Original Contribution

Dietary nitrite restores NO homeostasis and is cardioprotective in endothelial nitric oxide synthase-deficient mice

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Abstract

Endothelial production of nitric oxide (NO) is critical for vascular homeostasis. Nitrite and nitrate are formed endogenously by the stepwise oxidation of NO and have, for years, been regarded as inactive degradation products. As a result, both anions are routinely used as surrogate markers of NO production, with nitrate as a more sensitive marker. However, both nitrite and nitrate are derived from dietary sources. We sought to determine how exogenous nitrite affects steady-state concentrations of NO metabolites thought to originate from nitric oxide synthase (NOS)-derived NO as well as blood pressure and myocardial ischemia–reperfusion (I/R) injury. Mice deficient in endothelial nitric oxide synthase (eNOS) demonstrated decreased blood and tissue nitrite, nitrate, and nitroso proteins, which were further reduced by low-nitrite (NOx) diet for 1 week. Nitrite supplementation (50 mg/L) in the drinking water for 1 week restored NO homeostasis in eNOS−/− mice and protected against I/R injury. Nitrite failed to alter heart rate or mean arterial blood pressure at the protective dose. These data demonstrate the significance of dietary nitrite intake on the maintenance of steady-state NO levels. Dietary nitrite and nitrate may serve as essential nutrients for optimal cardiovascular health and may provide a novel prevention/treatment modality for disease associated with NO insufficiency.

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Nitric oxide (NO) is a multifunctional signaling molecule, intricately involved with maintaining physiological processes such as host defense, neuronal communication, and the regulation of vascular tone [1]. NO is produced from the terminal guanidine nitrogen of L-arginine through an N^\text{\text{-}}^\text{\text{-}}^-\text{hydroxy-L-arginine} intermediate yielding L-citrulline [2], a reaction catalyzed by nitric oxide synthase (NOS) [3]. Three distinct isoforms of NOS have been identified, each with different tissue localization, catalytic properties, regulation, and inhibitor sensitivity. Four NOS isotypes (NOS I, II, III) homozygous (eNOS−/−) lines have thus far been generated, along with heterozygous and double NOS knockouts and, most recently, a triple NOS knockout that is surprisingly still viable [8]. Although the development of transgenic mice lacking the NOS gene has greatly advanced understanding of the physiological roles of NOS, it has also increased the realization of the complex interplay between the various NOS isoforms and, most recently, the confounding effects of nitrate and nitrite acting as NOS-independent sources of NO [9]. It is becoming increasingly apparent that compensatory mechanisms may develop during the loss of a particular NOS gene which may involve either NOS-dependent or NO-dependent, but NOS-independent, mechanisms or a combination [10–12]. Because of these adaptive mechanisms or environmental and dietary factors, it is not uncommon for transgenic animals to lack overt phenotypes. Research by several groups has demonstrated that both nitrite and nitrate derived from both endogenous and exogenous sources (i.e., diet) can be recycled back to NO and other bioactive nitrogen oxides [13–16]. Therefore, detailed biochemical and functional analysis of the eNOS knockout mouse under conditions of changing nitrate availability is required for a better understanding of these compensatory mechanisms by NOS-independent sources of NO.

Although previous functional studies have provided vital data on the physiological consequences of abolished eNOS activity [4,17–19], a detailed analysis of steady-state nitrite, nitrate, and nitrosothiol production in the face of changes in exogenous nitrite availability is lacking. Using established methodology we sought to provide a comprehensive assessment of NO metabolite levels in tissue and blood, as well as recovery from myocardial ischemia–reperfusion injury, from transgenic mice lacking eNOS compared with control mice with or without supplemental nitrite.

Abbreviations: NOS, nitric oxide synthase; eNOS, endothelial NOS; RSNO, mercury-sensitive NO adduct; RNNO, mercury-insensitive NO adduct; I/R, ischemia–reperfusion; LCA, left coronary artery; AAR, area at risk; LV, left ventricle.

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Methods

Animals

Ten-week-old mice deficient in eNOS (NOS III, eNOS^{−/−}; originally provided by Paul Huang, Massachusetts General Hospital, Boston, MA, USA) were compared with age-matched C57BL/6J mice as wild type (Jackson Laboratory, Bar Harbor, ME, USA). Animals were maintained on a standard chow diet (Purina 5001) and kept on a normal 12/12 light cycle with a minimum of 10 days allowed for local vivarium acclimation prior to experimental use. All experiments complied with federal and state regulations in accordance with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council). None of the animals were fasted prior to blood and tissue harvest in order to determine true steady-state levels.

Dietary supplementation and depletion of tissue nitrite

For dietary supplementation studies, sodium nitrite was added to the drinking water of mice for 1 week at 50 mg/L concentration. For nitrite depletion studies, mice were kept on the standard rodent diet (Purina 5001) and tap water for 9 weeks before switching over to an amino acid diet (TD 99396, Harlan Teklad) with a matched L-arginine content and MilliQ water. The average nitrite/nitrate content of this diet was found to be considerably lower (20.5±0.7 nmol/g nitrite and 503.1±17.9 nmol/g nitrate) than that of Purina rodent chow (104.3±4.7 nmol/g nitrite and 6275±50.7 nmol/g nitrate). There was no difference in daily chow consumption or weight gain between the groups. Nitrite restoration was achieved by administering 50 mg/L sodium nitrite in the drinking water to mice on low-NOx diet for 1 week.

Tissue NO products/metabolite determination

Biological specimens were harvested after 10 weeks of standard diet (Purina 5001) or 9 weeks on standard diet followed by 1 week on low-NOx diet (Harlan 99366) for quantitative analyses of nitroso species and oxidation products of NO as detailed elsewhere [20,21]. Briefly, nitroso compounds were measured by the addition of acidified sulfanilamide and injection of biological or diet samples into a triiodide-containing reaction mixture continuously purged with nitrogen. No attempt was made to differentiate between a mercury-sensitive (RSNO) and mercury-insensitive (RNNO) adduct due to the limited blood and tissue volume from the mouse. Evolved NO was quantified in the gas phase using an ozone-based chemiluminescence analyzer (Eicom, Kyoto, Japan) [20]. Plasma nitrite was obtained by centrifugation at 800 g and 4°C for 10 min.

Myocardial infarction protocol

Surgical procedures used in the myocardial I/R protocol were similar to methods described previously [22,23]. The surgeon was blinded to dietary interventions. Briefly, mice were anesthetized, orally intubated, and placed on a rodent ventilator. A median sternotomy was performed, and the left coronary artery (LCA) was visualized and ligated proximally using a 7-0 silk suture mounted on a BV-1-tapered needle. The LCA was completely occluded for 45 min, and reperfusion (24 h) was initiated by removal of the 7-O suture. The chest incision was reaproximated, and mice were allowed to recover in a temperature-controlled setting and supplemented with 100% oxygen.

Myocardial infarct size determination

Following 24 h of reperfusion, mice were anesthetized and a polyethylene catheter was placed in the right carotid artery. The LCA was religated at the same location as before, and Evan’s blue dye (4%) was perfused to delineate the area not at risk. The heart was then excised, sectioned, and stained for 5 min in 1% 2,3,5-triphenyltetrazolium chloride (TTC). The myocardial slices were then digitally imaged and weighed. Areas of infarction, risk, and nonischemic left ventricle were assessed by a blinded observer using computer-assisted planimetry (NIH Image J 1.37) [23].

Hemodynamic measurements

Mean arterial blood pressures and heart rate were measured in eNOS^{−/−} mice maintained on a standard diet and in mice supplemented with dietary nitrite in the conscious state using radiotelemetry techniques. Mice were surgically implanted with the PA-C10 radiotelemeter pressure transducers (DSI, St. Paul, MN, USA), and catheters were placed in the aorta as previously described [24] 7 days prior to any dietary manipulations. Once a stable baseline was achieved, both blood pressure and heart rate data were collected for a period of 3 minutes, six times each 24-hour cycle, over a 7 day sampling period.

Nitrite reductase assay

Mice were anesthetized, and after thoracotomy, a 21-gauge needle was inserted into the left ventricle and all organs were flushed free of blood with air-equilibrated phosphate-buffered saline, pH 7.4. Blood-free heart and liver samples were homogenized under ice in phosphate-buffered saline (1:5, w/v) with a Polytron (PT10-35) homogenizer. Just prior to use, tissue homogenate was brought with PBS to a sixfold final dilution (v/v). For incubation, an equivalent tissue sample volume was placed into the light-protected reaction vessel of an ozone-chemiluminescence NO analyzer. The vessel was maintained at 37°C, and the same fresh biological sample was purged with N₂ (anoxia). NO generation was continuously monitored online for 5 min by the analyzer on addition of 200 μM NaNO₂ (final concentration). This NO concentration has been established as sufficient substrate to support vasorelaxation in vitro, regional vasodilation in vivo, and Hb nitrosylation and S-nitrosation in vitro and in vivo [25].

Statistical analysis

All data in this study are expressed as means±SEM. Differences in data between the groups were compared using Prism 4 (GraphPad Software, Inc) with Student’s paired two-tailed t test or one-way analysis of variance (ANOVA) where appropriate. For the ANOVA, if significant variance was detected, the Tukey test was used as the post hoc analysis. A P value less than 0.05 was considered significant.

Results

Steady-state concentrations of nitrite, nitrate, and nitroso proteins are affected by both NOS and dietary nitrite

Consistent with previous results [26], eNOS^{−/−} mice exhibit a 50% reduction in steady-state nitrite and nitrate in plasma compared with age-matched controls (C57BL6J), both on standard Purina 5001 rodent chow (Fig. 1). We also observed a significant decrease in cardiac nitrite in eNOS^{−/−} mice, with no difference in cardiac nitrate. Interestingly, both plasma nitrite and nitrate can be further reduced by switching the eNOS^{−/−} mice to a low-NOx diet. Although the sample size investigated here did not reach statistical significance, there is a slight reduction in plasma NOX as a result of changes in dietary NOX intake. There was no difference in plasma or cardiac nitroso proteins between the two groups of mice on standard diet. However when the eNOS^{−/−} mice were placed on low-NOX diet, plasma nitroso proteins completely disappeared, and there was a significant reduction in cardiac nitroso proteins. Steady-state plasma and cardiac NOX could not be measured.
be restored and nitroso proteins enhanced in the eNOS\(^{-/-}\) mice by supplementing their drinking water with 50 mg/L sodium nitrite for 7 days. This dose has been shown to be cardioprotective in healthy mice [27]. Interestingly this low dose of dietary nitrite supplementation simply restores NO biochemistry without increasing levels from the value seen in C57 mice on standard diet. This demonstrates that we can recapitulate NO physiology through low nitrite supplementation in the diet and reveals the influence of changes in dietary NO\(_x\) intake on the steady-state concentration of metabolites commonly used to assess NO\(_x\)-derived NO.

**Supplemental nitrite masks the underlying NO biochemistry**

Next we were interested in determining if we could still uncover the changes in NO biochemistry in C57 versus eNOS\(^{-/-}\) mice on NO\(_x\) deficiency and supplemented nitrite ingestion. C57 and eNOS\(^{-/-}\) mice were fed either standard rodent chow or low-NO\(_x\) diet along with 50 mg/L nitrite in the drinking water for 7 days. Supplemental nitrite masked the underlying NO biochemistry differences between the C57 and eNOS\(^{-/-}\) mice on both diets. As shown in Fig. 2, there was no difference between plasma and cardiac NO\(_x\) and nitroso proteins in C57 and eNOS\(^{-/-}\) mice given nitrite orally for 7 days. However, although there were no differences between the C57 and eNOS\(^{-/-}\) mice on either diet, there were significant differences in both mouse strains on the low-NO\(_x\) diet compared with standard diet. Both strains demonstrated a diminished response to the supplemental nitrite. Both strains of mice on low-NO\(_x\) diet with nitrite supplementation had decreased plasma and cardiac nitrite and nitrate compared with the same mice given standard diet with supplemental nitrite. Mice typically consume 15 g/100 g body wt food per day and drink 15 mL/100 g body wt per day. Given this estimation, a 30-g mouse would typically consume 4.95 g of chow per day. As such the mice on low-NO\(_x\) diet consumed 101.5 nmol nitrite per day and 2490 nmol nitrate per day compared with 516 nmol nitrite and 29,806 nmol of nitrate per day, with a net daily difference of 415 nmol of nitrite and 27,316 nmol nitrate. The 50 mg/L nitrite supplementation equates to a net daily intake of 250 µg, which is more than enough to account for the difference in diet composition. Therefore, there appears to be a change in metabolism of nitrite in the mice given the low-NO\(_x\) diet. Current studies are ongoing to investigate these changes.

**Nitrite supplementation protects against myocardial I/R injury**

Our previous results demonstrated that a low-NO\(_x\) diet exacerbates I/R injury and dietary nitrite can protect the heart from I/R injury in mice with functional NOS [27]. Furthermore, eNOS\(^{-/-}\) mice suffer greater I/R injury than healthy age-matched C57 controls [19]. However, it is not known if nitrite protects in animals without functional endothelial NOS. The potential cardioprotective effects of nitrite supplementation were tested in a well-established murine model of in vivo myocardial I/R. These data are illustrated in Fig. 3. For these experiments, nitrite (50 mg/L) was administered in the drinking water.

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**Fig. 1.** Steady-state plasma and heart nitrite, nitrate (NO\(_x\)), and nitroso proteins in C57 and eNOS\(^{-/-}\) mice. C57 mice were fed a standard rodent chow and eNOS\(^{-/-}\) mice were fed either Purina 5001 or low-NO\(_x\) diet±50 mg/L nitrite supplementation for 7 days, at which time steady-state levels of plasma and heart nitrite (A), nitrate (B), and nitroso proteins (RXNO) (C) were measured. eNOS\(^{-/-}\) mice have a 50% reduction in plasma nitrite and nitrate, which is further reduced by feeding a low-NO\(_x\) diet. These biochemical effects can be reversed by giving supplemental nitrite to eNOS\(^{-/-}\) mice. Values are means±SEM from \(n=4-9\) mice in each group. \(*P<0.05\) versus C57 on standard diet.

**Fig. 2.** Steady-state plasma and heart nitrite, nitrate (NO\(_x\)), and nitroso proteins in C57 and eNOS\(^{-/-}\) mice given supplemental nitrite for 7 days. C57 and eNOS\(^{-/-}\) mice were fed either standard rodent chow or low-NO\(_x\) diet±50 mg/L nitrite supplementation for 7 days, at which time steady-state levels of plasma and heart nitrite (A), nitrate (B), and nitroso proteins (RXNO) (C) were measured. Supplemental nitrite masks the underlying NO biochemistry of the eNOS\(^{-/-}\) mouse. Both strains of mice demonstrate a dampened response to supplemental nitrite when on a low-NO\(_x\) diet. Values are means±SEM from \(n=4-9\) mice in each group. \(*P<0.05\) versus mice on standard diet with no supplemental nitrite.
of eNOS−/− mice on a standard rodent chow for 7 days. On the seventh
day, the mice were subjected to 45 min of LCA ischemia followed by 24 h of reperfusion. The extent of myocardial infarction was evaluated at 24 h of reperfusion. As shown in Figs. 3A–C, nitrite supplementation in eNOS−/− mice increased plasma and cardiac nitrite (A), nitrate (B), and nitroso proteins (C) in eNOS−/− mice. Representative midventricular photomicrographs of hearts from mice maintained on a standard chow and from mice supplemented with nitrite in their drinking water. (D) Area-at-risk (AAR) with respect to the left ventricle (LV) was similar in the two groups. Nitrite supplementation significantly attenuated myocardial infarct size (Inf) by 49% (P<0.05) with respect to both the area-at-risk (Inf/AAR) and the left ventricle (Inf/LV) (E). Values are means±SEM. Numbers inside bars indicate the number of animals investigated in each group. *P<0.05 versus eNOS−/−.

Myocardial nitrite reductase is unaffected by deficiency in eNOS

It was recently reported the NOS can reduce nitrite to NO under anoxia [28]. Given these data, one might expect no benefit from nitrite treatment in eNOS knockout mice if this was the mechanism of nitrite reduction. However, our clear reduction in infarct size from nitrite treatment in eNOS−/− mice indicates otherwise. To determine if eNOS contributes to nitrite reduction under anoxia, we compared the nitrite reductase activity of both heart and liver from C57 and eNOS−/− mice.

As shown in Fig. 4, there was no difference in NO production from nitrite in the hearts of C57 and eNOS−/−. However, the liver of eNOS−/− mice tended to produce more NO from the same amount of nitrite.
than did the liver of C57 mice. These data reveal that there are other mechanisms of nitrite reduction that may be increased in the liver as a result of NO deficiency to provide an alternative NOS-independent means of NO production.

**Effect of dietary nitrite on blood pressure and heart rate in eNOS<sup>−/−</sup> mice**

It has previously been reported that nitrite can act as an alternate source of NO and reverse the hypertensive effects when co-administered with a NOS inhibitor [29]. However, the concentration of nitrite needed to detect any changes in systolic blood pressure was 1000 mg/L, a 20-fold greater nitrite dose than was used in the present study to confer cardioprotection. To determine if this relatively low dose of nitrite could ameliorate the hypertension in eNOS<sup>−/−</sup> mice and heart rate, eNOS<sup>−/−</sup> mice were implanted with radiotelemetry pressure transducers (Fig. 5). Mice supplemented with nitrite displayed no significant differences in mean arterial blood pressure or heart rate when compared with mice maintained on normal water during the 7-day observation period. Therefore, the doses of nitrite shown to be cardioprotective in this study do not affect blood pressure.

**Discussion**

Our results indicate that any dietary changes that increase nitrite availability affect the steady-state concentrations of products and metabolites commonly used to assess endothelial NO production from NOS and mask the underlying biochemistry of NOS-derived NO. Consistent with our recent results demonstrating that dietary nitrite can protect the heart from I/R injury [27], nitrite is similarly efficacious in eNOS<sup>−/−</sup> mice, reducing injury by 49%. Given the hosts of disease states associated with disruption in NO production from insufficient production in endothelial dysfunction, it is critical to be able to accurately and specifically determine changes in NO output from eNOS to determine optimal therapy. The results from this study reveal that one must carefully consider dietary influences of nitrite and/or sources of nitrite and suggest that nitrite can recapitulate NO biochemistry in the face of endothelial dysfunction. As the main source of dietary nitrite is nitrate [13], differences in diets can have a significant effect on steady-state concentrations of nitrite and nitrate that are not derived from NOS. Analysis of a number of standard rodent chows reveals as much as a 10-fold difference in NOx content between different rodent diets (Bryan, unpublished observations).

It is widely accepted in cardiovascular physiology that the production of NO from eNOS is critical not only for its maintenance of vascular tone but also for its antithrombotic, antiproliferative, and anti-inflammatory actions [30]. eNOS knockout mice are viable but are hypertensive [4–7]. The also exhibit normal flow responses in various tissue beds [6,31–34]. These findings have most commonly been attributed to compensatory mechanisms including upregulation of other NOS isoforms, but some of these effects may be due to the nitrite and nitrate found naturally in their diet. In fact, co-administration of nitrite has been shown to reverse the hypertensive effects of NOS inhibitors [29]. How dietary nitrite affects endogenous-specific cellular signaling events is a topic of intense research. Bryan et al. first described nitrite as an endogenous signaling molecule that can function in both NO-dependent and NO-independent pathways [35]. Shiva et al. revealed that nitrite affords its cytoprotective properties through modulation of mitochondrial electron transfer [36]. Many of the proposed mechanisms of nitrite are based on reduction back to NO. Many systems have been demonstrated to be capable of reducing nitrite to NO, including the mitochondrial electron transport system [37–41], simple protonation [42,43], deoxyhemoglobin [25,43], deoxymyoglobin [44], and xanthine oxidase [45–47]. Most recently, NO has been implicated as a nitrite reductase under anoxic conditions [28]. Our data on heart and liver from eNOS<sup>−/−</sup> mice reveal no difference in NO formation from nitrite under anoxic conditions. In fact, the liver from eNOS<sup>−/−</sup> mice may have higher nitrite reductase activity than from liver from C57 mice. This would argue for a compensatory mechanism to produce NO from nitrite when NOS is absent or inactive. Transgenic animals reveal the immense complexity of the regulatory pathways used to maintain physiological processes. Transgenic mice lacking functional eNOS provide an ideal model to highlight the importance of maintaining physiological levels of NO.
metabolites and support the notion of alternative NO-producing compensatory mechanisms including dietary intake of nitrite and nitrate.

The amount of nitrite used in this study did not elicit a significant change in systemic blood pressure in eNOS-deficient mice. A recent report by Cosby et al. [25] suggests that nitrite can dilate vessels at “near-physiological” levels. However, in an earlier study, Lauer et al. reported that nitrite lacked intrinsic vasodilatory properties [48]. More recent studies reveal that nitrite preferentially dilates under hypoxic conditions in humans [49]. The use of nitrite as a vasodilator is not novel. As early as 1880, nitrite was described in terms of its vasodilatory abilities [50], and much later, Furchgott and Bhardwakom used acidified sodium nitrite to relax precontracted aortic strips in 1953 [51]. Both studies used supraphysiological concentrations of nitrite. Furthermore, it has been known for years that nitrite is a very poor vasodilator and activator of soluble guanylyl cyclase in comparison to standard nitrosopsvasodilators such as NO [52]. It was previously demonstrated that nitrite can exert cellular signaling effects at doses that do not affect blood pressure [35]. Together these results suggest that nitrite has other important physiological actions independent of blood pressure regulation that occur at much lower concentrations that can easily be achieved through diet or increase in NO output from NOS. Furthermore, our results suggest dietary nitrite/nitrate intake masks differences in the products of NO metabolism between wild-type and eNOS−/− mice and changes in nitrite/nitrate concentration affect endogenous metabolism or clearance of nitrite and nitrate. Dietary NOx may provide a further compensatory mechanism to maintain the viability of these transgenic animals lacking functional NOS genes. The chronic loss of gene function in transgenic animals leaves ample time for the development of compensatory mechanisms. These experiments were performed on animals receiving a subchronic (7 days) reduction in dietary nitrite/nitrate. It is likely that physiological differences between eNOS−/− and wild-type mice would become more apparent when placed on a reduced NOx diet from birth, therefore removing the ability of dietary NOx-dependent adaptive mechanisms to develop that could effectively compensate for the loss of a specific gene. Longer-term studies are needed to confirm this. The findings of this study, however, may be interpreted to better represent the effect of diet on the physiological situation whereby a genetic disposition leading to reduced levels of NO (endothelial dysfunction) could lead to compensatory mechanisms, which eventually lead to susceptibility to or protection from a disease state. Diet is becoming increasingly important in the nitric oxide field, with recent research suggesting dietary nitrate and nitrite can be systematically reduced to NO [13] and affect a host of physiological systems [27,35,53–55].

There has recently been a number of studies demonstrating beneficial properties of both dietary nitrite and nitrate [27,53,55,56]. Since the early 1980s there have been numerous reports on the association of N-nitrosamines and human cancers [57,58] but a causative link between nitrite and nitrate exposure and cancer is still missing [59]. Furthermore, a 2-year study on the carcinogenicity of nitrite by NIH has conclusively found that there was no evidence of carcinogenic activity by sodium nitrite in male or female rats or mice [60]. These negative connotations of nitrite and nitrate have led the government to regulate and restrict the levels in food and drinking water. The recent report by Webb et al. [56] demonstrates that dietary nitrate, through its reduction to nitrite, can lower blood pressure, prevent i/R-mediated endothelial dysfunction, and attenuate platelet aggregation in humans. Collectively, these studies clearly reveal the benefits of nitrite and nitrate from the diet as a means to restore or enhance NO bioavailability and/or homeostasis. A report from the National Research Council (The Health Effects of Nitrate, Nitrates, and N-Nitroso Compounds, NRC, 1981) estimated, on the basis of food consumption tables, that the average total nitrite and nitrate intake in the United States was 0.77 and 76 mg, respectively. If we assume a human body (70 kg) produces 1.68 mmol NO per day (based on 1 µmol/kg/h NO production), an average daily intake of 0.77 mg of nitrite would equate to 11.1 µmol/day and 76 mg nitrate would equate to 894 µmol/day or roughly 1 mmol NOx per day from diet. This almost matches what our body makes from NO if we assume most of the NO goes to stepwise oxidation to nitrate and nitrite. Therefore, almost 50% of our steady-state levels of NOx, which are routinely used as clinical biomarkers of NO activity, come from diet. There should be a reevaluation of current nitrate and nitrite consumption analysis based on 21st-century dietary habits. The strategy of avoiding nitrite- and nitrate-rich foods imposed in the 1970s due to their propensity to form N-nitrosamines may prove to be unwarranted and even detrimental to cardiovascular health.

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