Enjoy your heart-beets. The role of dietary inorganic nitrate in cardiovascular health

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INTRODUCTION
According to the World Health Organisation, since 2014, cardiovascular diseases (CVD) have been the primary cause of death, not only in developed countries, but also worldwide. Most CVD [1–3] and conditions such as post-ischaemic inflammation and no-reflow phenomenon [4, 5] are linked to disturbances in the function, structure, and integrity of arterial endothelium, collectively described as a pro-inflammatory and a pro-atherosclerotic phenotype of the endothelium.

A current view is that the diseased endothelial phenotype is a consequence of an increased vascular generation of reactive oxygen species (ROS) mediated by CVD risk factors, ROS-induced inactivation of the endothelium-derived nitric oxide (NO), and a resulting imbalance between the cellular signalling by NO and ROS [1–3, 6, 7]. Antioxidants failed to prevent CVD in clinical trials [8]. Instead, strategies intended to boost the NO signalling have emerged as a promising therapeutic objective for the prevention and treatment of CVD.

Vascular NO is generated though: (i) the classic L-arginine-NO synthase pathway and (ii) the newly described nitrate-nitrite-NO pathway in which the dietary inorganic nitrate (NO$_3^-$) (e.g. present in beetroot) undergoes in vivo conversion to nitrite (NO$_2^-$) and then to NO. Importantly, dietary NO$_3^-$ and NO$_2^-$ have been demonstrated to improve NO signalling and to induce beneficial cardiovascular effects [9–12]. Herein, current evidence is reviewed regarding: the role of NO and ROS in the mechanism of CVD, the role of NO$_3^-$/NO$_2^-$ bio-activation in vivo NO homeostasis, and the pro-health potential of the dietary NO$_3^-$/NO$_2^-$.

CLASSIC L-ARGININE/NO SYNTHASE PATHWAY
The major sources of NO in vivo are three NO-synthase (NOS) isoforms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) isoform. The large mass of the endothelium causes eNOS to be the major NO producer. NOS uses NADPH, O$_2^-$ and tetrahydrobiopterin (BH$_4^-$) to convert L-arginine to L-citrulline with a concomitant release of NO (Fig. 1).
However, under conditions of limited availability of L-arginine and/or BH₄, NOS activity switches from the NO to superoxide (O₂⁻) generation (a process known as NOS uncoupling). Remarkably, the CVD risk factors are associated with eNOS uncoupling mediated by BH₄ deficiency (BH₄ undergoes peroxynitrite-mediated inactivation, see later) and/or increased production of a range of endogenous NOS false substrates, including asymmetric dimethyl arginine (ADMA) [13, 14].

The eNOS releases NO intra- and abnormally. It is expressed in the endothelium of large arteries, and its expression decreases in small resistance arteries and veins and is virtually absent in the capillaries [15]. The laminar blood flow and the related endothelial shear stress are the major eNOS activators. Short-lasting and long-lasting increases in the shear stress (e.g. accompanying physical exertion) stimulate the eNOS activity and the expression, respectively. Also, erythrocytes express eNOS and produce NO, although the significance of these phenomena remains unclear [16].

Nitric oxide is a signalling molecule preferentially targeting heme proteins and protein cysteine residues (Fig. 2, left-hand side). Binding of NO to the Fe²⁺-heme of soluble guanylyl cyclase activates this enzyme to produce cyclic guanosine 3,5-monophosphate (cGMP), cGMP in turn activates protein kinase G, which mediates phosphorylation of proteins in vascular smooth muscle (phosphatases, ion channels) (Fig. 3A), resulting in vascular relaxation. The in vivo half-life of NO is < 1 s; therefore, its cGMP-dependent effects (elicited in an autocrine/paracrine manner) are rather local. However, NO exerts most of its effect via two more stable mediators S-nitrosothiols and NO₂⁻ (the latter being a physiological store of NO, see later) that act in an endocrine- and cGMP-independent manner. S-nitrosothiols are products of the reaction of protein S-nitrosylation, which involves addition of the nitrosyl (–N=O) moiety to the cysteine thiol (–SH group) side chain of a protein (Fig. 3B). The nitrosyl group can then be exchanged between cysteine thiols of the same or neighbouring proteins. The process of transnitrosylation, has the effect that circulating S-nitrosothiols may accumulate in the peripheral tissues, and that they are relatively stable, which explains the wide range of cellular effects of the endothelial NO within and outside the cardiovascular system. The S-nitrosylation results in protein posttranslational modifications and eventually may have an effect on gene expression. The activation of endothelial cells has been reported to mediate the S-nitrosylation of > 100 proteins, including mitogen-activated protein kinases, thyrosine kinases, phosphatases, transcription factors, innate immune system receptors, and β₂-adrenergic receptors [17].

The majority of the endothelium-derived NO is rapidly inactivated to biologically inactive NO₃⁻ by haemoglobin and myoglobin present in erythrocytes and muscle cells, re-
spectively. However, a small fraction of the NO is converted to a biologically active NO$_3^-$ either by ceruloplasmin [18] or via NO autoxidation (Fig. 2). Thereby, serum NO$_3^-$ levels are ~100 times higher than NO$_2^-$ in healthy subjects (~30 μM vs. 150–300 nM) [19].

The biologically favoured NO reaction is that with O$_2^-$ (NO + O$_2^-$ → ONOO$^-$) (Fig. 2). Consequently, the reported effects of vascular O$_2^-$ overproduction (as associated with the CVD risk-factors) included: (i) impaired vascular NO bioavailability (seen as the endothelial dysfunction in the FMD test); (ii) vascular overproduction of the toxic peroxynitrite (ONOO$^-$) mediating BH$_4^-$ inactivation, the eNOS uncoupling and further impairment of the NO availability, and (iii) decreased S-nitrosothiol and NO$_2^-$ production and impairment of their signalisation [15]. Peroxynitrite may cause nitration of protein tyrosine residues to form 3-nitrotyrosine (Fig. 3B). While protein phosphorylation and S-nitrosylation are part of the normal cellular regulatory mechanism, protein nitration is an irreversible toxic process. Actually, plasma and/or tissue 3-nitrotyrosine is a biomarker of the nitrosative stress.

**O$_2^-$ AND THE REACTIVE OXYGEN SPECIES PATHWAY**

A major source of the vascular O$_2^-$ is NADPH oxidase (Nox), particularly its isoforms Nox1 and Nox2, expressed in the endothelial and vascular smooth muscle cells. Nox1/2 constitutively generate O$_2^-$, which in turn stimulates O$_2^-$ generation by other enzymatic systems (mitochondria, xanthine oxidase, and eNOS) [3, 20, 21]. Once generated, O$_2^-$ initiates a cascade of oxidative reactions causing the generation of other ROS (Fig. 2, right-hand side). The reaction: O$_2^-$ + NO → ONOO$^-$ is biologically favoured. Much slower are two O$_2^-$ dismutation reactions (one catalysed by the superoxide dismutase [SOD] and the other spontaneous) yielding hydrogen peroxide (H$_2$O$_2$), which in turn may become a source of a toxic hydroxyl radical.

The major mechanism of the cellular signalisation by O$_2^-$ and H$_2$O$_2$ involves oxidative modification of protein cysteine thios (Fig. 3C). Thus, nitrosative (Fig. 3B) and oxidative modifications of cysteine thios compete with each other. With the increase in ROS availability, cysteine thios undergo the modifications progressing from sulphenic acid (S-OH) via disulphide (S-S) and sulphinic acid (S-O$_2$H) up to irreversible sulphonic acid (S-O$_3$H). Disulphide formation can be internal, or mixed between proteins. These oxidative modifications represent a graded transition from normal signalling functions, through adaptation to oxidative stress (e.g. S-S), and finally to toxicity (sulphonic acid) [22, 23].

**NITRIC OXIDE AND ROS INTERACTIONS VS. THE PRO-ATHEROSCLEROTIC ENDOTHELIAL PHENOTYPE**

The endothelium maintains vascular and systemic homeostasis through multiple interactions with cells within the vessel wall and the blood (Table 1). These interactions are mediated mainly by endothelium-derived autacoids such as NO and O$_2^-$, but also by prostaglandins, endothelin-1, and others. The endothelial homeostasis is disrupted in CVD and states such as ischaemia-reperfusion, and the resulting changes in the endothelial phenotype can be identified as:
Figure 4. Vascular endothelium phenotype as determined by the balance between reactive nitrogen species (RNS)- and reactive oxygen species (ROS)-mediated cellular signalisation. The balance is preserved (left) in healthy endothelium, resulting in its anti-atherosclerotic phenotype. The balance is disrupted (right) by the cardiovascular disease risk factors, which, via several mechanisms, boost ROS pathway and downregulate RNS pathway, altogether mediating the development of the pro-atherosclerotic endothelial phenotype (see text for more details)

(i) an impairment of endothelium-dependent vasodilatation;
(ii) a pro-inflammatory state; (iii) a pro-thrombotic state, and
(iv) a state promoting arterial wall proliferation [15, 24].

The endothelial phenotype is currently viewed as a product of the competition between endothelial signalling mediated predominantly by NO and its reactive metabolites (reactive nitrogen species [RNS]) and by $O_2^-$ and its reactive metabolites (reactive oxygen species [ROS]). An emerging paradigm is that (Fig. 4):

1. Even healthy vessels produce some basal amounts of NO and $O_2^-$ mediating the physiological endothelial signalling functions.

2. A major source of the vascular NO and $O_2^-$ are eNOS and Nox. Major inducers of the activity and the expression of eNOS and Nox1/Nox2 are exercise and agonists such as angiotensin II and endothelin-1, respectively. In addition, RNS and ROS pathways mutually inhibit their activity and expression.

3. While RNS pathway dominates in the healthy endothelium, the diseased endothelial phenotype is a consequence of an imbalance between the endothelial signalling by RNS and ROS caused by the vascular ROS overproduction [1–3, 6, 7].

The CVD risk factors (hypercholesterolaemia, hypertension, diabetes, and others) acting mostly via angiotensin II and endothelin-1 [3, 7], and ischaemia/reperfusion acting via endothelin-1 [4, 5], all impair the endothelial NO bioavailability via four potential mechanisms: (i) the agonists mediated up-regulation of the vascular Nox1/Nox2 expression and increased production of an excess vascular $O_2^-$, which
in turn inactivates NO to form ONOO• [1–3, 6, 7]; (ii) oxidative stress-mediated downregulation of the eNOS expression; (iii) oxidative stress-mediated eNOS inhibition related to the up-regulated production of the endogenous eNOS inhibitors (e.g. ADMA) [14]; and (iv) eNOS uncoupling caused by ADMA and/or ONOO−-induced BH4 inactivation [13].

Exercise training is a major natural activator of eNOS, an inhibitor of Nox, an inducer of anti-atherosclerotic endothelial phenotype, and hence an effective protective measure against CVD [25]. This is probably related to the fact that exercise, by increasing arterial laminar blood flow velocity and endothelial shear stress, up-regulates eNOS and endothelial NO production, which in turn down-regulates vascular Nox and vascular O2− generation [2, 25, 26]. In addition, exercise training was shown to normalise, in an endothelium-dependent manner, increased sympathetic nervous system activity and increased renin–angiotensin system activity in patients with CVD [25]. Actually, a prolonged pharmacological inhibition of eNOS resulted in increased vascular expression of Nox1/Nox2 and in oxidative stress in experimental models, altogether supporting the concept that the endothelial ROS and RNS pathways control each other (Fig. 4).

Currently, the endothelial phenotype can only be indirectly clinically assessed. One approach is to evaluate the endothelial NO-availability by assessing the flow-induced and NO-dependent vasodilation (using the FMD test, for instance). It appears that the impairment of such a response, dubbed “endothelial dysfunction”, correlates with the presence and the progression of various forms of CVD, and that the measures preventing CVD also reduce the endothelial dysfunction [24, 27], supporting the view that disrupted endothelial homeostasis underlies the mechanism of CVD.

THE NO3−-NO2−-NO PATHWAY

Recently, it has become clear that: (i) inorganic NO3− is a substrate for endogenous NO2−-reductases and their in vivo production of authentic NO; (ii) ~70% of NO3− present in the blood and/or stored in tissues is derived from the L-arginine/NOS pathway and the remaining 30% is acquired through dietary intake; (iii) NO2− and NO3− ingestion increases plasma levels of NO3−/NO2− in humans, and a diet depleted of the NO3−/NO2− decreases these levels (at least in animals); (iv) estimates of NO3− intake from food are 93–124 mg/day in Europe (60–80% of this from vegetables and the reminder from drinking water) and as much as ~1100 mg/day in Japan [28]; and (v) the NO3− component of vegetables contributes to the beneficial health effects of this food group, including protection against CVD [10, 11, 23, 29].

The ingested NO3− is absorbed in the upper gastrointestinal tract and reaches peak plasma concentration 30–60 min after ingestion. Within a 24-h period ~75% of the absorbed NO3− is excreted by the kidneys. The remaining ~25% is taken up by the salivary glands and then gradually secreted with the saliva into the oral cavity, where commensal bacteria on the tongue convert ~20% of the NO3− present in the saliva (i.e. ~5% of the originally ingested NO3−) to NO2− [10, 11, 23]. The NO2− is swallowed, and its proportion is protonated in the stomach, forming nitrous acid, which in turn decomposes to NO, which ensures the normal gastric mucosa physiology and serves the first-line host defence against pathogens [29]. The NO2− that escaped protonation in the stomach enters the systemic circulation (reaching the peak plasma NO2− concentration 2–3 h after NO3− ingestion) and is partially stored in the peripheral organs, enabling its local and/or systemic (endocrine) activity [10, 11, 23]. The biological activity of the NO3− is related to the fact that it, per se, induces protein S-nitrosylation [30] and/or undergoes enzymatic reduction to NO (Fig. 5) [10, 11, 23, 29].

Nitrite is reduced to bioactive NO by deoxyhaemoglobin (in erythrocytes), deoxymyoglobin (in cardiomyocytes and skeletal muscle cells), xanthine oxidase (in erythrocytes and endothelial cells), and some mitochondrial enzymes [10]. Importantly, the effectiveness of these systems to reduce NO2− to NO increases in acidic conditions, such as those associated with tissue ischaemia/hypoxia. In contrast, ischaemia/hypoxia impair the NO formation by NOS [10].

THERAPEUTIC UTILITY OF THE NO3−-NO2−-NO PATHWAY

A number of epidemiological studies have shown that fruit- and vegetable-rich diets (e.g. DASH diet, traditional...
of a beetroot juice (1395 mg NO₃⁻/NO₂⁻ normotensive subjects: an increase in serum NO₂⁻ levels and caused the following in ameliorating myocardial ischaemia/reperfusion injury and/or in reducing infarct size in animal models [10, 11, 23]. Conversely, cardiac and hepatic ischaemia/reperfusion injury were increased and serum and tissue levels of NO₂⁻ were decreased in animals fed with NO₃⁻/NO₂⁻ depleted food [30]. Overall, these findings indicated NO or NO₂⁻ as inducers of the protection, and became a basis for further clinical studies. In one such study, patients with ST elevation myocardial infarction were given NaNO₂ (70 μmol IV) 5 min before the reperfusion, and no beneficial effects on infarct size and long-term prognosis were observed [38]. However, it is conceivable that NO₂⁻ takes a much longer time to develop its action. The results of a similar study in which NO₂⁻ was given directly to the coronary are awaiting publication [39]. Likewise, a randomised, double-blind, placebo-controlled trial testing the effect of NaNO₃ pretreatment (700 mg on the eve of the coronary artery bypass grafting and then 3 h prior to the surgery) on surgery-induced myocardial injury is also awaiting publication (clinicaltrials.gov: NCT01348971).

**NO₃⁻/NO₂⁻ vs. exercise tolerance**

Several studies in healthy untrained and trained subjects revealed that dietary NO₃⁻ supplementation (NaNO₃ or beetroot juice) dose-dependently increased exercise capacity and simultaneously decreased the oxygen cost of the exercise [11, 40, 41]. This latter effect is probably attributable to the fact that enzymes of the mitochondrial respiratory chain act as NO₂⁻ reductases to produce NO [10], and that NO favour-
ably modifies the mitochondrial oxygen cost of adenosine-5'-triphosphate formation [11]. In this context, it has been shown that dietary NO\textsubscript{3} supplementation resulted in a 19% improvement in the efficiency of mitochondria (P/O ratio) isolated from skeletal muscles of healthy volunteers [42]. Likewise, a single dose of NO\textsubscript{3} (558 mg) was reported to increase by \(-20\%\) the walking distance in patients with peripheral arterial disease [43]. However, it is unclear if this effect was due to improved energetics or some anti-isaemic effect. Also, the long-term effectiveness of the NO\textsubscript{3}/NO\textsubscript{2} treatment needs to be investigated.

**NO\textsubscript{3}/NO\textsubscript{2} vs. insulin resistance**

Impaired NO availability (seen as endothelial dysfunction), such as that usually accompanying the CVD risk factors, is always associated with insulin resistance. Interventions improving endothelial function were shown to reduce insulin resistance and, vice versa, interventions improving tissue insulin sensitivity improve also the endothelial function [44]. These data suggested the existence of a cause-effect relationship between the disturbances in the NO, insulin, and glucose metabolism, a view supported by experimental studies [23, 45]. For instance, insulin has been shown to stimulate endothelial NO generation and skeletal muscle blood flow, which in turn is a major determinant of insulin-dependent glucose uptake in skeletal muscles [44]. On the other hand, mice with the eNOS gene knockout were shown to develop a metabolic syndrome-like phenotype, which could be prevented by dietary NO\textsubscript{3} supplementation [46]. It has also been demonstrated that, at least in rats, NO\textsubscript{3} and NO\textsubscript{2} increase pancreatic insulin secretion [47], which altogether suggests that NO and insulin mutually stimulate their generation. Nevertheless, the only study aimed at clinical verification of these encouraging experimental data suggested the existence of a cause-effect relationship between the disturbances in the NO, insulin, and glucose metabolism, a view supported by experimental studies [23, 45].

**PHARMACOKINETICS AND TOXICOLOGY OF NO\textsubscript{3}/NO\textsubscript{2}**

The salutary effects of NO\textsubscript{3} vs. NO\textsubscript{2} seen in experimental and small clinical studies might eventually translate into their use as pharmaceutical agents. The practical aspects of this would be as follows.

1. NO\textsubscript{3} and NO\textsubscript{2} are cheap and easily applicable (diet supplemented with vegetables and/or inorganic NO\textsubscript{3}/NO\textsubscript{2}), and an equipotent hypotensive effect of beetroot juice and NaN\textsubscript{3} was reported [10].

2. In contrast to organic nitrates (e.g. nitroglycerin) [49], inorganic NO\textsubscript{3}/NO\textsubscript{2} does not result in the nitrate-tolerance phenomenon. Furthermore, NO\textsubscript{3}/NO\textsubscript{2} prevents, but does not induce, vascular oxidative stress [11, 37].

3. Although the therapeutic potential of NO\textsubscript{3} and NO\textsubscript{2} is similar, a therapeutic profile of NO\textsubscript{3} seems to be more favourable. Thus, inorganic and dietary NO\textsubscript{3} have a much longer half-life in human plasma (~6 h) compared to NO\textsubscript{2} given either by oral or IV routes (15–45 min), meaning that NO\textsubscript{3}, in contrast to NO\textsubscript{2}, could be given as a once-daily dosing regimen [11].

4. The L-arginine/NOS pathway is oxygen-dependent. The NO\textsubscript{2} conversion to NO increases with increasing acido- and hypoxia. Therefore, manipulations of the latter pathway may be particularly suited to conditions with accompanied organ ischaemia/hypoxia.

5. In the 1970s a debate was initiated as to the safety of the ingested NO\textsubscript{3} or NO\textsubscript{2}, particularly those used in the process of cured meat preparation. It was argued that NO\textsubscript{2} and NO\textsubscript{3} may become a source of carcinogenic N-nitrosamines in food, and the use of NO\textsubscript{3}/NO\textsubscript{2} for curing was almost banned. However, according to recent epidemiological studies, there is no evidence for the carcinogenicity of NO\textsubscript{3}/NO\textsubscript{2} in food, meaning that current recommendations as to the permissible concentration of NO\textsubscript{3}/NO\textsubscript{2} in food and water may be too restrictive [11, 28]. Instead, emerging evidence suggests therapeutic utility of NO\textsubscript{3} and NO\textsubscript{2}, a prospect awaiting verification in large-scale clinical trials.

**Conflict of interest:** none declared

**References**


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