

Roles of dietary inorganic nitrate in cardiovascular health and disease

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Abstract

Inorganic nitrate from dietary and endogenous sources is emerging as a substrate for *in vivo* generation of nitric oxide (NO) and other reactive nitrogen oxides. Dietary amounts of nitrate clearly have robust NO-like effects in humans, including blood pressure reduction, inhibition of platelet aggregation, and vasoprotective activity. In animal models, nitrate protects against ischaemia–reperfusion injuries and several other types of cardiovascular disorders. In addition, nitrate most surprisingly decreases whole body oxygen cost during exercise with preserved or even enhanced maximal performance. Oxidative stress and reduced NO bioavailability are critically linked to development of hypertension and other forms of cardiovascular diseases. Mechanistically, a central target for the effects of nitrate and its reaction products seems to be the mitochondrion and modulation of oxidative stress. All *in vivo* effects of nitrate are achievable with amounts corresponding to a rich intake of vegetables, which are particularly rich in this anion. A theory is now emerging suggesting nitrate as an active component in vegetables contributing to the beneficial health effects of this food group, including protection against cardiovascular disease and type-2 diabetes.

Keywords

Nitrite • Nitric oxide • S-Nitrosothiol • DASH • Type 2 diabetes

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1. Introduction

Dietary inorganic nitrate (NO_3^-) and nitrite (NO_2^-) have for half a century been considered toxic constituents in our diet because of their proposed role in the development of gastric cancer and other malignancies.^{1,2} For this reason, the levels of these anions are strictly regulated in our food and drinking water. Apart from the dietary aspects of nitrate and nitrite, the interest in their biological role was sparked by the findings in the mid-1980s showing that these anions are generated endogenously in our bodies.³ A few years later, the L-arginine–nitric oxide synthase (NOS) pathway was discovered and this system was found to be the major source of endogenous nitrate and nitrite, since nitric oxide (NO) is rapidly oxidized to these higher nitrogen oxides.^{4–6} This fact has led to the extensive use of nitrate and nitrite as markers of NO production, with the notion that they are solely inactive and stable end-products of NO metabolism. However, this view is currently changing since it is now clear that nitrate and nitrite can be serially reduced *in vivo* back to NO and other bioactive nitrogen oxides.^{7–12} Several lines of research now converge into a picture describing a nitrate–nitrite–NO pathway with physiological, nutritional, and therapeutical importance. This pathway is complementing the ‘classical’ L-arginine–NOS–NO

pathway, especially under hypoxic conditions when the oxygen-dependent NOS isoforms may be dysfunctional.⁷ Thus, instead of just wasting oxidized NO, our bodies are actively recycling it. Nitrite reduction to NO was first described in the stomach, where salivary nitrite forms NO non-enzymatically via acid-catalysed reduction.^{13,14} Soon after, Zweier *et al.*¹⁵ described NOS-independent nitrite reduction in the ischaemic and acidic heart, and some years later, a physiological role for nitrite in hypoxic and metabolic vasoregulation was suggested.¹¹ Subsequent studies show that a variety of enzymes and proteins can catalyse the one-electron reduction of nitrite to NO in blood and tissues (Figure 1). This review focuses on recent developments in nitrate and nitrite research. We discuss the sources and biological fate of nitrate as well as its physiological, nutritional, and therapeutical roles, with special emphasis on dietary inorganic nitrate in the cardiovascular systems.

2. Diet and NO synthase: the two major sources of nitrate

As already mentioned, the L-arginine–NOS system significantly contributes to the overall nitrate and nitrite production. NO is a reactive

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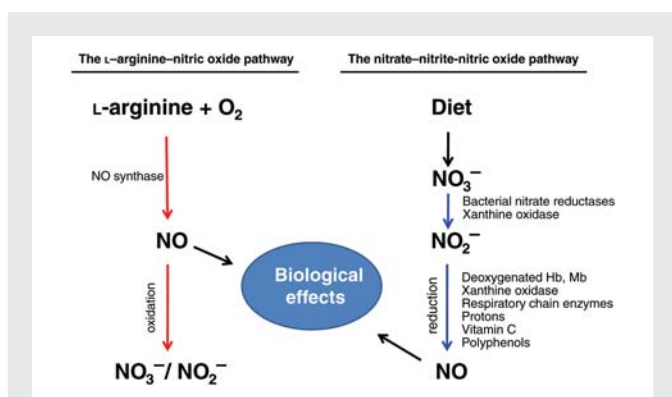


Figure 1 Two parallel pathways for NO formation in mammals. NO synthases (NOS) catalyse the formation of NO from the substrates L-arginine and molecular oxygen. NO is rapidly oxidized to form nitrite (NO₂⁻) and nitrate (NO₃⁻), but a recycling of these anions may occur by which NO is formed again. Nitrate reduction to nitrite is mainly carried out by commensal bacteria in the oral cavity and to a lesser extent also by mammalian enzymes in tissues (xanthine oxidase). Once nitrite is formed, several pathways exist with the capacity to further metabolize nitrite to NO and other biologically active nitrogen oxides. Most of these pathways are greatly accelerated under hypoxic conditions. Thus, nitrite reduction represents an alternative to the classical NOS pathway for the generation of NO. Our diet (mainly green leafy vegetables) is a major contributor to the body pool of nitrate and ingestion of nitrate may fuel the nitrate–nitrite–NO pathway. Dietary nitrate supplementation is associated with robust NO-like effects including a reduction in blood pressure and inhibition of oxygen consumption in humans. In addition, nitrate or nitrite administration is protective in numerous animal models of cardiovascular disease.

free radical with a biological half life in the millisecond range, and a major metabolic route is through oxidation thereby forming higher nitrogen oxides including nitrite and nitrate. Nitrate is clearly the major end-product of NO oxidation, and the levels in blood and tissues exceed those of nitrite by at least two orders of magnitude (μM vs. nM).⁶ In addition to the NOS system, our everyday diet represents an equally important source of nitrate and nitrite. Vegetables are by all means the dominant source of nitrate in the diet (>80%) and leafy green vegetables, in particular, contain high amounts of this anion.⁷ The relative contribution of nitrate from endogenous vs. exogenous sources naturally varies depending on diet, drugs, disease, and more. Inflammatory conditions with massive activation of inducible NOS (iNOS or NOS2)¹⁶ or physical exercise with activation of endothelial NOS (eNOS or NOS3)¹⁷ are associated with increased endogenous levels of nitrate. Conversely, dietary intake of nitrate can increase systemic nitrate levels dramatically. For instance, one serving of lettuce, spinach, or beetroot contains more nitrate than what is generated by the entire NOS system over a day.¹⁸

3. The biological fate of nitrate

Nitrate circulates in plasma, distributes to the tissues and has a half life of approximately 5 h. By not yet fully defined mechanisms, circulating nitrate is actively taken up by the salivary glands and concentrated in the saliva (10–20-fold higher than in blood).¹⁹ This massive concentration of nitrate can result in salivary nitrate levels of several

millimolar.²⁰ Up to 25% of all circulating nitrate enters this peculiar enterosalivary cycle, while the rest is excreted by the kidneys. In the oral cavity commensal facultative anaerobic bacteria reduce nitrate to nitrite by the action of nitrate reductase enzymes.^{19,21} In the absence of oxygen, these bacteria use nitrate as an alternative electron acceptor to gain adenosine triphosphate (ATP). Thus, bacterial enzymatic activity may also contribute to the very high salivary levels of nitrite. When swallowed, nitrite in the saliva is metabolized to NO and other reactive nitrogen oxides locally in the acidic environment of the stomach and a number of physiological roles for gastric NO have been proposed which are discussed elsewhere.^{22–26} Of relevance to this particular review is the fact that large amounts of swallowed nitrite survives gastric passage and enters the systemic circulation.²⁷ This is of great interest since several mechanisms for nitrite reduction to NO and other bioactive nitrogen oxides have been described in blood and various tissues.^{10,18,28,29} These findings demonstrate that a complete reverse pathway (nitrate–nitrite–NO) exists in mammals. This was very surprising as nitrate has been universally considered an inert end-product ever since NO formation in mammals was discovered 25 years ago.

4. Nitrate and ischaemia–reperfusion injury

Treatment of myocardial ischaemia due to coronary artery occlusion is aimed to re-establish perfusion with minimal heart injuries. However, reperfusion *per se* may contribute to myocardial damage beyond that induced by ischaemia, referred to as ischaemia–reperfusion (IR) injury. Several factors are suggested to contribute to development of myocardial IR injury, including endothelial and microvascular dysfunction, pro-inflammatory activation, and oxidative stress.³⁰ Reduced NO bioavailability is a central event in IR injury and importantly contributes to the vascular dysfunction.

In 2004, Webb *et al.*³¹ reported protective effects of nitrite in isolated perfused heart preparations subjected to IR injury. Under ischaemic conditions, both rat and human myocardium generated NO from nitrite. Although NO was produced in a dose-dependent manner (10–100 μM), the degree of protection did not differ, suggesting that the beneficial effect of nitrite treatment was reached already at low concentrations. Furthermore, the authors showed that the conversion to NO was dependent on xanthine oxidoreductase (XOR), since co-administration of allopurinol or BOF-4272 attenuated nitrite-dependent NO formation. XOR is generally thought to contribute to IR injuries via production of reactive oxygen species (ROS), including superoxide ($\text{O}_2^{\cdot-}$). However, the findings by Webb *et al.* suggest that during hypoxic conditions, nitrite supplementation may partly shift the activity of XOR from generation of damaging $\text{O}_2^{\cdot-}$ to protective NO.

Soon after, Duranski *et al.*³² demonstrated potent cytoprotective effects of low dose nitrite using *in vivo* mouse models of myocardial infarction and liver ischaemia. The nitrite-mediated effects were independent of NOS and abolished by co-administration of the NO scavenger carboxy-PTIO (cPTIO), suggesting NO as an active mediator. Furthermore, the efficiency profile of nitrite therapy on liver and heart function were U-shaped, with a maximum protective effect at a dose of 48 nmol nitrite. This supports the idea that cytoprotection is reached already at low NO elevations (nano- to low micromolar range), whereas high, non-physiological levels (high micro- to

millimolar range) may promote cellular apoptosis and necrosis.^{32,33} Interestingly, the L-arginine-NOS pathway for NO production is oxygen dependent, whereas the nitrate and nitrite-mediated NO production occurs at lower oxygen tensions.⁷ Therefore, the nitrate–nitrite–NO pathway can be viewed as a back-up system to ensure sufficient NO levels during ischaemic conditions.

A number of subsequent studies in different animal species have confirmed protective effects of low dose nitrite in various settings of IR injury, including models of stroke,³⁴ kidney ischaemia,³⁵ lung injury,^{36,37} acute myocardial infarction,³⁸ cardiac arrest,³⁹ and chronic limb ischaemia.⁴⁰ Gonzalez *et al.*³⁸ revealed that nitrite dosing during the last 5 min of a 120 min occlusion period, reduced myocardial infarction size from 70 to 36% in a canine model. Together, these findings clearly indicate a potential role for nitrite as a useful adjunctive therapy in preventing IR injuries in several organs and tissues.

Besides XOR for nitrite reduction, a number of different pathways have been described in tissues, including deoxygenated myoglobin,^{41,42} respiratory chain enzymes of mitochondria,⁴³ aldehyde oxidase,⁴⁴ carbonic anhydrase,⁴⁵ and even NO synthase.⁴⁶ In addition, reducing agents such as vitamin C⁴⁷ and polyphenols^{48,49} catalyse non-enzymatic reduction of nitrite (Figure 1).

In 2004, our group demonstrated that ingestion of nitrate in dietary amounts resulted in sustained elevation of circulating nitrite in humans.²⁷ The observed increase in plasma nitrite was absent if subjects avoided swallowing after nitrate ingestion. Also, rinsing the oral cavity with an antiseptic mouthwash had the same blocking effect on nitrite elevation.²⁰ This demonstrates that the acute increase in plasma nitrite after nitrate ingestion is dependent on enterosalivary recirculation of the nitrate and reduction to nitrite by oral commensal bacteria. In addition, Jansson *et al.*⁵⁰ more recently reported that a mammalian nitrate reductase activity is present in tissues from both rodent and human. It is intriguing to compare the systemic nitrite load provided by a nitrate-rich meal with the amount of nitrite needed to protect tissues from IR injury. As mentioned, those studies demonstrated maximal protective effects of exogenous nitrite already at very modest doses.³² In fact, a similar or even higher systemic load of nitrite is achieved by ingestion of no more than 100 g of vegetables such as spinach or beetroot.⁹ Indeed, several studies have now confirmed that orally administered nitrate and nitrite are protective in IR injury.^{18,51,52}

Although the mechanism of nitrate-nitrite-mediated cytoprotection is unknown, NO is considered a mediator of the ischaemic preconditioning cell-survival program. The underlying mechanism by which NO provides tissue protection in IR is also not clear, but activation of soluble guanylate cyclase (sGC), inhibition of cytochrome c, inhibition of deleterious mitochondrial calcium uptake,³² and inhibition of mitochondrial complex I and subsequent reduction in ROS production⁵² have been suggested.

5. Blood pressure lowering and cardioprotective effects of inorganic nitrate

Hypertension affects more than one billion individuals worldwide and remains the most common risk factor for cardiovascular morbidity and mortality. NO is a key regulator of renal and cardiovascular function, and emerging evidence shows that oxidative stress and subsequent NO-deficiency in the kidney are critically associated with

the development of hypertension and other forms of cardiovascular disease.⁵³ Therefore, treatment modalities that reduce oxidative stress and/or increase NO production may have important implications in preventing and treating cardiovascular disease.

If nitrate can be converted to nitrite and NO *in vivo*, it is reasonable to assume that vasodilatation mediated by this messenger would lower blood pressure. We tested this hypothesis in a double-blind, cross-over designed study in healthy volunteers⁵⁴ (Table 1). The subjects received sodium nitrate (NaNO₃) or equimolar amounts of NaCl (placebo) for 3 days in a dose of 0.1 mmol kg⁻¹ day⁻¹, corresponding to an intake of 100–300 g of nitrate-rich vegetables. Indeed, nitrate supplementation reduced diastolic blood pressure (DBP) by 4 mmHg compared with placebo suggesting formation of vasodilatory NO. The same group confirmed this finding in a similar conducted study with greater number of participants.⁵⁵ In this study, a blood pressure lowering effect of nitrate was also observed for systolic blood pressure (SBP). Webb *et al.*⁵⁶ used beetroot juice as a natural source of nitrate to study the same phenomenon in healthy volunteers. Subjects drank 500 mL of either the juice (0.3 mmol nitrate kg⁻¹) or water, and blood pressure was monitored repeatedly over a 24 h period. An impressive reduction in both SBP (10 mmHg) and DBP (8 mmHg) was noted within 3 h of ingestion, an effect that correlated with maximum elevations in plasma nitrite concentration. A significant blood pressure reduction was still present 24 h following single administration. Interestingly, the blood pressure lowering effects were abolished if the subjects avoided swallowing for a period after drinking the juice, again demonstrating a central role of enterosalivary circulation in bioactivation of nitrate. In the same study, Webb *et al.* demonstrated inhibitory effects of nitrate on *ex vivo* platelet aggregation. Moreover, they could show that the same dose of nitrate prevented endothelial dysfunction after a mild ischaemic insult in the forearm. In a randomized cross-over study, the same group recently demonstrated robust blood pressure lowering effects also with a considerably lower dose of beetroot juice, and effects were similar to those observed with equimolar amounts of potassium nitrate salt.⁵⁷ This demonstrates that nitrate is indeed the active component of the juice. In the same study, the authors detected increases in plasma cyclic guanosine monophosphate (cGMP) after nitrate ingestion, which strongly suggests increased NO formation.

In Japan, the occurrence of cardiovascular diseases is low, and Japanese longevity is the highest in the world. In a recent study, Sobko *et al.*⁵⁸ examined the blood pressure effects of a 10-day period with traditional Japanese diet rich in vegetables compared with western-type diet in 25 healthy volunteers. The traditional Japanese diet is naturally very rich in nitrate, and this was reflected in greatly increased levels of nitrate and nitrite in plasma and saliva compared with the control diet. DBP decreased on average 4.5 mmHg with the Japanese diet (18.8 mg nitrate kg⁻¹ bw⁻¹ day⁻¹) compared with the control diet. Again, these findings support the importance of dietary nitrate on blood pressure regulation, and give one possible contributing factor to the healthy aspects of traditional Japanese food.

Traditional organic nitrates such as nitroglycerine are classically subjected to development of tolerance after repeated administration. To test whether dietary inorganic nitrate supplementation has sustained blood pressure lowering effects, we measured blood pressure telemetrically in conscious rats during a 5-day period.⁵⁹ Nitrate-treated (1.5 mg nitrate kg⁻¹ bw⁻¹ day⁻¹) animals displayed a reduction in mean arterial pressure and DBP over the entire

Table 1 Physiological and therapeutic effects of dietary inorganic nitrate in humans and in animal models

Nitrate source	Nitrate dose (mmol kg ⁻¹ day ⁻¹)	Organ system or disease model	Species	Placebo	Effect	References
Sodium nitrate	0.1	Cardiovascular	Human	NaCl	DBP ↓ DBP, SBP ↓	Larsen et al. ⁵⁴ Larsen et al. ⁵⁵
Potassium nitrate	0.06–0.35			Water	DBP, SBP ↓	Kapil et al. ⁵⁷
	0.03				Platelet aggregation ↓	Richardson, 2002 ⁹⁶
Beetroot juice	0.3	Cardiovascular	Human	Water	DBP, SBP ↓ Platelet aggregation ↓ Endothelial dysfunction ↓	Webb et al. ⁵⁶
Beetroot juice	0.07–0.15			Black-currant juice	SBP ↓	Bailey et al. ⁷¹
Japanese traditional diet	0.3			Western diet	DBP ↓	Sobko et al. ⁵⁸
Sodium nitrate	0.1	Musculo-skeletal	Human	NaCl	Oxygen consumption ↓	Larsen et al. ⁵⁵
Beetroot juice	0.07–0.15	Musculo-skeletal	Human	Black-currant juice	Oxygen consumption ↓	Bailey et al. ⁷¹
						Bailey et al. ⁷³ Vanhatalo et al. ⁷²
Sodium nitrate	1	Acute aortic cross clamp	Rat	NaCl	Post-ischaemic blood flow ↑ ADMA ↓	Jansson et al. ⁵⁰
Sodium nitrate	1	Cardiac IR injury	Mouse	Water	Infarct size ↓	Bryan et al. ⁵¹
Sodium nitrate	1	Cardiovascular	Rat	Water	MAP ↓	Petersson et al. ⁵⁹
Sodium nitrate	0.1–1	Stomach	Rat	NaCl	Mucosal blood flow ↑ Mucus generation ↑	Björne et al. ²²
		Ulcer			Ulcer formation ↓	Miyoshi et al. ²⁵ ; Jansson et al. ⁵⁰ ; Petersson et al. ⁵⁹
Sodium nitrate	0.1	eNOS knockout with features of metabolic syndrome	Mouse	Water	Body weight ↓ Visceral fat ↓ Triglycerides ↓ Glucose tolerance ↑	Carlström et al. ⁸²

For comparison, a dose of 0.1 mmol kg⁻¹ day⁻¹ nitrate corresponds in humans to an intake of 100–300 g of a nitrate-rich vegetable such as beetroot, spinach, or lettuce. DBP, diastolic blood pressure; SBP, systolic blood pressure; MAP, mean arterial blood pressure; ADMA, asymmetric dimethyl arginine.

observation period. Similar observations have been reported in non-human primates with repeated administration of inorganic nitrite, indicating no development of tolerance.⁶⁰

As described, clinical and experimental studies during the last years have independently reported that dietary nitrate supplementation reduces blood pressure in healthy normotensive individuals. Considering the important link between NO deficiency and development of hypertension and other forms of cardiovascular disease, it is reasonable to assume that stimulation of the nitrate–nitrite–NO pathway may boost NO production and thus have beneficial effects.

In a recent study, we tested this hypothesis by studying the effects of dietary nitrate in a rat model of renal and cardiovascular disease, induced by early unilateral nephrectomy in combination with chronic high-salt diet for 10 weeks (Carlström et al., unpublished results). Control rats displayed several features of renal and cardiovascular dysfunction, including hypertension, cardiac hypertrophy and fibrosis, proteinuria and histological as well as biochemical signs of renal damage, and oxidative stress. In animals receiving nitrate, blood pressure was dose-dependently lowered with no signs of tolerance. Strikingly, proteinuria and histological signs of renal injury were

almost completely prevented and the cardiac hypertrophy and fibrosis was attenuated. Mechanistically, dietary nitrate increased or restored tissue levels of bioactive nitrogen oxides and reduced the levels of oxidative stress markers in plasma and urine.

Chronic blockade of NOS with N^{ω} -nitro-L-arginine methyl ester (L-NAME) results in severe hypertension and progressive kidney damage, and has been used as model for renal and cardiovascular disease. Studies by Tsuchiya *et al.*⁶¹ and Kanematsu *et al.*⁶² demonstrated that chronic nitrite supplementation (100 mg/L drinking water) attenuated hypertension, and that a very low dose of oral nitrite (1 mg/L) protected against L-NAME-induced kidney injuries without significant changes in blood pressure.

The renal microvasculature plays an important role in blood pressure regulation, and increased preglomerular resistance has been demonstrated in models for hypertension.^{63,64} To further address the role/mechanisms of nitrate–nitrite–NO pathway in the kidney, we have performed experiments with nitrite in isolated and perfused renal afferent arterioles. Preliminary results show that extraluminal nitrite application (10 μ M) dilates arterioles and attenuates angiotensin II-mediated contraction by increasing NO bioavailability (Carlström *et al.*, unpublished results). The mechanism for increased NO generation in the renal microvasculature is NOS-independent and rather involves enzymatic reactions with XOR and activation of cGMP. This finding supports a novel role of nitrite in regulation of renal microcirculation and blood pressure.

In addition to the diet, recent research suggests that the skin can be a considerable source of compounds with NO-like activity.^{65,66} Specifically, it has been proposed that NO-related species from dietary and endogenous sources are stored in the skin and then mobilized by sunlight and delivered to the systemic circulation to exert coronary vasodilator and cardioprotective as well as antihypertensive effects. Oplander *et al.*⁶⁵ demonstrated that moderate whole body UVA irradiation (20 J/cm²) induced formation of S-nitrosothiols in the blood of healthy subjects. Furthermore, UVA irradiation caused a rapid decrease in SBP and DBP which correlated ($R^2 = 0.74$) with enhanced plasma concentrations of nitrosated species.

Taken together, dietary nitrate may fuel the nitrate–nitrite–NO pathway and partly compensate for disturbances in endogenous NO generation from NOS (Table 1). The mechanisms for nitrate-mediated antihypertensive effects and renal and cardiac protection require further investigations, but modulation of NO bioavailability and reduction in oxidative stress have been suggested.

6. Nitrate, mitochondria, and oxygen consumption

Recent data suggest that many of the biological effects of nitrite^{52,55} involve interaction with mitochondria. In the past two decades, it has been established that the mitochondrion is a physiological target for NO.⁶⁷ NO binds to cytochrome c oxidase, the terminal respiratory complex in the mitochondrial electron transport chain, in competition with oxygen. The binding of NO to cytochrome c oxidase, even at concentrations below those inhibiting respiration,⁶⁸ elicits intracellular signalling events, including elongation of cellular oxygen gradients but also the generation of ROS with potentially damaging effects. These NO-elicited events act as triggers by which mitochondria modulate signal transduction cascades involved in the induction of cellular

defence mechanisms and adaptive responses, particularly in response to hypoxia and other environmental stressors.⁶⁹

A muscle cell under high metabolic demand is featured by a very low pO_2 , and pH is substantially decreased; conditions which favour nitrite conversion to NO. With this in mind, we designed a study to look at effects of dietary nitrate during physical exercise. Healthy volunteers performed graded exercise on a cycle ergometer while oxygen consumption and circulatory and metabolic parameters were measured. We found that the metabolic cost of performing standardized constant load exercise was reduced after supplementation with 0.1 mmol kg⁻¹ day⁻¹ sodium nitrate for 3 days compared with placebo.⁵⁵ This highly surprising effect occurred without any change in venous lactate concentration, indicating that there was no compensatory increase in glycolytic energy contribution and thus metabolic efficiency seemed to be improved. These results have subsequently been confirmed in and extended using beetroot juice as the nitrate source as well as sodium nitrate salt.^{70–72} These studies do not only show that nitrate reduces oxygen cost but intriguingly, the time-to-exhaustion was extended,^{71,72} possibly due to a reduced ATP cost of muscle force production⁷³ or as a direct effect of the improved metabolic efficiency. The molecular mechanisms behind the effect of nitrate on metabolism have not been settled in detail but research points towards the mitochondrion as the central target, with a reduction in proton leak as a major contributing factor (Larsen *et al.*, unpublished results).

Further evidence that the NO pathway is a fundamental characteristic in cardiovascular health and athletic performance comes from the finding that performance and maximal oxygen uptake (VO_2 peak) correlates robustly with the increase in plasma nitrite after an exercise challenge.^{74,75} Taken together, these findings may have implications, not only for exercise physiology, but also for disease conditions associated with dysfunctional mitochondria and reduced oxygen availability.

7. Nitrate, eNOS, and the metabolic syndrome

Obesity and hypertension are increasing problems worldwide, resulting in an enormous economic burden to the society.^{76,77} The metabolic syndrome is a combination of medical abnormalities including central obesity, dyslipidaemia, hyperglycaemia, and hypertension, and it is estimated that around 25% of the world's adult population have the metabolic syndrome.⁷⁷ This clustering of metabolic abnormalities that occur in the same individual appear to present a considerably higher cardiovascular risk compared with the sum of the risk associated with each abnormality.

Mice lacking the gene for eNOS do not only develop hypertension, but do also display key features of the metabolic syndrome including dyslipidaemia, obesity, and insulin resistance.^{78–80} The striking clustering of these particular abnormalities in eNOS-deficient mice has lead authors to suggest that a reduced NO bioavailability is a central event in the pathogenesis of metabolic syndrome. In support of this, a polymorphism in the eNOS gene is associated with metabolic syndrome in humans.⁸¹ In a recent study, we investigated the long-term cardiovascular and metabolic effects of nitrate supplementation (0.1 mmol kg⁻¹ day⁻¹) in mice lacking eNOS.⁸² Previously, Bryan *et al.*⁸³ reported that eNOS-deficient mice have lower levels of nitrite and other nitrogen oxides, which could be restored by nitrite supplementation. Remarkably, Carlström *et al.*⁸² showed that inorganic nitrate supplementation

for 10 weeks reduced visceral fat accumulation, lowered circulating levels of triglycerides, and reversed the pre-diabetic phenotype in these animals. These results suggest that stimulation of the nitrate–nitrite–NO pathway can partly compensate for disturbances in endogenous NO generation from eNOS. These findings may have implications for novel nutrition-based preventive and therapeutic strategies against cardiovascular disease and type 2 diabetes.

Interestingly, the dose of dietary nitrate was chosen only to replace what is being generated by eNOS under normal conditions.⁸⁴ The fact that this very modest amount had such profound biological effects supports the intriguing possibility that endogenous nitrate levels are already sufficient to affect cellular processes. Thus, in addition to the second by second regulation of vascular tone by eNOS-derived NO, its oxidized end-product nitrate may serve as a long-lived reservoir for NO-like bioactivity in tissues. Carlström et al. also noted increased plasma and tissue levels of S-nitrosothiols and nitrosylation products with dietary nitrate supplementation. These bioactive compounds may be mediating the observed effects, however the exact mechanism for this and the signalling pathways involved remain to be elucidated.

8. Nutritional implications

Epidemiological studies have convincingly shown that a diet rich in fruits and vegetables, such as traditional Mediterranean or Japanese diets, protects against development of cardiovascular disease and type 2 diabetes.^{85–88} In addition, intervention studies, e.g. the classical Dietary Approaches to Stop Hypertension (DASH) trial, have shown blood pressure lowering effects of these kind of diets.⁸⁹ However, the specific component(s) attributed with this protection is yet to be pinpointed and trials have generally failed to link the effects with a single nutrient. With accumulating data showing beneficial effects of nitrate in the cardiovascular system, researchers have suggested that nitrate might be one active constituent in these healthy diets.^{9,90–92} This development is quite astonishing considering that this particular anion for more than two decades has been viewed as an unwanted and potentially harmful compound.¹ Although much more research is needed before we can draw any firm conclusions, it is striking that the observed blood pressure reduction with modest dietary nitrate supplementation^{54,56} is similar or even greater than that seen with vegetable and fruit in the DASH study.⁸⁹ In a very recent meta-analysis, Carter et al.⁹³ studied the influence of fruit and vegetables on the incidence of type 2 diabetes. They found that there were no significant benefits of increasing the overall consumption of vegetables, fruit, or fruit and vegetables combined. However, further sub-analysis specifically showed that a greater intake of green leafy vegetables was associated with a 14% reduction in risk of type 2 diabetes. The possibility of boosting NO production by dietary intervention may have important implications for public health, in particular cardiovascular disease. Future clinical studies are warranted to elucidate if nitrate can offer a nutritional approach to prevention and treatment of cardiovascular disease, and if the beneficial effects will outweigh any negative health effects traditionally attributed to this anion. If such investigations point towards an overall protective effect of nitrate, we should reconsider today's strict regulations of nitrate levels in food and drinking water.

9. Summary and future perspectives

Accumulating evidence indicates that inorganic nitrate, a supposedly inert anion abundant in vegetables, can be converted to nitrite, NO, and other bioactive nitrogen oxides *in vivo*. The central role of commensal bacteria in bioactivation of nitrate is intriguing and suggests a symbiotic host-microbial relationship involved in the regulation of cardiovascular function.⁹ Modest dietary intake of nitrate reduces blood pressure, inhibits platelet function, and prevents endothelial dysfunction after a mild ischaemic insult in humans. Moreover, nitrate reduces oxygen cost during exercise and has effects on fundamental mitochondrial functions. In animal studies, nitrate has prolonged blood pressure lowering effects, protects against drug- and salt-induced renal and cardiac injuries, enhances post-ischaemic blood flow, protects against IR injury, and reverses features of the metabolic syndrome. While these effects of exogenously delivered nitrate are unequivocal, the physiological relevance of endogenously generated nitrate and nitrite is still to be settled. Nevertheless, experiments using extremely low doses of nitrate or nitrite indicate bioactivity also in the physiological range, and studies with nitrate in amounts titrated to replenish endogenous nitrate generation in eNOS-deficient mice further strengthen this notion.

Although this field of research is still in an early phase, it is tempting to speculate that the salutary effects of nitrate and nitrite seen in animal studies, and in small trials in healthy volunteers, can be utilized also in patients in prevention and treatment of cardiovascular disease. As pharmaceutical agents, inorganic nitrate and nitrite should not be confused with organic nitrates such as nitroglycerine or isosorbide mononitrate which are commonly used in cardiovascular medicine.⁹⁴ The latter drugs are lipophilic potent vasodilators that act via rapid generation of NO in smooth muscle. Inorganic nitrate and nitrite also generate NO, but the pharmacokinetic profiles are profoundly different and their potency as vasodilators is orders of magnitude lower. However, a great advantage with nitrate and nitrite as potential drug candidates is that the final step in their bioactivation to NO and other short-lived bioactive nitrogen oxides is pH- and pO₂-dependent, thus greatly enhanced where NO is most needed (i.e. ischaemic tissue). These properties of nitrate and nitrite further strengthen their therapeutical role, for example in myocardial infarction and ischaemic events, as formation of bioactive nitrogen oxides predominantly should be located the disease area without generalized systemic vasodilatation and hypotension. In addition to these properties, the effects of nitrate and nitrite seem to be devoid of tolerance.^{59,60} The reason for this is currently unclear but could be related to the different pharmacodynamic profiles of inorganic nitrate and organic nitrates, for example in relation to NO release kinetics and influence on sGC redox state and activity.⁹⁵ Also, one should note that many of the effects of nitrate and nitrite may in fact be unrelated to classical NO–cGMP signalling. As an example, nitrate and nitrite reaction products directly modulate mitochondrial function via various mechanisms.

Finally, the nutritional implications of nitrate and nitrite biology are among the most intriguing in this area of research. The amounts of these anions needed for the effects on the cardiovascular system, described in this review, are readily achieved via our everyday diet, most easily via a rich intake of fruits and vegetables. If the cardiovascular benefits of this healthy diet turn out to be related to their high

amount of nitrate, we have to reconsider our current thinking and realize that inorganic nitrate may not necessarily be a threat to human health. Instead, in some years, we might even consider this anion as an essential nutrient.

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