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Review

Clinical evidence demonstrating the utility of inorganic nitrate in cardiovascular health

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ABSTRACT

The discovery of nitric oxide and its role in almost every facet of human biology opened a new avenue for treatment through manipulation of its canonical signaling and by attempts to augment endogenous nitric oxide generation through provision of substrate and co-factors to the endothelial nitric oxide synthase complex. This has been particularly so in the cardiovascular system and it is well recognized that there is reduced bioavailable nitric oxide in patients with both cardiovascular risk factors and manifest vascular disease. However, these attempts have failed to deliver the expected benefits of such an approach. Recently, an alternative pathway for nitric oxide synthesis has been elucidated that can produce authentic nitric oxide from the 1 electron reduction of inorganic nitrite. Furthermore, it has long been known that symbiotic, facultative, oral microflora can facilitate the reduction of inorganic nitrate, that is ingested in the average diet in millimolar amounts, to inorganic nitrite itself. Thus, there exists an alternative reductive pathway from nitrate, via nitrite as an intermediate, to nitric oxide that provides a novel pathway that may be amenable to therapeutic manipulation. As such, various research groups have explored the utility of manipulation of this nitrate–nitrite–nitric oxide pathway in situations in which nitric oxide is known to have a prominent role. Animal and early-phase human studies of both inorganic nitrite and nitrate supplementation have shown beneficial effects in blood pressure control, platelet function, vascular health and exercise capacity. This review considers in detail the pathways of inorganic nitrate bioactivation and the evidence of clinical utility to date on the cardiovascular system.

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1. Introduction

The elucidation of the identity of EDRF as NO and its protean roles in human physiology and pathophysiology has been one of the seminal discoveries in biology in the late 20th century. Within the cardiovascular system, basal endothelial NO release plays a critical role in sustaining cardiovascular health and it does this in many ways including by exerting vasodilator [1–3], anti-platelet [4,5], anti-proliferative [6,7] and anti-leucocyte phenotype [8].

These beneficial properties of NO in the cardiovascular system, coupled with evidence of reduced, bioavailable NO in patients with classic cardiovascular risk factors, such as hypertension [9,10] and hypercholesterolaemia [11], and endothelial dysfunction [12], have highlighted the obvious therapeutic potential of NO and NO donors [13]. The organic nitrates, such as GTN and isosorbide mononitrate, represented the first class of NO donors to reach the clinical setting, although tachyphylaxis [14] and induced endothelial dysfunction [15] after prolonged use have limited their clinical utility and likely partly explains the lack of efficacy of organic nitrates in large scale clinical trials [16]. However, the discovery of authentic NO production from the 1 electron *reduction* of NO_2^- [17–19] and demonstration that symbiotic, facultative, anaerobic, oral bacteria can reduce NO_3^- to NO_2^- [20–24] has provided a further avenue within which to explore NO-based therapeutics.

Today, this NO_3^-–NO_2^-–NO pathway has been proposed to act as a *back-up* system for NO generation [25] in situations where the conventional pathways for NO synthesis may be compromised, such as in cardiovascular disease [9,26,27]. Perhaps more importantly recent evidence suggests that this pathway also plays a significant role in maintaining levels of bioactive NO that underlie its critical role in cardiovascular homeostasis [28]. These advances have led to a radical revision of the pathways that govern endogenous NO generation and NO metabolism, previously viewed as a one-way, linear termination of activity by the oxidation of NO to both NO_2^- and NO_3^-. However, this novel paradigm reveals the 2 species to be in a 'NO cycle' [29] that can be potentiated through the provision of inorganic NO_3^-, given either by dietary or inorganic supplementary route. This review will discuss the evidence testing the utility of this *alternative* pathway for NO synthesis in cardiovascular health and disease.

2. Bioactivation of NO_3^- to NO

Until recently, a widely-held view of mammalian NO biology included the production of NO uniquely from the 5 e^- *oxidation* of the amino acid, L-arginine by NOS enzymes [30]. The termination of action of NO *in vivo* is achieved through its oxidation. In pure aqueous solutions, the oxidation of NO occurs slowly and the primary product is nitrite (NO_2^-) [31]. However, in biological systems, NO reacts preferentially with oxyhaemoproteins, such as oxyhaemoglobin (oxyHb) and produces NO_3^- and methaemoglobin (metHb) [31] as first described more than 140 years ago by Hermann in 1865 (cited in Gladwin et al. [32]). There is recent evidence of a NO oxidase/NO_2^- synthase function of the multi-function Cu-containing enzyme, ceruloplasmin [33] but the importance of this pathway in terminating NO activity and regulating basal NO levels is unclear at this time. As such, NO_3^- is the predominant oxidative metabolite of NO in biological systems [31]. However, the view that the oxidation of NO to NO_2^- and NO_3^- represents a termination of the pathway has needed revision with the publication of numerous studies demonstrating bioactivity of a reductive pathway prevalent *in vivo* whereby NO_3^- is reduced to NO_2^- and thence to the biologically active NO (Fig. 1).

NO_3^- is found in the plasma of healthy individuals with concentrations measured ranging between 20 and 40 μM during fasting

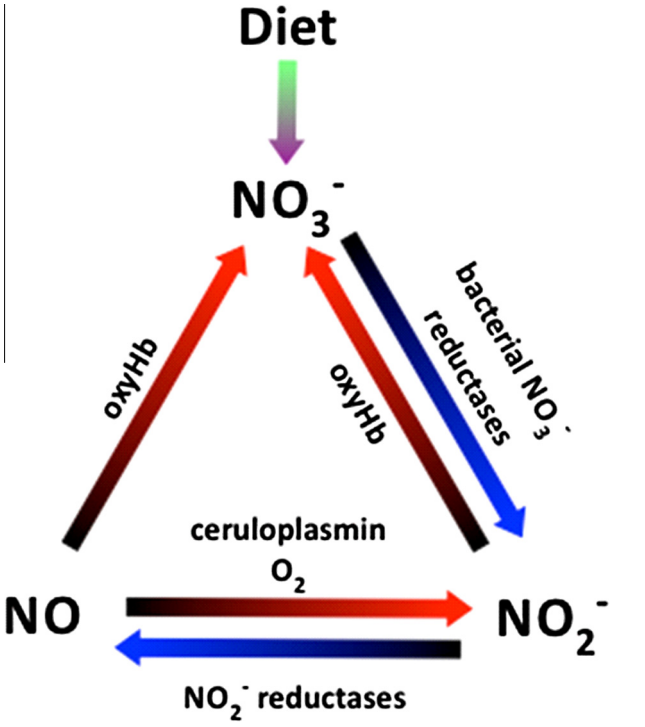

Fig. 1. The alternative pathway for nitric oxide production. (NO = nitric oxide; NO_3^- = nitrate; NO_2^- = nitrite; NOS = nitric oxide synthase; O_2 = oxygen; oxyHb = oxyhaemoglobin).

conditions [34–36]. *In vivo*, NO_3^- originates from two potential sources i.e. from the oxidative metabolism of NO, but also from NO_3^- ingested orally in the diet, predominantly in green-leafy vegetables (such as rocket and beets) [37]. Nitrogen-balance studies lasting up to 14 weeks, have estimated from 24 h urine NO_3^- collections that endogenous biosynthesis of NO_3^- is ~0.6–0.9 mmol (~37–56 mg) per day in healthy people [38]. Daily intakes of NO_3^- in European populations are estimated to be 1.5–2 mmol (93–124 mg) [39].

Orally ingested NO_3^- is absorbed across the upper GI tract [40–42] rapidly [35,36,43,44] and bypasses first-pass metabolism with close to 100% bioavailability [44]. The effective $t_{1/2}$ for NO_3^- in plasma, after consumption of different vegetable sources of NO_3^- (spinach, lettuce and beetroot), has been calculated to be 5.7–6.7 h [44].

Up to ⅔ of the absorbed NO_3^- is excreted unchanged in the urine [38,45–47]. Most of the remaining NO_3^- is concentrated from the circulation in the salivary glands of NO_3^- [48,49] by a sialic acid transporter, sialin (a $2NO_3^-/H^+$ co-transporter) [50], and is then secreted into the oral cavity as NO_3^--rich saliva [21,51,52] at concentrations up to 10-fold higher than plasma [35,51]. This process, and the further metabolism of orally-secreted NO_3^- has been dubbed the entero-salivary circulation [20] (Fig. 2).

Analysis of saliva taken directly from salivary gland ducts, as opposed to mixed saliva in the oral cavity, revealed that within the salivary gland there is no NO_2^-, suggesting that conversion of NO_3^- to NO_2^- within the oral cavity was responsible for salivary NO_2^- levels [53]. Ishiwata et al. were the first to suggest that the appearance of NO_2^- in the saliva may be due to the presence of NO_3^--reducing bacteria [53] similar to symbiotic, NO_3^- reducing bacteria identified in the GI tract [54–56]. It is recognized that some anaerobic bacteria can use NO_3^- as a terminal electron donor in respiration instead of O_2 and, thereby, reduce NO_3^- to NO_2^- (as reviewed in [57,58]).

The first species of human NO_3^--reducing bacteria isolated from the oral cavity was *Bacillus coagulans* [22] and later, *Veilonella*,