Therapeutic effects of inorganic nitrate and nitrite in cardiovascular and metabolic diseases

S. A. Omar1, A. J. Webb2, J. O. Lundberg1 & E. Weitzberg1


Nitric oxide (NO) is generated endogenously by NO synthases to regulate a number of physiological processes including cardiovascular and metabolic functions. A decrease in the production and bioavailability of NO is a hallmark of many major chronic diseases including hypertension, ischaemia–reperfusion injury, atherosclerosis and diabetes. This NO deficiency is mainly caused by dysfunctional NO synthases and increased scavenging of NO by the formation of reactive oxygen species. Inorganic nitrate and nitrite are emerging as substrates for in vivo NO synthase-independent formation of NO bioactivity. These anions are oxidation products of endogenous NO generation and are also present in the diet, with green leafy vegetables having a high nitrate content. The effects of nitrate and nitrite are diverse and include vasodilatation, improved endothelial function, enhanced mitochondrial efficiency and reduced generation of reactive oxygen species. Administration of nitrate or nitrite in animal models of cardiovascular disease shows promising results, and clinical trials are currently ongoing to investigate the therapeutic potential of nitrate and nitrite in hypertension, pulmonary hypertension, peripheral artery disease and myocardial infarction. In addition, the nutritional aspects of the nitrate–nitrite–NO pathway are interesting as diets suggested to protect against cardiovascular disease, such as the Mediterranean diet, are especially high in nitrate. Here, we discuss the potential therapeutic opportunities for nitrate and nitrite in prevention and treatment of cardiovascular and metabolic diseases.

Keywords: beetroot, blood pressure, ischaemia, nitric oxide, vasodilatation.

Introduction

Nitric oxide (NO) is an important and unique signalling molecule that plays a key role in numerous physiological processes, particularly in the cardiovascular system [1, 2]. This small gaseous radical, generated by specific NO synthases (NOSs), is implicated in the proliferation of vascular smooth muscle cells and angiogenesis, endothelial integrity through its anti-adhesive and anti-aggregatory properties and the maintenance and regulation of vascular tone and blood pressure [3, 4]. Reduced NO bioavailability, either through decreased production or through increased consumption, has been associated with endothelial dysfunction and implicated in the development of numerous cardiovascular disease (CVD) processes, including renal disease [5–7]. Thus, the restoration of NO signalling provides an attractive mechanism to positively influence and modify CVD processes. Considerable scientific research over the last few decades has increased our understanding of the highly complex and redox-dependent biochemistry and signalling properties of NO. Reduced availability of substrates and cofactors, oxidation and uncoupling of NOS function and generation of reactive oxygen species (ROS) that react with and disrupt NO signalling are all important factors for the development/pathogenesis of CVD.

A recently discovered pathway for NO generation is the serial reduction of the inorganic anions nitrate (\(\text{NO}_3^-\)) and nitrite (\(\text{NO}_2^-\)) [8]. These inorganic anions are generated by oxidation of endogenous NOS-derived NO but are also present in the diet, with green leafy vegetables being particularly high in nitrate [9]. The nitrate–nitrite–NO pathway acts independently from the NOSs and is enhanced in
hypoxia/ischaemia. It is therefore a highly interesting source of NO bioactivity in certain CVDs, most notably in conditions involving ischaemia–reperfusion injury. It is now evident that this pathway has the capacity to generate NO-like effects in the cardiovascular system, making it therapeutically intriguing; it is currently being investigated for the treatment of hypertension, pulmonary hypertension, peripheral artery disease and myocardial infarction. In addition, the nutritional aspects of the nitrate–nitrite–NO pathway are interesting as diets suggested to protect against CVD, such as the Mediterranean diet, are especially high in inorganic nitrate.

Here, we will describe physiological and pathophysiological NO signalling and discuss the relevance of the nitrate–nitrite–NO pathway in the cardiovascular system in health and disease.

**NO generation**

In 1916, Mitchell et al. [10] inferred that nitrate was produced endogenously by demonstrating that the quantities of nitrate excreted in urine exceeded those obtained through dietary ingestion. This was confirmed 65 years later by Green et al. [11] in N15 isotopic studies to investigate the putative carcinogenic effects of nitrate and nitrite in germ-free rats and in humans [12]. These observations provided early descriptions of the mammalian generation of nitrogen oxides but did not identify NO as a signalling molecule with physiological relevance.

**NOSs**

The seminal discovery in 1980 by Furchgott and Zawadski showing that the vascular endothelium could generate a vasorelaxing substance triggered intense activity to identify this endothelium-derived relaxing factor (EDRF) [13]. In the late 1980s, it was shown that EDRF was the tiny gaseous radical NO that was generated from the amino acid L-arginine and molecular oxygen by specific NOSs [1, 3]. In addition to these substrates, several cofactors are needed including reduced nicotinamide adenine dinucleotide phosphate (NADPH) and terahydrobiopterin (BH4) [14]. Three NOS isoforms exist of which two are constitutively expressed [NOS 1/neuronal NOS (nNOS) and NOS 3/endothelial NOS (eNOS)], generating moderate amounts of NO involved in various physiological processes [2]. The third isoform [NOS 2/inducible NOS (iNOS)] is induced by bacteria and cytokines and produces significantly higher amounts of NO compared to the constitutive NOSs [15]. iNOS is a part of the innate immune system through its ability to kill bacteria and viruses and is present in various inflammatory conditions. However, the role of iNOS in inflammation is still debated as it is now evident that this isoform can also be constitutively expressed in noninflamed normal tissues [16]. The early availability of L-arginine analogues as NOS inhibitors and later the possibility of specific NOS gene deletion have been instrumental in improving knowledge about the physiological and pathophysiological role of the various NOS isoforms.

In the cardiovascular system, and especially in the vasculature, eNOS has a central role in the regulation of vascular tone and maintenance of endothelial homeostasis. Its activity is regulated by humoural, mechanical (shear stress) and pharmacological stimuli. The phosphorylation state of specific serine, threonine and tyrosine residues of the enzyme is central in the regulation of eNOS activity [14]. In the circulation, eNOS-derived NO is oxidized to nitrite in the presence of the multicopper metalloenzyme ceruloplasmin [17] and to nitrate in the presence of haemoglobin in red blood cells (Fig. 1). Hence, these anions, often together termed NOx, have been extensively used as surrogate markers of NO generation. For example, circulating levels of nitrite are inversely correlated with CVD risk [18]. However, because of the known substantial dietary contribution of nitrate to circulating nitrate and nitrite levels, such measurements are not without limitations [19].

Decreased availability of substrates or cofactors (the latter often as a consequence of oxidation by ROS) leads to eNOS uncoupling, a state in which the enzyme switches from generation of NO to production of superoxide (O2·) [20, 21]. This process represents a major pathological mechanism underlying the progressive decrease in vascular NO bioavailability observed during ageing and CVD.

**The nitrate–nitrite–NO pathway**

The discovery by three groups in the mid-1990s that NO can be generated from inorganic nitrate and nitrite in mammals [22–24] caused substantial scientific interest. Historically, however, the Chinese used inorganic nitrate in the treatment of coronary disease as recorded in a document dating back to 700 AD [25]. Nitrate was used as an
anti-anginal to relieve the symptoms of chest pain by placing it under the tongue. Inorganic nitrite was first produced in its pure form in 1777 by the Swedish chemist Carl Scheele [26]. In 1880, the effects of potassium nitrite were identified by Reichert and Mitchell in a number of organ systems including the cardiovascular system [27], and its use as a treatment for angina was described by Hay in 1883 [28].

The widespread use of sodium nitrite, even in an injectable form, became prevalent for the treatment of anti-anginal to relieve the symptoms of chest pain by placing it under the tongue. Inorganic nitrite was first produced in its pure form in 1777 by the Swedish chemist Carl Scheele [26]. In 1880, the effects of potassium nitrite were identified by Reichert and Mitchell in a number of organ systems including the cardiovascular system [27], and its use as a treatment for angina was described by Hay in 1883 [28].

The widespread use of sodium nitrite, even in an injectable form, became prevalent for the treatment of cardiovascular disease (CVD) [29].

Fig. 1 Pathways for generation and inhibition of nitric oxide (NO) in the vasculature. Vascular NO is generated by endothelial NO synthase (eNOS) which uses L-arginine and molecular oxygen (O₂) as substrates. The cofactor tetrahydrobiopterin (BH4) is essential for dimerization of the enzyme and binding of L-arginine. NO diffuses to the underlying vascular smooth muscle where it activates soluble guanylyl cyclase (sGC) leading to cyclic GMP (cGMP) formation and vasodilatation. Phosphodiesterase V (PDE V) regulates these effects by breakdown of cGMP. An alternative pathway for NO generation is the serial reduction of nitrate (NO₃⁻) and nitrite (NO₂⁻). Circulating nitrate, both from the diet and from oxidized NO, is actively taken up by the salivary glands and concentrated in saliva. Oral commensal bacteria reduce salivary nitrate to nitrite which after swallowing reaches the systemic circulation and is subsequently reduced to NO and other reactive nitrogen species (RNS) by deoxyhaemoglobin and tissue nitrite reductases. In cardiovascular disease (CVD), there are several factors contributing to reduced NO bioavailability. Increased generation of superoxide (O₂⁻) by NADPH oxidases leads to BH4 oxidation and generation of dihydrobiopterin (BH2). This contributes to eNOS uncoupling which leads to further O₂⁻ generation. NO and O₂⁻ generate the oxidant and nitrating agent peroxynitrite (ONOO⁻) which can uncouple eNOS and oxidize sGC (Ox-sGC), making the latter unresponsive to NO. In addition, arginase, which competes with eNOS for L-arginine, is upregulated in several CVDs, leading to eNOS substrate deficiency which can contribute to reduced activity and uncoupling. Finally, the endogenous NOS inhibitor asymmetric dimethyl arginine (ADMA) is increased in CVD which may further decrease eNOS activity.
of hypertension and angina in addition to a number of other diseases including epilepsy [29]. However, due to such indiscriminate prescribing and the use of high doses, patients suffered a number of side effects including severe hypotension and lethal methaemoglobinaemia [30, 31]. The appearance of organic nitrate (nitroglycerin) and organic nitrite (amyl nitrite) which had more predictable activity profiles almost completely displaced inorganic nitrate and nitrite from medical therapy [32]. However, inorganic nitrite continued to be used in a medicinal capacity as a therapeutic antidote for two toxic agents, cyanide [33] and hydrogen sulphide [34].

**Enterosalivary circulation of nitrate**

The two major sources of inorganic nitrate in mammals are NOS-derived oxidized NO and the diet (Fig. 1). As nitrate avoids first-pass liver metabolism, ingestion causes a rapid increase (within 15 min) in circulating levels of nitrate peaking at 90 min [35]. The levels remain high for several hours following ingestion (plasma half-life 5–6 h) after which they slowly decline, remaining elevated from baseline for up to 24 h [35]. Within this 24-h period, ~75% is excreted by the kidneys; thus, the long half-life of inorganic nitrate is a result of its low urinary clearance (~26 mL min⁻¹) [36]. Although nitrate is readily filtered through the glomerulus, it is actively reabsorbed through the proximal renal tubules, for example by up to 90% in dogs [37]. Trace amounts of the remaining 25% nonrenally excreted nitrate are secreted via the sweat glands with the bulk being concentrated in the salivary glands [38, 39]. Salivary nitrate levels can reach 10 mmol L⁻¹, which is ~10- to 100-fold greater than those of plasma (20–40 μmol L⁻¹), although plasma nitrate can increase to ~400 μmol L⁻¹ after a dietary nitrate load [38]. This nitrate-rich saliva is then excreted into the oral cavity where it is reduced to nitrite via the action of nitrate reductases of commensal bacteria located on the dorsal part of the tongue [40]. These bacteria are facultative anaerobes and are able to switch to nitrate-dependent respiration at low oxygen tensions [41, 42]. Following bacterial nitrate reduction, salivary nitrate levels can increase to >1 mmol L⁻¹ which is >1000-fold greater than in the circulation (100–300 nmol L⁻¹) [38]. Once swallowed, this nitrite is rapidly absorbed across the upper gastrointestinal (GI) tract (bioavailability ~95–98%) [43], leading to a rise in plasma nitrite levels after 30 min and peaking at 3-h postingestion [38, 44]. The circulating levels then decline steadily returning to baseline after 24 h [35]. Nitrate and nitrite are distributed rapidly within tissues, reaching near steady-state levels within 5 min [44]. A proportion of the swallowed nitrite-rich saliva is protonated in the acidic environment of the stomach forming nitrous acid (HNO₂) which in turn breaks down to form NO and other nitrogen oxide moieties [22,24]. This reaction is enhanced by increasing concentrations of nitrite, as well as by a low stomach pH and the presence of ascorbic acid [45] or polyphenols [46, 47]. The gastric generation of NO has been shown to promote effects to support gastric mucosal integrity, including increased mucosal blood flow and mucus generation [48, 49].

Although this enteral-salivary circuit provides the main pathway for the reduction of nitrate to nitrite in mammals, Bernheim and Dixon demonstrated in 1928 that nitrate is reduced to nitrite in rat muscle and ox liver [50]. Exactly 80 years later, using mouse, rat and liver tissue homogenates, Jansson et al. [51] established that xanthine oxidoreductase (XOR) catalyses nitrate reduction in mammalian cells. More importantly, in vivo mammalian nitrate reductase activity was confirmed in germ-free mice receiving intraperitoneal injections of nitrate, resulting in an increase in circulating nitrite. With the knowledge that nitrate and nitrite may be recycled back to bioactive NO, it is interesting to note that physical exercise, dietary habits, health status and lifestyle greatly affect plasma levels of nitrate and nitrite.

**Mechanisms of reduction of nitrite to NO**

The oxidation of NO to yield nitrate and nitrite was thought to be a one-way reaction, producing essentially physiologically inactive metabolites. However, numerous studies have now demonstrated that nitrite (and nitrate via nitrite) can in fact be reduced back to NO [52]. This can occur through the activity of a number of enzymes and proteins, which have been shown to possess nitrite reductase capability, such as the haem-containing globins (haemoglobin, myoglobin, cytoglobin and neuroglobin) [53–56], mitochondrial proteins [57, 58], molybdenum-containing enzymes (XOR, aldehyde oxidase and sulphite oxidase) [59–61], NOS enzymes [62] and cytochrome P450 [63]. In contrast to NOS-dependent NO generation which requires oxygen, these pathways for nitrite reduction are enhanced during hypoxic conditions. [64] This offers an alternative role for nitrate and nitrite,
as a reservoir for NO generation during conditions of hypoxia/ischaemia. Indeed, one of the major therapeutic possibilities that has been investigated with regard to nitrate and nitrite is in the area of cardiovascular ischaemia–reperfusion injury.

Nitrite reduction also occurs via simple acidic disproportionation [65, 66]. In the presence of protons, nitrite exists in equilibrium with nitrous acid, which in turn coexists in equilibrium with other nitrogen oxides. The intermediate dinitrogen trioxide (N₂O₃) dissociates to form nitrogen dioxide (NO₂) and NO, according to the following equations:

\[
H^+ + NO_2 \leftrightarrow HNO_2 \leftrightarrow NOOH
\]

\[
NOOH + NO_2 \leftrightarrow N_2O_3 + OH^- \leftrightarrow NO_2 + NO + OH^-
\]

As a consequence of this, even moderate reductions in pH will promote NO generation from nitrite. In conditions of low perfusion and ischaemia due to disease or during heavy physical exercise, reduced tissue pH will promote NO generation from nitrite. As in the case of hypoxic enhancement of nitrite reduction, it has been suggested that NO generation from nitrite will occur selectively in hypoxic/ischaemic tissues [53, 67]. This mechanism suggests a clear advantage compared to systemic delivery of an NO-donating drug without this redox-regulated mode of action.

Nitrite reduction follows exposure to ultraviolet (UV) light [68]. Human skin tissue contains about 4–6 μmol L⁻¹ nitrite [69] acquired via oxidation of endogenously produced NO and from bacterial reduction of nitrate delivered from the sweat glands [70]. The exposure of human skin to UVA light was shown to reduce the nitrite contained within it to vasoactive NO [68]. In addition, this produced a significant 2.3-fold increase in cutaneous S-nitrosothiol formation [68]. Of interest, Liu et al. [71] were able to demonstrate a blood pressure-lowering effect after exposure to UV light in healthy volunteers and this was coupled to an increase in circulating nitrite levels.

**NO signalling**

The activation of soluble guanylyl cyclase (sGC) leading to formation of cyclic GMP (cGMP) is a major NO signalling pathway in the cardiovascular system (Fig. 1) [72, 73]. This pathway underlies the vasodilatory action of NO as well as many other important physiological events including nerve signalling, mitochondrial biogenesis and angiogenesis. The radical nature of NO makes it very reactive with transition metals such as the iron-containing haem in sGC and iron–sulphur clusters in aconitase. Another example of this type of reaction is the binding to the ferrous (Fe²⁺) haem in cytochrome c oxidase resulting in reversible inhibition in competition with oxygen [74–76]. This high affinity of NO to ferrous haem explains the potent scavenging effect of circulating haemoglobin. Based on the redox-sensitive chemistry of the NO radical, two other pathways of NO signalling can be described. NO can react with other radicals in a rapid, diffusion-limited manner as exemplified by the reaction with thyl radicals (RS') to form S-nitrosothiols (R-SNOS) [77]. NO can also be subjected to oxidation by oxygen or ROS which can generate reactive nitrogen species with nitrosothiols (R-NO), dinitrogen trioxide or nitrogen dioxide chemical properties, which can nitrosate thiols [77] or nitrate tyrosine residues (R-ONO) [78], respectively, thereby mediating protein post-translational modification (Table 1) [79]. Circulating nitrosothiols can transmit NO-like bioactivity in an endocrine fashion and are able to transfer the NO adduct moiety to other thiol-containing proteins through transnitrosation. NO reacts extremely rapidly with superoxide to form peroxynitrite (ONOO⁻). In turn, peroxynitrite can isomerize to form nitrate or if protonated (ONOOH) decompose to NO₂ and OH radicals with potent oxidant and nitrating properties, potentially leading to damage of a wide array of molecules in cells, including DNA and proteins [80].

All these signalling modes of NO are highly redox-sensitive and governed by the actual concentrations of NO and ROS. Adding to this complex chemistry is the fact that nitrate and nitrite are sources of NO and several of the reactive nitrogen oxides described above (Table 1). It is therefore difficult to determine the actual final mediator of observed effects in in vivo experiments or in clinical studies. Moreover, the fact that nitrite reduction to NO cannot be effectively blocked pharmacologically, because there are several pathways for its bioactivation, adds further complexity to the identification of nitrate- and nitrite-dependent signalling.
Cardiovascular drugs that enhance NO bioavailability

Several of the most commonly used pharmaceutical agents to prevent or treat CVD enhance NO signalling. This is achieved by different modes of action including formation of NO, inhibition of NO breakdown or direct stimulation of downstream signalling pathways. The classical organic nitrates (e.g. nitroglycerin), used for more than a century, act via release of nitrite and NO [81]. However, these drugs are limited by the development of tolerance which is not the case with inorganic nitrate and nitrite (Table 2) [82]. Direct delivery of NO gas by inhalation is an established treatment for pulmonary hypertension in the newborn [83]. However, in studies in adults with pulmonary hypertension, inhaled NO has not been equally effective [84]. Phosphodiesterase V inhibitors are used in patients with erectile dysfunction and pulmonary hypertension [85]; by inhibiting cGMP breakdown, they amplify the vasodilatory effect of NO. Angiotensin-converting enzyme inhibitors and angiotensin receptor blockers reduce NADPH oxidase activity which leads to reduced superoxide formation and increased NO bioavailability [86, 87]. At the same time, less peroxynitrite is formed with reduced nitrosative stress. In addition, the important cofactor BH4 will remain in its reduced form, thereby limiting eNOS uncoupling (Fig. 1). Novel beta-receptor antagonists such as nebivolol promote NO generation by stimulating eNOS and probably also via their antioxidant properties [88]. Statins are pluripotent drugs that, in addition to their cholesterol-lowering effect, increase NO bioavailability by enhancing eNOS gene expression [89] and activity [90], supporting BH4 synthesis [91], inhibiting NADPH oxidase activity and stimulating catabolism of the endogenous NOS inhibitor asymmetric dimethyl arginine (ADMA) [92]. Recently, a direct sGC stimulator (riociguat) was registered for the treatment of pulmonary hypertension with some advantages compared to existing drugs [93]. It is highly likely that additional indications in the treatment of CVD will be found for this class of drugs. From a dietary perspective, polyphenols are interesting because they enhance NO bioavailability by several mechanisms. Their direct antioxidant properties reduce NO scaveng-

### Table 1

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<tr>
<th>Nitric oxide</th>
<th>NO synthesis</th>
<th>NO synthase</th>
<th>NO oxidation</th>
<th>SNO decomposition</th>
<th>Nitrite reduction</th>
<th>Nitric oxide</th>
<th>NO synthase</th>
<th>NO oxidation</th>
<th>SNO decomposition</th>
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<td>Nitrate</td>
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<td>S-nitrosothiol</td>
<td>R-SNO</td>
<td>NO synthase</td>
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<tr>
<td>Peroxynitrite</td>
<td>ONOO−</td>
<td>Reaction of superoxide and NO</td>
<td>Reaction of superoxide and NO</td>
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<td>Nitroalkene</td>
<td>NO2</td>
<td>NO oxidation</td>
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<tr>
<td>8-nitro-cGMP</td>
<td>C10H12N5O7P</td>
<td>Formed from iNOS-derived NO</td>
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<td>N-nitrosamine</td>
<td>R2N-N</td>
<td>NO oxidation</td>
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Table 2  Characteristics of organic nitrates and inorganic nitrate/nitrite

<table>
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<tr>
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<th>Organic nitrates</th>
<th>Inorganic nitrate/nitrite</th>
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</thead>
<tbody>
<tr>
<td>Chemistry</td>
<td>Synthetic esters of nitric acid (RONO₂) where R is an organic residue</td>
<td>Naturally occurring water-soluble anions. Present in high concentrations in certain vegetables and generated endogenously</td>
</tr>
<tr>
<td>Pharmacokinetics</td>
<td></td>
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<tr>
<td>Absorption</td>
<td>Lipophilic and readily absorbed via sublingual, transdermal or rectal routes. Variable oral bioavailability due to extensive hepatic first-pass metabolism</td>
<td>Absorbed in the upper GI tract with almost 100% bioavailability. Nitrite can also be administered intravenously or by inhalation</td>
</tr>
<tr>
<td>Distribution</td>
<td>Rapidly distributed throughout the tissues</td>
<td>Distributed throughout the tissues. Nitrate undergoes enterosalivary circulation and 25% accumulates in saliva</td>
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<tr>
<td>Metabolism</td>
<td>Rapidly metabolized by various enzymes including cytochrome P450s and ALDH-2. Varying half-life ranging from 1–4 min (nitroglycerine) to 5 h (5-ISMN)</td>
<td>Nitrate is reduced to nitrite by oral commensal bacteria and to some extent by tissue XOR. The half-life of nitrate is 6 h</td>
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<tr>
<td>Excretion</td>
<td>Nitrogen oxides generated from organic nitrates are eventually excreted as inorganic nitrate by the kidney</td>
<td>Nitrite is converted nonenzymatically to NO and a variety of bioactive nitrogen oxides in the acidic stomach. In blood and tissues, nitrite is reduced to NO by a variety of haem- and molybdenum-containing enzymes. The half-life of nitrate is around 30 min The majority of ingested nitrate (60–70%) is excreted by the kidney</td>
</tr>
<tr>
<td>Pharmacodynamics</td>
<td>Potent vasodilator causing rapid (within minutes) selective venodilatation and large artery dilatation (low dose), versus vasodilatation of resistance arterioles (high dose) with a concomitant fall in preload/afterload and BP. At lower doses, selective lowering of central aortic BP</td>
<td>Effects of nitrate are dependent on its metabolism to (and accumulation of) nitrite. Nitrite is a moderately potent vasodilator causing rapid, selective venodilatation and (recently discovered) normoxia-dependent selective large artery dilatation at low doses, versus hypoxia-dependent vasodilatation of resistance arterioles. Nitrite appears to selectively lower central aortic BP. Nitrate lowers peripheral BP with a slow onset (2–3 h) and long duration</td>
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ing [94], but they also inhibit NADPH oxidase-dependent superoxide generation [95]. Moreover, data suggest that they increase vascular eNOS activity via p38 MAPK and the PI3/AKT pathways [96]. In addition, they enhance nitrite reduction to NO [9]. Together, the evidence suggests that several pharmaceutical agents and dietary constituents, including polyphenols, nitrate and nitrite, promote NO signalling which probably contributes to their beneficial effects.

Cardiovascular effects of nitrate and nitrite

The first report that dietary nitrate could exert NO-like effects in the cardiovascular system was from the study by Larsen et al. [97] in 2006 which showed that intake of sodium nitrate for 3 days lowered blood pressure in young healthy individuals. This was later confirmed in studies using beetroot juice as a natural source of inorganic nitrate [35, 98]. Since then, numerous studies in animals and humans have investigated the effects of dietary nitrate on ischaemia–reperfusion, hypertension, renal function, oxidative stress and tolerance to hypoxia [9]. In parallel, the effects of direct nitrite administration on cardiovascular function in animal disease models and in humans have been investigated [99]. There are currently several ongoing phase II trials to explore the nutritional and therapeutic opportunities of stimulating the nitrate–nitrite–NO pathway. Below, we review the current knowledge of the role of nitrate and nitrite in several important areas related to the cardiovascular system.

Endothelial dysfunction

A hallmark of endothelial dysfunction is a reduced bioavailability of NO, either through reduced eNOS activity or expression, or via increased NO consumption by free radicals and ROS [100]. Endothelial dysfunction is thought to be a precursor to the onset of CVD, and a dysfunctional endothelium, in humans most often evaluated by brachial artery flow-mediated dilatation (FMD), is a major predictor of atherosclerotic disease progression and outcome in coronary artery disease [100–102].

Animal models

Using a murine model in which endothelial dysfunction was induced through a high-cholesterol diet leading to hypercholesterolaemia, Stokes et al. [103] demonstrated that supplementation with dietary nitrite preserved endothelial function in addition to inhibiting the development of microvascular inflammation and the rise in C-reactive protein. Similarly, using dietary nitrite in a mouse model of age-related endothelial dysfunction,

Table 2 (Continued)

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<thead>
<tr>
<th>Organic nitrates</th>
<th>Inorganic nitrate/nitrite</th>
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<tr>
<td>Inhibits platelet aggregation</td>
<td>Nitrate improves endothelial function and increases mitochondrial efficiency</td>
</tr>
<tr>
<td>Nitrate and nitrite inhibit platelet aggregation</td>
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Therapeutic applications for cardiovascular disease

Registered for use in treatment of angina pectoris

Currently, in clinical trials for acute MI, systemic hypertension, pulmonary hypertension, heart failure and peripheral artery disease

Side effects

Headache, hypotension, development of tolerance

Prolonged use may cause endothelial dysfunction

With very high doses of nitrite risk of methemoglobinemia. Chronic ingestion of nitrate/nitrite may cause formation of nitrosamines with proposed carcinogenic properties

GI, gastrointestinal; ALDH-2, aldehyde dehydrogenase 2; XOR, xanthine oxidoreductase; BP, blood pressure; MI, myocardial infarction.
Sindler et al. [104] found that circulating and tissue levels of nitrite were reduced by ~45% in older mice. Following supplementation with nitrite, circulating levels of nitrite returned to those of younger mice and age-related arterial stiffing, oxidative stress and endothelial dysfunction were reversed [104].

Clinical studies
It has been shown that ingestion of sodium nitrate improves endothelial function in healthy subjects at doses resembling the mean daily intake of nitrate via the diet in Western countries (Fig. 2) [105]. Moreover, intake of nitrate either as nitrate salt or in the form of beetroot juice mitigated the deleterious effects on endothelial function as a consequence of a mild ischaemia–reperfusion (I/R) injury induced in the forearms of healthy subjects and assessed via the endothelium-dependent reactive hyperaemia response [35, 106]. The induction of ischaemia–reperfusion injury significantly reduced FMD of the radial artery by ~60%; however, this was reversed by the ingestion of nitrate, restoring FMD to near baseline levels [35]. A recent meta-analysis based on 12 studies of the vascular effects of nitrate or beetroot juice showed a dose-dependent improvement in vascular function due to these interventions [107].

Similar beneficial effects were demonstrated with sodium nitrite infused at a dose of 1.5 μmol min⁻¹ for 20 min prior to ischaemia–reperfusion injury in the forearm of healthy volunteers. This treatment reduced the deleterious effects of ischaemia–reperfusion injury resulting in a near pre-ischaemic FMD response [108]. However, the protective effects of nitrite were abolished when the nitrite infusion was initiated during the ischaemic event [108]. Rodriguez-Mateos et al. [109] recently demonstrated a dose-dependent improvement in FMD by dietary nitrate. It is interesting that the effects on FMD were seen at very low doses, corresponding to only a small serving of a nitrate-rich vegetable. Moreover, the authors demonstrated additive effects when nitrate was combined with cocoa flavanols, demonstrating that nutrient–nutrient interactions modulate vascular function. In healthy overweight and slightly obese men, a single dose of beetroot juice attenuated the post-prandial impairment of FMD following a mixed meal [110].

Fig. 2 Putative mechanisms underlying the protective effects of nitrogen oxides in ischaemia–reperfusion (I/R) injury. The nitrate–nitrite–nitric oxide (NO) pathway generates not only NO but also other reactive nitrogen species (RNS) that are able to nitrosate (-SNO) and nitrate (-NO₂) proteins, thereby altering their function. Through these various signalling possibilities, nitrate, nitrite and NO have been shown to be protective against IR injury. RNS are also involved in pre-, post- and remote conditioning. Mitochondria are central targets of the RNS in IR injury. Nitrite reduces superoxide generation via reversible nitrosation of complex I. NO is also involved in opening of ATP-sensitive potassium channels which is a central mechanism in preconditioning. The reversible binding of NO to cytochrome C oxidase, leading to inhibition of mitochondrial respiration, is another putative protective mechanism. NO has been shown to be involved in inhibiting the opening of the mitochondrial permeability transition pore with attenuated cytochrome C release and reduced apoptosis. Dietary nitrate improves mitochondrial efficiency through reduced expression of uncoupling proteins. In addition to these mitochondrial effects, vasodilatation of coronary arteries, inhibition of platelet aggregation and leukocyte adhesion may also contribute to the protective effects of nitrate, nitrite and NO. Finally, inhibition of arginase by dietary nitrate may lead to better endothelial and inducible NO synthase function during I/R. NO₃⁻, nitrate; NO₂⁻, nitrite; iNOS; inducible NO synthase; eNOS, endothelial NO synthase; KATP, ATP-sensitive potassium channel.
Although several studies have demonstrated improvements in FMD by dietary nitrate, the findings of two studies showing no effects have also been published. Bahra et al. [111] found that, whereas nitrate ingestion improved vascular compliance in healthy volunteers, it did not alter FMD. Moreover, Gilchrist et al. [112] found no effect of dietary nitrate on FMD in patients with type 2 diabetes.

**Vasodilatation**

**Animal models**

In 1927, Densham used a cat model to demonstrate the vasodilatory effects of sodium nitrite, comparing it to the actions of acetylcholine [113]. However, Densham only noted a dilatory effect when the animals were preperfused with lactic acid. He suggested that the H⁺ ion was required to ‘unlock’ the dilatory activity of nitrite [113]. In 1953, Furchgott and Bhadrakom showed that strips of rabbit aorta were relaxed by nitrite under normal pH; however, high doses of nitrite were used (100 and 1000 µmol L⁻¹) [114]. In 2001, Modin and co-workers tested lower concentrations of nitrite on rat aortic rings in aerobic conditions but at pH levels similar to those seen in tissues during hypoxia/ischaemia [115]. The threshold for the onset of relaxation at pH 6.6 was found to be only 2.5 µmol L⁻¹, which at the time was regarded as physiological levels. This relaxation was associated with an increase in NO production and was abolished by the addition of the sGC inhibitor ODQ and enhanced by vitamin C. Thus, the authors speculated that endogenous nitrite could be involved in the physiological phenomenon known as metabolic vasodilatation.

The sensitivity to nitrite may differ between different types of vessels. To assess the dilatory effect on various vessel types, nitrite (10 µmol L⁻¹) was added to rabbit aorta, inferior vena cava and pulmonary artery resulting in dilatation in all vessels [116]. Under normoxic conditions, the pulmonary artery dilated more than the aorta (the inferior vena cava response was variable). However, under conditions of hypoxia, dilatation of the aorta was significantly greater than of the pulmonary artery and the inferior vena cava [116]. The dilatory response during hypoxia was enhanced in all vascular beds.

It has recently been shown that the renal microcirculation is exquisitely responsive to nitrite. Indeed, in larger vessels such as the aorta, the threshold for dilatation by nitrite under normoxic conditions is >10⁻⁵ mol L⁻¹, whereas significant vasodilatation was observed in renal arterioles at 10⁻⁷ mol L⁻¹, which is well within the physiological range. Moreover, other vascular beds (carotid, renal interlobar and mesenteric arteries) were unresponsive to nitrite under similar conditions.[117]. The reason for variable responses to nitrite in different vascular beds may be related to the abundance of nitrite reductases in the vascular wall as well as the degree of superoxide generation by NADPH oxidases.

It is likely that the majority of nitrite-induced effects on vessel tone in vitro require its reduction to NO and subsequent activation of sGC. This is based on studies showing the absence of effects of nitrite in the presence of ODQ. However, this is complicated by the results of a very recent study showing that nitrite-dependent dilatation under hypoxic conditions was ODQ-insensitive in the low physiological ranges of nitrite [118].

The conditions that govern reduction of nitrite to NO are complex and have not been fully elucidated. Nitrite reduction to NO probably occurs through numerous pathways including xanthine oxidase, myoglobin and haemoglobin as discussed above. The dominating nitrite reductase pathways probably differ depending on a multitude of factors including species, type of vascular bed, redox conditions and oxygenation. In all cases, nitrite reduction to NO and vasoactivity is enhanced by hypoxia and at reduced pH.

**Clinical studies**

In 2001, Lauer et al. could not observe any vasodilator effect of intra-arterial nitrite infused into the forearm of volunteers at doses of 36 µmol min⁻¹. However, the infusion was only continued for a total of 1 min [119]. Based on these data, they concluded that nitrite was inert in vivo. In 2003, Cosby et al. [53] demonstrated a dose-dependent increase in forearm blood flow following infusion of 36 µmol min⁻¹ nitrite for 5 min into the brachial arteries of healthy volunteers, which was enhanced with arm exercise (intravascular concentration of nitrite ~200 µmol L⁻¹). The significant increase in forearm blood flow, suggesting a vasodilatory effect of nitrite on resistance arterioles, was associated with the formation of nitrosylated haemoglobin HbNO. Thus, the authors postulated that the effects of nitrite were mediated
by its reduction to NO [53]. Furthermore, the group demonstrated that the reduction of nitrite to vasoactive NO was catalysed by deoxyhaemoglobin [53]. Based on this hypothesis, Maher et al. [120] postulated that the vasoactive effects of nitrite would be more pronounced in the venous segment of the vascular tree. Indeed, they found that under normoxic conditions, small doses of nitrite had no effect on the arterial system but caused significant dilatation in the venous system [120]. Only with higher doses of nitrite was an increase in forearm blood flow precipitated. However, under hypoxic conditions, vasodilator effects of nitrite on the arterial system were enhanced causing an increase in forearm blood flow at lower doses [120]. Hypoxia had little effect on venodilatation. As the venous tree can contain up to 70% of the circulating blood volume, small changes in the tone of the venous capacitance vessels would lead to an intense fall in blood pressure by reducing venous return and cardiac preload.

Recently, it was shown that sodium nitrite infused, at pharmacological and near-physiological doses, into the brachial arteries of healthy volunteers resulted in significant selective dilatation of the radial artery, but not of the resistance arterioles, as assessed by forearm blood flow [121]. This demonstrates that nitrite preferentially dilates muscular conduit vessels in humans in vivo, compared with skeletal muscle resistance arterioles; surprisingly, contrary to the recently described key differences between organic and inorganic nitrates and nitrites [32], nitrite showed a similar degree of selectivity as the organic nitrate nitroglycerin. Furthermore, this dilatory effect of nitrite was optimal during normoxia and was attenuated under conditions of both hyperoxia and hypoxia. Therefore, different mechanisms appear to operate in different vessel types and species and under varying conditions, and the hypoxia dependency of the effects of nitrite is not universal.

**Blood pressure**

**Animal models**

In early studies, Classen et al. demonstrated persistent blood pressure-lowering effects of very high doses of oral nitrite [122]. They also speculated that dietary nitrate might have the same effects, but this theory was never tested [123]. Kanematsu et al. [124] later studied the effects of oral nitrite in NOS-inhibited hypertensive rats and saw a drop in blood pressure following a high dose of nitrite. Then, Petersson et al. [125] used telemetric devices in rats to study the effects of a low dietary dose of nitrate on blood pressure. They found that nitrate consistently reduced mean arterial pressure over the 3-day study period. It is interesting that when rats were treated twice daily with a commercial antiseptic mouthwash, the nitrate-induced increase in plasma nitrite and the decrease in blood pressure were abolished, illustrating the central involvement of oral nitrate-reducing bacteria in bioactivation of nitrate. Carlstrom et al. [126] investigated the effects of nitrate in a model of chronic salt-induced hypertension and demonstrated that nitrate treatment (0.1 or 1 mmol kg⁻¹ per day; with the lower dose resembling the nitrate content of a diet rich in vegetables) attenuated hypertension in a dose-dependent manner with no signs of tolerance. In addition, nitrate treatment prevented proteinuria and histological signs of renal injury, and cardiac hypertrophy and fibrosis were attenuated. Mechanistically, dietary nitrate restored the tissue levels of bioactive nitrogen oxides and reduced the levels of oxidative stress markers in plasma and urine. Recent studies from Carlstrom’s group and others have demonstrated that vascular NADPH oxidases in the renal vasculature are major targets for the beneficial effects of nitrate in chronic models of hypertension and that xanthine oxidase plays a central role in bioactivation of nitrite [104, 127–129]. Besides acting on the renal vasculature, nitrite was recently shown to inhibit NADPH oxidase-derived superoxide generation in activated macrophages [130]. Whether this contributes to the anti-inflammatory effects of nitrite is yet to be determined.

Recently, crosstalk between vascular eNOS and the nitrate–nitrite–NO pathways was described by Carlstrom et al. [131]. It was shown that long-term treatment with high doses of dietary nitrate in rats was associated with a paradoxical increase in blood pressure which was coupled to inhibition of vascular eNOS activity. Based on this, the authors speculated that the beneficial effects of nitrate on cardiovascular function are likely to be more pronounced in situations in which endogenous NO synthesis from NOS is already compromised such as in CVD or during normal ageing. In younger healthy individuals, the effects of nitrate may be less pronounced or even reversed, in particular if the dose is very high.

When nitrate is given orally, a number of factors will determine how effectively it is converted to NO-
like bioactivity in the circulation. These factors include uptake of nitrate in the salivary glands and reduction of nitrate to nitrite by oral bacteria. Moreover, the nitrite then formed needs to be absorbed in the GI tract and then metabolized to a bioactive nitrogen oxide in the vessel to induce vasodilatation. Pinheiro et al. [132] have suggested that intact gastric acid secretion is necessary for effective bioactivation of orally derived nitrite. Using a proton pump inhibitor to increase gastric pH, they demonstrated attenuation of nitrite-mediated blood pressure reduction in rats. It is currently not clear how acid promotes the effects of nitrite. Possible mechanisms include direct formation and export of NO or S-nitrosothiols in the gastric lumen [133], or the acid may simply enhance absorption of nitrite through protonation (HNO$_2$ formation), thereby causing it to become uncharged and facilitating transport across biological membranes.

**Clinical studies**

In 1880, Reichert and Mitchell found that oral administration of nitrite (~30 mg) resulted in an initial rise in blood pressure followed by a moderate decrease over time. The degree of blood pressure drop was intensified by the use of higher doses [27]. In 1933, Weiss and Ellis examined the effects of sodium nitrite on the cardiovascular and renal systems in healthy volunteers and in patients with hypertension or renal disease [134]. They found that oral sodium nitrite resulted in a drop in blood pressure that occurred 5–15 min after ingestion. Although the percentage change in blood pressure was similar between healthy and hypertensive individuals, the absolute drop and duration of this drop was greater in patients with hypertension [134]. Furthermore, changes in systolic blood pressure were greater than those in diastolic blood pressure. Weiss and Ellis suggested that the differences in response between systolic and diastolic blood pressure and between healthy and hypertensive individuals were related to the higher pre-existing vascular tone in patients with hypertension. In addition, nitrite had either no effect or depressed renal function.

Larsen et al. [97] investigated the effects of dietary nitrate on blood pressure in healthy volunteers. Dietary supplementation with 0.1 mmol kg$^{-1}$ per day sodium nitrate (a dose equivalent to the ingestion of 200–300 g of spinach or 2–3 beetroots) for 3 days resulted in a significant reduction in diastolic blood pressure by 3.7 mmHg. Later, using a larger study cohort, the same group also demonstrated effects on systolic blood pressure [135]. Webb et al. [35] determined the effects of a natural nitrate source. The ingestion of a single dose of ~35–45 mmol L$^{-1}$ inorganic nitrate (in the form of beetroot juice) by healthy subjects resulted in a significant (10 mmHg) drop in systolic and diastolic blood pressure. The drop in systolic blood pressure correlated with the rise in plasma concentrations of nitrite, and interruption of the enterosalivary circuit (by spitting) resulted in the abolition of these blood pressure-lowering effects and plasma nitrite did not increase. The drop in blood pressure was evident 60 min after ingestion of the nitrate load with the greatest effect occurring after 2.5–3 h. Of interest, blood pressure was still decreased 24 h after ingestion of nitrate [35]. A number of subsequent studies have confirmed the blood pressure-lowering effects of dietary nitrate, and a meta-analysis of the results was recently published [98, 136].

Whether endogenously produced nitrate, generated by the NOSs, would be sufficient to have a physiological role in the regulation of blood pressure was investigated by Kapil and co-workers [137]. Healthy subjects were given a low nitrate diet for 1 week, and by the concomitant use of an antibacterial mouthwash, the possibility of recycling nitrate to nitrite, which is normally performed by oral bacteria, was inhibited. This procedure led to an increase in blood pressure which was correlated with the decrease in plasma nitrite level. The results of this study suggest that endogenously produced nitrate contributes to blood pressure regulation and that the oral microflora is involved in modulating cardiovascular function (Fig. 3).

Influenced by the blood pressure-lowering effect of nitrate in healthy subjects, a phase II study of dietary nitrate has recently been completed in hypertensive subjects [138]. Beetroot juice (250 mL per day) was used as the source of nitrate and 24-h ambulatory systolic blood pressure was reduced by a mean of 8 mmHg, compared to the placebo group receiving a newly developed nitrate-depleted beetroot juice. The effect persisted throughout the 4-week intervention period. These findings are promising and provide opportunities for the development of dietary supplements or novel drugs containing inorganic nitrate. Furthermore, these findings are intriguing from a nutritional viewpoint as nitrate is abundant in vegetables, a food group associated with protection...
from cardiovascular disease [139]. Ongoing studies are being conducted to examine whether, and if so to what extent, nitrate contributes to the positive health effects of diets rich in vegetables such as the Mediterranean [139] or traditional Japanese diets [140]. If nitrate is indeed a major contributor, the current view of nitrate as an unwanted residue in the food chain will have to change and a higher intake of specific food items that are naturally high in this anion may be recommended.

**Ischaemia–reperfusion injury and myocardial infarction**

It seems clear that increasing the bioavailability of NO in ischaemia–reperfusion injury is associated with protective effects in animal models [141]. In 1995, Zweier et al. [23] demonstrated NOS-independent generation of NO from nitrite under hypoxic conditions. This led to studies of the effects of nitrate and nitrite in models of cardiac ischaemia.

**Animal models**

In 2004, Webb et al. [142] investigated the effects of sodium nitrite in an *ex vivo* rodent model of myocardial infarction. They found that nitrite substantially reduced infarct size by a mechanism involving XOR-catalysed formation of NO. The following year, using an *in vivo* mouse model, Duranski et al. [143] demonstrated a dose-dependent protective effect of intraventricular nitrite. Again the protective effects were NO dependent, as demonstrated by a loss of protection in the presence of the NO scavenger CPTIO. In the same study, the authors also demonstrated cytoprotective effects of nitrite in liver ischaemia–reperfusion injury [113]. Since then, the effects of nitrite have been demonstrated in myocardial infarction in multiple animal models [144, 145] as well as in a variety of organs including brain [146], liver [143], kidney [147] and hindlimb [148]. Nitrite also preserved neurological and cardiac function in a model of global ischaemia after cardiac arrest and resuscitation [149].

The mechanism underlying the cytoprotective effects of nitrite involves interaction of nitrite reaction products with mitochondria (Fig. 4). Specifically, nitrite S-nitrosates complex I of the respiratory chain results in inhibition of respiration and reduction in the formation of harmful ROS at this complex, most commonly during reperfusion [150, 151]. Additional mitochondrial effects that may have a role include improvements in mitochondrial efficiency caused by a reduction in proton leak, which is likely to help to maintain ATP levels during ischaemia [152]. NO can also modify cardiac energetics by binding to mitochondrial cytochrome c oxidase [54]. Interestingly, it was recently suggested that circulating nitrite may contribute to the protective effects of remote preconditioning [153].

As for other tissues, there are a number of potential candidates that may catalyse the reduction of nitrite to NO in the ischaemic heart. XOR clearly plays a role as discussed above. In addition, myoglobin has been shown to reduce nitrite to NO in the myocardium [54, 154].

**Clinical studies**

The encouraging effects of nitrate and nitrite in animal models have stimulated the investigation of these compounds in the clinical setting. In one
study, a mild ischaemic insult in the forearm of healthy subjects significantly reduced FMD by ~60%. Supplementation with dietary nitrate (in the form of beetroot juice) imparted a significant protective effect preventing endothelial dysfunction, and restoring FMD to near baseline levels [35]. In a small pilot study in patients with peripheral artery disease, dietary nitrate (beetroot juice) improved exercise capacity measured as longer walking distance and peak walking time [155].

Recently, two phase II studies have been conducted to investigate the effects of nitrite therapy for acute myocardial infarction. Siddiqi et al. [156] administered sodium nitrite intravenously immediately prior to reperfusion in patients with acute ST-segment elevation myocardial infarction and found no protective effect as measured by creatine kinase, troponin I or infarct size. Jones et al. [157] examined the effects of intracoronary nitrite given prior to balloon dilatation in patients with acute myocardial infarction and found no effect on creatine kinase release, troponin T or cardiac magnetic resonance imaging-assessed infarct size. By contrast, there was an increase in myocardial salvage index and a reduction in major adverse cardiac events (MACEs) at 1 year in the nitrite group. Moreover, in a large subgroup of patients with severely obstructed coronary blood flow (TIMI grade flow ≤1), nitrite reduced infarct size and MACEs and improved myocardial salvage index. The reason for the discrepancy in the results between these two studies is not clear but could be related to patient selection, doses of nitrite and/or mode of administration.
Pulmonary arterial hypertension (PAH) is characterized by increased pulmonary vascular resistance and pulmonary artery pressure leading to impaired function of the right ventricle, reduced cardiac output and death. Loss of NO bioactivity is thought to contribute to the pathogenesis of PAH, and agents that augment pulmonary NO signalling, including inhaled NO gas and phosphodiesterase inhibitors, are clinically effective [158]. Recently, the therapeutic effects of nebulized nitrate have been evaluated in an attempt to increase pulmonary NO signalling.

**Animal models**

Gladwin *et al.* [159] were the first to demonstrate beneficial effects of inhaled nitrite in PAH. They showed that administration of nebulized nitrite to newborn lambs ameliorated hypoxia-induced pulmonary artery pressure by up to 60%. These effects were more pronounced than those of inhaled NO and were strongly correlated with reduced pH and oxygen levels. The more prolonged effects of nitrite compared to inhaled NO are likely to be secondary to its more stable structure. Blood *et al.* [160] demonstrated that the pulmonary vasodilating effect of inhaled nitrite results from its conversion to NO in airway and parenchymal lung tissue and is not dependent on reactions with deoxyhaemoglobin in the pulmonary circulation.

Baliga *et al.* [161] examined the effects of dietary nitrate and nitrite in a model of PAH induced by chronic hypoxia. They showed that nitrate reduced right ventricular pressure and hypertrophy, and pulmonary vascular remodelling, and these effects were largely mirrored by dietary nitrite. These cytoprotective effects of dietary nitrate were associated with increased plasma and lung concentrations of nitrite and cGMP but were attenuated in mice lacking endothelial NOS or after treatment with the XOR inhibitor allopurinol. A similar XOR-dependent effect of nitrite in PAH was also demonstrated in an earlier study in rodents [162]. Similarly, Pankey *et al.* [163] demonstrated protective effects in a rat model of PAH following intraperitoneal injection of nitrite.

Together, these findings show that nitrite and dietary nitrate are protective in numerous models of PAH regardless of administration mode. For NO, the inhalation route represents a major advantage due to its relative lung selectivity with minor effects on systemic blood pressure. It is likely that the relatively lower potency of nitrate/nitrite compared to NO, along with their selective bioactivation in hypoxic areas, enable these compounds to effectively lower pulmonary artery pressure without systemic vascular collapse. However, how effectively nitrate and nitrite will be metabolized to NO in the pulmonary circulation of patients with PAH remains unclear, given the much greater abundance of XOR in rodents compared to humans.

**Clinical studies**

A phase I study to examine the pharmacokinetic and pharmacodynamic characteristics, safety and tolerability of nebulized sodium nitrite was recently conducted [164]. Nebulized sodium nitrite was well tolerated following repeated dosing, and circulating markers of NO formation increased. At higher dosage, hypotension with tachycardia developed but venous methaemoglobin did not exceed 3.0%. Neither the tolerability nor pharmacokinetics of nitrite were affected by conditions of mild hypoxia, or co-administration of sildenafil, supporting the safe use of inhaled nitrite in the clinical setting of PAH. As mentioned above, the sGC stimulator riociguat was recently registered for the treatment of PAH. Directly targeting downstream NO signalling may have several advantages compared to current treatment modalities, and additional sGC stimulators or activators are likely to enter clinical practice.

Leucocyte and platelet activation

**Animal models**

The effects of nitrate and nitrite on leucocyte function have been investigated in rodent models. Nitrite administered in the drinking water inhibited leucocyte adhesion and emigration and prevented arteriolar dysfunction in mice fed a cholesterol-rich diet [103]. This was associated with decreased levels of C-reactive protein. Jadert *et al.* [165] used a mouse cremaster model of cytokine-induced microvascular inflammation to study the effects of dietary nitrate on leucocyte function. The authors demonstrated that dietary nitrate markedly reduced leucocyte recruitment in response to the proinflammatory chemokine MIP-2 in a process involving attenuation of P-selectin and ICAM-1 upregulation.

**Clinical studies**

Mathru *et al.* [166], investigating the effects of inhaled NO on the systemic inflammatory response
during surgery, found that this intervention attenuated the inflammatory response characterized by reduced expression of CD11b/CD18, P-selectin and nuclear factor kappaB compared with the control group. These effects were accompanied by increased plasma levels of nitrate and nitrite.

Early studies had demonstrated that NO inhibits platelet activation albeit not very potently [167]. Richardson et al. [168] showed that oral potassium nitrate ingestion by healthy human volunteers inhibited ex vivo platelet activation as assessed by measuring the aggregation effect of collagen. The mechanism underlying this effect was thought to be related to the formation of nitrosothiols; however, this could not be demonstrated in the systemic or portal circulation. The role of nitrite was subsequently discovered by Webb et al., who showed that dietary nitrate consumed as beetroot juice by healthy volunteers inhibited platelet aggregation 3 h after ingestion. These effects were inhibited when the enterosalivary circuit was interrupted by spitting, demonstrating the central role of salivary nitrate reduction to nitrite by oral bacteria in the bioactivation of nitrate [35]. More recently, Velmurugan and colleagues investigated the effects of nitrate on platelet function in healthy subjects. Beetroot juice as well as potassium nitrate capsules attenuated ex vivo platelet aggregation responses to ADP and collagen but not to adrenaline. These inhibitory effects were associated with reduced platelet P-selectin expression and elevated platelet cGMP levels [169].

Heart failure

In 2007, Larsen et al. [170] demonstrated that inorganic nitrate decreased the oxygen cost during physiological exercise. This highly surprising effect occurred in the absence of any changes in lactate indicating that mitochondrial respiration had become more efficient. The oxygen sparing effect, which is also coupled to increased performance, have been confirmed in numerous studies using either a nitrate salt or a natural vegetable source of nitrate [171]. Mechanistically, nitrate reaction products including NO affect mitochondrial efficiency through downregulation of proteins involved in proton leak (ANT and UCP-3) [152].

Animal models

It has been shown in rat experiments that nitrate also enhances the force of skeletal muscle contraction, particularly of fast-twitch fibres [172], through the modulation of calcium-handling proteins. This implies that beyond effects on skeletal muscle function, dietary nitrate might also have an impact on cardiac function, which would be of interest, for example, for the treatment of patients with heart failure. Few studies have investigated the direct effects of nitrate or nitrite on cardiac function, and the data have been inconsistent. In an isolated Langendorff rat heart model, Pellegrino et al. [173] found that nitrite exhibited potent negative inotropic and lusitropic effects via cGMP/protein kinase G-dependent signalling, shown as a decrease in left ventricular pressure and relaxation. However, the same group later showed that nitrate is a positive modulator of the Frank–Starling response in the rat heart [174]. More recently, it was shown that dietary nitrate alleviates metabolic abnormalities in the hypoxic rat heart, which was evident as reduced oxidative stress, improved mitochondrial respiration rates and increased ATP levels compared to control hypoxic rats [175].

Clinical studies

Zamani and colleagues recently demonstrated that dietary nitrate increased exercise capacity in patients with heart failure with preserved ejection fraction (HFpEF) by targeting peripheral abnormalities [176, 177]. Nitrate increased exercise vasodilatory and cardiac output reserves and reduced arterial wave reflections, which are linked to left ventricular diastolic dysfunction and remodelling. The mechanism underlying the reduced arterial wave reflections/augmentation index may also be related to the effects of nitrate-derived nitrite on conduit arteries [121]. Future longer-term trials will be needed to determine whether inorganic nitrate could be useful as a therapy for patients with HFpEF.

Metabolic effects

Animal models

In addition to elevated blood pressure throughout life, mice lacking endothelial NOS develop a metabolic syndrome-like phenotype over time. This finding led Carlstrom et al. [178] to investigate whether dietary nitrate would affect any of these characteristics. Indeed, in aged eNOS-deficient mice, nitrate reversed the metabolic syndrome phenotype including improvements in glucose handling, overall weight loss, decreases in abdominal fat accumulation and a drop in circulating triglyceride levels. It is interesting that the dose of
sodium nitrate used was similar to the amounts of nitrate generated endogenously by mice with normal eNOS levels, indicating that NOS-derived nitrate may serve a physiological role in modulating metabolic functions. In a later study, Nystrom et al. [179] demonstrated that nitrite acutely increased the blood flow of the endocrine pancreas and stimulated insulin release from isolated pancreatic islets. Recently, these antidiabetic effects of nitrate were reproduced with nitrite in a mouse model of type-2 diabetes [KKAy mice] [180]. In addition, Essawy et al. [181] used a high-fructose diet to induce metabolic syndrome in rats and found that 10 weeks of dietary nitrate reversed several of its features including reduced insulin resistance and lower adiposity index.

A possible mechanism for the effects on glucose handling may be nitrite-mediated induction of GLUT-4 translocation to enhance cellular glucose uptake. Sodium nitrite was administered via the drinking water to db/db diabetic mice for 4 weeks. After treatment, these mice experienced less weight gain, improved fasting glucose levels and reduced insulin levels. The results of cell culture experiments suggested that nitrite improved insulin signalling through restoration of NO-dependent nitrosation of GLUT4 signalling translocation [182]. In addition, the stimulation of insulin release and the increase in tissue blood flow induced by nitrate/nitrite may have also contributed to the antidiabetic effects. Moreover, recent data suggest that dietary nitrate may promote browning of white adipose tissue with putative antiobesity and antidiabetic effects [183].

Clinical studies
Whereas beneficial effects of nitrate and nitrite are seen in several animal models of diabetes, such effects have yet to be demonstrated in humans. Gilchrist et al. [112] examined the effects of 2-week supplementation with beetroot juice on blood pressure, endothelial function and insulin sensitivity in patients with type 2 diabetes but found no significant changes despite increases in plasma nitrite levels.

Summary
The inorganic anions nitrate and nitrite have emerged as substrates for in vivo formation of NO and related bioactive nitrogen oxides with promising beneficial effects on cardiovascular and metabolic function. This provides an opportunity for drug development as these anions have proven effective in a multitude of animal models of cardiovascular and metabolic disease including systemic and pulmonary hypertension, ischaemia–reperfusion injury and diabetes. This area of research is particularly intriguing given that nitrate is an everyday dietary constituent. Clinical trials are now underway; some of these have already shown encouraging results. However, negative findings have also been reported from some clinical studies and clearly, much further work is needed before any promising findings can be translated into effective, preventive and therapeutic strategies for use in clinical practice.

Conflict of interest statement
Drs Lundberg and Weitzberg are co-inventors named on patent applications for the therapeutic use of nitrate and nitrite.

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References


Review: Nitrate and nitrite in cardiovascular disease


80 Beckman JS, Beckman TW, Chen J, Marshall P, Freeman BA. Apparent hydroxyl radical production by peroxinitrite: implications for endothelial cell injury from nitric oxide. Proc Natl Acad Sci USA 1990; 87: 1620–4.


Review: Nitrate and nitrite in cardiovascular disease


Review: Nitrate and nitrite in cardiovascular disease


134 Weiss S, Ellis LB. Influence of sodium nitrite on the microvasculature is a primary target for blood pressure-lowering effects by inorganic nitrate and nitrite. Hypertension 2015; 65: 320–7.

135 Classen HG, Stein-Hammer C, Thoni H. Hypothesis: the intake of nitrate affects the activity of NADPH oxidase and exerts lowering effects by inorganic nitrate and nitrite. Hypertension 2011; 574.


139 Omar SA, Fok H, Tilgner KD et al. Inorganic nitrate in the renal microvasculature is a primary target for blood pressure-lowering effects by inorganic nitrate and nitrite. Hypertension 2011; 574.


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