Chapter 3
Nitrite-Dependent Nitric Oxide Production Pathway: Diversity of NO Production Systems

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Abbreviations

AsA	Ascorbate
DHA	Dehydroascorbate
dNiR	Dissimilatory nitrite reductase
eNOS	Endothelial nitric oxide synthase
HO1	Heme oxygenase-1
iNOS	Inducible nitric oxide synthase
MDA	Monodehydroascorbate
NADPH	Nicotinamide adenine dinucleotide phosphate
NiR	Nitrite reductase
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitrate reductase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
US EPA	United States Environmental Protection Agency
XOR	Xanthine oxidoreductase

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H. Tsukahara and K. Kaneko (eds.), *Studies on Pediatric Disorders*, Oxidative Stress in Applied Basic Research and Clinical Practice, DOI 10.1007/978-1-4939-0679-6_3,

3.1 Introduction

Nitrite has long been recognized for its toxicity as the causative agent of methemoglobinemia, also called cyanosis or "blue baby syndrome." The condition was first reported by pediatric resident Hunter Comly [1] in rural areas having nitrate-contaminated groundwater. The ingested nitrate is reduced by bacteria of the gastrointestinal tract to nitrite. Once in the bloodstream nitrite oxidizes hemoglobin (Fe^{2+}) to methemoglobin (Fe^{3+}), giving rise to the characteristic blue-hued skin. Infants under 6 months of age are particularly prone to methemoglobinemia since they have a low activity of circulating methemoglobin reductase [2]. Later, the use of nitrite in curing meat drew concern due to the formation of mutagenic nitrosamines during cooking [3] although extensive subsequent animal and epidemiological studies have not indicated that nitrite in meat leads to carcinogenesis [4].

More recently we have come to appreciate other, often beneficial, aspects of nitrite as a nitric oxide (NO) precursor in a range of physiological functions, which have recently been reviewed by Lundberg et al. [4] and are covered in Chap. 2 of this volume. At room temperature and pressure, pure NO is a gaseous free radical. However, as a biological mediator, it is an aqueous solute that exhibits versatile functions not only in humans but probably in all organisms [5–7].

Figure 3.1 illustrates major sources of nitrite for NO synthesis in humans. Unlike L-arginine-dependent NO production, which is a substrate-specific reaction in a local microenvironment, NO production from nitrite involves multiple routes

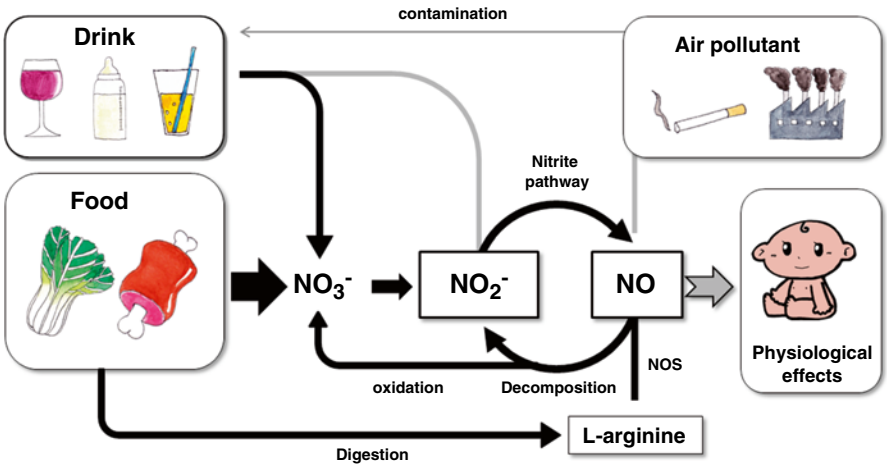

Fig. 3.1 Exogenous and endogenous sources of nitrite. Physiologically available nitrite is supplied mostly as nitrate contained in dietary foods and drinks. Leafy vegetables and seaweeds are major sources of nitrate. Nitrite is also obtained from processed food such as meat products and, in some cases, contaminated groundwater. In addition to these exogenous sources, nitrite can be endogenously synthesized as an oxidation product of NO produced by NO synthase (NOS). The relationship between nitrite and NO shows circularity

(exogenous and endogenous) and mechanisms (chemical and enzymatic). Nitrite and nitrate are oxidation products of NO synthesized by nitric oxide synthase (NOS). The relationship between nitrite and NO shows a circularity in terms of NO production, giving rising to the "chicken and egg" issue [8]. The multiplicity and circularity characteristic of nitrite may lead current researchers to a state of confusion. To help integrate new findings into our body of knowledge, in this chapter we summarize our current understanding of this alternative route for NO production from a biological perspective.

3.2 Dietary Sources of Nitrate and Nitrite

Nitrite is an inorganic nitrogen compound that exists as ionic form in a solution (NO_2^-; the p*K*a of nitrous acid, HNO_2, is approximately 3.2). Although nitrite is an intermediate metabolite in the plant nitrate assimilation pathway that synthesizes amino acids, nitrite occurs only at very low to undetectable levels in plants [9, 10]. Thus, natural foods such as leafy vegetables and seaweeds provide little nitrite but high levels of nitrate [11], a possible health benefit of Japanese and Mediterranean diets [12–14]. Bioavailable nitrite is mostly supplied exogenously via nitrate contained in foods but nitrite itself may also be obtained from processed foods or cured meats.

3.2.1 Vegetables

Vegetables are major dietary sources of nitrate. More than 80 % of nitrate ingested can be attributed to vegetables [11]. Absorbed by roots and translocated through the plant vascular system, nitrate can become highly concentrated in plant tissues, even to the point of crystallization [15]. Nitrate concentrations vary widely between plant species [9, 10] and even within the same tissue types of the same species, most likely due to differences in nitrate fertilization during cultivation [16]. A recent survey found a hundredfold variation in average nitrate levels in cooked greens, ranging from 4,850 mg kg^{-1} in English spinach to 48 mg kg^{-1} in iceberg lettuce [9].

3.2.2 Meat Curing Ingredients

Nitrate and nitrite are important meat preservatives, having a long history going back as far as 5,000 years throughout civilizations worldwide [17]. The original "curing" agent saltpeter (potassium nitrate) relies on bacterial conversion of nitrate to nitrite within the meats [18]. Since the late nineteenth century sodium nitrite instead of nitrate has been directly used to certain meat products, such as ham, bacon, and sausage [19]. The U.S. Federal Code of Regulations (21CFR

172.170 and 172.175) requires levels of nitrate and nitrite not to exceed 365 and 146 mg kg^{-1} in the finished product, respectively, similar to regulations prescribed by other countries.

3.2.3 *Drinking Water*

Water supplies, especially near agricultural areas, are often contaminated with nitrate derived from crop fertilizer. In many countries the content of nitrate in drinking water is regulated. A limit of 44 mg nitrate L^{-1} has been set by the United States Environmental Protection Agency (US EPA) for drinking water, for example [20]. In addition to direct contamination, nitric oxides (NOx) in industrial air pollution indirectly increase nitrate and nitrite content of stock water (Fig. 3.1).

3.2.4 *Human Breast Milk*

In the context of pediatrics, milk should be discussed separately from drinking water. Milk is a highly evolved product designed by millions of years of natural selection to maximize survival of newborns. Mammals secrete nitrate into milk at levels ranging from 1.4 to 11.2 mg nitrate L^{-1} [11, 21, 22]. The highest levels of nitrate are released 2–5 days postpartum. Nitrite is typically only found in milk at barely or non-detectible levels except early after birth, with reports of 2.1 mg L^{-1} 2–5 days postpartum and 0.8 mg L^{-1} 1–3 days postpartum [23]. The provisioning of nitrate and nitrite in milk at levels substantially exceeding that found in plasma [11] implies a physiological benefit of these components. The inability to produce nitrite due to the low level of nitrate reductase (NR) activity in the mouths of infants [24] explains the inclusion of nitrite in early breast milk. Mammals also secrete into milk substantial nanomolar levels of phytochemicals, including flavonoids, which are reported to enhance bioconversion of nitrite to NO [25].

3.3 Nitrite as a Degradation Product of NO, an Endogenous Source of Nitrite

The presence of endogenous nitrite formation in human body was first noted by Mitchell and coworkers a century ago [26] but the endogenous source of this nitrite remained unknown until the 1980s [27]. Up until then it had been known that exogenously supplied nitrate can be converted to nitrite by the nitrate reductase activity of oral bacteria (3.1).

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \tag{3.1}$$

In addition to exogenous dietary sources of nitrate intake, recent studies have confirmed that nitrite can be generated through the oxidation of NO produced by the L-arginine-dependent NOS pathway (see Chap. 2), thereby explaining the mechanism for endogenous nitrite generation that had been unknown for over 60 years.

In the presence of O_2, NO can be oxidized (3.2).

$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \tag{3.2}$$

Some proteins such as ceruloplasmin are reported to facilitate oxidation of NO to nitrite [28]. Nitrite is formed stoichiometrically by autoxidation of NO, thereby allowing the widely available Griess assay to be applied for measurement of NOS activity [29].

3.4 Nitrite Pathway: An NOS-Independent NO Production System

Recent studies have provided strong evidence for the presence of L-arginine-independent NO-generating systems that are as important as the NOS system, especially under hypoxic conditions. Contrary to the view of nitrite simply as a "waste" product of the NOS reaction, it is now understood that nitrite is an alternative source of NO in our body. For simplification we will refer to this newly recognized NOS-independent NO-generating pathway hereafter as "the nitrite pathway" [8].

3.5 Chemical Mechanisms for NO Production from Nitrite

In contrast to enzymatic NO synthesis with L-arginine, the basic chemistry of NO production from nitrite is rather simple: one-electron reduction of nitrite produces NO. Because of this simplicity, multiple routes and mechanisms, including enzymatic and nonenzymatic (or chemical) reactions, are possible.

3.5.1 Acid (Proton)

Nitrite can be converted to NO without the aid of enzymes, an important feature not observed of L-arginine-dependent NO production. Nitrite ion (NO_2^-) exists in equilibrium with its conjugate acid nitrous acid (HNO_2) (3.3). Formation of the HNO_2 anhydride gives dinitrogen trioxide (N_2O_3) (3.4) and NO is then generated through decomposition of N_2O_3 (3.5).

$$NO_2^- + H^+ \leftrightarrow HNO_2 \tag{3.3}$$

$$2HNO_2 \leftrightarrow N_2O_3 + H_2O \tag{3.4}$$

$$N_2O_3 \leftrightarrow NO + NO_2 \tag{3.5}$$

Spontaneous NO production from nitrite is prominent under acidic conditions due to the HNO_2 p*K*a of 3.1–3.5. In mammalian systems, gastric fluid fits this requirement. Within hours after the clearance of slightly alkaline ammonic fluid from the stomach its contents become acidified to pH 1–3, sufficiently low to drive nonenzymatic production of NO from nitrite [11]. It needs to be stressed, however, that the formation of N_2O_3 from HNO_2 is second order and will only be relevant under conditions of high NO_2^- and H^+ concentrations. Since spontaneous NO production from nitrite under acidic conditions is relatively slow [30], it may have minimal physiological relevance.

3.5.2 Reductants (Antioxidants)

A large amount of NO can be generated by the reduction of acidified nitrite solution in the presence of reductants. It has long been known that NO can be generated by the reduction of nitrite in the presence of iodide or ferrocyanide in acidified solution and historically this reaction was used to quantify nitrate in urine [26]. This chemical production of NO is almost stoichiometric, thereby being widely used as a convenient method to calibrate NO concentrations in a solution even today [31]. A more biologically relevant reductant, ascorbate (AsA), an essential antioxidant in vegetables [32], has also been reported to induce chemical NO production from nitrite [33, 34]. One-electron reduction of nitrite produces NO and dehydroascorbate (DHA) through the formation of monodehydroascorbate (MDA) radical:

$$2HNO_2 + AsA \rightarrow 2NO + DHA + 2H_2O \tag{3.6}$$

Figure 3.2a shows pH dependence of chemical NO production from nitrite with potassium iodide (KI) and AsA. Both dependences clearly follow the Henderson–Hasselbalch equation when the p*K*a of HNO_2 is assumed to be 3.2 [6], indicating the requirement for acidic conditions (or HNO_2). In daily diets, phytochemicals such as polyphenols of vegetables are also strong antioxidants [35, 36] and can act, in addition to AsA, to reduce nitrite to NO [37].

3.6 Enzymatic Mechanisms for NO Production from Nitrite

Under hypoxic conditions, with lower competition for binding sites by O_2, enzymatic reduction of nitrite to NO takes place more readily thereby allowing for production of NO without the need for the NOS activity, which requires O_2. Accordingly, organisms commonly recognize NO as a signal of hypoxic conditions [38, 39]. Previously, enzymatic NO production from nitrite was only appreciated in denitrifying and nitrifying bacteria (Fig. 3.3) with little seeming relevance to human physiology. Recent investigations have revealed that multiple enzymes or proteins in mammalian systems are also capable of NO production from nitrite. Interestingly, many of these are well-known classical enzymes and proteins.

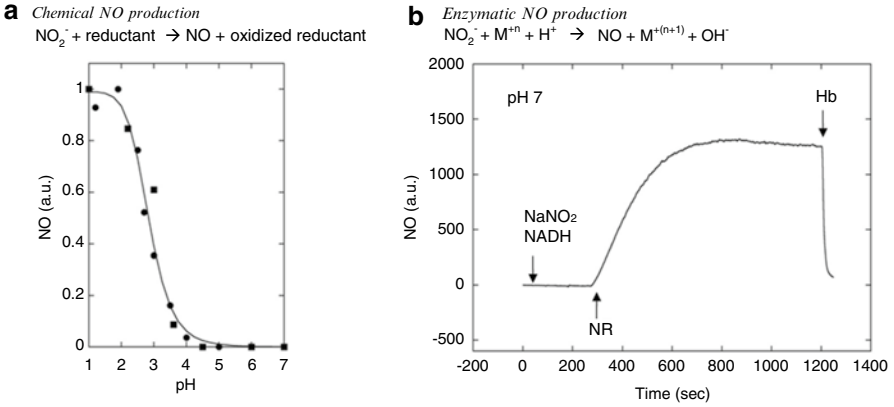

Fig. 3.2 NO production from nitrite. Chemical NO production with nitrite strongly depends on pH (**a**). Since HNO_2 (nitrous acid) is required to produce NO, the pH dependence follows the Henderson–Hasselbalch equation. In the presence of a reductant such as ascorbate, the rate of NO production becomes much faster. Whatever the reductants (*filled circle*, ascorbate; *filled square*, KI), the pH dependence shows identical *curves*. At a neutral pH, chemical NO production is not significant even in the presence of the reductant NADH (**b**). By adding enzyme, e.g., nitrate reductase (**b**), nitrite is rapidly converted to NO in a solution. NO was electrochemically detected with an NO-specific electrode. *M* metal-containing enzyme, *NR* nitrate reductase, *Hb* bovine hemoglobin. The graphs are redrawn after Yamasaki (2000) [6] and Yamasaki and Sakihama (2000) [101] for (**a**) and (**b**), respectively

3.6.1 Hemoglobin and Myoglobin

During hypoxia, deoxyhemoglobin in erythrocytes [40] and deoxymyoglobin in myocardial cells [41] can reduce nitrite to NO to form methemoglobin and metmyoglobin, respectively. In isolated erythrocytes the intracellular reduction of nitrite has been shown not to be limited by transport of nitrite across the membrane, which occurs through a combination of simple diffusion of HNO_2 and facilitated diffusion [42]. Recently, similar NO-producing nitrite reductase (NiR)-like activities have been found in neuroglobin [43], cytoglobin [44], and plant hemoglobins [45]. It appears that such NiR-like activity is conserved throughout these hemoglobin families [46] but it remains to be convincingly demonstrated whether the kinetic constraints on the reaction observed in purified systems are overcome under physiological conditions.

3.6.2 Xanthine Oxidoreductase

Xanthine oxidoreductase (XOR) functions in the catabolism of purines in organisms across the phylogenic spectrum. Under hypoxic conditions XOR can act as an NiR [47, 48], utilizing xanthine as an electron donor and at nitrite concentrations

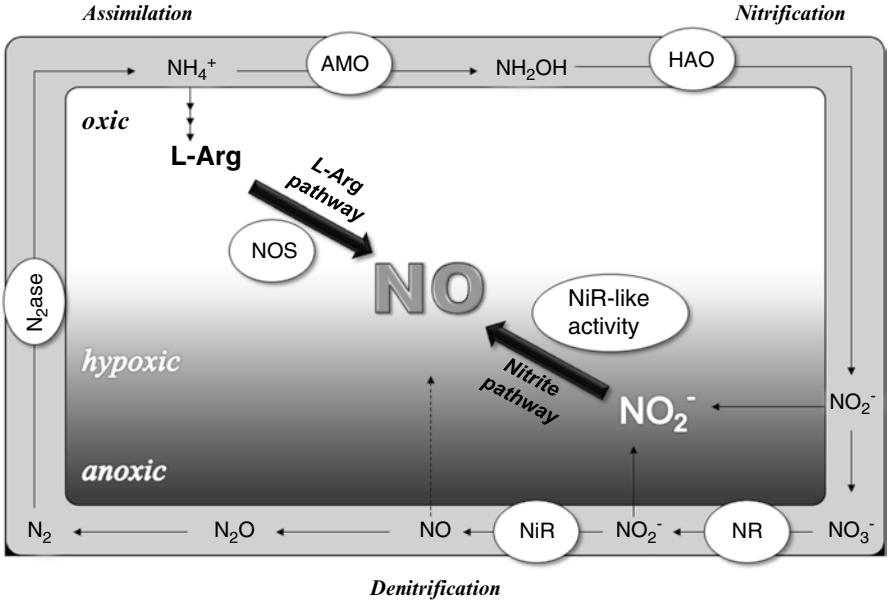

Fig. 3.3 Two pathways for enzymatic NO production in the bacterial nitrogen cycle. Various bacterial metabolisms can perform all the steps of the biological nitrogen cycle, processing nitrogen through oxidation states ranging from 3− (e.g., ammonia) to 5+ (e.g., nitrate). Nitrate is formed in nature primarily by nitrification, the sequential oxidation of ammonia by ammonia- and nitrite-oxidizing bacteria as a means to obtain energy. Under anaerobic conditions some bacteria are capable of inducing protein complexes that act in series, in a process termed denitrification, to reduce nitrate to N_2 gas. A portion of the nitrogen flowing through both nitrification and denitrification is released as NO. In animals, some nitrate ingested from foods is converted only to nitrite by bacteria in the mouth and gut. Nitrite produced from nitrate can be used to form NO either by nonenzymatic (chemical) or by enzymatic mechanisms using reductants. This nitrate–nitrite–NO pathway (nitrite pathway) complements the L-arginine-dependent NO production pathway driven by the NOS enzymes. An important difference between the two NO-producing systems is the oxygen requirement. Selected enzymes shown (in *ovals*) are: *N_2ase* nitrogenase, *AMO* ammonia monooxygenase, *HAO* hydroxylamine oxidoreductase, *NiR* nitrite reductase, *NR* nitrate reductase, *NOS* nitric oxide synthase

(5–40 μM) that are typically found in myocardial tissue [49]. Together, both XOR and deoxymyoglobin appear to be responsible for the majority of nitrite-responsive vasodilation activity although their apparent relative contributions vary among studies [41, 50, 51].

3.6.3 Mitochondrial Electron Transport

Under hypoxic conditions the electron transport systems of mitochondria throughout the Eukarya domain show evidence of their denitrifying heritage by reducing nitrite to NO at complex IV [52, 53], as well as at complex III and cytochrome *c*

[54]. Exposure to nitrite also dampens mitochondrial respiratory activity during anoxia, presumably by *S*-nitrosation of complex I, and thereby lowers production of damaging reactive oxygen species (ROS) by mitochondria during reperfusion [13].

3.6.4 Dissimilatory Nitrite Reductase

NO is a product of dissimilatory nitrite reductase (dNiR) in both denitrifying bacteria and ammonia-oxidizing bacteria [55]. NO released by these bacterial pathways has local effects on neighboring organisms (see below) and constitutes a major biogeochemical source of nitrogen oxides that return to terrestrial and marine environments [56]. Only a fraction of the NO formed in denitrifying bacteria diffuses from cells. The majority is a substrate for NO reductase, which is the evolutionary antecedent of the terminal oxygen-reducing cytochrome *c* oxidase in mitochondria and its bacterial relatives [52]. In the ancient atmosphere, before cyanobacterial photosynthesis generated the oxic atmosphere, NO was probably the predominant terminal electron acceptor for respiratory bacteria [57]. Thus, modern metabolic strategies for handling O_2, including cytochrome oxidases and globin proteins, are adaptions built on an NO-metabolizing framework [58].

3.6.5 Nitrate Reductase

Nitrate reductase (NR) can reduce nitrite to NO in plants [59, 60], fungi [6], and bacteria [61, 62] under high nitrite concentrations. Analogous to leukocyte inducible NOS (iNOS) activity in the innate immunity of animals [63], production of NO by nitrate reductase in plants stimulates expression of pathogen defense genes [64–67]. Using nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor, molybdenum (Mo^{2+}) at the reaction center appears to reduce nitrite, similar to the reaction catalyzed by XOR [68].

3.7 Other Key Reactions of Nitrite

It is evident that nitrite serves as an alternative NO source. Because of the unique chemistry of nitrite, some reactions should be considered separately from those of NO.

3.7.1 HNO Production from Nitrite

HNO, or nitroxyl, the one-electron reduction product of NO, reacts with heme proteins to exert important physiological effects that are unique from those of NO [69–71]. HNO is produced naturally within organisms but the biological

mechanism of its formation remains unknown largely due to its short half-life. Based on the results of in vitro experiments it is conceivable that endogenous production of HNO could proceed by the reaction of nitrite with AsA [72] and subsequent decay of *O*-nitroascorbate to DHA and HNO [73]. Thus, nitrite may also act as a precursor to HNO in addition to NO.

3.7.2 Nitration and Nitrosation

The reaction between NO and the ROS superoxide (O_2^-) produces peroxynitrite ($ONOO^-$), a potentially toxic reactive nitrogen species (RNS). In the presence of catalytic CO_2, $ONOO^-$ nitrates the amino acid tyrosine to form nitro-tyrosine [74]. Nitration ($-NO_2$) of biomolecules can also involve nitrite itself through distinctive routes and mechanisms [75]. In macrophages, myeloperoxidase catalyzes the formation of nitro-tyrosine with nitrite and hydrogen peroxide (H_2O_2) [76].

In the oxidation of NO, N_2O_3 is generated. Reactions between N_2O_3 and biomolecules including amino acids result in nitrosation ($-NO$) of the residues at nucleophilic sites. The reaction is catalyzed by thiocyanate contained in the cabbage family and is inhibited by antioxidants including AsA [77]. Since the 1850s, the nitrosation reaction that produces nitrosoamines has attracted much attention as a potential carcinogenic effect of nitrite [11]. Although there has been long debate on the potential toxicity of nitrite in carcinogenesis, extensive animal as well as epidemiological studies have suggested that nitrite in diets does not in fact lead to carcinogenesis in normal conditions [4].

3.8 Bacterial Associations with the Nitrite Pathway

To fully grasp the function of nitrite pathway in vivo, knowledge of human physiology must be complemented with an understanding of nitrite processing by the human microbiome.

3.8.1 Nitrate Reduction by Gastrointestinal Bacteria

Approximately, one-third to one-half of nitrate ingested by mammals exits the body through bacterial denitrification to gas as well as via assimilation into bacterial biomass that is ultimately eliminated in feces [11]. Some bacteria that colonize the lumen of the gastrointestinal tract are capable of switching from aerobic to nitrate respiration, reducing nitrate to N_2 but often releasing a substantial portion of N from the pathway as NO [78]. Certain others, while in the nitrate respiratory mode,

produce only nitrate reductase, thereby releasing nitrite for intestinal absorption or acid-mediated conversion to NO [4, 79].

NO production via the nitrite pathway is a common trait of the commensal bacteria of the intestine, especially among the *Lactobacillus* and *Bifidobacterium* genera [80]. Though some commensal bacteria consume NO, overall there is net production of NO by bacteria in the gastrointestinal tract [81, 82], which helps to explain the significant loss of intestinal NO emission in antibiotic-treated neonates compared to their untreated counterparts [81].

Of the nitrate absorbed from the intestine approximately one-quarter is returned to the upper gastrointestinal tract via saliva, presumably to permit reduction of nitrate to nitrite by mouth flora. Some of this nitrite will undergo acid-mediated reduction to NO in the stomach while the rest is absorbed into the bloodstream. Interestingly this nitrite-production activity in the mouth appears to exert a significant blood pressure-lowering effect on the host. Subjects that lowered their oral nitrite-producing activity by 90 % through use of an antibacterial mouthwash showed 2–3.5 mg Hg increases in systolic and diastolic blood pressure that correlated with a 25 % reduction in plasma nitrite levels [83], consistent with previous studies showing that dietary supplementation with nitrate lowers blood pressure [84].

These findings linking nitrite levels with blood pressure may imply a mechanism for the association between breastfeeding and lower systolic and diastolic pressure sustained into adulthood identified in a meta-analysis study [85] and thereby explain the observation of particularly high levels of nitrate in early postpartum milk. Nitrate in milk could conceivably favor the colonization of the mouth by nitrate reducing bacteria [24]; it would be interesting to learn whether the low levels of nitrate in certain baby formulas alter the ultimate composition of the microbial mouth flora.

3.8.2 Nitrite-Derived NO in Biofilm Dispersal: Ecological Aspects

Biofilms are multispecies assemblages of microbes that display coordinated metabolism and development and resilience in the face of physiochemical stresses and antibiotic treatment [86]. They are associated with increased virulence of a variety of infections that are particularly baneful to pediatric medicine, including dental carries and upper respiratory tract and medical device-associated infections. The denitrifying bacterium *Pseudomonas aeruginosa* is a common member of pathogenic biofilm communities [87–89] that releases NO under denitrifying conditions. NO has properties ideally suited for signaling within biofilms: it is membrane diffusible and can interact with several cellular targets including heme groups, iron sulfur clusters, and thiols [90], allowing it to exert a variety of post-transcriptional influences.

3.9 Behind the Multiplicity in a Simple System: Evolutional Aspects

Looking back on a long research history, one can notice that our society's recognition of the simple molecule nitrite (or nitrate) has changed over time. There have been contradictory findings and interpretations regarding this ubiquitous molecule. Two opposed effects of nitrite (carcinogenic or therapeutic) may lead nonspecialists to a state of confusion: is it good or bad? Moreover, the circularity of the relationship between nitrite and NO gives rise to the chicken and egg issue: substrate or product? For understanding the nature of ubiquitous and essential biomolecules, including O_2, NO, and nitrite, knowing their fine "balance" is more valuable than strict categorization [4, 8, 91]. As Fritjof Capra introduced the parallelism between modern physics and eastern philosophies [92], the application of "Yin–Yang" (shadow and light in a unity) philosophy may be of help in illustrating the opposing behaviors of the key players in oxidative stress [35] (Fig. 3.4).

The diversity in the effects of nitrite and NO can also be explained from an evolutionary perspective. High concentrations of NO arising from lightening the ancient atmosphere [93] and the consequently formed NO_2^- (reaction 3.2) would have bound iron, inhibiting electron transport processes, while the species N_2O_3 and nitrogen

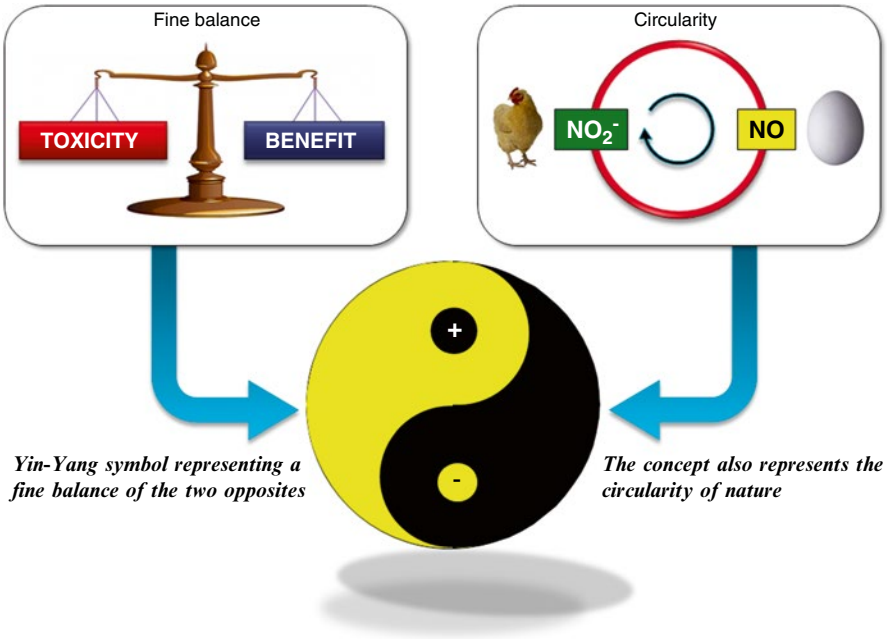

Fig. 3.4 Philosophical aspect of nitrite's dualism. Yin–Yang philosophy is one of the oldest concepts in Chinese medicine and Chinese food therapy. The philosophy is often helpful to understand contradictory features (good and bad) of ubiquitous molecules including nitrite

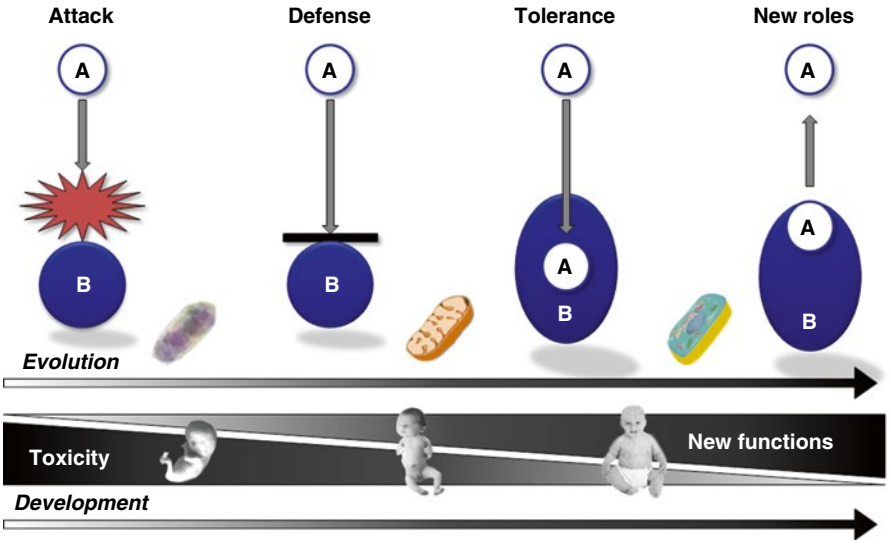

Fig. 3.5 Evolutional aspect of nitrite's dualism. This illustration shows conceptual steps representing how living organisms have evolved to cope with unfavorable exogenous chemicals. Suppose the element A is harmful to the element B. At the beginning, B suffers due to the attack by A (attack stage). Then, B develops protection mechanisms (defense stage) and further evolves tolerance mechanisms (tolerance stage). Finally, A plays new roles for B (new roles stage). Eventually, B is able to actively produce A to utilize it. This concept can account for the dualism of ROS, RNS, H_2S, and even for biological interactions. The figure is redrawn from Yamasaki (2005) [8]

dioxide (NO_2), formed in reactions (3.4) and (3.5), respectively, would have nitrosated proteins, causing general toxicity. Tolerance would have been gained through evolution of means to reduce NO_2^- and NO to N_2O or N_2 gas, in conjunction with deployment of cytosolic thiols, as in glutathione. It is conceivable that the need for tolerance to nitrogen oxides drove the evolution of their corresponding reductases. Subsequent development of protein complexes capable of linking proton pumping with the reduction of NO_2^- and NO would have allowed cells to take advantage of the positive redox potential of these compounds and thereby extract more cellular energy from their electron donors [57]. Free atmospheric NO would have plummeted with the rise in the atmosphere of O_2 resulting from oxygenic photosynthesis; NO could then serve as a signal of hypoxic conditions. Cells already well adapted to NO would find the need to synthesize it for certain physiological purposes, leading to the evolution of NOSs.

Figure 3.5 is generalized model for how biological relationships of two elements change in the process of evolution and development [8]. Suppose that in an early stage hostile elements (invaders) solely cause harm to living organisms (stage 1; attack). Later, living organisms evolve protective survival mechanisms (stage 2; defense). After the acquisition of the protecting mechanism, invaders may exist inside and may even function for mutual benefit (stage 3; tolerance). At the final stage, living organisms actively reproduce the past-invader to provide new

functions (stage 4; new roles). This sequential change in relationship can be found in many biological interactions, e.g., pathogenesis, parasitism, and symbiosis. Human beings are snapshots of life's evolution, encompassing both "primitive" and "advanced" relationships and mechanisms that explain the dualism of simple molecules such as O_2, H_2S, and NO [8].

3.10 Implications for Therapeutic Application of Nitrite

It is evident that nitrite can supply NO to blood and tissues through endogenous and exogenous pathways. Because of the difficulty in directly treating patients with NO gas, nitrite has now been extensively studied as an alternative NO delivery agent for stroke, myocardial ischemia, hypertension, and transplantation therapy and for promoting angiogenesis [94]. Although the effects of nitrite therapy on those disorders have not been fully elucidated, there is accumulating evidence that the cytoprotective effects of nitrite therapy are mostly ascribable to the action of NO derived from nitrite and are independent of endothelial NOS (eNOS) and heme oxygenase-1 (HO1) activities [94].

It should be noted again that nitrite and nitrate are found at a high concentration (submillimolar) in postpartum breast milk [95]. In particular, colostrum contains the highest amount of nitrite [23]. Accordingly, a concentration of NO in stomach gas is higher than 7 ppm between postpartum days 2 and 5, which is not observed in neonates fed on low-nitrite formulas [96]. The gastrointestinal tract of the neonates must be sterile until the successful colonization of commensal bacteria that originate from the mother and environments [23]. It is most likely that a high concentration of nitrite in the early postpartum period is needed until the gut microbial flora establishes to metabolize nitrate as the substrate [23].

It appears that an early exposure to nitrite influences subsequent disease risk of children. Breastfeeding has been shown to reduce the risks of the infant developing asthma and allergies [97] as well as childhood acute leukemia [98]. Cardiovascular disease risk is also reduced through the reduction of obesity, blood pressure, and cholesterol. Interestingly, breastfeeding during infancy is associated with a reduction in risk of ischemic cardiovascular disease later in life [99]. It is well appreciated that immunoglobulins transferred from mother to neonate are one of the strong benefits of colostrum. Likewise it appears that human breast milk may supplement insufficient NO production capacity in neonates by providing nitrite orally, a new beneficial role of human breast milk.

3.11 Future Prospects

Nitrate–nitrite–NO metabolism in mammals has been scientifically investigated for more than 100 years. The long-standing question—what is the endogenous source of nitrate and nitrite?—can be now answered: it is the NO produced by host NOS

systems and commensal bacterial activities. Autoxidation of NO to nitrite and nitrate can thus be understood to constitute a simple mechanism for recycling NO-generating capacity for the nitrite pathway.

As foods and drinking water change bacterial flora in gut [100], cytoprotective effects of nitrite may be altered among individuals depending on health conditions, age, daily diet, and local food customs. To determine an appropriate dose of nitrite for an individual patient, finding the balance point for attaining the maximal beneficial effect will be necessary. Although development of such personalized medicine must wait for future research, integration of our diverse knowledge of nitrite will be necessary to find the "Yin–Yang" balance of this essential inorganic molecule.

Acknowledgements Work in the authors' laboratory (H.Y.) was supported by a Grant-in-Aid for Scientific Research (B) from Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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