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REVIEW

Nitrite in organ protection

Tienush Rassaf[1], Peter Ferdinandy[2,3] and Rainer Schulz[4]

[1]*Department of Medicine, Division of Cardiology, Pulmonary and Vascular Medicine, University Hospital Düsseldorf, Düsseldorf, Germany,* [2]*Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary,* [3]*Pharmahungary Group, Szeged, Hungary, and* [4]*Department of Physiology, Justus-Liebig-University, Giessen, Germany*

Correspondence
Prof Dr Tienush Rassaf, Division of Cardiology, Pulmonary and Vascular Medicine, Medical Faculty, University Hospital Düsseldorf, Moorenstrasse 5, D-40225 Düsseldorf, Germany.
E-mail: tienush.rassaf@med.uni-duesseldorf.de

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In the last decade, the nitrate-nitrite-nitric oxide pathway has emerged to therapeutical importance. Modulation of endogenous nitrate and nitrite levels with the subsequent S-nitros(yl)ation of the downstream signalling cascade open the way for novel cytoprotective strategies. In the following, we summarize the actual literature and give a short overview on the potential of nitrite in organ protection.

Abbreviations

CypD, cyclophilin D; eNOS, NOS III, endothelial nitric oxide synthase; Hb, haemoglobin; iNOS, NOS II, inducible nitric oxide synthase; Mb, myoglobin; MPTP, mitochondrial permeability transition pore; Ng, neuroglobin; nNOS, NOS I, neuronal nitric oxide synthase; XOR, xanthine oxidoreductase

Sources of nitrite

Three sources of nitrite have been identified in mammalian physiology. The first, endogenous source is the oxidation of the nitric oxide (NO) radical to nitrite. NO is produced endogenously from the amino-acid L-arginine by the NO-synthases (NOSs). Three different NOSs exist: the endothelial NOS (eNOS, NOS III), the inducible NOS (iNOS, NOS II) and the neuronal NOS (nNOS, NOS I). The genes for the three different NOS-isoforms are located on different chromosomes. eNOS was first discovered in the vascular endothelium, nNOS in the brain and iNOS in macrophages. Whereas eNOS and nNOS are constitutively expressed in calcium and calmodulin-dependent, transcription of iNOS is induced by cytokines and lipopolysaccharides. The enzymatic production of NO contains a five-electron transfer and requires the presence of several substrates and cofactors, such as L-arginine, oxygen, tetrahydrobiopterin and reduced nicotinamide adenine dinucleotide phosphate (for review see: Moncada and Higgs, 1993). NO is a highly reactive gaseous molecule with one unpaired electron. NO acts mainly in auto/paracrine fashion, and signalling is limited by its rapid oxidation to nitrite and nitrate, and its rapid non-enzymatic reaction with superoxide to yield peroxynitrite (for review see: Ferdinandy and Schulz, 2003). NO rapidly reacts with oxyhaemoglobin to form methemoglobin and nitrate. Oxidation of NO to nitrite is enhanced by the multicopper oxidase ceruloplasmin, catalyzing the oxidation of NO to NO^+ which is rapidly hydrolyzed to nitrite (Shiva *et al.*, 2006).

The second major source is nitrite reduced from nitrate (Figure 1). Green leafy vegetables, such as lettuce, spinach and beetroot all contain high concentrations of nitrate. One serving of such a vegetable contains more nitrate than what is endogenously formed by all three NOS isoforms during 1 day in humans (Lundberg *et al.*, 2009). After ingestion of nitrate and effective absorption in the upper gastrointestinal tract, concentrations of nitrate in the saliva reach millimolar concentrations. In the oral cavity, commensal anaerobic bacteria reduce nitrate to nitrite by their nitrate reductase enzymes. When swallowed, due to the acidic gastric milieu, part of the nitrite is immediately protoned to nitrous acid, which then decomposes to NO and other nitrogen oxides. Most of the swallowed nitrite escapes the acidic milieu and enters the systemic circulation. Reduction of nitrate to nitrite with the consecutive increase in circulating nitrite levels is highly dependent on the oral commensal bacteria. Avoidance

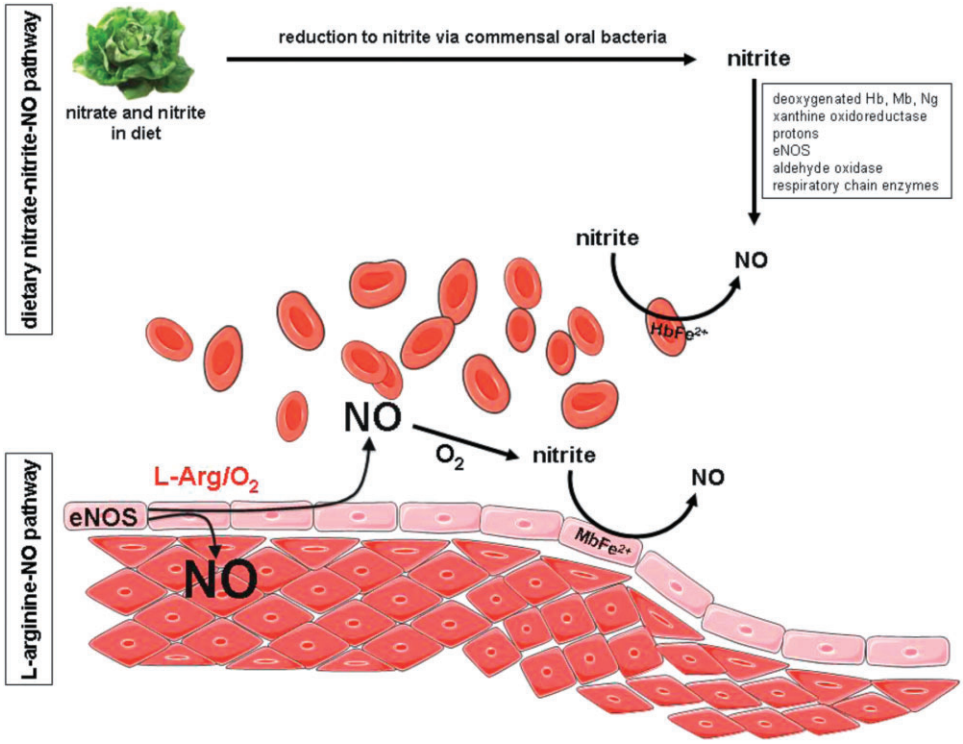

Figure 1

Sources for nitric oxide (NO) formation in mammals. NO is formed by the endothelial NOS (eNOS) using L-arginine as a substrate in an oxygen-dependent manner. Dietary nitrate is reduced to nitrite via commensal bacteria in the oral cavity. Nitrite can be reduced to NO in eNOS independently via deoxygenated myoglobin (Mb), haemoglobin (Hb), neuroglobin (Ng), xynthin oxidoreductase, protons, aldehyde oxidase and enzymes of the respiratory chain to bioactive NO.

of swallowing (Lundberg and Govoni, 2004) and the use of an antibacterial mouthwash (Hendgen-Cotta *et al.*, 2012) abolish the conversion of nitrate to nitrite and the consecutive increase in plasma nitrite.

The third source is dietary ingestion of nitrite. Cured meat contains nitrite which is used to protect from bacterial contamination and to give the meat the fresh red colour. Furthermore, baked goods, beets, corn and spinach are all major sources of nitrite (Lundberg *et al.*, 2009; van Faassen *et al.*, 2009).

Nitrite levels in mammals

All three above described sources contribute to the circulating nitrite pool. Physical exercise, dietary habits, health status and lifestyle lead to a variability in measured nitrite levels. Plasma nitrite levels in healthy fasted humans range from 0.1 to 0.5 uM (Rassaf *et al.*, 2002; 2003; 2006a; 2007a; 2010); lower levels of nitrite have been described in patients with myocardial infarction and endothelial dysfunction (Kehmeier *et al.*, 2008), often as a result of hypertension and diabetes mellitus (Fujiwara *et al.*, 2000). Physical exercise increases plasma nitrite levels in healthy subjects (Rassaf *et al.*, 2006b; 2007a), but not in patients with cardiovascular disease and/or endothelial dysfunction (Rassaf *et al.*, 2010). Plasma nitrite levels may be lowered by about 50% by dietary restrictions (Gladwin *et al.*, 2000) and can be increased by diet rich in nitrate (van Velzen *et al.*, 2008; Heiss *et al.*, 2012). Nitrite levels in erythrocytes have been described to be higher than in plasma (Bryan *et al.*, 2004; Dejam *et al.*, 2005). A recent study investigating the NO-metabolism in Tibetan highlanders – a population well adapted to environmental hypoxia associated with high altitudes – revealed plasma nitrite levels of about 10 uM, exceeding the plasma nitrite levels of humans living at sea level 50-fold (Erzurum *et al.*, 2007). This high nitrite concentration was associated with increased blood flow in this population. Circulating nitrite levels in rodents are higher than those in normal humans and have been described as high as 10 uM (Feelisch *et al.*, 2002; Rodriguez *et al.*, 2003; Bryan *et al.*, 2004), thereby matching the levels seen in humans in high altitude. One reason for the higher nitrite levels in rodents may be that NO-synthase activity is several folds higher in mice compared to humans (Wickman *et al.*, 2003).

Toxic levels of nitrite and nitrate

The US Food and Drug Administration and the European Food Safety Authority considered a dose of 22 mg sodium/nitrite/kg as lethal for adults due to the complications that arise from methemoglobinemia. Lower dosages apply for infants, who are more susceptible and vulnerable than adults.

A major health concern is also that dietary nitrite and nitrite derived from dietary nitrate may lead to cancer, due to a proposed association between nitrate intake and the formation of the carcinogen substances N-nitrosamines (Spiegelhalder *et al.*, 1976). Nitrite – in contrast to nitrate – undergoes nitrosative chemistry; this is prevented by ascorbic acid and therefore nitrite added to meat products contains supra-stoichiometric erythorbate or ascorbic acid. Experimental and epidemiologic studies, however, failed to show an increased risk of cancer with increasing consumption of nitrate (van Loon *et al.*, 1998; Pannala *et al.*, 2003; Hord *et al.*, 2009; Tang *et al.*, 2011) and the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives concluded in 2003 that there was no evidence that nitrate was carcinogenic in humans. Epidemiological studies mostly indicate that abundant consumption of vegetables reduce the risk of cancer (Block *et al.*, 1992; Terry *et al.*, 2001). It should be noted that high doses of nitrite preparations are widely used for acute care in cyanide poisoning (see for review: Gracia and Shepherd, 2004).

Bioactivation of nitrite

For many years nitrite has been regarded as an inert by-product of the NO-pathway. However, experimental and clinical studies over the past years challenged this dogma. Several endogenous pathways exist that provide the reduction of nitrite to NO, with haemoglobin, myoglobin, neuroglobin, cytoglobin, xanthine oxidoreductase, eNOS and mitochondrial enzymes being involved (for reviews see: van Faassen *et al.* 2009; Lundberg *et al.*, 2009). The extent of contribution of the different pathways depends on the tissue, the pH, oxygen tension and redox status (Feelisch *et al.*, 2008).

Under normoxia, oxygenated haemoglobin rapidly reacts with nitric oxide to form methemoglobin and nitrate. Hypoxia, however, leads to an alteration of haemoglobin conformation and oxygen binding status, affecting its ability to reduce nitrite (Huang *et al.* 2005a,b). During rapid desoxygenation, nitrite reductase activity increases, reaching a peak at pO_2 (Jansson *et al.*, 2008), that is the moment when 50% of the haemoglobin is saturated with oxygen (Huang *et al.*, 2005b). Through this allosterically controlled bioactivation of nitrite, erythrocytes possess a kind of oxygen sensor, which may enable them to modulate microvascular flow by eliciting nitrite-derived NO and vasodilation in areas of poor oxygenation. Whereas the exact role of erythrocytes in the regulation of vascular flow is still under debate, it was shown in a recent study with myoglobin-deficient mice that vascular myoglobin may be responsible at least in part for the regulation of hypoxic vasodilation (Totzeck *et al.*, 2012a). Myoglobin has also been identified to play a role in cardiac nitrite bioactivation. Under normoxic conditions, oxymyoglobin protects the heart from the deleterious effects of excessive NO (Godecke *et al.*, 2003). Under hypoxia, however, myoglobin changes its role from an NO scavenger to an NO producer. Deoxymyoglobin reduces nitrite to bioactive NO thereby leading to an adaptation of cardiac energetics to myocardial function under hypoxic and ischaemic conditions (Rassaf *et al.*, 2007b; Hendgen-Cotta *et al.*, 2010a,b; Totzeck *et al.*, 2012b).

In myocardial ischaemia/reperfusion, NO accumulation by electron-spin-resonance spectroscopy has been shown during ischaemia in spite of the lack of oxygen in rat hearts, which has been suggested to be via a non-enzymatic mechanism (Csonka *et al.*, 1999; Zweier *et al.*, 1999). Later it has been shown that there are nitrite reductase activities in the myocardium. In myocardial ischaemia/reperfusion injury, myoglobin-mediated NO formation has been shown to be cytoprotective and important for improvement of left ventricular function after infarction (Hendgen-Cotta *et al.*, 2008). Other heme-proteins that elicit nitrite-reductace activity under hypoxic conditions are neuroglobin and cytoglobin (Petersen *et al.*, 2008; Tiso *et al.*, 2011; 2012). The relevance and function of these nitrite-reductases have still to be elucidated.

Another nitrite reductase is the xanthine oxidoreductase (XOR), which fulfils its role in purine catabolism and generates the reactive oxygen species superoxide anions, hydroxyl radicals and hydrogen peroxide. XOR, which is up-regulated in ischaemia and inflammation (Harrison, 2004), reduces inorganic nitrite (Godber *et al.*, 2000) and nitrate (Jansson *et al.*, 2008) to NO.

eNOS itself has also been identified as a nitrite reductase (Gautier *et al.*, 2006; Vanin *et al.*, 2007) under anoxia and under acidic conditions. This function enables eNOS to produce NO under both normoxic and hypoxic conditions.

Mitochondria are important targets for NO. NO binds to the complexes of the respiratory chain and thus inhibits respiration (Bolanos *et al.*, 1994; Brown and Cooper, 1994; Cleeter *et al.*, 2001). Under hypoxia, however, several mitochondrial complexes like complex III, complex IV and ubiquinone/cytochrome b_1 can reduce nitrite (671; 672; 673). Another mitochondrial enzyme which reduces nitrite to NO is the aldehyde oxidase (Li *et al.*, 2008).

Cytochrome P450, a family of enzymes which are involved in drug metabolism, have been shown to exert nitrite reductase activity (Kozlov *et al.*, 2003). The physiological relevance of this novel function is still under investigation. Furthermore, the ubiquitous enzyme carbonic anhydrase has been shown to generate NO from nitrite (Aamand *et al.*, 2009), which may be important for the regulation between blood flow and metabolic activity in tissues.

Last but not least, in the stomach (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994) and under acidic conditions nitrite can undergo protonation to nitric oxide (Zweier *et al.*, 1995; 1999).

In summary, several pathways have been identified for the reduction and thus bioactivation of nitrite to NO. In the following, the relevance of the respective reactions in organ protection will be discussed.

Nitrite in organ protection

As early as 1956, nitrite alone was demonstrated to protect phages against X-ray radiation injury, presumably acting as a reducing agent (Bachofer, 1956). Later on, the presence of nitrite, but not nitrate, reduced the extent of apoptosis in cultured endothelial cells during UVA-irradiation in a concentration-dependent manner by inhibiting lipid peroxidation; this protective effect was abolished by simultaneous

administration of a NO scavenger (Suschek *et al*., 2003) suggesting that nitrite-derived NO may contribute to protection against UV-induced cell damage (Suschek *et al*., 2006). Nitrite, generated from nitrate by oral bacteria 'the so called enterosalivary cycle', and then converted to NO (Benjamin *et al*., 1994; Lundberg *et al*., 1994; 2009; 2006; 2008; Kapil *et al*., 2010a) in the stomach was also suggested to play an important role in the protection of gastric mucosa from hazardous stress (Miyoshi *et al*., 2003). Indeed, the stomach content, but also the plasma, heart (Samouilov *et al*., 2007) and liver nitrite levels were significantly reduced after dietary nitrate and nitrite depletion (Feelisch *et al*., 2002), and could be restored to normal levels with nitrite supplementation (Bryan *et al*., 2007).

As mentioned above, dietary nitrate is an important source of the endogenous nitrite pool, with vegetables being the main source of nitrate in our diet. Epidemiologic studies have demonstrated that diets rich in vegetables and fruits (i.e. the Mediterranean diet) protect against cardiovascular (Willett, 1994) diseases and first interventional trials have demonstrated that such diets lower blood pressure (Liese *et al*., 2009). Whereas the active compound being responsible for this protection has not been identified so far, it is important to note that high dietary nitrate concentrations reduce blood pressure to a level similar to that achieved with a Mediterranean diet.

Lundberg and Weitzberg were the first to describe a blood pressure lowering effect of inorganic sodium nitrate in healthy volunteers (Larsen *et al*., 2006). Diastolic blood pressure was reduced by 4 mmHg after ingestion of a sodium-nitrate drink compared to placebo. The authors suggested that the formation of vasodilatory nitric oxide was responsible for this effect. The results have been corroborated by other groups investigating the effects of beet root juice – which contains high amounts of nitrate – on blood pressure (Webb *et al*., 2008; Kapil *et al*., 2010b). In a recent study, it has been demonstrated that nitrate has also blood pressure lowering effects in humans with hypertension when applied in lower doses (Ghosh *et al*., 2013). In further studies, Larsen *et al*. found that the oxygen cost during standardized exercise was reduced after dietary nitrate supplementation compared to placebo (Larsen *et al*., 2007; Weitzberg *et al*., 2010). No differences in lactate formation were measured, indicating that there was no compensatory increase in glycolic energy contribution, and thus metabolic efficiency seemed to be improved (Weitzberg *et al*., 2010).

Applying the protocol of Larsen *et al*. (2006), the effects of dietary nitrate supplementation on healthy subjects with endothelial dysfunction was investigated; nitrate supplementation reversed endothelial dysfunction, an effect that was associated with an increase in circulating vascular progenitor cells (Heiss *et al*., 2012), endogenous nitrite and S-nitrosothiol levels. In a mouse hind-limb model, dietary nitrate improved vascular regeneration compared to placebo (Hendgen-Cotta *et al*., 2012). The cytoprotective effects of nitrate were completely abolished, however, when mice received a mouthwash twice daily, using a commercially available antibacterial solution that eradicated the commensal bacterial flora which is necessary to reduce nitrate to nitrite (Hendgen-Cotta *et al*., 2012).

Cardiovascular

Acidified sodium nitrite, a releaser of NO, reduced infarct size (expressed as percentage of the area at risk) in a cat model of 90 min ischaemia and 270 min reperfusion when intravenous infusion of acidified sodium nitrite was started 30 min after coronary artery occlusion (Johnson *et al*., 1990).

Since the rate of NO generation from nitrite depends on the reduction in oxygen and pH, nitrite could be reduced to NO in ischaemic tissue and exert protective effects (for review, see van Faassen *et al*., 2009). Therefore, sodium nitrite (without acidification) was administered in mice undergoing ischaemia/reperfusion and indeed nitrite reduced myocardial infarct size by 67%. Consistent with hypoxia-dependent nitrite bioactivation, nitrite was reduced to NO, S-nitrosothiols, N-nitrosamines and iron-nitrosylated heme proteins during early reperfusion (for review, see Tiravanti *et al*., 2004). Nitrite-mediated protection was independent of endothelial nitric oxide synthase (Webb *et al*., 2004; Duranski *et al*., 2005). These findings were confirmed in rat hearts *in vitro* and *in vivo*, which also demonstrated that intravenous nitrate infusion – when given at the same dose as nitrite – conferred no reduction in infarct size following ischaemia/reperfusion (Baker *et al*., 2007). Bioactivation on nitrite required increased activity of xanthine dehydrogenase and xanthine oxidase during ischaemia in rats (Baker *et al*., 2007) and the presence of myoglobin in mice (Rassaf *et al*., 2007b), since the reduction in infarct size following administration of nitrite was completely abolished in myoglobin knockout mice (Hendgen-Cotta *et al*., 2008). However, more nitrite reducing pathways are available under ischaemic/hypoxic conditions (for review, see Dezfulian *et al*., 2007; Sinha *et al*., 2008; Shiva *et al*., 2011; Tota *et al*., 2011). The mechanism how nitrite exerts its cytoprotective effects has been described earlier (Shiva *et al*., 2007); nitrite modifies and inhibits complex I by post-translational S-nitrosation. This dampens electron transfer and reduces reactive oxygen species generation and ameliorates oxidative inactivation of complexes II–IV and aconitase. This prevents mitochondrial permeability transition pore opening and cytochrome c release (Shiva *et al*., 2007).

Another potential mechanism of nitrite-induced protection relates to the modification of the mitochondrial permeability transition pore (MPTP) opening, which plays a critical role in mediating cell death during ischaemia/reperfusion injury. Cyclophilin D (Cyp D), which accelerates MPTP opening, undergoes S-nitrosylation on cysteine 203 of Cyp D, leading to reduced MPTP opening in mice wild-type fibroblast but not in Cyp D knockout fibroblast (Nguyen *et al*., 2011). In our recent experiments, nitrite reduced infarct size following ischaemia/reperfusion in wild-type mice but not in Cyp D knockout mice suggesting that the above mechanism might hold true also in hearts *in vivo* (Figure 2).

Also mice fed a standard diet with supplementation of nitrite in their drinking water for 7 days exhibited significantly higher plasma and myocardial levels of nitrite, nitroso and nitrosyl–heme and displayed a 48% reduction in infarct size following ischaemia/reperfusion. Supplemental nitrate in the drinking water for 7 days also increased blood and tissue NO products and significantly reduced infarct size (Bryan *et al*., 2007). Nitrite supplementation in the drinking water

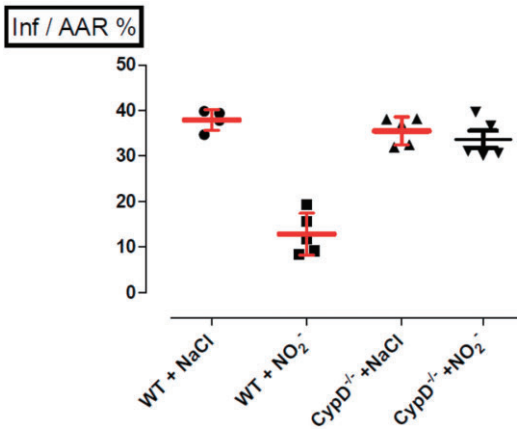

Figure 2

Blockade of mitochondrial permeability transition pore opening reduces cytochrome c loss from the intermembrane space and prevents apoptosis (for review see Schulz *et al.*, 2004; Heusch *et al.*, 2008; Calvert and Lefer, 2009). Indeed, in our recent unpublished experiments in anaesthetized mice nitrite reduced infarct size following 30 min coronary occlusion and 120 min reperfusion in wild-type mice ($P < 0.05$). This reduction in infarct size by nitrite was not seen in cyclophilin D knockout mice.

for 1 week restored NO homeostasis also in eNOS knockout mice, normally having reduced NO and NO metabolites, and protected against ischaemia/reperfusion injury (Bryan *et al.*, 2008). In humans, endothelial dysfunction is also associated with reduced circulating nitroso compounds (Heiss *et al.*, 2006) and the lack to increase plasma nitrite levels during exercise (Lauer *et al.*, 2008; Rassaf *et al.*, 2010), the latter contributing to protection of the heart against myocardial ischaemia/reperfusion injury by exercise (Calvert *et al.*, 2011) (for review, see Calvert, 2011).

Cardioprotective signalling of nitrite in rats involved the activation of NADPH oxidase and ATP-dependent potassium channels (Baker *et al.*, 2007). NO-dependent activation of PKG through the soluble guanylate cyclase-cGMP pathway has been shown to open mitochondrial ATP-dependent potassium channels which results in a mild increase in reactive oxygen species generation (Philipp *et al.*, 2006), a modest dose-dependent depolarization of the mitochondria, reduced mitochondrial calcium accumulation and finally prevention of the opening of the mitochondrial permeability transition pore. Furthermore, nitrite affects cytochrome P450 activities, heat shock protein 70 and heme oxygenase-1 expression in a variety of tissues (Bryan *et al.*, 2005).

In hyperlipidaemia, when NO bioavailability of the heart is diminished possibly due to oxidative stress-induced formation of peroxynitrite (Onody *et al.*, 2003; Csont *et al.*, 2007), NO-mediated cardioprotective pathways are disrupted (Giricz *et al.*, 2009; Kupai *et al.*, 2009); (for reviews see Ferdinandy, 2003; Ferdinandy *et al.*, 2007), and heat-shock protein 70 response is diminished (Csont *et al.*, 2002). One could speculate that nitrite treatment to replenish NO formation in the heart would be a plausible therapeutic option. This, however, has not been investigated so far. In a clinical study by Higashino *et al.* (2007), a male group of hypertensive patients with complex co-morbidities of diabetes, hyperlipidaemia and renal disorder had higher nitrate/nitrite levels compared with a normotensive control group. This suggest that in co-morbid states with oxidative stress, NO may be oxidized to nitrite/nitrate. Whether nitrite treatment would reverse this process and increase formation of cardiac NO is not known.

Nevertheless, it should be noted here that pathological accumulation of NO in myocardial ischaemia may yield high flux of non-enzymatic formation of peroxynitrite upon reperfusion when there is a burst of superoxide generation. This mechanism has been shown to contribute to reperfusion injury of the heart (Yasmin *et al.*, 1997; Csonka *et al.*, 1999; 2001). Therefore, the protective or detrimental effect of NO from whatever sources in ischaemia/reperfusion may largely depend on the local concentrations NO and superoxide and their ratio, if it favours formation of pathological concentrations of peroxynitrite or not (see for reviews: Ferdinandy and Schulz, 2003; Ferdinandy, 2006; Pacher *et al.*, 2007). Whether nitrite treatment may lead to pathological accumulation of NO in the ischaemic myocardium is not known. However, it may be a limitation of nitrite treatment in pathologies where the non-enzymatic reaction of NO and superoxide yields pathological concentrations of peroxynitrite.

EC barrier function and vessel response

Under hypoxic conditions, caspase-3 was de-nitrosylated and the resulting activation of caspase-3 and subsequent cleavage of β-catenin was critical for hypoxia-induced increased endothelial permeability. Nitrite treatment led to S-nitros(yl)ation and the inactivation of caspase-3, suppressing the barrier dysfunction of endothelia caused by hypoxia. This process required the conversion of nitrite to bioactive nitric oxide in a nitrite reductase-dependent manner (Lai *et al.*, 2011). The supplementation of a low dose of nitrite through local intra-arterial infusion, attenuated ischaemia-reperfusion-induced vasoconstriction, arteriole stagnation, and capillary no-reflow during reperfusion (Wang *et al.*, 2011) and prolonged application of nitrite improved revascularization in chronically ischaemic peripheral muscles (Hendgen-Cotta *et al.*, 2012), potentially through mobilization of circulating angiogenic cells (Heiss *et al.*, 2012).

Brain

In a cerebral ischaemia/reperfusion injury model, using the intraluminal occlusion of the middle cerebral artery for 90 min in rats, intravenous nitrite infusion at the time of reperfusion reduced infarction volume (measured at 24 h) and enhanced local cerebral blood flow and functional recovery. Carboxy-PTIO, a direct NO scavenger, abolished the neuroprotective effects of nitrite (Jung *et al.*, 2006). Varying the time point of infusion further indicated that nitrite reduced the infarction volume and enhanced functional recovery when nitrite was administered within 3 h after transient intraluminal occlusion and 1.5 h in the permanent occlusion model in rats respectively (Jung *et al.*, 2009). However, these results were not confirmed in a study using intravenous sodium nitrite as adjuvant to recombinant tissue plasminogen activator in cerebral artery occlusion with 2 and 6 h of ischaemia followed by reperfusion in rats. Nitrite treatment did not reduce infarct volume at 48 h reperfusion compared

with saline-treated placebo groups receiving recombinant tissue plasminogen activator only (Schatlo *et al.*, 2008).

In cynomolgus macaques, subarachnoid haemorrhage-induced vasospasm created via implantation of a blood clot was reversed by intravenous nitrite infusion (27 vs. 46% in vehicle) (Pluta *et al.*, 2005; Fathi *et al.*, 2011). Furthermore, a single dose of intravenous nitrite given at cardiopulmonary resuscitation improved cardiac function, survival and neurological outcomes in a placebo-controlled study in a mouse model of cardiac arrest (Dezfulian *et al.*, 2009). When nitrite was injected intravenously 3 h after intracerebral haemorrhage induction in rats, most doses of nitrite provided no beneficial effect on behavioural deficits, brain oedema and hematoma volumes. A high dose of nitrite, however, decreased hematoma volume, but not brain oedema (Jung *et al.*, 2011).

Thus, depending on the timing of application nitrite might not only reduce irreversible brain injury following ischaemia/reperfusion but also vasospasm following cerebral haemorrhage.

Liver

In hepatic ischaemia/reperfusion injury in mice, nitrite exerted profound dose-dependent protective effects on cellular necrosis and apoptosis, with highly significant protective effects observed at near-physiological nitrite concentrations. Nitrite-mediated protection of the liver was dependent on NO generation and independent of endothelial NO synthase and heme oxygenase-1 enzyme activities (Duranski *et al.*, 2005). In patients undergoing orthotopic liver transplantation, inhaled NO doubled plasma nitrite levels, which improved liver function and reduced liver injury (Lang *et al.*, 2007). Although the authors stated that not all effects of inhaled NO may be mediated by nitrite, it seems to be obvious that protective effects of nitrite in ischaemia/reperfusion injury may be translated into humans (Lang *et al.*, 2007).

Nitrite can also convey NO bioactivity in an endocrine fashion. It can be transported in blood, metabolized in remote organs (see above), and mediate cytoprotection in the setting of ischaemia/reperfusion injury. Indeed, in mice with cardiac-specific overexpression of the human endothelial NO synthase gene nitrite, nitrate and nitrosothiols levels were increased in the heart, plasma and liver. These mice displayed a significant reduction in hepatic ischemia/reperfusion injury compared with wild-type littermates (Elrod *et al.*, 2008).

Finally, liver ischemia/reperfusion injury is a major cause of primary graft non-function or initial function failure post-transplantation. Liver enzyme release was significantly reduced with nitrite supplementation, the protective effect being more efficacious with longer cold preservation times. Liver histological examination demonstrated better preserved morphology, and less apoptosis with nitrite treatment and liver graft acute function post-transplantation was improved (Li *et al.*, 2012).

Lung

In a mouse model of pulmonary arterial hypertension, inhaled nebulized nitrite has been demonstrated to be a potent pulmonary vasodilator that can effectively prevent or reverse pulmonary arterial hypertension (Zuckerbraun *et al.*, 2011). Treatment with nebulized nitrite, either once or three times per week prevented the development of pulmonary arterial hypertension. Additionally, nitrite treatment 2 weeks into the hypoxic exposure, after the establishment of pulmonary hypertension, halted the progression of pulmonary hypertension and reversed increases in right ventricular pressure (Zuckerbraun *et al.*, 2010; 2011). Further experimental studies demonstrated that nitrite protects against ventilator-induced lung injury in rats (Pickerodt *et al.*, 2012).

Translation of those experimental results into the clinical practise is ongoing. In an actual phase II trial ('Inhaled nitrite in subjects with pulmonary hypertension', NCT01431313), researchers from the University of Pittsburgh, PA, USA investigate the effects of inhaled nitrite delivered in a dose-escalation manner on the change in pulmonary vascular resistance in subjects with pulmonary arterial hypertension undergoing right heart catheterization.

Kidney

In rats subjected to 60 min of bilateral renal ischaemia and 6 h of reperfusion sodium nitrite administered topically 1 min before reperfusion significantly attenuated renal dysfunction and injury, an effect that was abolished by pretreatment with a NO scavenger. Renal tissue homogenates produced NO from nitrite mainly through the activity of xanthine oxidoreductase (Tripatara *et al.*, 2007). Similarly, in mice subjected to bilateral renal ischaemia for 30 min and 24 h reperfusion, renal dysfunction, damage and inflammation were increased; these effects were all reduced following nitrite treatment 1 min prior to reperfusion. Within 1 min of reperfusion kidney nitrite levels were raised. The beneficial effects of nitrite were absent or reduced in mice deficient for endothelial NO synthase and nitrite treatment under these conditions even enhanced renal dysfunction (Milsom *et al.*, 2010).

Thus, sufficient metabolism of nitrite to NO appears to be a prerequisite to obtain kidney protection. In rat kidney, NO was generated from nitrite during following 40 min of ischaemia (which was independent from NO synthase activity and thus differs from mice) (Okamoto *et al.*, 2005). Not surprisingly then that in male rats undergoing unilateral nephrectomy followed by 45 min of ischaemia of the contralateral kidney, nitrite infusion before or during ischaemia did not attenuate the loss of brush border, the extent of tubular necrosis or red blood cell extravasation 24 and 48 h after acute renal injury. Interestingly, nitrate infusion appeared to worsen renal injury (Basireddy *et al.*, 2006). However, in rats subjected to unilateral nephrectomy and chronic high-salt diet, dietary nitrate prevented proteinuria and histological signs of renal injury (Carlstrom *et al.*, 2011). Moreover, signs of cardiac hypertrophy and fibrosis were attenuated (Carlstrom *et al.*, 2011).

Crush syndrome and shock

Limb muscle compression and subsequent reperfusion are the causative factors in developing a crush syndrome. In rats subjected to bilateral hind limb compression for 5 h followed by reperfusion for 0 to 6 h, nitrite administration reduced the extent of rhabdomyolysis markers such as potassium, lactate

dehydrogenase and creatine phosphokinase. Nitrite treatment also reduced the inflammatory activities in muscle and lung tissues, finally resulting in a dose-dependent improvement of survival rate (Murata *et al.*, 2012). Similarly, in a mouse shock model induced by a lethal tumour necrosis factor challenge, nitrite treatment significantly attenuated hypothermia, mitochondrial damage, oxidative stress and dysfunction, tissue infarction and mortality. Nitrite-dependent improvement in symptoms was not associated with inhibition of mitochondrial respiratory complex activity, but was dependent on the soluble guanylate cyclase system. Nitrite could also provide protection against toxicity induced by Gram-negative lipopolysaccharide (Cauwels *et al.*, 2009) (for further review please see Cauwels and Brouckaert, 2011).

Taken together, the nitrate-nitrite-NO pathway appears to play a crucial role in protecting the heart, vessel, brain, kidney and lung against ischaemia/reperfusion injury. Nitrite treatment may be advantageous in well-known NO deficient states such as, for example hyperlipidaemia. Timing and dose of nitrite application as well as the potential to convert nitrite to NO in the tissue are important to obtain a reduction in injury.

Conflict of interest

The authors declare that no conflicts of interest exist.

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