Inorganic nitrite supplementation for healthy arterial aging

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Sindler AL, DeVan AE, Fleenor BS, Seals DR. Inorganic nitrite supplementation for healthy arterial aging. J Appl Physiol 116: 463–477, 2014. First published January 9, 2014; doi:10.1152/japplphysiol.01100.2013.—Aging is the major risk factor for cardiovascular diseases (CVD). This is attributable primarily to adverse changes in arteries, notably, increases in large elastic artery stiffness and endothelial dysfunction mediated by inadequate concentrations of the vascular-protective molecule, nitric oxide (NO), and higher levels of oxidative stress and inflammation. Inorganic nitrite is a promising precursor molecule for augmenting circulating and tissue NO bioavailability because it requires only a one-step reduction to NO. Nitrite also acts as an independent signaling molecule, exerting many of the effects previously attributed to NO. Results of recent studies indicate that nitrite may be effective in the treatment of vascular aging. In old mice, short-term oral sodium nitrite supplementation reduces aortic pulse wave velocity, the gold-standard measure of large elastic artery stiffness, and ameliorates endothelial dysfunction, as indicated by normalization of NO-mediated endothelium-dependent dilation. These improvements in age-related vascular dysfunction with nitrite are mediated by reductions in oxidative stress and inflammation, and may be linked to increases in mitochondrial biogenesis and health. Increasing nitrite levels via dietary intake of nitrate appears to have similarly beneficial effects in many of the same physiological and clinical settings. Several clinical trials are being performed to determine the broad therapeutic potential of increasing nitrite bioavailability on human health and disease, including studies related to vascular aging. In summary, inorganic nitrite, as well as dietary nitrate supplementation, represents a promising therapy for treatment of arterial aging and prevention of age-associated CVD in humans.

arterial stiffness; vascular endothelial dysfunction; oxidative stress; inflammation; mitochondrial biogenesis; nitric oxide

AGING, ARTERIAL DYSFUNCTION, AND CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVD) remain the leading cause of morbidity and mortality in modern societies, and most forms of CVD are linked to dysfunction of arteries (59, 111). Aging is the primary risk factor for CVD, and therefore, most forms of CVD are diseases of aging (59, 98, 112, 136, 137). Thus there is something about aging that causes dysfunction of arteries, which in turn, leads to an increased risk for CVD (100). Given the changing demographics of aging worldwide, these events represent a pathophysiological platform for a new baby boom-driven epidemic of CVD in the near future. Indeed, a recent projection suggests that without effective intervention, 40% of U.S. adults will have at least one form of CVD by 2030, and medical costs for these disorders will triple, primarily as a result of population aging (68). As such, establishing the efficacy of treatments that prevent and/or reverse age-associated arterial dysfunction is a high biomedical priority because it has the potential to prevent age-associated CVD (91, 117, 155).

ARTERIAL DYSFUNCTION WITH AGING

Aging causes numerous changes to arteries that increase the risk for CVD, but two key contributors are stiffening of the large elastic arteries (aorta and carotid arteries) and the development of vascular endothelial dysfunction (100, 142, 164, 194).

Large Elastic Artery Stiffness

Large elastic artery stiffening with aging is mediated by changes in both structural and functional factors (99, 136, 195). Structural factors include changes to the extracellular matrix involving increases in collagen (fibrosis) and reductions in elastin (i.e., the major structural proteins in the arterial wall that confer stiffness and elasticity, respectively) (41). The cytokine growth factor, transforming growth factor-β (TGF-β), may play an important role in signaling collagen synthesis with aging (13, 163). The formation of advanced glycation end products (AGEs), glucose-derived molecules that cross-link structural proteins in the arterial wall, also increases with age and contributes to large elastic artery stiffening (62). Functional influences on arterial stiffness involve factors that modulate vascular smooth muscle tone, including paracrine molecules, of which endothelial cell-synthesized nitric oxide (NO) is among the most important (42, 52, 123, 173, 191, 195). In vivo, the gold standard for assessing large elastic artery stiff-
ness is aortic pulse wave velocity (PWV) (105, 126); the greater the PWV, the greater the stiffness. Intrinsic stiffness of these arteries also can be assessed ex vivo in aortic rings (1, 53, 73).

Vascular Endothelial Dysfunction

Reduced bioavailability of NO as a result of decreased production by endothelial NO synthase (eNOS), increased degradation, or both, also is the key mechanism mediating vascular endothelial dysfunction with aging (118, 164, 177). The vascular endothelium is a single cell layer at the interface between the flow of blood in the lumen of the vessel and the vessel wall. These cells produce a remarkable variety of biologically active molecules that act in autocrine and/or paracrine fashion to regulate the function and health of the vasculature. Endothelial dysfunction can be defined as any alteration in the phenotype of the normal healthy endothelium (36, 48, 133). However, endothelial function is most commonly assessed as the dilation produced by a mechanical or chemical (e.g., acetylcholine) stimulus that evokes increased production of NO by the endothelial cells, leading to activation of guanylate cyclase, increases in cyclic GMP, and relaxation of vascular smooth muscle (55, 75). The greater the endothelium-dependent dilation (EDD) observed, the greater the endothelial function (health).

Large elastic artery stiffness and vascular endothelial dysfunction are not independent functions. Endothelial dysfunction is believed to contribute to large elastic artery stiffness (173), and stiffening of arteries likely restricts EDD. Inadequate NO would serve as a critical factor linking these events (173, 195).

Mechanisms of NO Insufficiency

The key mechanism for reduced NO bioavailability with aging is oxidative stress (49, 164, 177). Oxidative stress can be defined as increased bioactivity of reactive oxygen species (ROS) relative to antioxidant defenses (97). Oxidative stress reduces NO bioavailability via excessive production of superoxide, which reacts with NO to form peroxynitrite. Peroxynitrite in turn causes nitration of tyrosine residues on proteins (nitrotyrosine), providing a useful cellular marker of oxidative stress (107, 159), and also oxidizes tetrahydrobiopterin (BH4), an essential cofactor for NO synthesis by eNOS, to its inactive form, BH2 (54, 165). This reduction in bioavailability of BH4 leads to the uncoupling of eNOS, which produces more superoxide and less NO in a vicious cycle that further reduces NO bioavailability (101, 106). Increased superoxide production by oxidant enzymes such as NADPH oxidase and reduced expression/activity of endogenous antioxidant enzymes that remove superoxide (e.g., superoxide dismutases; SOD) are among the key players contributing to oxidative stress, reduced NO, and vascular dysfunction with aging (15, 64, 130). Consistent with the known synergistic interaction between oxidative stress and inflammation (181), vascular inflammation develops with aging and also contributes to arterial dysfunction (34, 44, 108, 149, 186). Finally, recent evidence suggests that mitochondrial dysfunction may play an important role in large elastic artery stiffening and endothelial dysfunction with aging via modulation of oxidative stress (33, 35, 56, 198).

In summary, on the basis of our present knowledge of the mechanisms underlying vascular aging, it is reasonable to postulate that treatments that increase NO bioavailability, reduce oxidative stress and inflammation, and perhaps improve mitochondrial health, have the potential to improve arterial dysfunction in middle-aged and older adults.

NO, NITRITE, AND NITRATE

NO is a gaseous signaling molecule that plays an essential role in regulating systemic physiological function, and maintaining NO homeostasis is essential for optimal function and health (116, 134, 178). NO has a very short half-life (i.e., milliseconds) and is rapidly and sequentially oxidized to nitrite (NO2⁻) and nitrate (NO3⁻). These molecules have much longer half-lives (minutes to hours) and can be measured in the plasma, where in particular, nitrite has been established as a marker of NO flux/formation (93, 104).

Two major pathways contribute to systemic NO formation. The most well-known pathway is the production of NO from L-arginine in the presence of oxygen by isoforms of the enzyme NO synthase (L-arginine-NO pathway) (Fig. 1, left side) (114). In the presence of several cofactors, most notably BH4, eNOS constitutively produces small amounts of NO (inducible NOS can synthesize large amounts of NO in certain inflammatory conditions) (165). In healthy young adults, constitutive NO production by eNOS is sufficient for normal physiological function in most cases. However, NOS is dysfunctional in most if not all physiological and pathophysiological states associated with chronic NO insufficiency, including aging (10, 27, 184). Indeed, in these states activation of NOS often results in increased formation of superoxide instead of NO due to inadequate availability of BH4, further exacerbating oxidative stress (90, 106). Thus activation of NOS may not be an effective strategy to boost NO bioavailability in such settings (47).

More recently, an alternative pathway of NO formation, the nitrate-nitrite-NO pathway, has been identified (Fig. 1, right)
side) (114, 178). In this pathway, nitrate and nitrite are boosted by consuming foods rich in these compounds (e.g., green leafy vegetables, beets, etc.) (71, 160) or administering nitrate or nitrite salts orally (63, 74, 86); or through inhalation (200), infusion (121, 151), or topical application (144). Nitrate and nitrite are then sequentially reduced to the same bioactive form of NO that is synthesized by NOS. Nitrate is readily converted to nitrite by commensal bacteria in the mouth (115), and the reduction of nitrite to NO occurs systemically, mediated by a variety of endogenous nitrite reductases (17, 26). Therefore, nitrate and nitrite are physiologically recycled in the blood and tissues, acting as precursors that can be easily converted to bioactive NO on demand (178).

By whatever means of delivery, nitrite is an attractive candidate for restoring physiological NO signaling in states of chronic NO insufficiency such as aging because it is rapidly absorbed from the circulation by peripheral tissues and stored in cells until conversion to NO is needed (21, 51, 138). Nitrite has an immense capacity to produce NO, in some tissues synthesizing 10,000 times the amount of constitutive NO formation from NOS (66, 178). Importantly, at physiological concentrations, nitrite, but not nitrate, acts as an independent signaling molecule, performing many of the same actions previously attributable to NO (21, 160, 178). Thus increasing nitrite bioavailability likely has dual-acting effects on vascular and systemic physiological function and health in states of NO insufficiency.

Growing evidence supports the clinical efficacy and safety of inorganic nitrite. Nitrite is classified by the Food and Drug Administration as “Generally Recognized as Safe” for use as a food additive and for the treatment of cyanide poisoning (9, 23, 183). Over the last decade, it has been shown that earlier health concerns regarding nitrite intake are not supported by the data (16, 18, 67, 115, 170), and nitrite is well tolerated with little or no side effects in humans and nonhuman primates when administered at nontoxic doses (Table 1; note that wide ranging doses were used in these studies, in many cases given acutely to assess dose-response and safety). Inorganic nitrite and nitrate also have different chemical compositions and physiological effects than organic nitrite or nitrate (143). Most health care professionals are familiar with organic nitrates that have been used for many years in medicine, such as nitroglycerin for the treatment of angina. Although highly effective under such acute conditions, chronic use of organic nitrates and nitrates leads to tolerance and adverse physiological effects such as endothelial dysfunction (131, 132, 143). Furthermore, organic nitrates and nitrates also have potent vasodilatory and blood pressure lowering effects, whereas inorganic nitrates and nitrites are milder vasodilators and have small or no effects on blood pressure in nonhypertensive adults (39, 63, 141).

Several recent investigations suggest that administration of nitrate through consumption of green leafy or root vegetables (e.g., via beetroot juice) has therapeutic potential to treat a variety of clinical diseases and improve exercise performance, likely via nitrate conversion to nitrite within the body (11, 87, 88, 109, 116, 152). Because the cardiovascular effects of nitrate supplementation have been recently reviewed elsewhere (109), the focus of this synthesis article will be on nitrite supplementation. In general, both approaches appear to have physiological and clinical benefits in settings of NO insufficiency, and each has relative strengths and weaknesses, conceptually. Sodium nitrite has been advanced as a more direct strategy for increasing nitrite concentrations in the body because it involves only a one-step reduction to NO. Direct supplementation with nitrite also evades the need for increased consumption of green leafy vegetables and/or beets/beetroot juice, which like other dietary interventions, may not be sustainable for many individuals (although pharmacological supplementation of nitrate also would obviate this potential limitation). Sodium nitrite also may show greater efficacy for increasing nitrite concentrations in some populations, as has been reported in older adults (124). Moreover, reductions of nitrate to nitrite depend entirely on oral commensal bacteria, and individual differences in microflora may affect nitrate reduction (61, 86, 174). Indeed, there may be sex differences in endogenous nitrate processing involving the entero-salivary circulation (86). In addition, the use of mouthwash and/or antibiotic medications may disturb the oral bacterial flora required for nitrate reduction, thereby attenuating the NO-dependent benefits of nitrate supplementation (61). These issues may be avoided by supplementing with inorganic nitrite. Dietary supplementation via increased intake of foods high in nitrate, on the other hand, may be considered a more natural approach to boosting nitrite and NO bioavailability (72, 119).

Currently, however, there is an absence of information from studies comparing the health benefits of nitrite vs. nitrate supplementation. This is an important area of future investigation.

INORGANIC NITRITE AS AN ORGAN-PROTECTIVE MOLECULE

Ischemia/Reperfusion Injury in the Heart

Much of the initial work on the cardiovascular effects of nitrite shows that pretreatment with this compound, delivered as a salt in the form of sodium nitrite, protects against ischemia/reperfusion (I/R) injury in the heart in preclinical models (7, 19, 20, 40, 46, 60, 79, 189). In agreement with this, in healthy young humans, acute venous infusion of sodium nitrite increases resistance to temporary (reversible) I/R injury in the peripheral circulation, as assessed noninvasively by measuring the decrease in brachial artery flow-mediated dilation from normal baseline levels following cuff inflation-induced blood flow occlusion to the forearm (76). In adults with inducible myocardial ischemia, venous infusion of sodium nitrite improves functional cardiac responses during dobutamine stress echocardiography, suggesting that nitrite acts as a coronary vasodilator (76). The results of studies using nitrate administration also lend support to the concept that nitrite-boosting interventions promote resistance to I/R injury (86, 190).

Several ongoing clinical trials are currently investigating the therapeutic effects of nitrite administration during coronary angioplasty (80) (clinical trial NCT01584453), coronary artery bypass surgery (clinical trial NCT01098409), and acute myocardial infarction (clinical trials NCT01388504, NCT00924118, and NCT01178359).

Injury in Other Organs/Tissues

Numerous investigations have reported that nitrite is protective against I/R and other forms of injury in multiple tissues
### Table 1. Sodium nitrite administration in humans and nonhuman primates

<table>
<thead>
<tr>
<th>Administration</th>
<th>Dose</th>
<th>Duration per dose</th>
<th>Sample size</th>
<th>Population</th>
<th>Peak MetHb, %</th>
<th>Avg ↓ MAP, mmHg</th>
<th>Side effects</th>
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<td>NR</td>
<td>NR</td>
<td>(32)</td>
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<td>Cynomolgus primates</td>
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<td>NR</td>
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<td>Acute (5 min)</td>
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<td>NR</td>
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<td>MA &amp; older adults with diabetes</td>
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<td>Acute</td>
<td>9</td>
<td>Young healthy adults</td>
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<td>−14</td>
<td>Mild headache (n = 5); nausea (n = 1)</td>
<td>(74)</td>
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<tr>
<td>Oral</td>
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<td>Acute</td>
<td>9</td>
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<td>NS</td>
<td>Mild headache (n = 4)</td>
<td>(74)</td>
</tr>
<tr>
<td>Oral</td>
<td>290–380 mg</td>
<td>Acute</td>
<td>9</td>
<td>Young healthy adults</td>
<td>≤11.0</td>
<td>−11</td>
<td>Mild headache (n = 4); nausea (n = 2)</td>
<td>(74)</td>
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<td>Young healthy men with inducible myocardial ischemia</td>
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<td>NS</td>
<td>NR</td>
<td>(77)</td>
</tr>
<tr>
<td>Venous infusion</td>
<td>~2 mg</td>
<td>Acute (20 min)</td>
<td>10</td>
<td>MA &amp; older adults with inducible myocardial ischemia</td>
<td>NR</td>
<td>NS</td>
<td>NR</td>
<td>(76)</td>
</tr>
<tr>
<td>Venous infusion</td>
<td>290–380 mg</td>
<td>Acute (30 min)</td>
<td>9</td>
<td>Young healthy adults</td>
<td>&lt;13.0</td>
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<td>Mild headache (n = 5); nausea (n = 1)</td>
<td>(96)</td>
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<tr>
<td>Oral</td>
<td>140–190 mg</td>
<td>Acute</td>
<td>9</td>
<td>Young healthy adults</td>
<td>&lt;5.0</td>
<td>−8</td>
<td>Mild headache (n = 3)</td>
<td>(96)</td>
</tr>
<tr>
<td>Oral</td>
<td>290–380 mg</td>
<td>Acute</td>
<td>9</td>
<td>Young healthy adults</td>
<td>&lt;12.0</td>
<td>−11</td>
<td>Mild headache (n = 3); nausea (n = 2); vomiting (n = 1)</td>
<td>(96)</td>
</tr>
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<td>Venous infusion</td>
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<td>3</td>
<td>Young healthy adults</td>
<td>&lt;4.0</td>
<td>−10</td>
<td>Mild dizziness (n = 1); tiredness (n = 1)</td>
<td>(95)</td>
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<td>Venous infusion</td>
<td>90–130 mg</td>
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<td>(95)</td>
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<td>Mild dizziness (n = 1); arm tingling (n = 1)</td>
<td>(95)</td>
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<td>Arterial infusion</td>
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<td>21</td>
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<td>(121)</td>
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Continued
Table 1.—Continued

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<tr>
<th>Administration</th>
<th>Dose</th>
<th>Duration per dose</th>
<th>Sample size</th>
<th>Population</th>
<th>Peak MetHb</th>
<th>Avg ↓ MAP, mmHg</th>
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<td>10.8</td>
<td>−17</td>
<td>NR (150)</td>
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<td>14 days</td>
<td>3</td>
<td>Cynomolgus primates</td>
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<td>NR (150)</td>
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<td>180 min</td>
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<td>Acute (bolus)</td>
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<td>Young healthy adults</td>
<td>NR</td>
<td>NR</td>
<td>NR (158)</td>
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<tr>
<td>Oral</td>
<td>200–300 mg</td>
<td>Acute (30 min)</td>
<td>10</td>
<td>Young &amp; MA healthy adults</td>
<td>NR</td>
<td>−13</td>
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<td>(192)</td>
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<tr>
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<td>Acute (30 min)</td>
<td>14</td>
<td>Young &amp; MA with renal disorders or nephrectomy</td>
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<td>−12</td>
<td></td>
<td>(192)</td>
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<td>Oral</td>
<td>200–300 mg</td>
<td>Acute (30 min)</td>
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<td>Young, MA &amp; older with Stage 1 &amp; Stage 2 hypertension</td>
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<td></td>
<td>(192)</td>
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<td>NC</td>
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</tbody>
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MetHb, methemoglobin; MAP, mean arterial pressure; MA, middle-aged (40–60 yr); NR, not reported; NS, no significant change; NC, not clear.

and organs (81, 125, 188), as reviewed extensively elsewhere (157). Indeed, nitrite administration reduces skeletal muscle (188), renal (125, 179), and liver (46, 154, 169) injury after ischemia. Infarct size and hematoma volume decreases and blood flow enhances in the brain after stroke with nitrite pretreatment (81, 83), and a nitrate-rich diet has been reported to increase cerebral perfusion in older adults (152). Within the lung, nitrite, nitrate, or both limits injury and enhances recovery after ischemia (140, 176) and ameliorates pulmonary hyperperfusion in experimental animal models (8, 22, 145). In humans, nitrite reduces pulmonary arterial pressure during hypoxia (77) and improves cell activation and wound healing in human airway epithelial cells (187). Nitrite treatment lessens renal injury in hypertensive rats (84) and can protect against brain death-mediated renal injury while also improving posttransplant renal function (89). Nitrite, but not nitrate, therapy is effective in restoring blood flow and stimulating vascular remodeling, thereby preventing tissue necrosis in aged-diabetic mice after unilateral femoral ligation (12). Recent studies in humans (39, 77) and ongoing clinical trials (NCT01431313, NCT01725256, NCT01725269, NCT01715883, and NCT01316796) may provide evidence in humans supporting these preclinical findings.

Cardiovascular Disease and its Risk Factors

The effects of dietary nitrate on cardiovascular outcomes have been reviewed elsewhere, and generally support the efficacy of this approach (109). Regarding inorganic nitrate, multiple studies in preclinical models suggest that nitrite also may be effective for the prevention and/or treatment of many cardiovascular disorders or risk factors for CVD including peripheral artery disease (4, 69, 146), atherosclerosis (2, 3, 5, 127), chronic heart failure (32, 122), stroke (50, 82, 150), diabetes (138), and hypertension (22, 57, 58, 128). Presently, there is some evidence that treatment with sodium nitrite may have beneficial cardiovascular effects in humans by acutely (by either mildly or moderately) decreasing blood pressure (32, 37, 63) (Table 1); however, many of these studies administered much higher acute doses of nitrite than are currently being used in clinical trials. In addition, acute infusions of sodium nitrite increase forearm blood flow under normoxic (32, 74, 122, 151) and hypoxic (77, 122) conditions in healthy adults and in patients with heart failure (121). Recently published studies in humans provide additional evidence for nitrite or nitrate as a therapeutic agent for the prevention and treatment of cardiovascular disorders or risk factors for CVD, and ongoing clinical investigations will extend present insight (clinical trials...
Despite accumulating evidence suggesting that acute nitrite administration may be broadly effective in treating cardiovascular disease (4, 24, 114), little preclinical or clinical information is available with regard to chronic nitrite therapy and arterial function/health per se (128, 175). In the present synthesis article, we summarize the results of recent work aimed at establishing the efficacy of inorganic nitrite as a treatment for arterial aging and discuss the potential physiological mechanisms suggested.

INORGANIC NITRITE: AN ANTI-AGING TREATMENT FOR ARTERIES?

The potential efficacy of sodium nitrite in vascular aging was initially assessed in a preclinical investigation in which sodium nitrite was added to the drinking water (50 mg/l) for 3 wk in young (4–6 mo) and old (26–28 mo) male C57/BL6 mice (53, 171). Nitrite concentrations in the plasma and large elastic arteries (aorta and carotid arteries) were lower in older animals, but sodium nitrite supplementation increased nitrite concentrations in the old mice up to or above those of young control animals (Fig. 2) (171). Thus sodium nitrite supplementation was effective in restoring circulating and tissue nitrite bioavailability in old mice. Because sodium nitrite had no effect on arterial function in the young mice in this study (171), and constitutive NO production by eNOS is sufficient in young animals/humans in most cases, only the influence of nitrite therapy on arterial function of old mice will be described in the sections below.

Large Elastic Artery Stiffness

Among the measurements of large elastic artery stiffness, aortic PWV has emerged as the strongest and most consistent predictor of cardiovascular events and risk with aging (126, 185). In this initial study (171), aortic PWV was markedly higher (i.e., the aorta was stiffer) in old compared with young control mice (Fig. 3A). Sodium nitrite treatment reduced aortic PWV in old mice to well below old controls and not significantly different from that in young animals. These results suggested that sodium nitrite supplementation, started late in life, can substantially reduce large elastic artery stiffness in old mice.

In a follow-up study (53), the potential mechanisms of this destiffening effect by sodium nitrite were investigated. Aortic PWV was higher in old mice and this was associated with higher adventitial TGF-β and collagen levels, and lower medial elastin and higher adventitial and medial abundance of AGEs in the aorta. Sodium nitrite reduced aortic PWV in old mice, but did not influence aortic TGF-β, collagen, or elastin. However, nitrite supplementation normalized total, adventitial, and medial AGEs in the aorta of old mice (Fig. 3B). In aortic rings from young mice studied ex vivo, administration of AGEs directly induced stiffening, an effect prevented by incubation with sodium nitrite (Fig. 3C).
To our knowledge, the effects of inorganic nitrite supplementation on arterial stiffness have not been investigated in humans. However, 4 wk of sodium nitrate supplementation was reported to decrease aortic PWV in older adults with risk factors for CVD (156). In addition, several studies have investigated the effects of acute nitrate administration in human subjects. An acute oral dose of potassium nitrate was found to decrease aortic PWV in young healthy adults (6), whereas a single dose of beetroot juice reduced aortic PWV in patients with Stage 1 essential hypertension (57). A meal high in nitrate had no significant effect on aortic PWV in a group of healthy adults, although an indirect measure of arterial stiffness derived from measurements at the radial artery was found to decrease (110).

In summary, these findings indicate that short-term supplementation with sodium nitrite reduces arterial stiffness in old mice, perhaps by reversing AGEs formation. Moreover, there is some evidence from preliminary studies that suggests that boosting nitrite bioavailability via dietary nitrate intake may hold promise for reducing large elastic artery stiffness in humans.

Vascular Endothelial Dysfunction

In an initial preclinical study (171), vascular endothelial function as assessed by carotid artery EDD in response to acetylcholine, was impaired in old compared with young control animals (Fig. 4, top). Inhibition of NO using the NO synthase inhibitor L-NAME, markedly reduced EDD in young animals while having less effect in old mice, indicating that the impaired EDD in old animals was mediated by reduced NO bioavailability. Sodium nitrite treatment restored EDD in old mice by increasing NO-dependent dilation (Fig. 4, bottom). Nitrite did not affect endothelium-independent dilation to sodium nitroprusside (171), indicating that the improvements in EDD were not induced by increased vascular smooth muscle sensitivity to NO. Thus nitrite supplementation appears to ameliorate vascular endothelial dysfunction in old animals by restoring endothelial NO bioavailability.

These results obtained in a setting of primary aging are in agreement with previous observations in young hypercholesterolemic mice (175), and also are consistent with the vasodilatory effects and vascular protection against I/R injury (76, 86, 190) afforded by dietary nitrite (32, 37, 120–122) and nitrate (14, 69) administration in healthy adults, as well as patients with risk factors for CVD (156). Together, these findings suggest that inorganic nitrate or nitrite supplementation may have broad therapeutic efficacy for treating arterial endothelial dysfunction in a number of clinical states.

Potential Mechanisms of Action

Oxidative stress. Recent preclinical investigations found that the staining intensity for nitrotyrosine, a cellular marker of oxidant modification of proteins, was markedly greater in aorta of old compared with young control mice (53, 171) (Fig. 5A). Sodium nitrite treatment normalized aortic nitrotyrosine staining in old mice, indicating amelioration of age-associated arterial oxidative stress. This is consistent with recent observations that sodium nitrite supplementation reduces markers of oxidative stress in a model of experimental hypertension (128), after cerebral or cardiac I/R injury (81, 92), following alcohol-induced liver injury (81, 113), and after femoral ligation in diabetic mice (12). Nitrate supplementation reduced oxidative stress in spontaneously hypertensive rats (30), in rats with unilateral nephrectomy that consumed a high-salt diet (25), and in a mouse model of cardiomyopathy (196, 199). Collectively, this work supports the concept that nitrite and nitrate have strong antioxidant effects in a variety of tissues, including arteries, under physiological and pathophysiological conditions associated with oxidative stress.

Superoxide production. To gain insight into the source of arterial oxidative stress with aging and its mitigation with nitrite administration, aortic superoxide production was directly assessed using electron paramagnetic resonance spectroscopy in the above referenced studies (53, 171). Superoxide production was much greater in aorta from old compared with young control mice, and short-term treatment with sodium nitrite completely normalized superoxide formation in old animals (53, 171) (Fig. 5B). These observations indicate that sodium nitrite treatment reverses arterial oxidative stress at least in part by reducing superoxide production, and are in agreement with recent findings in hypertensive rats showing reduced staining for superoxide in aortic rings following nitrite supplementation (128, 129).

To link these age- and nitrite-related effects on arterial superoxide production and oxidative stress to arterial function, the superoxide scavenger TEMPOL (4-hydroxy-2,2,6,6-
tetramethylpiperidine-\(N\)-oxyl) was administered to carotid arteries ex vivo and EDD was assessed (171). TEMPOL had no effect on EDD in old mice treated with sodium nitrite, but completely restored EDD in old control animals (i.e., the group with excessive superoxide-associated oxidative stress at baseline). Overall, these results are consistent with the idea that sodium nitrite treatment restores NO-mediated EDD in old mice by reducing superoxide production and oxidative stress.

**Tetrahydrobiopterin and eNOS uncoupling.** To determine whether age-associated oxidative stress and its reversal with sodium nitrite might be influencing arterial function via BH4 deficiency-dependent eNOS uncoupling, carotid arteries have been treated ex vivo with sepiapterin (171), which increases BH4 bioavailability, recouples eNOS, and boosts NO production in states of BH4 deficiency (38). Sepiapterin restored acetylcholine-induced EDD selectively in carotid arteries of old control mice although it had no effect on EDD in old mice treated with sodium nitrite (171). These data suggest that superoxide-related oxidative stress suppresses arterial function in old control mice by causing oxidation of BH4 and uncoupling eNOS, thus reducing NO production. Sodium nitrite restores NO-mediated vascular endothelial function in part by reversing these effects of aging on superoxide formation and the oxidation of BH4, thus preserving eNOS-mediated NO production. These observations are consistent with recent findings of increases in BH4 and the BH4/BH2 ratio in the liver after sodium nitrite treatment for 3 wk in mice fed a high-cholesterol diet (175), and further support the concept that nitrite has antioxidant actions in vivo.

**NADPH oxidase.** To determine the source of superoxide-associated oxidative stress with arterial aging and the therapeutic effects of sodium nitrite supplementation, the contribution of the major oxidant-producing enzyme in vascular tissue, NADPH oxidase has been assessed (171). Protein expression of NADPH oxidase was threefold greater in aorta of old mice compared with those of young control mice, but this was normalized after sodium nitrite treatment (Fig. 5C). To link NADPH oxidase-associated superoxide production to age- and sodium nitrite-related effects on arterial function, the influence of inhibiting the enzyme with apocynin was assessed on carotid artery EDD ex vivo (171). Inhibition of NADPH oxidase with apocynin had no effect on EDD in arteries of old animals treated with sodium nitrite, but restored EDD in old control mice (171). These observations support the postulate that sodium nitrite treatment ameliorates superoxide-dependent impairment of NO-mediated vascular endothelial function in old mice at least in part by reducing superoxide production by NADPH oxidase.

Taken together, these results are consistent with recent findings in an experimental model of hypertension in which sodium nitrite supplementation reduced NADPH oxidase activity and apocynin decreased staining for superoxide in aortic rings of control and/or hypertensive rats (128). In subsequent in vitro experiments (liver homogenates) from that study,
nitrite administration did not exert direct antioxidant effects, inhibit the oxidant enzyme xanthine oxidase, or suppress mitochondrial superoxide production, but rather appeared to exert its antioxidant influence via inhibition of NADPH oxidase protein expression and activity (128).

Antioxidant defenses. Arterial antioxidant enzyme defenses also have been assessed in response to nitrite treatment in old mice (171). The activity of the major antioxidant enzyme SOD was found to be ~50% lower in aorta of old compared with young control mice, and short-term treatment with sodium nitrite restored SOD activity in aorta of old mice to levels observed in young controls (Fig. 5D). These data provided the first evidence for an antioxidant-boosting effect of sodium nitrite, and suggest that activation of SOD may play a significant role in the ability of nitrite supplementation to reverse age-associated oxidative stress and improve arterial function. These findings are supported by recent work demonstrating that nitrite normalizes SOD activity in a model of reno-vascular hypertension (129), and in gills and liver of fish (31). Nitrite supplementation also preserves or increases antioxidant capacity in the heart after an acute nitrite exposure under basal conditions (43, 89, 113, 148), after I/R injury (37), and in a rat model of high altitude-induced hypoxia (172). Finally, nitrite protects the kidneys after brain death-induced renal injury (74) and liver after an acute alcohol-induced injury (97) by enhancing antioxidant defenses.

Inflammation. Chronic low-grade inflammation develops in arteries with aging (44, 180) and is a key mechanism mediating age-related arterial dysfunction (34, 45, 149, 162). Consistent with these earlier observations, expression of the proinflammatory cytokines interleukin (IL)-1β, IL-6, interferon-γ, and tumor necrosis factor-α (TNF-α) were shown to be greater in aorta of old mice than young control mice (171) (Fig. 6). Most importantly, nitrite supplementation completely reversed this age-associated arterial inflammation (171). These findings are in agreement with a report showing that sodium nitrite treatment normalizes plasma C-reactive protein concentrations, leukocyte markers of inflammation, and improves vascular endothelial function in hypercholesterolemic mice (175). Consistent with this finding, sodium nitrite decreases plasma IL-6 concentrations and increases survival rate in a crush injury model in rats (134), and reduces liver granulocyte infiltrates after endotoxemic shock (28, 29, 65). Nitrite also lessens inflammation caused by ischemia in various organs (89, 125, 140, 146, 147, 176, 197) and normalizes inflammation in a mouse model of inflammatory bowel disease (139). Moreover, studies conducted in microvascular endothelial cell culture demonstrate that nitrite prevents TNF-α-induced upregulation of intracellular adhesion molecule type 1, providing evidence of a direct anti-inflammatory effect of nitrite in vascular cells (78). Taken together, these results support the view that nitrite therapy exerts a potent systemic and vascular anti-inflammatory effect that is associated with improvements in arterial function.

Mitochondrial function. Accumulating evidence implicates mitochondrial dysregulation in impaired arterial function both in animal models of cardiovascular disease (94, 161, 182) and human diabetes mellitus (166). Emerging evidence suggests that NO deficiency is associated with impaired mitochondrial function and that restoration of NO bioavailability induces mitochondrial biogenesis (127, 135, 167, 168). A recent study reported nitrite-induced stimulation of mitochondrial biogenesis is associated with increases in adenosine monophosphate-activated protein kinase and peroxisome proliferator-activated receptor gamma coactivator 1-α signaling in aortic smooth muscle cells in an experimental model of carotid artery injury (127). Pretreatment with nitrite under normoxic conditions protects cardiomyocytes from cell death after a hypoxic challenge and is dependent on activation of protein kinase A, which inhibits dynamin-related protein-1 leading to enhanced mitochondrial fusion (153). Nitrite also minimizes mitochondrial damage in a mouse shock model induced by a lethal dose of TNF-α (29), and nitrate normalizes mitochondrial-specific antioxidant proteins in a cardiotoxicity model in mice (196). In humans, boosting inorganic nitrite either from oxidation of endogenously produced NO or from dietary supplementation of nitrate, enhances mitochondrial function and efficiency by improving oxidative phosphorylation (102, 103). Collectively, these observations have potentially important implications for the prevention and treatment of metabolic disorders, age-related physiological dysfunction, and chronic diseases. We expect future studies to determine the effects of nitrite on age-associated mitochondrial dysfunction and its role in vascular function.

**Integrative Working Hypothesis**

How might oral inorganic nitrite or nitrate supplementation produce the beneficial vascular effects observed in preclinical studies in old mice and, perhaps, in recent investigations in models of cardiovascular disease (3, 84, 129, 175)? Although the precise mechanisms of action are incompletely understood, we offer the following reasoned speculation as illustrated in Fig. 7. Dietary supplementation of inorganic nitrate that results in significant increases in circulating and tissue nitrite levels would presumably have the same actions.

To briefly review, aging leads to increases in vascular production of superoxide anion, resulting in a state of oxidative stress and causing synergistic development of vascular inflammation. The excessive vascular superoxide bioactivity induces

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**Fig. 6.** Expression of proinflammatory cytokines interleukin (IL)-1β, IL-6, interferon-γ (IFNγ), and tumor necrosis factor-α (TNF-alpha) in aorta from young and old control and old nitrite-supplemented (Old Nitrite) mice. Values are means ± SE. *P ≤ 0.05 Young Control vs. Old Control; †P ≤ 0.05 Old Control vs. Old Nitrite. Data were adapted from (171).
several changes to arteries including modifications of structural proteins (greater levels of collagen, fragmentation of elastin, formation of AGEs, etc.) and greater generation of the ROS peroxynitrite (via superoxide reaction with NO), with consequent oxidation of BH4 and uncoupling of eNOS. The latter series of events result in lower NO bioavailability due to the combination of direct superoxide reaction with NO and less eNOS production of NO. Reduced bioavailability of NO in turn results in less circulating nitrite because less NO is being metabolized into nitrite.

We postulate that nitrite supplementation reverses the vascular effects of aging, possibly by multiple mechanisms of action. Nitrite, perhaps in part through modulation of gene transcription (21, 85, 172), reduces superoxide by suppressing ROS production by the potent vascular oxidant enzyme NADPH oxidase and, possibly, by mitochondria, while boosting the activity of the important superoxide scavenging antioxidant enzyme, SOD. This normalization of vascular superoxide reverses the changes in structural proteins and its reaction with NO, reduces peroxynitrite formation, and recouples eNOS, such that NO bioavailability is increased via less destruction (by superoxide) and more production by eNOS. Endothelial NOS also produces less superoxide under these conditions (reversing the vicious cycle) and suppresses inflammation. In addition to these effects on superoxide, nitrite supplementation increases NO bioavailability via subsequent reductions in the circulation and tissues to NO, mediated by both enzymatic and nonenzymatic pathways (114). Thus, circulating and arterial concentrations of nitrite are restored in old animals and, collectively, these events lead to improvement, even normalization in some cases, of NO homeostasis and vascular function.

CONCLUSIONS AND CLINICAL IMPLICATIONS

In conclusion, nitrite and nitrate are biologically important precursors of NO that have the potential to restore NO bioavailability in states of NO insufficiency via the nitrate-nitrite-NO pathway. Recent findings provide experimental support that oral supplementation of inorganic nitrite or nitrate may have the ability to ameliorate key features of arterial aging that are believed to be clinically important antecedents of CVD, including large elastic artery stiffening, endothelial dysfunction, and vascular oxidative stress and inflammation. Nitrite supplementation also may induce mitochondrial biogenesis and improve mitochondrial health in arteries, and exert broadly beneficial actions in multiple cell stress pathways in the aging vasculature. Finally, nitrite therapy also holds promise for treating many other age-related clinical disorders associated with increased risk for CVD, including hypercholesterolemia (175), hypertension (58, 128), and chronic kidney disease (84).

Increasing the bioavailability of NO by boosting concentrations of its precursor, nitrite (either by direct supplementation of nitrite or via increased dietary nitrate intake), represents a new paradigm that has great potential for improving NO homeostasis and vascular function with aging. However, much of the work to date determining the beneficial effects of nitrite or nitrate supplementation per se has occurred using preclinical models or acute administration in humans. As such, more studies are needed to determine the broad therapeutic potential and feasibility with chronic nitrite or nitrate administration on physiological and pathophysiological outcomes. Several ongoing clinical trials assessing the efficacy of nitrite and nitrate supplementation should provide new insight into the potential pleiotropic effects of increasing nitrite bioavailability on age-associated physiological dysfunction and disease. Despite its potential clinical implications, additional research is needed to establish the efficacy of chronic nitrite or nitrate supplementation for the promotion of vascular health and the prevention and treatment of CVD.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Fig. 7. Proposed mechanisms for the detrimental effects of aging on the vasculature and possible beneficial effects of nitrite. O2·-, superoxide; AGEs, advanced glycation end-products; NO, nitric oxide; SOD, superoxide dismutase.
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