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Mechanisms impairing blood pressure responses to nitrite and nitrate

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ABSTRACT

Hypertension is a multifactorial disease associated with impaired nitric oxide (NO) production and bioavailability. In this respect, restoring NO activity by using nitrite and nitrate has been considered a potential therapeutic strategy to treat hypertension. This possibility is justified by the understanding that both nitrite and nitrate may be recycled back to NO and also promote the generation of other bioactive species. This process involves a complex biological circuit known as the enterosalivary cycle of nitrate, where this anion is actively taken up by the salivary glands and converted to nitrite by nitrate-reducing bacteria in the oral cavity. Nitrite is then ingested and reduced to NO and other nitroso species under the acid conditions of the stomach, whereas reminiscent nitrite that escapes gastric reduction is absorbed systemically and can be converted into NO by nitrite-reductases in tissues. While there is no doubt that nitrite and nitrate exert antihypertensive effects, several agents can impair the blood pressure responses to these anions by disrupting the enterosalivary cycle of nitrate. These agents include dietary and smoking-derived thiocyanate, antiseptic mouthwash, proton pump inhibitors, ascorbate at high concentrations, and xanthine oxidoreductase inhibitors. In this article, we provide an overview of the physiological aspects of nitrite and nitrate bioactivation and the therapeutic potential of these anions in hypertension. We also discuss mechanisms by which agents counteracting the antihypertensive responses to nitrite and nitrate mediate their effects. These critical aspects should be taken into consideration when suggesting nitrate or nitrite-based therapies to patients.

1. Hypertension: a major public health problem with complex pathophysiology

Cardiovascular disease is one of the most common causes of disability-adjusted life years and accounts for approximately 17 million deaths a year worldwide [1–3]. Complications of hypertension are responsible for 9.4 million deaths globally every year [3,4]. Studies predict that within the next 20 years, the number of subjects affected by hypertension will increase by 60% to a total of more than 1.5 billion individuals [5]. Despite the increasing public awareness of this disease and its complications, the rates of adequate blood pressure control (< 140/90 mmHg) among patients receiving antihypertensive treatment remain unsatisfactory [6]. The reasons for these disappointing outcomes are complex, but include the availability of only a relatively limited repertoire of antihypertensive treatments and the uncertainty about the etiology of hypertension [7].

Indeed, genetic predisposition and environmental factors such as excessive body weight, dietary sodium intake, and excessive alcohol consumption can increase the risk of developing of hypertension [8,9]. However, a clear single cause cannot be identified in the vast majority of individuals [7]. This obscure etiology is accompanied by a complex pathophysiology, which involves several mechanisms that probably act in concert, such as upregulation of the renin-angiotensin system, excessive activation of the sympathetic nervous system, and endothelial dysfunction [9]. In this respect, it is widely acknowledged that the integrity of the endothelium is essential for a healthy cardiovascular system, and dysfunctional endothelial cells trigger key mechanisms involved in the pathogenesis of many cardiovascular diseases, including hypertension [10]. Importantly, the endothelium controls the vascular tone, structure, and function by regulating the release of many vasoactive mediators including nitric oxide (NO), a small gaseous and lipophilic molecule produced by NO synthases or by recycling of its metabolites through a pathway now known as nitrate-nitrite-NO [11–16]. Here, we briefly review the biological functions of this pathway and the therapeutic potential of nitrate and nitrite in hypertension. Furthermore, this article will focus on major mechanisms

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impairing the cardiovascular benefits of nitrite and nitrate therapy in hypertension.

2. Nitric oxide plays a critical role in the control of blood pressure

The canonical synthesis of NO from L-arginine involves three different synthases: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) NO synthases [17]. While nNOS and eNOS are constitutively expressed and play critical roles in a plenty of physiological events, iNOS is mainly involved in host defense, airway epithelial NO production, and inflammatory processes [18,19].

In the vasculature, NO produced in endothelial cells diffuses across vascular smooth muscle cell membranes and promotes vasodilatation, and therefore this is a critical mechanism for the control of peripheral vascular resistance, an important determinant of blood pressure [20,21]. Additionally, NO production in the kidneys and the brain also contributes to control blood pressure. Indeed, NO plays an important role in renal perfusion and glomerular filtration in the kidneys by affecting the afferent arteriolar tone [22,23]. In the brain stem, neuronal NO affects vasomotor tone and blood pressure by modulating the central sympathetic neural outflow [24]. Taking into account all these physiological roles of NO in the blood pressure maintenance, it is now clear that impaired NO production or reduced NO bioavailability leads to hypertension.

It is important to note that NO plays important roles beyond activation of sGC, particularly mediated by S-nitrosation of proteins {Marozkina, 2012 #167}. This important post-translational modification may be very relevant in vascular regulation and is now being studied in hypertension. Recent studies showed impaired S-nitrosation in hypertension and strategies increasing this modification may result in antihypertensive responses [25]. Moreover, nitrosation affects signaling mediated by G protein-coupled receptors (GPCR) [26], as suggested by studies showing that it modifies the affinity of angiotensin II for angiotensin II type 1 receptors [27] and β-arrestin trafficking [28].

Many studies reported on the responses to NO synthase inhibitors, which cause hypertension and impair the cardiovascular homeostasis [29,30]. These findings agree with those reported with NOS3 knockout mice, which show increased blood pressure and enhanced susceptibility to stroke and other cardiovascular alterations [31]. Therefore, given the relevance of a fully functional NO formation and signaling in the control of blood pressure, restoring NO formation may be an effective approach to treat hypertension [32]. Indeed, a plenty of cardiovascular drugs are described to increase NO bioavailability, including antihypertensive drugs, statins, and antioxidants. For instance, angiotensin convert enzyme (ACE) inhibitors promote endogenous NO formation resulting from elevated levels of bradykinin (as a consequence of ACE inhibition), which activates bradykinin B2 receptor on endothelial cells and activates eNOS increasing NO production [33,34]. Accordingly, polymorphisms in the genes encoding eNOS and mediators of its signaling pathway affect the antihypertensive responses to ACE inhibitor enalapril [35–38], highlighting the relevance of NO biology on the effects of these drugs. In addition to ACE inhibitors, AT_1 receptor antagonists also increase the NO formation [39] and this effect is attributable to blockade of AT_1 receptors, thus allowing angiotensin II to activate the AT_2 receptors to produce vasodilatation by stimulating NO production [39]. Moreover, at least part of the antihypertensive effects of statins, such as atorvastatin and simvastatin, is associated with pleiotropic, cholesterol-independent effects including enhanced eNOS expression and NO formation [40,41]. Additionally, many drugs with antioxidant properties show protective effects against hypertension by reducing oxidative stress, thus improving NO bioavailability [42]. Taken together, this body of evidence suggests that hypertension can be counteracted by drugs improving NO signaling or restoring NO bioavailability.

3. Nitric oxide can be formed by NO synthase-independent pathways

As mentioned before, NO can be produced by nNOS, iNOS, and eNOS with oxidation of L-arginine to L-citrulline via NADPH and oxygen consumption [11,43]. Once produced, NO is readily oxidized to nitrite and nitrate [44,45], and therefore these anions have been employed as index of endogenous production of NO [11,43]. However, a large number of studies has now shown that nitrate and nitrite represent much more than inert end products of NO metabolism, as these two anions are physiologically recycled back to NO and other bioactive nitrogen oxides, therefore representing an important source of NO independent of NO synthases [13].

The recycling of nitrate and nitrite to NO (nitrate-nitrite-NO pathway) involves a complex biological circuit now known as the enterosalivary cycle of nitrate [13]. After the consumption of food rich in nitrate such as beetroot, lettuce, and spinach, this anion is rapidly absorbed in the upper gastrointestinal tract, and 24 h later approximately 75% of the nitrate is excreted unchanged in the urine [46–48]. Most of the residual nitrate is actively taken up by the salivary glands and secreted into the oral cavity, where commensal facultative anaerobic bacteria present in the dorsal part of the tongue promote the conversion of nitrate to nitrite by the action of nitrate reductases [13,48]. The salivary nitrite is then swallowed and readily protonated in the acidic gastric environment to produce nitrous acid, which decomposes further to generate NO and other bioactive nitrogen oxides [49,50]. Part of the salivary nitrite is not converted into NO in the stomach and is absorbed systemically. After absorption, nitrite can be reduced to NO by many proteins and enzymes in blood and tissues, such as hemeproteins, molybdenum-containing enzymes and mitochondrial enzymes [51].

The most studied hemeproteins that convert nitrite into NO are hemoglobin and myoglobin, which have nitrite-reductase activity in their deoxygenated states, as in their oxygenated states these proteins can oxidize nitrite to nitrate [52,53]. Indeed, studies have shown that intra-arterial infusion of nitrite at near-physiological concentrations promotes vasorelaxion through the reduction of nitrite to NO by deoxyhemoglobin in an arteriovenous gradient manner [54]. On the other hand, there is evidence showing that this effect is related to S-nitrosohemoglobin formation from nitrite by hemoglobin, resulting in the allosteric NO delivery to tissues [55,56]. Additionally, deoxymyoglobin also reduces nitrite to NO 30-times faster than deoxyhemoglobin, given its lower heme redox potential [57]. The physiological and pathophysiological relevance of this reaction were demonstrated by studies showing the beneficial effects of the reduction of nitrite by deoxymyoglobin on cardiac function, hypoxic-mediated vasodilatation and ischemia reperfusion cytoprotection [15,58,59].

Molybdenum-containing enzymes such as xanthine oxidoreductase (XOR) and aldehyde oxidase are also important nitrite-reductases [15,53]. XOR plays an important role in the metabolic pathway of purine degradation by promoting the oxidation of hypoxanthine to xanthine, and xanthine to uric acid [60,61]. Additionally, XOR catalyzes the reduction of oxygen to superoxide and hydrogen peroxide, and this mechanism can contribute to the nitroso-redox imbalance observed in several diseases, including hypertension [62–65]. Conversely, XOR can reduce nitrite to NO under certain circumstances, therefore contributing to the protective effects of this anion [66–68]. Furthermore, there are studies showing that XOR can also reduce nitrate to nitrite and then to NO [61,69,70]. Interestingly, considering that both nitrite and nitrate are substrates for XOR resulting in NO formation, it has recently been shown that nitrate may compete with nitrite for XOR, thus attenuating the vascular and hypotensive responses to nitrite [71]. By using either NADH or xanthine as reducing substrates, XOR promotes nitrite reduction to NO at the molybdenum site [72–74]. The significance of this reaction to the beneficial effects of nitrite has been shown in many diseases and conditions, such as pulmonary hypertension, myocardial ischemia-reperfusion damage, and arterial

hypertension [75–82]. In addition to XOR, aldehyde oxidase also reduces nitrite to NO by similar mechanisms [83], and the potential functional role of this enzyme was demonstrated in nitrite-induced hypoxic vasodilatation [84].

Regarding the role of mitochondria in the nitrite reduction, several studies have shown the nitrite reductase activity of terminal components of the mitochondrial oxidative chain: complex III, cytochrome *c* and cytochrome *c* oxidase (complex IV) [85–92]. Interestingly, the use of myxothiazol to inhibit complex III decreased NO formation from nitrite under anaerobic conditions, indicating a role for this complex in nitrite reduction [92]. In addition, studies have reported that liposomes containing cytochrome *c*, an inter-mitochondrial membrane protein that transfers electrons from complex III to complex IV, are able to reduce nitrite to NO in a reaction that depends on pH and nitrite levels and is modulated by cardiolipin [89–91]. Moreover, it has now been demonstrated that complex IV, the terminal part of the mitochondrial chain, can convert nitrite into NO in a reaction that depends on pH and cytochrome *c* [88]. Importantly, these mitochondrial components contribute to the protective effects of nitrite under certain conditions, such as ischemia/reperfusion and muscle metabolism [85–87].

4. The antihypertensive effects of nitrite and nitrate

4.1. Human and animal studies

Taking into account the importance of the nitrate-nitrite-NO pathway, many studies were carried out to show beneficial effects of nitrite and nitrate on cardiovascular diseases, particularly in hypertension [14]. The first study demonstrating antihypertensive effects of nitrite was carried out by Classen et al., who reported that a single oral dose of nitrite acutely decreased blood pressure in spontaneously hypertensive rats (SHR) [93]. Interestingly, this group observed that nitrite prevented further increases in blood pressure of SHR controls receiving water [94,95]. Similar effects were demonstrated in other experimental models of hypertension, such as L-NAME [75,96], two-kidney, one-clip (2K1C) [97–99], and deoxycorticosterone-salt (DOCA-salt) [100] models. Interestingly, the blood pressure lowering-effects of nitrite were also demonstrated in humans in a recent study reporting a reduction of about 6 mmHg in systolic blood pressure in normotensive subjects after acute oral administration of nitrite [101].

Likewise, the antihypertensive effects of nitrate were reported in clinical and experimental studies. Indeed, while these effects were observed in different animal models of hypertension, such as SHR [102], 2K1C [25,103], and L-NAME [104], these interesting findings have also been reproduced in humans. The first clinical evidence showing a blood pressure-lowering effects of nitrate was reported by Larsen et al., who observed decreases in diastolic pressure in healthy individuals after 3 days of sodium nitrate intake [105]. Accordingly, it was shown that consumption of nitrate-rich diet for 10 days promotes significant increases in plasma nitrite levels and lowers diastolic blood pressure by approximately 4.5 mmHg in healthy subjects [106]. Subsequent studies showed that nitrate is not only able to reduce blood pressure in healthy volunteers [107,108], but also to promote sustained antihypertensive effects in hypertensive patients [109]. Although XOR inhibition did not affect nitrite-dependent vasodilation in healthy individuals [110], this enzyme seems to contribute to the blood pressure lowering-effects of nitrate in hypertensive patients [77]. Despite these interesting findings, the mechanisms underlying the antihypertensive effects of nitrite and nitrate have not been completely elucidated.

4.2. Potential mechanisms

One important mechanism contributing to the antihypertensive effects of nitrite and nitrate is the attenuation of oxidative stress. Because oxidative stress decreases NO bioavailability, antioxidant effects exerted by these anions could increase NO bioavailability and contribute to its vasorelaxing and antihypertensive effects [100]. In fact, studies have shown that the antihypertensive effects of nitrite and nitrate are associated with reduced NADPH oxidase-derived oxidative stress [98,111–113]. This is particularly significant because NADPH oxidase plays a major role in the nitroso-redox imbalance commonly observed in hypertension [114]. Additionally, it was recently shown that nitrite also exerts antioxidant effects independent on its direct effects on blood pressure [115,116].

Another possible mechanism involved in the blood pressure responses is nitrite bioactivaction by XOR. In this respect, we have recently found that XOR contributes to the hypotensive effects of nitrite in 2K1C and L-NAME-induced hypertension [75,76] and that the antioxidant tempol increases XOR-mediated vascular responses to nitrite [76]. In addition, a recent study has shown that despite XOR upregulation observed in hypertensive mice, chronic dietary nitrate treatment decreases superoxide formation and enhances NO bioavailability in those mice, suggesting a switch of XOR function under nitrate supplementation [117]. This effect could be attributable to the fact that nitrate-derived nitrite may accept electrons at the molybdenum site of XOR resulting in decreased donation of electrons to oxygen. With fewer superoxide generation, NO scavenging is reduced and therefore its bioavailability should increase [117].

The mechanisms discussed above take into account the possibility that nitrite or nitrate could promote direct effects or form NO and affect critical mechanisms regulating blood pressure. However, dietary or exogenously administered nitrite promote gastric generation of several other bioactive nitrogen oxides such as the nitrosating species N_2O_3 and NO^+, which can react with thiol groups found in sulfur-containing glycoproteins in mucus or in glutathione produced by gastric epithelial cells and form S-nitrosothiols [96,118]. Interestingly, we have recently shown that the antihypertensive effects of nitrite orally administered were associated with increased circulating levels of S-nitrosothiols and enhanced vascular protein nitrosation, and both effects were attenuated by omeprazole, a proton pump inhibitor that increases gastric pH and therefore affects the chemical reactions that occur in the stomach [25,103]. In line with these findings, the inhibition of glutathione synthesis with increasing doses of buthionine sulfoximine attenuated the antihypertensive responses to oral nitrite in parallel with decreases in total plasma S-nitrosothiol concentrations in a dose-dependent manner [103]. Conversely, the intravenous administration of glutathione to increase thiol concentrations enhanced the antihypertensive responses to orally administered nitrite [103]. Collectively, these findings strongly suggest that the acidic conditions of the stomach favor the generation of NO-related species, particularly S-nitrosothiols, which contribute to the antihypertensive responses to oral nitrite [119].

5. Mechanisms precluding the antihypertensive effects of nitrite and nitrate

While there is now strong evidence supporting the antihypertensive effects of nitrite and nitrate, these responses can be dramatically impaired by certain dietary components and agents such as cigarette smoke, antiseptic mouthwash, proton pump inhibitors and XOR inhibitors. These agents are now known to disrupt the enterosalivary cycle of nitrate and the potential mechanisms underlying this effect will be discussed below.

5.1. Dietary and smoking-derived thiocyanate

As briefly described above, the remaining nitrate that is not excreted in the urine is actively taken up by the salivary glands and is then secreted into the oral cavity at levels that are 10–20-fold higher than in plasma [13]. While the exact mechanism by which nitrate is taken up is not completely understood, there is evidence that this process occurs in competition with other anions, including thiocyanate, which seems to have a higher affinity for transport into the salivary glands as compared

to nitrate [120]. Based on this evidence, recent studies have evaluated the effects of thiocyanate-rich compounds on the responses to nitrate [121,122].

While great amounts of nitrate are found in some vegetables, such as beetroot, lettuce and spinach, vegetables of the *Brassica* family such as cabbage, cauliflower and broccoli, have been described to increase serum thiocyanate [123]. This effect is explained by the fact that these vegetables are rich in glucosinolates, which are converted into thiocyanate by myrosinase during the mastication [124,125]. Taking this information into consideration, Dewhurst-Trigg et al. hypothesized that the co-ingestion of vegetables rich in glucosinolate/thiocyanate with nitrate-rich vegetables would result in impaired dietary nitrate metabolism and attenuation of the protective effects of this anion on the cardiovascular system [122]. Interestingly, it was found that the co-ingestion of nitrate-rich and thiocyanate-rich vegetables precludes the increases in salivary nitrite concentrations and the hypotensive effects observed with the same portion of nitrate-rich vegetables ingested without thiocyanate-rich vegetables [122]. However, contrary to authors' hypothesis, salivary nitrate levels increased with nitrate-rich vegetables ingestion independent of the co-ingestion of thiocyanate-rich vegetables, suggesting that dietary thiocyanate intake associated with consumption of glucosinolate-rich vegetables does not affect nitrate salivary uptake, but probably impairs the conversion of nitrate to nitrite in the oral cavity or promotes nitrite degradation (Fig. 1).

Cigarette smoking also increases the circulating levels of thiocyanate, as cyanide present in cigarette smoke is readily converted into thiocyanate by transsulfuration reactions catalyzed by the enzymes thiosulfate sulfotransferase and 3-mercaptopyruvate sulfurtransferase [121]. Consistently, it has been shown reduced salivary nitrate concentrations in cigarette smokers after nitrate ingestion, compared to non-smokers [126,127]. Indeed, the effects of nitrate ingestion on plasma and salivary nitrate, nitrite and thiocyanate levels, and on blood pressure were recently evaluated in cigarette smokers and non-smoking controls [121]. It was found that nitrate supplementation reduced blood pressure only in non-smokers and these findings were associated with higher plasma and salivary thiocyanate levels and attenuation of the increases in plasma and salivary nitrate concentrations in smokers compared to non-smokers [121]. This suggests that, unlike thiocyanate-rich vegetables, smoking-derived thiocyanate may counteract nitrate salivary uptake (Fig. 1). These conflicting findings could be explained by higher plasma and salivary thiocyanate concentrations observed in cigarette smokers as compared to the those found after consumption of thiocyanate-rich vegetables [121,122]. Notably, these findings suggest that in both conditions thiocyanate impairs nitrate metabolism and the blood pressure responses to this anion. Interestingly recent studies suggest that smokers have greatly impaired nitrite formation (< 20%) from nitrate in the mouth, thus showing that modifications in oral nitrate-reductase activity is an important pathophysiological mechanism [128].

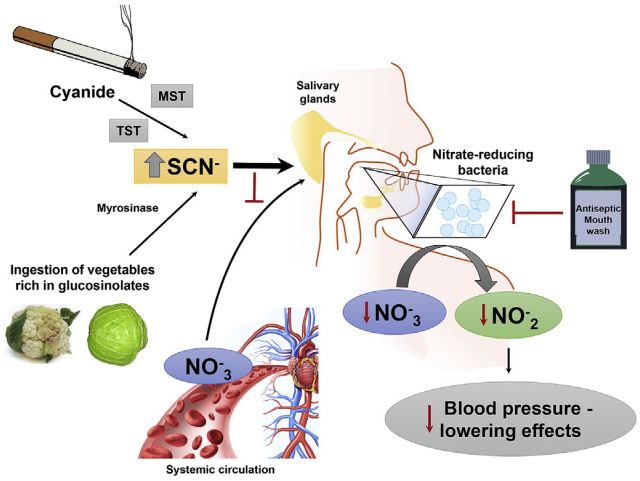

Fig. 1. Thiocyanate-rich compounds preclude the blood pressure responses to nitrate. The consumption of vegetables rich in glucosinolates increases thiocyanate (SCN^-) levels by the action of myrosinase, which converts glucosinolates into SCN^- during mastication. This mechanism is associated with impaired conversion of nitrate into nitrite in the oral cavity, thus attenuating the blood pressure responses to nitrate. Cigarette smoking also increases SCN^- concentrations, and this effect is attributable to cyanide present in cigarette smoke, which is readily converted into SCN^- by transsulfuration reactions catalyzed by the enzymes thiosulfate sulfotransferase (TST) and 3-mercaptopyruvate sulfurtransferase (MST). It has been suggested that nitrate uptake by salivary glands is blunted by cigarette smoking-derived SCN^-, resulting in decreased nitrate levels available to be reduced to nitrite, thereby precluding the hypotensive responses to nitrate. In addition to thiocyanate-rich compounds, antiseptic mouthwash impairs the blood pressure responses to nitrate treatment. Once nitrate is taken up by the salivary glands, it is converted into nitrite by nitrate-reducing bacteria in the oral cavity. This process is abolished by antiseptic mouthwash, which destroys those bacteria, leading to decreases in nitrite concentrations and counteracting the blood pressure-lowering effects of nitrate.

5.2. Antiseptic mouthwash

After nitrate is taken up by the salivary glands, the next step in the enterosalivary cycle is its conversion into nitrite by nitrate-reducing bacteria in the oral cavity. However, emerging evidence has demonstrated that this process is abolished by antiseptic mouthwash, decreasing salivary nitrite levels and counteracting the health benefits of nitrate therapy [25,107,129–135] (Fig. 1). In fact, the presence of commensal facultative anaerobic bacteria in the dorsal part of the tongue is essential to human nitrate reduction because mammalian cells are not capable of reducing this anion, thus suggesting a symbiotic relationship [13]. In this regard, it has been described more than 300 different bacterial species living in the human oral cavity, many of those with effective nitrate reductase activity, particularly *Veilonella* and *Actinomyces* spp [136,137]. These bacteria utilize nitrate as a terminal electron acceptor to form adenosine-5'-triphosphate (ATP) during respiration, thus promoting the reduction of salivary nitrate to nitrite by the action of nitrate reductases [138,139]. Interestingly, nitrite is formed in the oral cavity in proportion to the nitrate levels [140,141].

While the oral microbiota plays a major role in the enterosalivary cycle of nitrate, it has been estimating that antiseptic mouthwash eliminates about 94% of the salivary nitrate-reducing bacteria and reduces the rate of nitrate reduction by saliva by more than 85% [130]. The relevance of this effect to the cardiovascular health has been shown both in clinical and experimental studies. Indeed, antiseptic mouthwash administration in rodents treated with nitrate decreased plasma nitrite concentrations and blunted the effects of nitrate on blood pressure [25,129]. In addition, antiseptic mouthwash attenuated the increases in S-nitrosothiols and vascular S-nitrosation in rats receiving nitrate [25]. Interestingly, these effects were circumvented by oral nitrite treatment [25]. In agreement with these experimental findings, clinical studies have shown that plasma and salivary nitrite concentrations decreased after a dietary nitrate load in healthy subjects using antiseptic mouthwash [132,135]. In addition, it has been demonstrated that the antihypertensive responses to nitrate are impaired or completely eliminated with antiseptic mouthwash in humans [107,134]. Interestingly, the use of antiseptic mouthwash reduced both oral and plasma nitrite levels and resulted in a sustained increase in blood pressure in humans [131,133], suggesting that recycling of endogenously generated nitrate by oral bacteria has a physiological impact in the control of blood pressure.

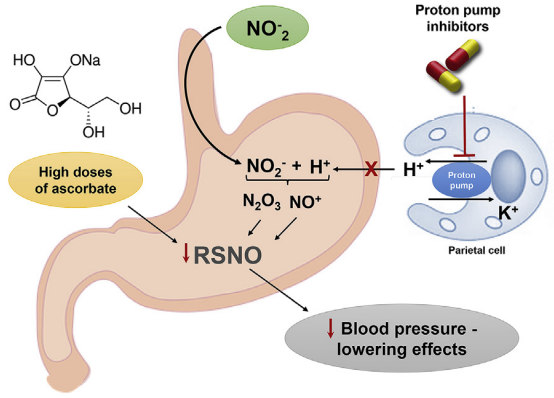

Fig. 2. Proton pump inhibitors (PPI) and high doses of ascorbate blunt the antihypertensive effects of nitrite. Nitrite protonation in the acid environmental of the stomach leads to the formation of nitric oxide and other nitrogen oxides, such as NO^+ and N_2O_3, thereby yielding to the generation of S-nitrosothiols (RSNO). These compounds have been associated with the antihypertensive effects of oral nitrite. However, because PPI inhibit acid secretion, the formation of RSNO by nitrite is counteracted with use of these drugs, resulting in impaired antihypertensive effects. Additionally, high concentrations of ascorbate have been shown to destroy RSNO, which also leads to impaired antihypertensive responses to nitrite.

5.3. Proton pump inhibitors

Proton pumps inhibitors (PPI) are over-the-counter drugs widely used worldwide, with more than $13 billion in sales each year [142]. They are the most effective gastric acid-suppressing agents for the treatment of several acid peptic diseases, such as peptic ulcer disease, gastroesophageal reflux disease, Zollinger-Ellison syndrome and idiopathic hypersecretion [143]. PPI bind to the gastric H^+/K^+-ATPases (proton pumps), preventing H^+/K^+ exchange within secretory canaliculi and thereby inhibit gastric acid secretion independently of the origin of the secretory stimuli [143]. Considering this mechanism of action, some studies have tested the hypothesis that PPI modify the effects of nitrite or nitrate [50,101,103,144]. This is because, as briefly discussed above, nitrite can produce NO and NO-related species under the acidic environmental of the stomach [13] (Fig. 2).

Indeed, the stomach has been considered a bioreactor in which NO and many other bioactive nitrogen oxides can be formed from oral nitrite [118]. In the acidic gastric milieu, nitrite is protonated to nitrous acid (HNO_2), which then decomposes into NO and other nitrosating species including N_2O_3 and NO^+ [96]. Interestingly, these nitrite-derived nitrosating species promote the formation of S-nitrosothiols, which have been associated with protective effects against cardiovascular and metabolic diseases, given that they maintain NO bioactivity by acting directly as a relatively stable NO donor or by transnitrosation reactions to produce new S-nitrosothiols [145].

The first evidence that proton PPI attenuate the gastric nitrite bioactivation was demonstrated by Lundberg et al. in a study showing that nitrite yields to gastric nonenzymatic NO formation, which is impaired by the PPI omeprazole [50]. The relevance of this effect on blood pressure has been consistently reported in animal and human studies. In this respect, pretreatment with omeprazole blunted the acute hypotensive effects of oral nitrite in L-NAME hypertensive rats, but not the hypotensive responses to nitrite administered intravenously, indicating that gastric pH has a major role in the blood pressure responses to oral nitrite [144]. In addition, omeprazole impaired the chronic antihypertensive effects of orally administered nitrite and nitrate in renovascular hypertensive rats and these effects were associated with reduced S-nitrosothiols levels, suggesting an involvement of these molecules in the observed effects [103]. In agreement with these experimental findings, a subsequent study enrolling healthy subjects treated with nitrite observed that pretreatment with the PPI esomeprazole precludes the hypotensive responses to orally, but not intravenously, administered nitrite [101]. Despite the potential role of S-nitrosothiols in the impairment of the antihypertensive effects of nitrite and nitrate by PPI, further studies are required to examine the exact mechanisms underlying those effects.

Importantly, besides its effects on blood pressure responses to nitrite and nitrate, the use of PPI has also been associated with increased risk of cardiovascular events and endothelial dysfunction [146–149]. Therefore, additional studies are necessary to evaluate the possible link between the effects of PPI on cardiovascular homeostasis and those related to impaired enterosalivary cycle of nitrate.

5.4. Ascorbate and other reducing compounds

In addition to gastric pH, the nitrite-induced generation of NO and nitroso compounds is influenced by ascorbate secreted together with gastric juice [150]. In agreement with *ex vivo* studies [151], it was recently reported that low doses of ascorbate increase the production of S-nitrosothiols promoted by oral treatment with nitrite and improve the antihypertensive responses to this anion [152], probably due to greater amounts of nitrite-derived NO formed in the gastric lumen, which diffuses towards gastric epithelial cells, and then is oxidized to produce nitrosating species such as N_2O_3. Interestingly, these findings are in line with the effects of tempol, an antioxidant that favors the gastric nitrite reduction and enhances the hypotensive responses to oral nitrite [153]. Additionally, it has been described that the consumption of foods and beverages rich in polyphenols, such as red wine, apple and black tea enhance the nitrite reduction in the stomach [154–156] and this interaction may enhance the blood pressure-lowering effects of nitrate [157]. On the other hand, high concentrations of ascorbate are associated with significant destruction of nitrite-derived S-nitrosothiols [158,159], and reduction of the antihypertensive effects of orally administered nitrite [152] (Fig. 2). Therefore, although the administration of low doses of ascorbate and other reducing agents such as tempol and polyphenols may improve the effects of oral nitrite, the use of ascorbate at very high concentrations may counteract this protective mechanism. Additional studies are required to explore the role of S-nitrosothiols or other potential mechanisms underlying the effects of reducing agents on the antihypertensive responses to nitrite and nitrate.

5.5. Xanthine oxidoreductase inhibitors

The ingested nitrite that escapes gastric reduction is absorbed systemically and can reach the vasculature and be converted into NO by XOR [51]. Therefore, XOR inhibitors can inhibit the formation of NO from nitrite by this mechanism [117,132,160–162]. Allopurinol and febuxostat are XOR inhibitors commonly prescribed to treat gout, which is a common rheumatic disorder involving inflammation triggered by the deposition of urate crystals secondary to chronic hyperuricaemia [160]. Allopurinol is a hypoxanthine analogue that reacts with XOR at the molybdenum site to form oxypurinol, which binds to XOR by direct coordination to molybdenum, thus preventing enzyme interaction with substrate [163]. Its use for gout therapy was approved by the FDA in 1966 and it remains the main treatment for hyperuricemia [164]. Febuxostat is another XOR inhibitor approved by FDA for gout treatment [165]. Its reaction with XOR is confined to electrostatic interactions with amino acid residues located very close to molybdenum, leading to an effective block of substrate access to the active site, which results in increased potency compared to allopurinol [165,166].

While XOR inhibitors are useful in gout treatment, the beneficial effects of nitrite and nitrate mediated by XOR may be cancelled with the use of XOR inhibitors [161] (Fig. 3). Indeed, it has been reported that allopurinol impairs the cardiovascular beneficial effects of nitrite and nitrate in different diseases and conditions [81,162]. With respect

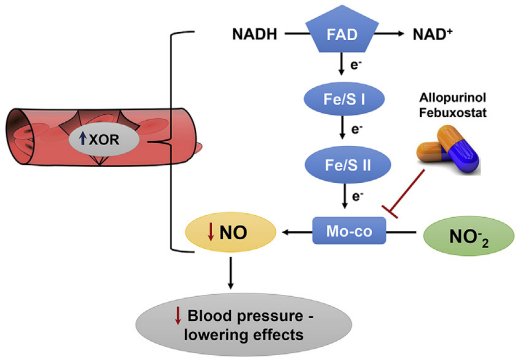

Fig. 3. Xanthine oxidoreductase (XOR) inhibitors counteract the anti-hypertensive effects of nitrite and nitrate. XOR consists of four redox centers: one FAD and two Fe/S clusters and a molybdenum cofactor (Mo-co). Nitrite reduction to nitric oxide occurs at Mo-co, but this event is prevented by XOR inhibitors such as allopurinol and febuxostat, which attenuate the anti-hypertensive effects of nitrite and nitrate.

to hypertension, many studies have consistently shown that treatment with allopurinol, oxypurinol or febuxostat prevents the blood pressure-lowering effects of nitrite and nitrate in rodents. In fact, it was shown that allopurinol impairs the acute hypotensive effects of nitrite in SHR and erythrocytic XOR was implicated in these effects [77]. Similar effects were found in L-NAME hypertensive rats pretreated with allopurinol, but in this study vascular XOR was associated with impaired blood pressure responses to nitrite [75]. Accordingly, we recently found increased vascular XOR activity in 2K1C hypertensive rats and treatment with oxypurinol impaired the vasodilatory and hypotensive responses to nitrite in those rats [76]. Moreover, a recent study observed that febuxostat blunts the antihypertensive effects of dietary nitrate in eNOS deficient mice [117]. It should be noted that the route used to administer nitrite (oral *versus* intravenously) may modify the interference of XOR inhibitors in the blood pressure responses to nitrite. Taken together, these findings suggest that it is possible that the wide use of XOR inhibitors such as allopurinol and febuxostat may suppress the beneficial effects of nitrite and nitrate. Clinical studies are necessary to examine this possibility.

6. Concluding remarks

Given the indisputable role of NO in the control of blood pressure, strategies to restore NO production and signaling may be effective in the hypertension therapy. In this respect, it is now clear that nitrite and nitrate are more than inert metabolites of NO and can be recycled back to this molecule by enzymatic and non-enzymatic mechanisms. This process involves the uptake of nitrate by salivary glands, the conversion of nitrate into nitrite by nitrate-reducing bacteria in the oral cavity, the reduction of nitrite to NO and other bioactive species under the acid conditions of the stomach, and the enzymatic NO formation from nitrite by nitrite-reductases in tissues. While restoring NO activity by using nitrite and nitrate has been considered a potential therapeutic approach to treat hypertension, the use of agents disrupting the enterosalivary cycle of nitrate may cancel the antihypertensive effects of these anions. The mechanisms underlying these detrimental effects are related to the particular step in the enterosalivary cycle of nitrate disrupted by each agent. Nitrate uptake by salivary glands is attenuated by cigarette smoking-derived thiocyanate; reduction of nitrate to nitrite in the oral cavity is impaired by antiseptic mouthwash; the gastric formation of NO and other bioactive nitrogen oxides from nitrite is blunted by PPI or by high concentrations of ascorbate; nitrite reduction by XOR is prevented by allopurinol and febuxostat. Altogether, these recent findings show evidence suggesting that nitrite and/or nitrate treatment would not be effective to treat hypertension in patients exposed to these interfering factors. The understanding of the mechanisms impairing blood pressure responses to nitrite and nitrate is critical to prevent potential therapeutic ineffectiveness with the use of this promising strategy in the hypertension treatment. These critical aspects should be taken into consideration when suggesting nitrate or nitrite-based therapies to patients and when interpreting the responses to this therapy in clinical studies.

Conflicts of interest

The authors report no conflict of interest.

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