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Cardioprotective actions of nitrite therapy and dietary considerations

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1. ABSTRACT

Nitrite is an intrinsic signaling molecule. Its ability to form nitric oxide (NO) under hypoxic conditions as well as its ability to form S-nitrosothiols under normoxic conditions has transformed this once inert anion into a critical molecule in maintaining NO and nitroso homeostasis throughout the entire physiological oxygen gradient *in vivo* in addition to serving as the endocrine mediator of NO. Steady state plasma nitrite has historically been used as a biomarker of NO availability. However, tissue specific metabolism of nitrite along with differences in nitrite and nitrate intake from diet warrant a careful assessment of plasma nitrite as an index of NO biochemistry. Metabolism and regulation of NO and nitrite are at the local cellular and tissue level dependent upon cellular redox status, redox active metal and thiol availability. Understanding nitrite metabolism and mechanisms of cytoprotection may offer novel means to prevent cardiovascular disease or limit injury from a cardiovascular event. Here the state of the art of nitrite in cytoprotection as well as maintenance of steady state blood and tissue nitrite is reviewed.

2. INTRODUCTION

Ischemic heart disease, including myocardial infarction, remains the leading cause of morbidity and mortality in all industrialized nations (1). There are two distinct components of damage to the heart in patients who experience acute myocardial infarction: ischemic injury and reperfusion injury. The myocardium is able to tolerate brief periods of ischemia as activation of inherent, adaptive mechanisms can preserve energy levels and prevent injury. These include switching metabolism to anaerobic glycolysis and fatty acid utilization, increasing glucose uptake, and decreasing contractility. If ischemia persists however, the myocardium will develop a severe ATP deficit, resulting in irreversible injury and culminating in cell death. Although reperfusion of ischemic tissues provides oxygen and metabolic substrates necessary for the recovery and survival of reversibly injured cells, reperfusion itself paradoxically results in the acceleration of cellular necrosis (2). Reperfusion is characterized by the formation of oxygen radicals upon reintroduction of molecular oxygen to ischemic tissues, resulting in widespread lipid and protein oxidative modifications,

mitochondrial injury, as well as tissue apoptosis and necrosis (3, 4).

The loss of NO generation as a result of a dysfunctional vascular endothelium is a very likely cause of heart disease (5). Continuous generation of NO is essential for the integrity of the cardiovascular system as a decreased production and/or bioavailability of NO is central to the development of cardiovascular disorders (6, 7). NO is a highly reactive and diffusible gas formed by three NO synthase (NOS) isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). NO has been extensively studied in the setting of ischemia-reperfusion (I/R) injury. Previous studies clearly demonstrate that the deficiency of eNOS exacerbates myocardial I/R injury (8, 9), whereas the overexpression of eNOS (10, 11), NO donor (12, 13) or inhaled NO gas (14) therapy significantly protect the myocardium (15). NO possesses a number of physiological properties that make it a potent cardioprotective-signaling molecule. These include vasodilation and the inhibition of oxidative stress, platelet aggregation, leukocyte chemotaxis and apoptosis (16-19). NO synthesis is critically influenced by various cofactors such as tetrahydrobiopterin (BH4), flavin mononucleotide and flavin adenine dinucleotide, the presence of reduced thiols, the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) and substrate and oxygen availability. Without an adequate delivery of substrate and co-factors (conditions that exist during ischemia), NOS no longer produces NO but instead transfers the free electrons to oxygen and thus produces oxygen free radicals (20). Therefore, alternate means to produce NO in ischemic tissues may limit I/R injury. Nitrite has evolved as a critical reservoir of NO activity particularly when NOS derived NO is dysfunctional.

The nitrite anion (NO_2^-) has moved to the forefront of NO biology (21) since nitrite represents a major storage form of NO in tissues (22). Nitrite, the oxidative breakdown product of NO, has been shown to serve as an acute marker of NO flux/formation (23); therefore a reduction in NO production results in a decrease in steady state concentrations of nitrite. Conversely, increased NO production leads to an increase in steady state nitrite. The primary target of NO is the enzyme soluble guanylyl cyclase, which when activated leads to the conversion of GTP to cGMP (24). It is through cGMP that NO is thought to elicit its primary cell signaling events. It has also been proposed that S-nitrosothiols (RSNOs) mediate the actions of nitric oxide synthase (25) whereby modification of critical cysteine residues by NO or NO related species modulate protein structure and function akin to phosphorylation (26). More recently nitrite has been implicated as one endocrine mediator of NO signaling (27, 28), although concrete experimental evidence has been lacking to demonstrate endocrine activity until recently (29). Nitrite has been shown to be an alternate source of NO during ischemia and hypoxia (30, 31) and is cytoprotective during I/R insult (32, 33). Nitrite can also form RSNOs along the physiological oxygen gradient (34, 35). Therefore its ability to act as a central reservoir for both NO and RSNO warrants careful consideration of the nitrite anion in health and disease.

A report by Kleinbongard *et al.* (36) demonstrates that plasma nitrite levels progressively decrease with increasing cardiovascular risk load. Risk factors considered include age, hypertension, smoking, and hypercholesterolemia, conditions all known for reduced bioavailability of NO. Although a correlation exists in the plasma, it is not known whether the situation is mirrored in the heart or other tissue at risk for ischemic injury or disease. If so, tissue nitrite may serve as an index of risk and restoring tissue nitrite may act as a first line of defense for protecting organs from ischemic and/or I/R injury. Since a substantial portion of steady state nitrite concentrations in blood and tissue are derived from dietary sources (34), modulation of nitrite intake may provide a first line of defense for cardiovascular disease (22).

This review will summarize the current state of the art of nitrite research including sources of nitrite, tissue specific metabolism of nitrite, mechanisms of activation to NO, RSNO and nitrate as well as discuss the mechanisms of cytoprotection. Finally, a discussion on the potential for changes in dietary nitrite and nitrate consumption as a natural and inexpensive means to prevent or treat cardiovascular disease or any disease associated with NO insufficiency.

3. NITRITE HOMEOSTASIS

3.1. Sources of steady state nitrite in blood and tissues

The endogenous production of NO by NOS has been established as playing an important role in vascular homeostasis, neurotransmission, and host defense mechanisms (37). The major pathway for NO is the stepwise oxidation to nitrite and nitrate (38). In plasma NO is oxidized almost completely to nitrite, where it remains stable for several hours (39, 40). In contrast, NO and nitrite are rapidly oxidized to nitrate in whole blood. NO can also be enzymatically oxidized to nitrite by cerulopasmin or other metal containing proteins (28). The half life of nitrite in human blood is about 110 seconds (41). Nitrate on the other hand has a circulating half life of 5-8 hours (42, 43). During fasting conditions with a low previous intake of nitrite/nitrate, enzymatic NO formation accounts for the majority of nitrite (44). On the basis of these studies, it was believed that NO is acutely terminated by oxidation to nitrite and nitrate. Early 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. It was these findings by Tannenbaum *et al.* (45) that significantly altered our conceptions of human exposure to exogenous nitrite and nitrates 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. Nitrite and nitrate salts are generally highly soluble in water. When taken orally, they are readily absorbed from the proximal small intestine (46). About 25% of orally ingested available nitrate is actively

secreted into the saliva. This nitrate is partially converted to nitrite by oral bacteria (47) and then disproportionates with formation of NO after entering the acidic environment of the stomach, helping to reduce gastrointestinal tract infection, increase mucous barrier thickness and increase gastric blood flow (46, 48-50).

In addition to the oxidation of NO, nitrite is produced by the consumption of food such as meat, vegetables and drinking water. Nitrite and nitrate have been used for centuries in curing and preserving meats and fish and in manufacturing certain cheeses (51). When added to foods such as cured meats, nitrite has at least 3 functions. Firstly it contributes to the flavor due to the inhibition of the development of rancidity (52). Secondly, it reacts with myoglobin to produce mononitrosylhemochrome (53), which results in the characteristic pink color of cured meat. Thirdly, it inhibits the growth of food spoilage bacteria *Clostridium botulinum,* being the most important. *C. botulinum* thrives under anaerobic conditions and produces a neurotoxin which is one of the most lethal natural products known (53). In this regard, nitrite is critical to the food industry for prevention of food borne illness.

Nitrite can also be derived from reduction of salivary nitrate by commensal bacteria in the mouth and gastrointestinal tract (55-58). Humans, unlike prokaryotes, are thought to lack the enzymatic machinery to reduce nitrate back to nitrite. However recent discoveries reveal a functional mammalian nitrate reductase (59). Commensal bacteria that reside within and on the human body can reduce nitrate thereby supplying another source of nitrite (55-58). Therefore dietary and enzymatic sources of nitrate are a potentially large source of nitrite in the human body. Nitrate is rapidly absorbed in the small intestine and readily distributed throughout the body (46). The concentrations of nitrate in drinking water are usually <10mg/L in the absence of bacterial contamination (60). Vegetables, especially beets, celery, and leafy vegetables like lettuce and spinach are rich in nitrates (46, 61, 62). 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. They report that the average total nitrite and nitrate intake in the U.S. was 0.77 mg and 76 mg, respectively per day. However, these data warrant careful reassessment based on more sensitive analytical methods and contemporary dietary habits.

It was once widely believed that nitrite is quickly oxidized to nitrate in biological media and nitrate represents an inactive breakdown product of NO synthesis or residual from dietary sources. However, it is now appreciated that nitrite or nitrate can be recycled to produce NO in various ways. Acidification of nitrite may release NO in the stomach (48). NO may be derived from urinary nitrate in infected urine (63). NO production from nitrite has been described in infarcted heart tissue (30). The process is independent of NOS since it is unaffected by specific NOS inhibitors. NO is also produced in the mouth of both rats (64) and humans (65), and in human sweat (66). In these instances, commensal bacteria may play a role in NO synthesis, and the NO that is produced may serve as a defense mechanism against pathogens representing a true symbiosis. More recently, nitrite has been implicated in acting as a bioactivable source of NO in the circulation. Nitrite has been traditionally considered a metabolic end-product of NO's reactions with oxygen devoid of intrinsic vasodilatory activity. However, recent studies have revealed a striking effect of nitrite infusions on forearm and systemic blood flow. Cosby *et al.* (67) suggested that nitrite is a large intravascular storage pool for NO and that nitrite bioactivation to NO could vasodilate regions with tissue oxygen debt in the human circulation. These studies, however, were performed using supraphysiological concentrations of nitrite (200µM). Nitrite is normally a very short-lived, highly regulated ion in the circulation (100-400nM) with a circulating half life in whole blood of 110 seconds (41). Steady state levels are determined by rates of production vs rates of metabolism or consumption.

3.2. Metabolism and consumption of nitrite

Nitrite is known to be metabolized by four pathways: metal-mediated oxidation, metal-mediated reduction, acid disproportionation and direct binding to molecular targets (68). Metal catalyzed oxidation of nitrite yields nitrate by the following reaction: (69):

$$2P\text{-}Fe^{2+}O_2 + 3NO_2^- + 2H^+ \rightarrow 2P\text{-}Fe^{3+} + 3NO_3^- + H_2O$$

or

$$4P\text{-}Fe^{2+}O_2 + 4NO_2^- + 4H^+ \rightarrow 4P\text{-}Fe^{3+} + 4NO_3^- + O_2 + 2H_2O$$

Nitrate is present in much higher concentrations in blood and tissues than nitrite. Possible explanations for the presence of predominately NO_3^- *in vivo* may have to do with the fact that the levels of NO produced by nitric oxide synthase *in vivo* would be much smaller and thus the half-life of NO would be much longer. In this case, NO would react directly and very rapidly with oxyhemoproteins ($P\text{-}Fe^{+2}O_2$) to yield NO_3^- before it has an opportunity to autoxidize to NO_2^-. Furthermore nitrate is present in much higher concentrations in the diet than is nitrite so a combination of oxidation reactions and the increased half life and stability of nitrate account for its higher steady state concentrations *in vivo*.

Much of the recent focus on nitrite physiology is due to its ability to be reduced to NO during ischemic or hypoxic events (22, 31, 70). Nitrite reductase activity in mammalian tissues has been described by the mitochondrial electron transport system (71-73), protonation (63), deoxyhemoglobin (67), deoxymyoglobin (74) and xanthine oxidase (33, 75, 76). Mitochondrial nitrite reduction has been shown to occur by ubiquinol (73, 77) and cytochrome c oxidase (78) with subsequent binding

of the NO produced to cytochrome bc1 site of complex III or complex IV resulting in oxygen-dependent reversible inhibition of mitochondrial respiration (79). All pathways described are redox active metal containing proteins. Much attention is currently placed on the role of hemoglobin in the reduction of nitrite to NO under hypoxia. Gladwin and colleagues proposed a role for Hb as a "NO_2^- reductase" able to generate sufficient NO to modulate blood flow in response to tissue O_2 demand (67). Others have also suggested that NO facilitates O_2 delivery by the RBC to hypoxic tissue through the formation of nitroso/nitrosyl hemoglobin adducts by intraerythrocyte NO_2^- reduction (35, 80). Mechanisms involving erythrocytes, however, have been questioned for their ability to deliver NO to vascular tissues without becoming inactivated by oxyHb or recaptured by deoxyHb within the cells (81). Mechanisms involving extravascular cells have also been proposed. For instance, NO formation has been attributed to acid-catalyzed NO_2^- reduction from xanthine or NADH by xanthine oxidoreductase (XOR) in the isolated, buffer-perfused ischemic heart (33, 75). Xanthine oxidase is a flavoprotein enzyme that is distributed in various mammalian tissues (82) and plays an important role in both physiology and pathophysiology. In addition to its known ability to reduce molecular oxygen to superoxide (O_2^-) (83), at low oxygen tensions and low pH xanthine oxidase can also reduce nitrite to NO at the molybdenum site of the enzyme (84-87). Oxygen acts as a strong competitive inhibitor of nitrite reduction by xanthine oxidase (75). Additionally, 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. Mitochondria have also been associated with nitrite reduction to NO. Under hypoxic conditions whereby mitochondrial electron carriers tend to remain reduced, isolated mitochondria produce NO when supplied with NO_2^- and a respiratory substrate (71). The suggested locus of NO_2^- reduction is the ubiquinone cycle of complex II (88) or cytochrome oxidase (89). Notably, mitochondrial control of hypoxic vasodilation is intrinsically tissue-based, since the RBC is devoid of mitochondria. Since all these 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 tension, substrate availability and perhaps even compartment specific needs representing a redundant system.

The acidic reduction of nitrite requires protonation and a one-electron reduction. The relatively low pKa of nitrite (3.34) (90) limits this activity in physiology but it can occur in the stomach or during ischemic events when tissue pH falls. This then raises the question of how nitrite is taken up and distributed when taken orally. Several reports clearly reveal that nitrite administered in the drinking water for several days results in an increase in steady state plasma and tissue nitrite without any effect on NOS expression (91, 92).

A comprehensive study by Feelisch *et al* reveals that differentiated tissues share a fundamental ability to reduce NO_2^- to NO that is facilitated with increased O_2 deprivation (93). Intrinsic tissue NO_2^- reduction far exceeds that by the RBC and can also be directly correlated with XOR activity and indices of oxidative metabolic capacity (mitochondrial membrane surface area and/or cytochrome oxidase activity). Together with mathematical modeling, this reveals nitrite reductase activity is derived from heme based mechanisms, which are distinct for the ferrous and ferric forms. The magnitude of tissue reductase activity, particularly in arterial tissue, suggests that NOS-independent NO formation from NO_2^- may indeed play an important physiological role in the O_2-responsive signaling regulating hypoxic vasodilation irrespective of the red cell. While nitrite has been identified to protect against myocardial and hepatic ischemia/reperfusion injury (32), little is known about specific enzymes or mechanisms involved. The liver is among the most effective organs to reduce nitrite to NO (34). Endogenous NO_2^--derived NO generation in hypoxic tissue obviates the fundamental conundrum as to how NO from RBC NO_2^- reductase activity could escape hemoglobin scavenging to reach and dilate local vascular beds (81, 94). The recent report by Bryan *et al* reveals that the vascular tissue has the greatest capacity to generate NO from nitrite (34, 93). Our demonstration that hypoxia-induced tissue NO_2^- reductase activity is accompanied by nitros(yl)ation of cellular targets suggests the possibility that some of the resulting nitroso/nitrosyl products may be NO_2^--related tissue effectors of (or markers for) hypoxic vasodilation. This notion gains support from recent demonstrations that NO_2^- itself may facilitate nitros(yl)ation reactions through unique heme- and thiol-dependent chemistries yet to be defined (34, 35, 80).

Nitrite has properties of a signaling molecule independent of the NO/cGMP pathway and nitrite can directly interact with molecular targets without the intermediacy of free NO (34) providing another route of metabolism for nitrite. Furthermore we and others have demonstrated that nitrite can transiently form nitrosothiols (RSNOs) in a first order reaction both under normoxic conditions and under hypoxia (31, 34, 35) and that changes in dietary nitrite and nitrate ingestion can affect protein expression and activity (34) as well as significantly protect from ischemia-reperfusion injury (91).

Nitrite and nitrate are excreted in the kidneys. Nitrate is excreted in the urine directly or after conversion to urea (95). Clearance of nitrate from blood to urine approximates 20ml/min in adults (96), indicating considerable renal tubular reabsorption of the ion. There is little detectable nitrite or nitrate in feces (97). Steady state concentrations of blood and tissue NO metabolites are a reflection of the rate of production and excretion. In order to get an insight into the renal excretion of nitrite and nitrate as a result of differences in dietary nitrite intake, urine was collected from mice on standard chow ± nitrite (50 mg/L) and low NOx diet ± nitrite (50 mg/L). As shown in Figure 1, after 5 days the mice on supplemental nitrite begin to excrete the extra nitrite given in the drinking water. Conversely, the mice on low NOx diet attempt to

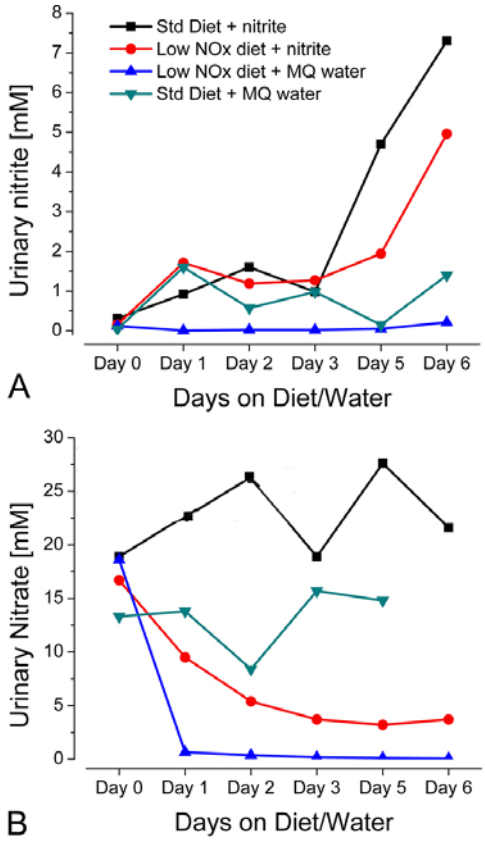

Figure 1. Urinary clearance of nitrite and nitrate. The urinary clearance of nitrite (A) and nitrate (B) was assessed daily for mice fed a standard chow ± nitrite supplementation and low NOx diet ± nitrite supplementation for 7 days. Data are average of 2 cages for each group with 3 mice in each cage (previously unpublished data).

preserve nitrite by limiting the excretion. The mice on low NOx diet rapidly adjust to the decreased NOx intake by decreasing their excretion of nitrate. Interestingly, the mice on low NOx diet with supplemental nitrite appear to slowly decrease the renal clearance of nitrate over time. These data demonstrate that the body rapidly responds to alterations in nitrite intake and therefore suggest an important regulation of nitrite and nitrate by the kidneys to maintain steady state nitrite and nitrate concentrations in blood and tissues. Therefore any compromise in kidney function may result in altered steady state blood and tissue NOx concentrations, which may lead to erroneous interpretation of data on total body NO production or availability. Some NOx is lost in sweat, however it is not a major route of excretion (66). The multiple levels of regulation of production and excretion of nitrite and nitrate warrant careful consideration when applying nitrite therapeutics or supplementation so as to not disturb normal cellular homeostasis.

4. NITRITE AS AN ENDOCRINE MEDIATOR OF NO SIGNALING

The secretion of a substance into the bloodstream that is transmitted to the tissue on which it has a specific effect describes an endocrine function. NO was discovered to be an extremely short lived gaseous free radical that signals through activation of soluble guanylyl cyclase (sGC) (24). More recent discoveries have revealed that NO acts not only as a paracrine mediator, but may exert effects distal to the site of production (98). Under normal physiological conditions, NO is produced by the endothelial cells from L-arginine and then diffuses into the underlying smooth muscle to activate sGC to form cyclic guanosine monophosphate (cGMP) and thus maintain basal vascular tone (99-101). Since NO is a freely diffusible gas it also diffuses into the blood vessel lumen where it is quickly inactivated by hemoglobin in red cells (102). For years it was thought that scavenging of NO by hemoglobin limited the activity of NO to the local environment. However, experiments using inhalative NO therapy reveal that NO activity can be transported (103) despite its short circulating half life (41). There is increasing appreciation that NO may be stabilized in blood by the formation of NO-modified proteins, peptides and lipids as well as oxidation to nitrite (27). The S-nitrosohemoglobin (SNO-Hb) hypothesis put forth by Stamler and colleagues provide an elegant and attractive model by which NO could be transported to oxygen deprived tissues by RBCs thereby facilitating more blood flow and hence more oxygen delivery. According to this model, NO binds cooperatively to the minor (~1%) population of deoxygenated heme

molecules in oxygenated red blood cells, forming iron-nitrosyl hemoglobin (104). The NO group is then transferred to the B93 cysteine residue to form SNOHb, which can then deliver NO in an allosteric-dependent manner, through a series of transnitrosation reactions, to promote the vasodilation of hypoxic tissues (104-107). More recently, nitrite has moved to the forefront as the endogenous modulator of distal NO bioactivity. Gladwin and colleagues proposed that nitrite transported in red cells are reduced to NO during hypoxia and therefore could account for the phenomenon of hypoxic vasodilatation and bring blood flow in line with tissue oxygen demand (67, 108). There remains a recurring fundamental gap in both of these proposed models. The unanswered question is how does the NO (either from SNO-Hb or NO from nitrite reduction) get released from the red cell before it is re-captured by hemoglobin (81). This is a fundamental obstacle in defining endocrine effects of NO mediated by red cells.

Nitrite is a relatively stable oxidation product of NO with a half life in whole blood around 110 seconds (39-41), long enough to be transported throughout the body. Nitrite is rapidly distributed into tissues, stored or metabolized as nitrosothiols (34) and has been shown as an endogenous signaling molecule independent of the NO-cGMP pathway (34). Furthermore, nitrite confers protection from ischemia-reperfusion injury in heart and liver (32, 33). Any NO therapy aimed to increase bioavailable NO also increases nitrite bioavailability. Collectively, these observations have led to a hypothesis that nitrite may act as an endocrine mediator of NO biology. Many of these interpretations of nitrite occur from studies which administered exogenous nitrite or NO under hypoxic conditions, i.e. non-physiological. Recent evidence by Elrod *et al* (29) demonstrates that enzymatic generation of NO in the heart is capable of modulating remote physiological actions and cell signaling events in distal tissues. Cardiac-specific eNOS overexpression results in significant increases in nitrite, nitrate and nitrosothiols in the heart, plasma and liver. CS-eNOS-Tg mice subjected to hepatic I/R insult displayed a significant reduction in hepatic I/R injury as compared to wild-type littermates. These findings demonstrate that endogenously derived NO is transported as nitrite and RSNO in the blood, metabolized in remote organs, and mediates cytoprotection in the setting of I/R injury. Both nitrite and RSNO were increased in plasma and liver of CSeNOS Tg mice and exogenous administration of both nitrite and GSNO confer protection from liver I/R injury. Therefore, it is difficult to ascertain at this time which molecule is the cytoprotective entity. However, this data suggests that nitrite can be given to provide a source of both NO and RSNO during ischemia and/or low molecular weight RSNOs can be administered to transnitrosate other thiols that may be involved in protein function/recovery during I/R injury. These are not mutually exclusive events, demonstrating the inherent biochemistry of nitrite and nitrosothiols and perhaps steady state equilibrium between the two molecules. This study by Elrod *et al* (29) represents the first clear evidence for an endocrine role of NO via transport in the circulation and metabolism within tissues.

5. DIETARY NITRITE AND NITRATE CONTRIBUTE TO ENDOGENOUS NO BIOCHEMISTRY

Several studies now demonstrate that exogenous nitrite contributes to whole body NO production and homeostasis and is an alternate source of NO *in vivo*. Co-administration of nitrite with a NOS inhibitor for 3 weeks significantly attenuates hypertension (109). 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 (48), nitrite can act as a circulating NO donor (110) and nitrite can itself perform many actions previously attributable to NO (21) without the intermediacy of NO (34). Nitrate also contributes to the NO status of mammals with the participation of commensal bacteria (111). Both nitrite and nitrate are common constituents of our diet. There are now several studies exploring the use of dietary nitrite and nitrate manipulations on endogenous NO biochemistry and changes in physiological outcome from myocardial ischemia-reperfusion injury (91, 92). There have recently been a number of studies demonstrating beneficial properties of both dietary nitrite and nitrate (91, 112-114). The recent report by Webb *et al* (112) demonstrates that dietary nitrate through its reduction to nitrite can lower blood pressure, prevent I/R mediated endothelial dysfunction and attenuate platelet aggregation in humans. Collectively, these studies clearly reveal the benefits of nitrite and nitrate from the diet as a means to restore or enhance NO bioavailability and/or homeostasis.

5.1. Nitrite administered in the drinking water affects steady state concentrations of NO products/metabolites

Recent investigations (91) reveal that dietary nitrite insufficiency through feeding a low NOx diet results in diminished circulating and cardiac steady state nitrite and nitrate concentrations in mice. On the contrary supplementation with 50mg/L nitrite in the drinking water restores nitrite status in mice fed the low NOx diet and actually increases nitrite concentrations in mice fed standard rodent chow (Purina 5001). Similar results were obtained in rats administered both nitrite and nitrate (10-1000 mg/L) in the drinking water (Bryan, unpublished observations). High doses of nitrite ingestion are associated with reducing hypertension in rats (109). These data reveal that changes in dietary nitrite consumption can affect steady state concentrations of blood and tissue NO products/metabolites commonly used to assess NO production and therefore contribute to NO homeostasis.

5.2. Nitrite insufficiency leads to increased cardiac ischemia-reperfusion injury in healthy mice which is reversed by nitrite supplementation in the drinking water

There is emerging evidence in the literature that increasing nitrite availability protects from hepatic and cardiac ischemia-reperfusion injury in otherwise healthy animals (32, 33). Shiva *et al* recently reported that ceruloplasmin deficient mice have decreased steady state plasma nitrite and sustain significantly more liver injury

than wild type mice after ischemia-reperfusion insult (28). However, since these mice also suffer from iron overload, which could contribute to I/R injury, the direct correlation between nitrite status and I/R injury is still missing. Using the approach of modulating dietary nitrite intake, we investigated if these biochemical changes had any physiological effect in the ability of these changes to affect myocardial I/R injury (91). These results reveal that a decrease in nitrite bioavailability through dietary nitrite insufficiency exacerbates myocardial injury. Infarct size, as measured by infarct size relative to area-at-risk, was found to increase by 36% in mice fed the low NOx diet as compared to control mice. The mice fed the low NOx di*et al*so had a higher mortality rate post myocardial infarction than mice on the standard rodent chow (56.5% survival vs 71.4% survival). Replenishing nitrite through supplementing drinking water reversed the increased myocardial infarct size incurred by nitrite insufficiency and resulted in increased survival back to control levels. These data unequivocally demonstrate physiological changes in outcome based on changes in dietary nitrite intake and resultant changes in blood and tissue nitrite. Since steady state concentrations of blood and tissue nitrite are affected by dietary changes in NOx, and these changes translate into a change in recovery from I/R, diet must play a critical role in the NO signaling pathway and cardiovascular health. Could modest changes in dietary habits to increase nitrite and nitrate ingestion affect outcome in humans suffering myocardial infarction or decrease the incidence for people at risk?

5.3. Dietary nitrite insufficiency unmasks NO biochemistry in eNOS -/- mice and supplementation restores NO biochemistry

Enzymatic NO insufficiency is a hallmark of a number of diseases including cardiovascular disease. Mice deficient in the endothelial isoform of NOS have decreased plasma nitrite when fasted (23). A recent study by Bryan *et al* (92) reveals that dietary nitrite supplementation can restore NO and nitrite status in eNOS knockout mice and protect from myocardial I/R injury. Our data reveal eNOS -/- mice have lower plasma nitrite concentrations consistent with earlier findings (23) but there is no significant difference in cardiac nitrite revealing that blood markers do not accurately reflect tissue status (22). Plasma nitrite could be further decreased by feeding eNOS-/- mice a low NOx diet demonstrating that plasma nitrite is a reflection of both NOS and diet. Feeding low NOx diet to eNOS-/- mice completely eliminated steady state concentrations of plasma nitroso without any effect on cardiac nitroso. Supplementation of 50 mg/L nitrite in the drinking water for 7 days restored plasma nitrite in eNOS-/- to control levels and increased both plasma and cardiac nitroso to above C57 control levels. These data are important because they indicate proof of principle that exogenous dietary nitrite can restore NO biochemistry under conditions of dysfunctional NOS. Furthermore, supplemental nitrite can restore NO/nitroso redox balance in eNOS knockout mice and therefore provide justification for studies on nitrite therapy in humans with diseases associated with NO insufficiency with the potential of exogenous nitrite to act as an alternate source of NO to restore NO homeostasis.

6. MECHANISM OF TISSUE PROTECTION

The precise mechanism of protection of nitrite is still unclear, though likely involves a variety of pathways and effects. The leading hypothesis is that nitrite is reduced to NO under ischemic conditions and therefore the protective effects of nitrite are simply through known NO biochemistry i.e. increases cGMP, inhibiting mitochondrial respiration through binding to cytochrome c. I/R injury is characterized by a number of cellular events including release of tissue enzymes, oxidation of essential protein and lipids and initiation of an inflammatory response that ultimately lead to cellular necrosis and apoptosis [2]. The mitochondrion is vital to tissue viability and damage to the mitochondrial machinery plays a central role in the progression of pathology following I/R. During I/R, mitochondrial ATP synthesis is decreased leading to depletion of tissue high energy phosphate stores (115), enzymes of the respiratory chain are damaged leading to diminished inner membrane potential (116, 117). The permeability transition pore is opened (118) and upon reperfusion reactive oxygen species generation is increased. Mitochondria have been shown to reduce nitrite to NO (89). Under hypoxic conditions whereby mitochondrial electron carriers tend to remain reduced, isolated mitochondria produce NO when supplied with NO_2^- and a respiratory substrate (71). NO is known to inhibit mitochondrial respiration (119-122), regulate ROS formation (123), initiate biogenesis (124) and limit apoptotic cytochrome c release (125, 126), so nitrite mediated cytoprotection is ideally suited to occur at the level of the mitochondrium. A recent report by Shiva *et al* demonstrates that nitrite augments tolerance to I/R injury by modulation of the mitochondrial electron transfer (127).

We have previously shown that nitrite can form nitrosothiols in a first order reaction requiring heme and thiols and can also be reduced to NO under anaerobic conditions (34). Since both NO and RSNOs have now been shown to be protective in the setting of I/R, nitrite now becomes the central molecule in that it can form both NO and RSNOs. I propose that nitrite serves two functions in the setting of ischemia and reperfusion. First, it serves as a NOS-independent source of NO by which nitrite is reduced to NO during ischemia when NOS is inactive. Secondly, nitrite reacts with critical thiols to form nitrosothiols under normoxic conditions and this conversion is more pronounced during ischemia (34). This nitroso modification acts as a reversible protective shield which prevents irreversible oxidation of proteins and lipids during the oxidative burst of reperfusion. Aside from "capping" critical thiols from oxidation, we propose that the nitroso products can then release NO or the NO^+ moiety during the reperfusion phase and act as a redox sensitive NO donor (128). Our biochemical data support this notion in terms of the increase in nitroso at the expense of nitrite followed by the decay of nitroso over time during reperfusion (91). Therefore, adding supplemental nitrite increases plasma and tissue nitrite also leading to an increase in steady state levels of nitroso products and thereby affording protection during I/R. Nitrite, then acts as a central molecule in I/R by its ability to protect against

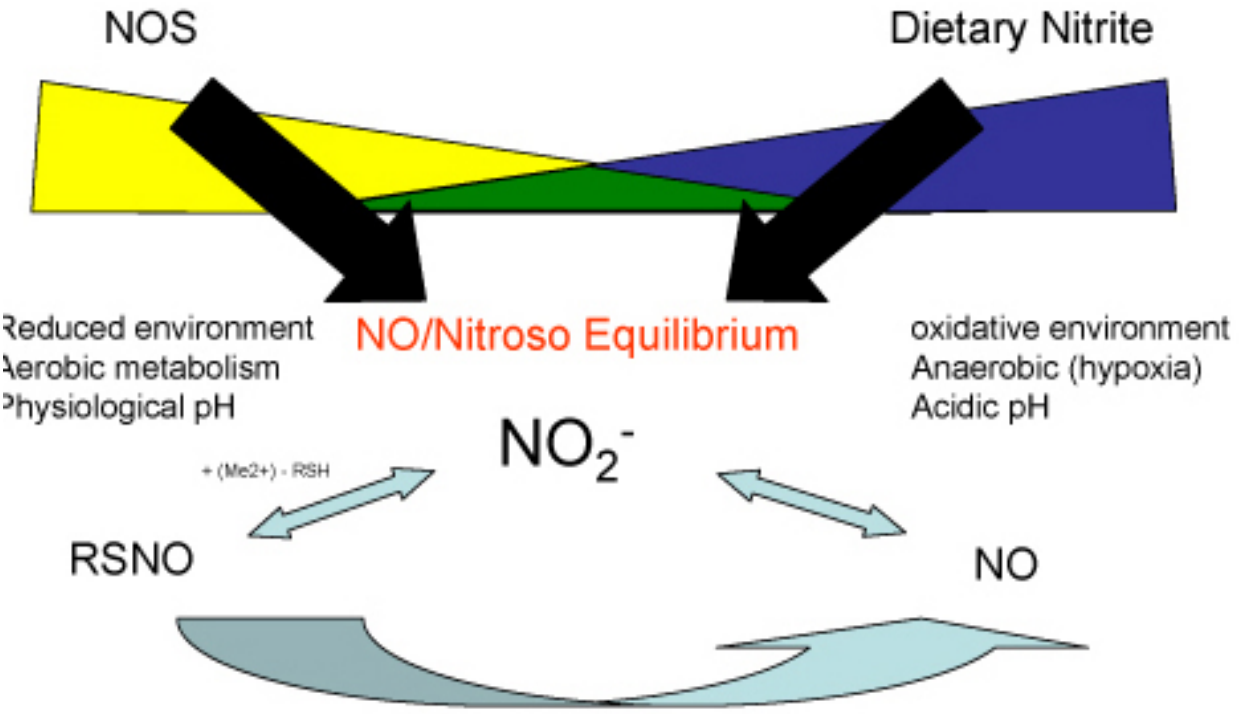

Figure 2. Both NOS and dietary sources provide NO and NOx for optimal NO/nitroso homeostasis. Under normal physiological conditions, reduced cellular environment and normal pH, NOS likely provides sufficient NO for homeostasis. These same conditions render nitrite extremely reactive with thiols to form nitrosothiols. On the contrary, under conditions when NOS is dysfunctional dietary sources of nitrite and nitrate can maintain NO-nitroso redox status and rescue the phenotype. These conditions favor the reduction of nitrite to NO.

both ischemic injury and reperfusion injury. On the contrary, nitrite insufficiency leads to increased injury because there are insufficient nitrite or nitroso products stored in blood or tissue to perform these protective actions. This concept is presented in Figure 2.

7. NITRITE THERAPEUTICS

There are a number of diseases associated or caused by an insufficiency of NO (129). NO status is routinely assessed by steady state nitrite and/or nitrate concentrations, therefore these diseases also suggest a deficiency in both anions which have recently shown biological activity; nitrite by itself (34) and nitrate through its ability to be reduced to nitrite and NO (112). The demonstration of NO formation by an enzyme in vascular endothelial cells in 1987 (100, 101) has since had profound implications in research and medicine. NO was shown to be a potent vasodilator, inhibitor of platelet aggregation, and active species of nitroglycerin before the discovery of EDRF in 1980 (37). It was subsequent studies that revealed that EDRF is NO and is synthesized by mammalian cells from L-arginine through a complex oxidation reaction catalyzed by the flavo-hemoprotein NO synthase (NOS). If any of the co-factors or prosthetics groups are not accessible or functional, NOS produces superoxide instead of NO. The complex regulation and production of NO provides many opportunities for malfunction, from high levels of asymmetric dimethyl arginine (ADMA), a competitive inhibitor of NOS, inefficient or lack of L-arginine transport and uptake, cofactor oxidation and enzyme uncoupling to name a few. Therefore, the notion that there may be an alternate source of NO from that produced by NOS is not revolutionary. Nitrite is now known to be an intrinsic signaling molecule (21, 34) capable of producing NO under appropriate conditions as well as form nitrosothiols (34, 35). Enhancing nitrite availability through therapeutic intervention by administering bolus nitrite prior to cardiovascular insult has shown remarkable effects in reducing the injury from myocardial infarction, ischemic liver and kidney injury, stroke and cerebral vasospasm (32, 33, 130-133) in animal models. These first reports on the efficacy of nitrite in cytoprotection have lead to nine current clinical trials for the use of nitrite and/or nitrate in both healthy volunteers and patients with specific cardiovascular complications (www.clinicaltrials.gov). Most recently nitrite has been shown to precondition the myocardium when given 24 hours prior to ischemic insult due to the modulation of mitochondrial electron transfer (127). Dietary nitrate supplementation has been shown to reduce diastolic blood pressure in healthy volunteers (112). Therapeutics is the branch of medicine concerned with the remedial treatment of disease. It is prudent at this juncture to take a step back and look at nitrite and nitrate as a means of prevention of a number of diseases associated with NO

insufficiency. Early intervention to restore NO/nitroso homeostasis through natural dietary means may prove to be a cost effective and natural means to prevent disease.

8. SUMMARY

The cardioprotective levels of nitrite and nitrate reviewed here can be easily achieved through increasing consumption of nitrite/nitrate rich foods. In fact, earlier reports by Lundberg and Govoni revealed that high intake of nitrate results in increased systemic nitrite levels (134) and most recently it has been reported that dietary nitrate reduces blood pressure in healthy volunteers (112). An optimal diet may then consist of a sufficient supply of nitrite and nitrate for health and protection from I/R injury. Regular intake of nitrite-containing food such as green leafy vegetables may ensure that blood and tissue levels of nitrite and NO pools are maintained at a level sufficient to compensate for any disturbances in endogenous NO synthesis (135). Since low levels of supplemental nitrite have been shown to enhance blood flow (67), dietary sources of NO metabolites could therefore improve circulation and oxygen delivery. This dietary pathway may not only provide essential nutrients for NO production but also provide a rescue or protective pathway for people at risk for cardiovascular disease (22). Moreover, any intervention, which increases blood and tissue concentrations of nitrite, may also provide protection against I/R injury.

Since the early 1980s there have been numerous reports on the association of N-nitrosamines and human cancers (136, 137) but a causative link between nitrite and nitrate exposure and cancer is still missing (138). Furthermore, a two year study on the carcinogenicity of nitrite by NIH has conclusively found that there was no evidence of carcinogenic activity by sodium nitrite in male or female rats or mice (139). A report from the National Research Council (*The Health Effects of Nitrate, Nitrite, and N-Nitroso Compounds*, NRC 1981) estimated based on food consumption tables that the average total nitrite and nitrate intake in the US was 0.77mg and 76 mg respectively. If we assume our body (70kg) produces 1.68mmole NO per day (based on 1µmole/kg/hr NO production). An average daily intake of 0.77 mg of nitrite would equate to 11.1µmoles per day and 76mg nitrate would equate to 894 µmoles per day or roughly 1 mmole NOx per day from diet. This almost matches what our body makes from NO if we assume most of the NO goes to stepwise oxidation to nitrite and nitrate. Therefore our steady state levels of NOx which are routinely used as clinical biomarkers of NO activity come almost 50% from diet. Moreover if nitrite were a carcinogen, we would be advised to avoid swallowing since saliva contains 50-100µM nitrite which can increase to near millimolar levels (48) after a nitrate rich meal. Assuming 50 µM nitrite in saliva and daily production of up to 1.5 liters per day the total nitrite exposure from saliva alone is 75 µmoles or 5.18 mg. The enterosalivary concentration and circulation of nitrate and ultimately nitrite provides an essential pathway for health and host defense. Even more, studies on high altitude natives of Tibet reveal that increasing nitrite and nitrate concentrations within the body are natural physiological responses that are devoid of harmful effects (140). There should be a re-evaluation of current nitrite and nitrate consumption analysis based on 21st century dietary habits. The strategy of avoiding nitrite and nitrate rich foods imposed in the 1970s due to the propensity to form N-nitrosamines may prove to be unwarranted and even detrimental to cardiovascular health.

This review documents the latest reports of the potential benefits of nitrites and nitrates in physiology. Although these agents have been used for hundreds of years as meat and food preservatives to minimize bacterial contamination, it has been feared that these substances could be carcinogenic. This misbelief has hopefully been corrected with current and ongoing studies. These are naturally occurring molecules in our diet as well as our body, and can function as reservoirs or prodrugs to be converted to nitric oxide. Nitric oxide is probably one of the most important signaling molecules in our body to regulate numerous physiological functions including blood flow to tissues and organs. The conversion of nitrite and nitrate to nitric oxide in our tissues is obviously an important finding. Interestingly formulations of topical nitrite preparations are effective in wound and burn healing. Clinical trials for such uses as well as diabetic skin ulcers are also underway. It appears that dietary manipulations of nitrites and their topical uses will be effective and inexpensive therapies due to their conversion to nitric oxide. The emerging physiological data on nitrite are strikingly analogous to a vitamin. A vitamin is by definition any of a group of organic substance essential in small quantities to normal metabolism, found in minute amounts in natural foods or sometimes produced synthetically: deficiencies of vitamins produce specific disorders. We know that nitrite is produced in relatively small quantities in normal metabolism of L-arginine and NO and is found in minute amounts in natural foods. Could it be then that if one does not eat sufficient foods rich in nitrite and nitrate specific disorders occur? Our animal studies reveal that nitrite insufficiency exacerbates I/R injury and a host of cardiovascular diseases are associated with decreased NO availability as measured by nitrite. Becoming more evident is the enormous benefit of exogenous dietary nitrite and nitrate in a number of disease models. A simple ubiquitous molecule we have been advised to avoid may be an indispensable nutrient that many are lacking. This is an exciting time in NO and nitrite based research.

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