Institution & Sponsor
NCT00048477 Phase II	Vessel dilation	Completed	42	Interventional, Safety/Efficacy	**1** forearm blood flow with exercise **2** plasma nitrite and MetHb	NHLBI
NCT00250185 Phase I	Ischemic stress	Completed	135	Interventional, treatment	**1** ischemic preconditioning **2** blood flow, mitochondrial function and inflammation	NHLBI
NCT00095472 Phase I	Sickle Cell Disease	Completed	18	Interventional, treatment	**1** vasodilation **2** reduce PA pressures and ischemia-reperfusion injury	NHLBI
NCT00102271 Phase I	Healthy subjects	Completed	36	Interventional, treatment	**1** changes in vascular tone +/− oxypurinol or ascorbic acid, PK data **2** alterations in response with hypoxia	NHLBI
NCT00105222 Phase I	Healthy subjects	Completed	15	Interventional, treatment	**1** exercise effects on circulating nitrite stores **2** effect on aerobic and anaerobic exercise capacity	NHLBI
NCT00103025 Phase I	Healthy subjects	Completed	12	Non-randomized, open label, uncontrolled safety	**1** blood pressure, heart rate, MetHb over sequential infusion	NINDS
NCT00098072 Phase I	Pulmonary Hypertension	Completed	27	Interventional, treatment	**1** inhalation of NO or aerosolized nitrite on lung and heart pressures **2** clinical chemistry and PK	NIHCC

Table 2

Active clinical trials investigating effects of sodium nitrite

Trial #/Phase	Condition	Status	Patient #'s	Study Design	Outcome Measures	Institution & Sponsor
NCT00924118 Phase II	Acute Myocardial Infarction	Recruiting	30	Randomized, interventional, open label with safety & efficacy	**1** infarct reduction per area at risk **2** LV volume, ejection fraction, MRI infarct size	Johns Hopkins University Hope Pharmaceuticals
NCT00873015 Phase II	Cerebral Vasospasm after Subarachnoid Hemorrhage	Recruiting	18	Randomized, interventional, single blind (subject) with safety	**1** PK of 14 day sodium nitrite infusion **2** safety and efficacy of 14 day sodium nitrite infusion	Univ. of Virginia Hope Pharmaceuticals NINDS
NCT01033227 Phase I Phase II	Sickle Cell Disease	Recruiting	30	Randomized, interventional, dose comparison, open label with safety & efficacy	**1** safety- vital signs, clinical lab, MetHb, and AE's **2** Pediatric pain tool, narcotic consumption, reticulocyte count, plasma Hb, serum LDH	Childrens Hospital Los Angeles Hope Pharmaceuticals
NCT00814645 Phase I	Pulmonary Arterial Hypertension	Not Recruiting	25	Non-randomized, interventional, placebo controlled, pharmacodynamics study	**1** measurement of PA pressure by ECHO **2** plasma PK, hematology and clinical chemistry, MetHb, O2 sat, ECG	Aires Pharmaceuticals
NCT01098409 Phase II	Coronary Artery Bypass Surgery	Recruiting	50	Randomized, interventional, placebo controlled, double blinded, efficacy	**1** troponin T 72 hrs post aortic cross clamp **2** troponin T, CKMB, NO metabolites, plasma 8-isoprostanes, and cardiac output at various times post bypass	Univ of Birmingham NHS Foundation Trust
NCT01178359 Phase I	Cardiac Arrest	Recruiting	16	Interventional, randomized, placebo controlled, single blinded, safety & efficacy	**1** blood pressure & blood nitrite	Univ. of Washington Medic One Foundation Institute of Translational Health Sciences
NCT01262521	Dietary Nitrate & CV Health	Recruiting	20	Interventional, randomized, active control, double blinded, efficacy	**1** circulating angiogenic cells	Heinrich-Heine Univ, Duesseldorf

Trial #/Phase	Condition	Status	Patient #'s	Study Design	Outcome Measures	Institution & Sponsor
					2 cytokines and endothelial function	