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Nitrite supplementation reverses vascular endothelial dysfunction and large elastic artery stiffness with aging

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Introduction

Cardiovascular diseases (CVD) remain the leading cause of death in modern societies (Lloyd-Jones *et al.*). Advancing age is the major risk factor for CVD (Lakatta 2002; Lakatta & Levy 2003). Most CVD are linked to disorders of arteries (Lloyd-Jones *et al.*) and it is now recognized that aging increases risk of CVD in large part by causing arterial dysfunction, which then leads to clinical vascular and cardiac diseases (Lakatta & Levy 2003).

Many changes to arteries likely contribute to the increased risk of CVD with aging. One of these is the development of vascular endothelial dysfunction, as most commonly indicated by impaired endothelium-dependent dilation (EDD) (Celermajer *et al.* 1994; Taddei *et al.* 1995; Lakatta & Levy 2003). Impaired EDD with aging is mediated by excessive superoxide, at least in part as a result of increases in the oxidant enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) (Zalba *et al.* 2000; Bedard & Krause 2007; Donato *et al.* 2007; Durrant *et al.* 2009). Increased superoxide reduces bioavailability of nitric oxide (NO) both by reacting with NO to form peroxynitrite and by oxidizing tetrahydrobiopterin (BH_4), an essential co-factor for NO production by endothelial nitric oxide synthase (eNOS) (Cosentino *et al.* 1998; Taddei *et al.* 2001; Landmesser *et al.* 2003; Blackwell *et al.* 2004; Shi *et al.* 2004). Another key vascular change with aging is the stiffening of large elastic arteries (Lakatta & Levy 2003), possibly linked in part to development of vascular endothelial dysfunction (Fitch *et al.* 2001; Wilkinson *et al.* 2004). Large elastic artery stiffness has emerged as a major independent risk factor for age-associated CVD and a key therapeutic target (Lakatta & Levy 2003; Nilsson *et al.* 2009; Mitchell *et al.*; Vlachopoulos *et al.* 2010).

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Author contributions

Amy L. Sindler, Bradley S. Fleenor, David J. Lefer and Douglas R. Seals contributed to the conception, experimental design and interpretation of the data. Amy L. Sindler and Melanie L. Zigler collected most of the data; David J. Lefer and John W. Calvert were responsible for the nitrite measures in arteries and tissue; Kurt D. Marshal assisted with the collection and analysis of the Western blot data. The manuscript was largely written by Douglas R. Seals and Amy L. Sindler; however, all authors were involved in the writing and in the final approval of the paper.

Oxidative stress and inflammation are believed to be important mechanisms contributing to these effects of aging on arteries (Brandes *et al.* 2005; Donato *et al.* 2007; Csiszar *et al.* 2008; Donato *et al.* 2008; Ungvari *et al.* 2010). Arterial oxidative stress develops with aging as a consequence of excessive production of superoxide by NADPH oxidase or other mechanisms (eNOS uncoupling, mitochondrial dysfunction) (Vasquez-Vivar *et al.* 1998; Ungvari *et al.* 2008; Durrant *et al.* 2009; Rippe *et al.* 2010) and possibly via selective reductions in antioxidant enzymes, including superoxide dismutase (SOD) (Sun *et al.* 2004; Rippe *et al.*). As a result of oxidative stress, injury or other causes, vascular inflammation also develops with aging, as indicated by increases in expression of pro-inflammatory cytokines such as interleukins 1 and 6 (IL-1, IL6), interferon γ (INFγ) and tumor necrosis factor α (TNFα) (Csiszar *et al.* 2008; Donato *et al.* 2008; Rippe *et al.* 2010; Ungvari *et al.* 2010).

Given the above, treatments that improve or reverse vascular endothelial dysfunction and large elastic artery stiffness in middle/older age may prevent much of the increase in CVD risk with aging (Lakatta & Levy 2003; Lonn *et al.* 2010; Seals 2010). In this context, the nitrite anion (inorganic nitrite) would seem to hold promise. Once considered an inert byproduct of NO metabolism, nitrite now is recognized as a physiologically important storage form of NO (Lundberg & Weitzberg 2008). Nitrite is a cytoprotective molecule considered to have broad therapeutic potential for the prevention and treatment of CVD (Calvert & Lefer; Lundberg & Weitzberg 2008). Sodium nitrite administration protects against ischemia/reperfusion injury (Bryan *et al.* 2007; Bryan *et al.* 2008; Gonzalez *et al.* 2008) and improves vascular endothelial dysfunction in hypercholesterolemic mice (Stokes *et al.* 2009). However, the efficacy of sodium nitrite for treating arterial aging is entirely unknown.

In the present study, we hypothesized that short-term oral sodium nitrite treatment would improve or reverse vascular endothelial dysfunction and large elastic artery stiffness in old mice while having no effects in young mice. As a secondary aim, we sought to gain initial insight into the mechanisms by which nitrite-induced improvements in function in the old mice might be mediated, particularly those related to reduced oxidative stress and inflammation.

Results

Animal characteristics

Characteristics of the groups are shown in Table 1. Body mass and heart mass were greater and gastrocnemius muscle mass was smaller in the old compared with the young groups ($p<0.05$). Carotid artery baseline diameter was greater in the old animals ($p<0.05$) and sodium nitrite treatment had no effect on these characteristics. Carotid artery preconstriction to phenylephrine was not different between groups.

Sodium nitrite treatment restores NO-mediated EDD in old mice

EDD to acetylcholine (ACh) was lower in old compared with young control mice ($p<0.05$) as a result of a smaller NO dilatory influence, as indicated by a smaller reduction in EDD in the presence vs. absence of the NO inhibitor N-G-nitro-L-arginine methyl ester (L-NAME) (Figure 1 A and B). Nitrite treatment restored EDD to ACh in old mice by restoring NO-mediated dilation, but had no effect in the young animals (Figure 1 A and B). There were no differences in NO-mediated EDD among the young control and young and old nitrite treated animals. Endothelium-independent dilation to sodium nitroprusside did not differ among the groups (Figure 1C). TEMPOL, a superoxide dismutase mimetic (Figure 2A), apocynin, a NADPH oxidase inhibitor (Figure 2B), and sepiapterin, a precursor of BH_4 (Figure 2C) each

restored EDD to ACh in old control animals, while not affecting responses in young control or old sodium nitrite treated animals. eNOS protein in the aorta was lower in the old vs. young control animals (Table 2), and this was not influenced by sodium nitrite treatment. Sodium nitrite supplementation increased eNOS expression in young mice. These data demonstrate that short-term sodium nitrite treatment restores NO-mediated EDD in old mice via a superoxide/NADPH oxidase/BH_4-related mechanism and not by affecting vascular smooth muscle sensitivity to NO or eNOS protein.

Sodium nitrite treatment normalizes aortic pulse wave velocity in old mice

Large elastic artery stiffness, as determined by aortic pulse wave velocity, was ~70% greater in old compared with young control mice ($p<0.05$) (Figure 3). Sodium nitrite treatment reversed the age-associated increase in aortic pulse wave velocity in old mice. These observations indicate that short-term treatment with sodium nitrite ameliorates age-associated stiffening of the aorta.

Sodium nitrite treatment reduces vascular oxidative stress and inflammation in old mice

Nitrotyrosine, a cellular marker of oxidative stress, was ~100% greater in the aorta of old compared with young control mice ($p<0.05$) (Figure 4A), and was associated with similarly greater aortic superoxide production, and an ~50% reduction in aortic SOD activity ($p<0.05$) (Figure 4 B and C). NADPH oxidase subunit p67 protein expression was ~300% greater in the aorta of old compared with young control mice ($p<0.05$) (Figure 4D). Nitrite treatment in old animals reduced aortic nitrotyrosine to levels observed in young control mice, and this was associated with complete reversal of the age-associated changes in superoxide production, SOD activity and aortic p67 expression. Protein expression of manganese SOD (MnSOD) in aorta was decreased in old control mice ($p<0.05$) and this was unaltered by nitrite supplementation; young nitrite supplemented mice had reduced MnSOD ($p<0.05$) (Table 2). Protein expression of the inflammatory cytokines IL-1β, IL6, INFγ and TNFα was increased in aorta of old compared with young mice. Short-term nitrite treatment reduced aortic inflammatory cytokines selectively in old mice, normalizing expression to levels observed in young controls ($p<0.05$, Figure 5). These observations indicate that short-term sodium nitrite treatment ameliorates arterial oxidative stress (by normalizing superoxide production and SOD activity) and inflammation with aging.

Sodium nitrite treatment restores the reductions in nitrite concentrations in plasma, large elastic arteries and heart aging

To obtain sufficient tissue to determine nitrite concentrations in large elastic arteries, carotid arteries and aorta were pooled from each group of mice (Figure 6). Nitrite concentrations in the large elastic arteries, heart and plasma were lower in old compared with young control animals ($p<0.05$). In the old mice, nitrite treatment restored nitrite concentrations to levels either not different from (elastic arteries and heart) or well above (plasma) those observed in young control mice ($p<0.05$). Nitrite supplementation also increased nitrite levels in young mice ($p<0.05$). These results show that nitrite concentrations in large elastic arteries, heart and plasma decrease with aging, and that short-term sodium nitrite treatment restores nitrite concentrations in old mice.

Discussion

The key finding of the present study was that 3 weeks of sodium nitrite treatment in old C57BL6 mice ameliorated carotid artery endothelial dysfunction and reduced large elastic artery stiffness to a level not different from young mice. Age is the major risk factor for CVD and vascular endothelial dysfunction and large elastic stiffening are believed to explain much of the increase in CVD risk with aging (Lakatta 2002; Vita & Keaney 2002;

Mitchell *et al.*). Our preclinical findings, therefore, suggest that sodium nitrite has intriguing translational potential as a treatment for the prevention of age-associated CVD in humans. The present results also provide novel insight into the mechanisms by which sodium nitrite reverses arterial aging, which include restoring NO and BH_4 bioavailability, suppressing superoxide-dependent oxidative stress by reducing NADPH oxidase and increasing SOD activity, and reducing inflammation. Finally, our results indicate that aging is associated with reduced concentrations of nitrite in large elastic arteries, the heart and plasma, providing additional rationale for therapies that enhance circulating and tissue nitrite stores.

Vascular endothelial dysfunction

The present results are consistent with previous reports from our laboratory (Durrant *et al.* 2009; Lesniewski *et al.* 2009; Rippe *et al.*) and others (Muller-Delp *et al.* 2002; Soucy *et al.* 2006) that vascular endothelial dysfunction develops with aging, as indicated by impaired EDD. The latter is mediated by reduced NO bioavailability (Luscher & Barton 1997; Spier *et al.* 2004; Sindler *et al.* 2009) as a result of NADPH oxidase-associated increases in superoxide (Hamilton *et al.* 2002; Rippe *et al.* 2010) and inadequate BH_4 bioactivity (Cosentino *et al.* 1998; Blackwell *et al.* 2004; Eskurza *et al.* 2005; Delp *et al.* 2008).

Recently it was shown that 3 weeks of sodium nitrite treatment reverses impaired EDD to ACh in cremaster muscle arterioles of hypercholesterolemic mice (Stokes *et al.* 2009). In the present study, we show that in a model of primary arterial aging, short-term treatment with sodium nitrite completely restores EDD in old mice to levels observed in young animals. Importantly, our findings show for the first time that sodium nitrite treatment restores EDD in a setting of impaired baseline function by restoring NO bioavailability (i.e., the NO-component of EDD) to normal control levels. eNOS protein in the aorta was reduced with aging, but was not influenced by sodium nitrite treatment in the older mice, consistent with earlier findings in myocardial tissue (Bryan *et al.* 2007).

The fact that administration of TEMPOL, a SOD mimetic, restored EDD in old control mice, while having no effect in young animals or old sodium nitrite treated mice, supports the idea that a reduction in superoxide was responsible for the normalization of endothelial function by sodium nitrite in old mice. That apocynin, an inhibitor of NADPH oxidase, selectively restored EDD in old control mice further suggests that the effects of sodium nitrite in old animals was mediated by reduced NADPH oxide production of superoxide. Lastly, sepiapterin, an exogenous donor of BH_4, restored EDD in old control, but not in old nitrite treated mice. This suggests that sodium nitrite may enhance NO bioavailability also by preserving BH_4, a critical co-factor for eNOS-dependent NO production, as shown recently in the liver of hypercholesterolemic mice (Stokes *et al.* 2009).

Together, our data are consistent with the possibility that short-term sodium nitrite treatment ameliorates vascular endothelial dysfunction in old mice by restoring NO bioavailability as a result of reduced NADPH oxidase superoxide production and enhanced bioactivity of BH_4.

Large elastic artery stiffness

Our finding that aortic pulse wave velocity was greater in old compared with young cage control mice is consistent with previous reports in both animals and humans that large elastic arteries stiffen with advancing age in the absence of disease (Reddy *et al.* 2003; Eskurza *et al.* 2004; Sutton-Tyrrell *et al.* 2005; Soucy *et al.* 2006). The present results extend these earlier findings by showing that 3 weeks of sodium nitrite therapy initiated in old age reduces aortic pulse wave velocity to levels that are no longer significantly different from those of young cage control and nitrite treated mice. Nitrite treatment had no effect on aortic pulse wave velocity in young mice, indicating that treatment selectively reduced large

elastic artery stiffness in old animals. Our findings in old mice are clinically important because recently it was shown that aortic pulse wave velocity is a major independent risk factor for incident CV events and all-cause mortality in older adults (Mitchell *et al.* 2010; Vlachopoulos *et al.* 2010). This provides experimental support for the idea that sodium nitrite treatment holds promise for reducing CVD and all-cause deaths in middle-aged and older humans.

Oxidant stress and inflammation

The present findings are consistent with previous work from our lab (Lesniewski *et al.* 2009; Rippe *et al.* 2010) and others (van der Loo *et al.* 2000; Csiszar *et al.* 2002; Yang *et al.* 2009) showing increased arterial oxidative stress with aging as indicated by a marked increase in aortic nitrotyrosine staining in old compared with young control mice. Nitrotyrosine is produced by nitration of tyrosine residues on proteins primarily by peroxynitrite, which is formed when superoxide reacts with NO (Radi 2004). The present findings also confirm recent results from our laboratory (Rippe *et al.* 2010) showing that this increase in arterial oxidative stress with aging in mice is associated with both increased superoxide production, as measured directly by spin trapping and electron paramagnetic resonance spectroscopy, and reduced activity of SOD, an important anti-oxidant enzyme.

Our findings here suggest that sodium nitrite treatment completely reversed the increase in arterial oxidative stress with aging, as indicated by a reduction of aortic nitrotyrosine abundance in nitrite treated old animals to levels observed in young control mice. An earlier report found that nitrite inhibits myeloperoxidase-mediated modification of low-density lipoprotein (Carr & Frei 2001), consistent with an antioxidant effect. Nitrite treatment appeared to reduce arterial oxidative stress in old mice, at least in part, by normalizing arterial superoxide production. Indeed, to our knowledge this is the first evidence that short-term sodium nitrite administration reduces superoxide formation in arteries, which is consistent with previous observations following ischemia/reperfusion injury in the heart (Dezfulian *et al.* 2009). Moreover, our results show that nitrite treatment restored SOD activity in aorta of old animals and this also may have contributed to reductions in arterial superoxide bioavailability and oxidative stress in this group. The effect appeared to be the result of an increase in activity of the enzyme and not due to changes in expression, as protein concentrations of the SOD isoforms were unaffected by sodium nitrite treatment in the old animals.

In the present study, we also show that sodium nitrite treatment reduced expression of several pro-inflammatory cytokines in aorta of old mice to concentrations observed in young controls. This is consistent with the results of a previous study in hypercholesterolemic mice, in which sodium nitrite treatment lowered plasma C-reactive protein and reduced leukocyte adhesion and infiltration through venular endothelium (Stokes *et al.* 2009). Taken together, our results provide the first evidence of a potent combined antioxidant and anti-inflammatory effect of sodium nitrite treatment on arteries.

Plasma and tissue nitrite concentrations

The present data are the first to show that nitrite concentrations are reduced with aging in large elastic arteries (−44% vs. young controls), the heart (−43%) and plasma (−46%). Reductions in nitrite in plasma and the heart have been documented previously in other mouse models of low NO bioavailability including hypercholesterolemia and eNOS deficiency (Bryan *et al.* 2008; Stokes *et al.* 2009). In the present study, nitrite supplementation in the drinking water increased arterial, cardiac and plasma nitrite concentrations in old mice to levels not different from (arterial and heart) or even greater than (plasma) young control mice. These findings are consistent with results of earlier

studies of nitrite supplementation in states in which baseline nitrite concentrations are reduced (Bryan *et al.* 2008; Stokes *et al.* 2009). We did not observe an increase in tissue nitrite above normal control concentrations reported previously in hypercholesterolemic mice (Stokes *et al.* 2009), but rather only in plasma levels in our old mice. Overall, our results demonstrate that short-term nitrite therapy in old mice restores (arterial and cardiac) or enhances above normal (plasma) nitrite concentrations compared with levels observed in young controls.

Conclusions

The results of the present study show for the first time that nitrite concentrations are reduced with aging in the circulation and cardiovascular tissues, and that short-term sodium nitrite therapy ameliorates vascular endothelial dysfunction and de-stiffens large elastic arteries, two clinically significant expressions of arterial aging, in old C57BL6 mice. Our findings also provide evidence that the improvements in vascular endothelial function in old mice treated with sodium nitrite are mediated by increased NO bioavailability as a result of reduced NADPH oxidase-dependent superoxide bioavailability and enhanced BH_4 bioactivity. Moreover, our data provide the first evidence for anti-oxidant and anti–inflammatory influences of sodium nitrite on arterial tissue. Overall, these findings from a preclinical model establish an experimental basis for translational research aimed at determining the efficacy of nitrite therapy for reversing arterial aging and reducing the risk of age-associated CVD in humans.

Experimental procedures

Animals

Young (4-6 months) and old (26-28 months; ~50% survival) male C57BL6 mice were obtained from the National Institute on Aging rodent colony and were fed normal rodent chow *ad libitum*. After an acclimation period of 2 weeks, the young and old mice were divided into two subgroups: control animals continued on regular drinking water and the other animals that had nitrite supplemented (50 mg/L) drinking water for three weeks. All mice were housed in an animal care facility at the University of Colorado at Boulder on a 12 h:12 h light-dark cycle. All animal procedures conformed to the *Guide to the Care and Use of Laboratory Animals* (NIH publication n. 85-23, revised 1996) and were approved by the UCB Animal Care and Use Committee.

Carotid artery vasodilatory responses

EDD and endothelium-independent dilation were determined *ex vivo* in isolated carotid arteries as previously described (Durrant *et al.* 2009; Lesniewski *et al.* 2009; Rippe *et al.*). Briefly, mice were anesthetized using isoflurane and euthanized by exsanguination via cardiac puncture. The carotid arteries were carefully excised, cannulated onto glass micropipettes and secured with nylon (11-0) suture in myograph chambers (DMT Inc.) containing buffered physiological saline solutions. The arteries were pressurized to 50 mmHg at 37° C and were allowed to equilibrate for 1 h. After submaximal preconstriction with phenylephrine (2 μmol/L), increases in luminal diameter in response to acetylcholine (ACh: 1×10^{-9} - 1×10^{-4} mol/L) with and without co-administration of the NO synthase inhibitor N-G-nitro-L-arginine methyl ester (L-NAME), (0.1 mmol/L, 30 min incubation), or the SOD mimetic, TEMPOL, (1 mmol/L, 60 min incubation), were determined. EDD also was determined in the presence of the NADPH oxidase inhibitor, apocynin, (1 mmol/L, 60 min incubation) and the exogenous BH_4 donor, sepiapterin, (1 mmol/L, 60 min incubation). Endothelium-independent dilation was determined by vasodilation in response to sodium nitroprusside (SNP: 1×10^{-10} - 1×10^{-4} mol/L). All dose response data are presented on a

percent basis. Preconstriction was calculated as a percentage of maximal diameter according to the following formula:

$$\text{Preconstriction}\,(\%) = (D_m - D_b)\,/D_m \times 100$$

Because of differences in maximal carotid artery diameter between young and old animals, vasodilator responses were recorded as actual diameters expressed as a percentage of maximal response according to the following formula:

$$\text{Relaxation}\,(\%) = (D_s - D_b)\,/\,(D_m - D_b) \times 100$$

Where D_m is maximal inner diameter at 50 mmHg, D_s is the steady-state inner diameter recorded after the addition of drug, and D_b is the steady-state inner diameter following preconstriction before the first addition of drug.

NO-dependent dilation was determined from the maximal EDD in the absence or presence of L-NAME according to the following formula:

$$\text{NO-dependent dilation}\,(\%) = \text{Maximal dilation}_{\text{ACh+}L\text{-NAME}} - \text{Maximum dilation}_{\text{ACh}}$$

In vivo aortic pulse wave velocity

Aortic pulse wave velocity was measured as described previously (Kim *et al.* 2009). Mice were anesthetized with 2% isoflurane and placed supine on a heating board with legs secured to ECG electrodes. Aortic velocity was measured with Doppler probes at the transverse aortic arch and abdominal aorta. Pre-ejection time, the time between the R-wave of the ECG to foot of the Doppler signal, was determined for each site. Aortic pulse wave velocity was calculated by dividing the distance between the transverse and abdominal probes by the difference in the thoracic and abdominal pre-ejection times.

Arterial superoxide production

Production of superoxide was measured by electron paramagnetic resonance *(*EPR) spectrometry using the spin probe 1-hydroxy-3-methoxycarbonly-2,2,5,5-tetramethylpyrrolidine (CMH, Alexis Biochemicals) as previously described (Rippe *et al.*). Two-millimeter aortic rings were incubated for 60 min at 37° C in 200 μl of Krebs-HEPES buffer containing 0.55 mmol/L CMH and analyzed immediately on an MS300 X-band EPR spectrometer (Magnettech, Berlin, Germany).

Arterial protein expression and enzyme activities

Aortas were used as a surrogate large elastic artery to provide sufficient tissue for analysis of protein expression by Western blot and enzyme activity as described previously (Durrant *et al.* 2009; Lesniewski *et al.* 2009; Rippe *et al.*). Aortas were excised, cleared of surrounding tissues and frozen in liquid nitrogen before storage at −80°C. The tissue was pulverized over liquid nitrogen and homogenized in ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors [Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN, USA) and 0.01% phosphatase inhibitor cocktail (Sigma, St. Louis, MO, USA)]. Ten micrograms of protein was loaded on 4-12% polyacrylamide gels, seperated by electrophoresis and transferred onto nitrocellulose membranes for Western blot analysis. Antibodies for Western blot analysis included anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH 1:1000, Cell Signaling), anti-nitrotyrosine (1:100, Abcam), anti-p67 phox (1:1000, Cell Signaling),

anti-MnSOD, anti-CuZn (1:2000 Stressgen), anti-ecSOD (1:500 Sigma) and anti-endothelial NO synthase (eNOS 1:500, BD Biosciences). Total SOD activity in aortic lysates (1 μg protein) was determined using the SOD Activity Assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Concentrations of the pro-inflammatory cytokines IL-1β, IL-6, IFNγ and TNF-α were determined in aortic whole cell lysates by multiplex ELISA (Searchlight Mouse Inflammatory Cytokine Kit; Aushon Biosystems; Billerica, MA) as previously described (Rippe *et al.*).

Analysis of nitrite in large elastic arteries, heart and plasma

Nitrite analysis procedures have been described in detail (Bryan *et al.* 2007; Bryan *et al.* 2008; Elrod *et al.* 2008). Nitrite concentrations were quantified by ion chromatography (ENO20 Analyzer, Eicom). Plasma was obtained by centrifugation at 800 g for 10 min.

Statistics

Results are presented as mean ± SEM. Statistical analysis was performed with SPSS 17.0 software. For the *ex vivo* vasodilatory dose response, group differences were determined by repeated measures ANOVA. A two-way ANOVA was used to analyze stiffness. For maximal dilation, protein expression, enzyme activities, superoxide production and animal characteristics comparisons between groups were made using ANOVA. Significance was determined using $p<0.05$.

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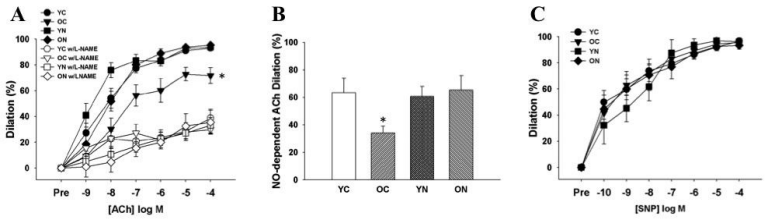

Figure 1. Endothelium-dependent, nitric oxide-dependent and endothelium-independent dilation

(A) Dose-responses to the endothelium-dependent dilator acetylcholine (ACh) in the absence and presence of the endothelial nitric oxide (NO) synthase inhibitor N-G-nitro-L-arginine methyl ester (L-NAME) in young and old control (YC and OC) and nitrite-supplemented (YN and ON) mice. (B) NO-dependent dilation (Max DilationACh – Max DilationACh+L-NAME). (C) Dose-responses to the endothelium-independent dilator sodium nitroprusside (SNP). Values are mean ± SEM. (n = 7 per group). * $p < 0.05$ vs. YC.

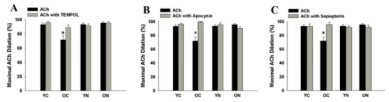

Figure 2. Superoxide-, NADPH oxidase- and tetrahydrobiopterin-dependent modulation of endothelial-dependent dilation

(A) Maximal dilation of carotid arteries to acetylcholine (ACh) and ACh + TEMPOL, a superoxide dismutase mimetic. (B) Maximal dilation of carotid arteries to ACh and to ACh + apocynin, a NADPH oxidase inhibitor. (C) Maximal dilation of carotid arteries to ACh and to ACh + sepiapterin, an exogenous tetrahydrobiopterin donor. Values are mean ± SEM. (n = 6 – 9 per group) * $p < 0.05$ vs. YC.

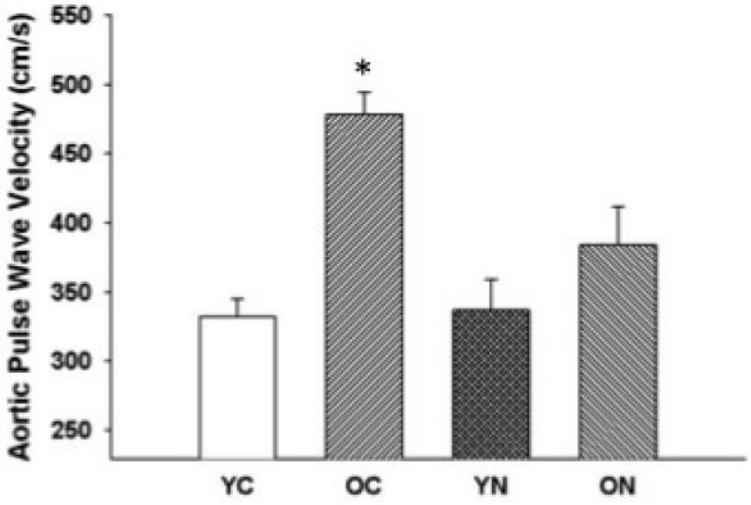

Figure 3. Large elastic artery stiffness

Aortic pulse wave velocity in young and old control (YC and OC) and young and old nitrite supplemented (YN and ON) mice. Values are mean ± SEM. (n = 5 per group) * $P < 0.05$ vs. YC.

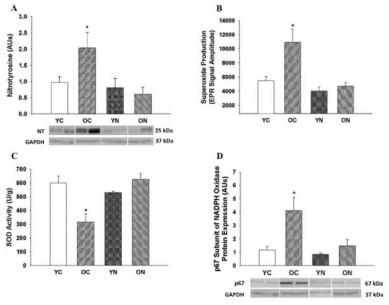

Figure 4. Aortic oxidative stress, superoxide production and oxidant/antioxidant enzymes

(A) Nitrotyrosine in aorta of young and old control (YC and OC) and young and old nitrite supplemented (YN and ON) mice. Data are expressed relative to GAPDH and normalized to YC mean value. Representative western blot images below. (B) Mean electron paramagnetic resonance (EPR) signal for superoxide from aortic rings. (C) Aortic superoxide dismutase (SOD) enzymatic activity. (D) Protein expression of p67 subunit of NADPH oxidase in aorta of young and old control (YC and OC) and young and old nitrite supplemented (YN and ON) mice. Data are expressed relative to GAPDH and normalized to YC mean value. Representative western blot images below. Values are mean ± SEM. (n = 6 – 9 per group) * $p < 0.05$ vs. YC.

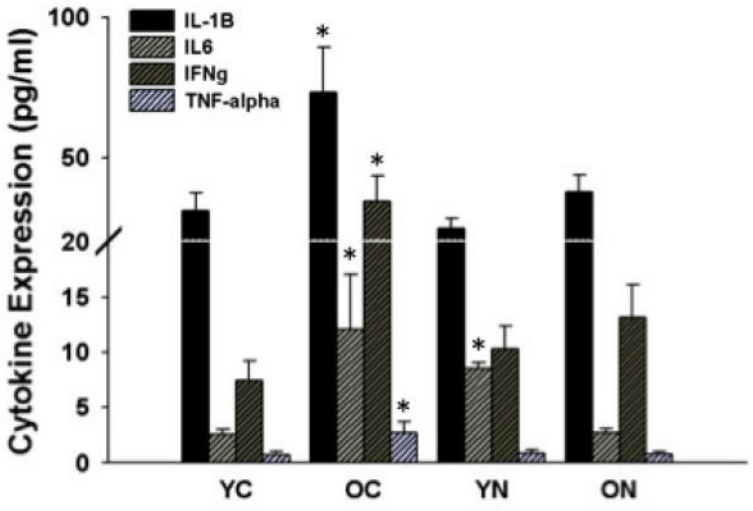

Figure 5. Aortic inflammatory cytokines

Expression of inflammatory cytokines IL-1β, IL-6, IFNγ and TNFα in aorta from young and old control (YC and OC) and young and old nitrite supplemented (YN and ON) mice. Values are mean ± SEM. (n = 5 – 8 per group) * $p < 0.05$ vs. YC.

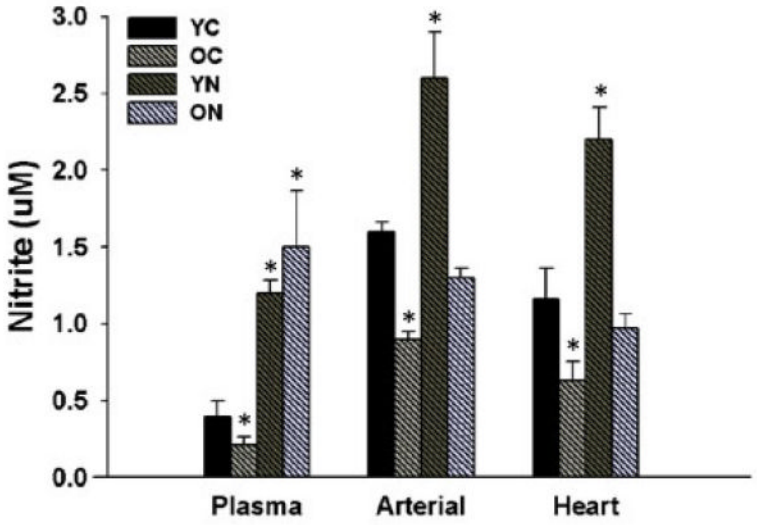

Figure 6. Circulating and tissue nitrite concentrations from plasma, pooled large elastic arteries and heart

Nitrite concentrations in plasma, large elastic arteries (pooled aortic and carotid arteries) and heart in young and old control (YC and OC) and young and old nitrite supplemented (YN and ON) mice. Values are mean ± SEM. (n = 8 animals per group) * $p < 0.05$ vs. YC.

Table 1

Animal characteristics

	YC	OC	YN	ON
Body mass (g)	23 ± 1	35 ± 1*	23 ± 1	34 ± 1*
Heart mass (mg)	136 ± 9	194 ± 8*	137 ± 1	189 ± 9*
Gastrocnemius mass (mg)	195 ± 9	156 ± 14*	191 ± 13	146 ± 7*
Carotid artery lumen diameter (μm)	389 ± 11	421 ± 8*	387 ± 10	410 ± 13*

Values are mean ± SEM.

* $p < 0.05$ vs. YC

Table 2

Protein expression in aorta

	YC	OC	YN	ON
MnSOD	1.0 ± 0.1	0.7 ± 0.1*	0.2 ± 0.05*	0.7 ± 0.1*
CuZnSOD	1.0 ± 0.3	1.2 ± 0.1	1.2 ± 0.2	1.1 ± 0.2
ecSOD	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.2
eNOS	1.0 ± 0.1	0.7 ± 0.1*	1.5 ± 0.1*	0.6 ± 0.1*

Values are mean ± SEM.

MnSOD, manganese superoxide disumtase; CuZnSOD, Copper Zinc superoxide dismutase; ecSOD, extracelluar superoxide dismutase; eNOS, endothelial nitric oxide synthase.

* $p < 0.05$ vs. YC.