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Dietary nitrite prevents hypercholesterolemic microvascular inflammation and reverses endothelial dysfunction

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Stokes KY, Dugas TR, Tang Y, Garg H, Guidry E, Bryan NS. Dietary nitrite prevents hypercholesterolemic microvascular inflammation and reverses endothelial dysfunction. *Am J Physiol Heart Circ Physiol* 296: H1281–H1288, 2009. First published February 27, 2009; doi:10.1152/ajpheart.01291.2008.—The nitrite anion is an endogenous product of mammalian nitric oxide (NO) metabolism, a key intermediate in the nitrogen cycle in plants, and a constituent of many foods. Research over the past 6 years has revealed surprising biological and cytoprotective activity of this anion. Hypercholesterolemia causes a proinflammatory phenotype in the microcirculation. This phenotype appears to result from a decline in NO bioavailability that results from a reduction in NO biosynthesis, inactivation of NO by superoxide, or both. Since nitrite has been shown to be potently cytoprotective and restore NO biochemical homeostasis, we investigated if supplemental nitrite could attenuate microvascular inflammation caused by a high cholesterol diet. C57Bl/6J mice were fed either a normal diet or a high cholesterol diet for 3 wk to induce microvascular inflammation. Mice on the high cholesterol diet received either nitrite-free drinking water or supplemental nitrite at 33 or 99 mg/l ad libitum in their drinking water. The results from this investigation reveal that mice fed a cholesterol-enriched diet exhibited significantly elevated leukocyte adhesion to and emigration through the venular endothelium as well as impaired endothelium-dependent relaxation in arterioles. Administration of nitrite in the drinking water inhibited the leukocyte adhesion and emigration and prevented the arteriolar dysfunction. This was associated with sparing of reduced tetrahydrobiopterin and decreased levels of C-reactive protein. These data reveal novel anti-inflammatory properties of nitrite and implicate the use of nitrite as a new natural therapy for microvascular inflammation and endothelial dysfunction associated with hypercholesterolemia.

nitrosothiols; tetrahydrobiopterin; C-reactive protein; leukocyte adhesion

THE VASCULAR ENDOTHELIUM produces constitutive levels of nitric oxide (NO) that maintain an anti-inflammatory and antithrombogenic phenotype in the healthy individual. In addition, NO is important in the maintenance of basal vascular tone and acts as a vasodilator in response to stimuli such as hypoxia and increased shear stress (49). Physical or biochemical injury to the endothelium impairs the production and/or function of endothelium-derived vasoprotective mediators of vascular health, such as NO, resulting in increased vascular contractions to vasoconstrictors such as endothelin-1, thromboxanes, and serotonin (38), enhanced thrombus formation, and exacerbated smooth muscle cell proliferation and migration (50). It is therefore not surprising that loss of endothelial NO function due to decreased production and/or to increased degradation of NO (18) is associated with several cardiovascular disorders, including atherosclerosis. One of the earliest pathophysiological responses to a wide array of injurious stimuli, including ischemia, cholesterol, diabetes, and atherosclerosis, is a reduction in NO bioavailability, and this has been implicated in the associated inflammatory phenotype that occurs in the microvasculature (28) and large vessels (18). The decrease in NO bioavailability can be attributed to several factors including impaired release of NO from endothelial NO synthase (eNOS), degradation of NO, or attenuated responses of the target cells to NO. In the case of cardiovascular disease, all of these have been identified, resulting in endothelial dysfunction, inflammation, platelet activation, and smooth muscle proliferation and migration. The restoration of NO bioavailability, for example, by the administration of NOS substrate L-arginine (17) or its cofactor tetrahydrobiopterin (BH_4) (31) and antioxidants (21), has proven effective in reducing the microvascular inflammation that develops early during hypercholesterolemia as well as the ensuing atherogenesis in animal models. Interestingly, one of the most widely prescribed class of drugs for hypercholesterolemia, statins, not only reduces cholesterol levels but protects against many inflammatory facets of cardiovascular disease through the activation of eNOS (35), further supporting the concept that therapies designed to maintain/increase NO bioavailability show promise for cardiovascular disease and many other disease processes in which inflammation plays a central role.

The biological actions of NO were once thought to be terminated by the oxidation of NO to nitrite and nitrate. The major pathway for NO metabolism is the stepwise oxidation to nitrite and nitrate. NO is oxidized almost completely to nitrite in plasma, where it remains stable for several hours (36). In contrast, NO and nitrite are rapidly oxidized to nitrate in whole blood. NO can also be enzymatically oxidized to nitrite by cerulopasmin or other metal-containing proteins (59). During fasting conditions with a low previous intake of nitrite/nitrate, the oxidation of NOS-derived NO accounts for the majority of nitrite (54). Nitrite is also an important antimicrobial additive to food products. Exogenous inorganic nitrite has been in use for as long as 5,000 years in the preservation of food. Nitrite in meat greatly delays the development of botulinum toxin, develops cured meat flavor and color, retards the development of rancidity during storage, inhibits the development of warmed-over flavor, and preserves flavors of spice and smoke (5). Nitrite in food controls and stabilizes the oxidative state of

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lipids in meat products (58), thus preventing lipid oxidation. Carr and Frei (14) have previously shown that nitrite inhibits myeloperoxidase-mediated low-density lipoprotein modification. The underlying chemistry of nitrite in meats that has been exploited for centuries may then have similar effects in human physiology.

Previously thought to be an inert product of NO metabolism, nitrite is now thought to be a source of NO in the vasculature by enzymatic reduction by heme proteins (15). Furthermore, exogenous nitrite has been shown to be protective in both hepatic and cardiac ischemia-reperfusion models in animals (19, 67) and can reverse hypertension due to NOS inhibition (65). We have previously shown that dietary nitrite and/or nitrate supplementation can limit myocardial ischemia-reperfusion injury (9, 10) and restore NO biochemistry in eNOS$^{-/-}$ mice (10). Dietary nitrate was first shown to reduce diastolic blood pressure in healthy individuals by Larsen et al. (39). Furthermore, the recent report by Webb et al. (68) demonstrates that dietary nitrate through its reduction to nitrite can lower blood pressure, prevent ischemia-reperfusion-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. It is well established that inflammation plays a central role in the pathogenesis of postischemic injury; however, whether these beneficial effects of nitrite were mediated through the reduction of inflammatory responses remains unclear. Therefore, the aim of this study was to determine if supplemental nitrite modulates the inflammatory responses. To do this, we employed a model of acute hypercholesterolemia and used intravital microscopy to monitor real-time inflammation in the microvasculature, which is the primary site of inflammation in a majority of disease processes. To gain insight into the mechanism underlying the nitrite-induced responses, we complemented these experiments with biochemical measurements of NO metabolites and the eNOS cofactor BH_4 as well as C-reactive protein (CRP), a useful index of cardiovascular risk.

MATERIALS AND METHODS

Animals. Male wild-type C57Bl/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). At 6–8 wk of age, mice were placed on either a normal diet (ND) or high cholesterol (HC) diet (Teklad TD94059 containing 1.25% cholesterol and 15.8% fat, Harlan Teklad) for 3 wk (n = 5–6 mice/group). Mice were given regular nitrite-free water or supplemented with 50 or 150 mg/l sodium nitrite (33 and 99 mg/l nitrite, respectively) in their drinking water ad libitum throughout the course of the HC diet.

Surgical protocol. Mice were anesthetized with ketamine hydrochloride (150 mg/kg body wt ip) and xylazine (7.5 mg/kg body wt ip). Core body temperature was maintained at 35 ± 0.5°C. The carotid artery was canulated for the measurement of mean arterial pressure (MAP). Animal handling procedures were approved by the Louisiana State University Health Sciences Center and The University of Texas Health Science Center at Houston Institutional Animal Care and Use Committee and were in accordance with guidelines of the American Physiological Society.

Intravital microscopy. The cremaster muscle was prepared for intravital microscopy and superfused with bicarbonate-buffered saline (BBS) solution 1 ml/min as previously described (61). Postcapillary venules (20–40 μm in diameter) with a wall shear rate (WSR) of ≥500 s^{-1} were studied. This threshold was selected based on previous reports describing a propensity for leukocytes to adhere in venules at low WSRs (57). The venule with the least number of adherent and emigrated leukocytes at the end of the 30-min stabilization period was chosen for study. One-minute recordings of the leucocytes were made of the first 100 μm of every 300 μm along the length of the unstimulated vessel, beginning as near to the source of the venule as possible. A leukocyte was considered adherent if it remained stationary for ≥30 s (leukocytes/mm^2) and was measured throughout the observation period. Leukocyte emigration was measured online at the end of each 1-min observation period. Emigrated leukocytes were expressed as the number of interstitial leukocytes per squared millimeter high-powered field of view adjacent to the segment under observation (leukocytes/mm^2). The mean value of each variable within a single venule was calculated, and comparisons were made between the experimental groups.

Once the venular data had been collected, animals were allowed to stabilize for 20–30 min, and arterioles with diameters between 15 and 40 μm and WSRs of ≥500 s^{-1} were chosen for study. Diameter and red blood cell velocity were measured in the chosen sections before and after a superfusion with 10^{-5} M of the endothelium-dependent vasodilator ACh for 5 min. After the vessel diameters returned to baseline with BBS superfusion, the nonendothelium-dependent vasodilator papaverine was superfused, and diameters were remeasured. Arteriolar vasorelaxation responses to ACh and papaverine are expressed as percent diameter changes versus baseline.

Circulating blood cell counts and plasma cholesterol levels. At the end of the experiment, blood was drawn from the right ventricle, and a sample was taken for circulating leukocyte and platelet counts. The rest of the blood was centrifuged, and plasma was frozen for the subsequent measurement of cholesterol levels using a spectrophotometric assay (Stanbio Laboratory, Boerne, TX). Circulating levels of triglycerides were measured using Infinity Triglyceride reagent (Sigma-Aldrich, St. Louis, MO).

Tissue NO product/metabolite determination. The plasma, heart, and liver from a separate subset of mice were harvested after 3 wk of ND or HC diet for quantitative analyses of nitrosothiols and oxidation products of NO as previously described (12). Briefly, nitrosothiols were measured by the addition of mercuric chloride with acidified sulphanilamide and injection of biological samples into a tri-iodide-containing reaction mixture continuously purged with nitrogen. Evolved NO was quantified in the gas phase using an ozone-based chemiluminescence detector (CLD 77am sp, EcoPhysics, Ann Arbor, MI). Nitrate and nitrite concentrations were quantified by ion chromatography (ENO20 Analyzer, Eicom, Kyoto, Japan) (12).

CRP. Plasma was obtained by centrifugation at 800 g and 4°C for 10 min. CRP was determined with a mouse ELISA kit from Helica Biosystems (Fullerton, CA).

HPLC analysis of BH_4. Hepatic BH_4 and dihydrobiopterin (BH_2) content was determined by modification of the method described by Whitsett et al.(69). First, snap-frozen hepatic tissues were quickly weighed while frozen and homogenized on ice at 80 mg/ml in 3.8% perchloric acid containing 1 mg/ml dithioerythritol (DTE) and 1 mg/ml diethylenetriaminepentaacetic acid (DTPA). Homogenates were centrifuged, and supernatants were collected for analysis by HPLC. Samples were analyzed using a Waters 2695 HPLC pump (Milford, MA) interfaced to an ESA Coularray four-channel electrochemical detector (Chelmsford, MA). The separation was accomplished using a 250 × 4.6-mm inner diameter Ultrasphere reversed-phase C_{18} column (Beckman Coulter, Fullerton, CA) and isocratic elution of 5% methanol and 95% of 83 mM sodium acetate containing 5.5 mM citric acid, 54 μM EDTA, and 160 μM DTE at a flow rate of 0.4 ml/min. The four channels of the electrochemical detector were set at 0, 150, 365, and 550 mV. BH_4 was quantitated by the addition of peak areas measured on the latter three channels, and BH_2 was quantitated on the latter two channels combined. Solutions of ultrapure BH_4 and BH_2 (Cayman Chemical, Ann Arbor, MI) freshly prepared in 0.1 N perchloric acid containing 1 mg/ml DTPA and 1 mg/m; DTE were used to generate a standard curve. BH_4 and BH_2 eluted at ~8.5 and 16 min, respectively, and both peaks were confirmed by spiking the tissue samples with authentic standard. The data collected were first normalized to tissue weights (i.e., mg pterin/mg liver) and then expressed as the ratio of BH_4 to BH_2.

Statistics. Significant effects of treatment were evaluated by one-way ANOVA using SPSS for Windows (San Diego, CA) or GraphPad Prism software. Where significant differences between data sets were observed, post hoc tests (e.g., Tukey or Scheffè) were performed to determine differences between individual data sets. In all cases, $P < 0.05$ was accepted as statistical significant.

RESULTS

Plasma cholesterol levels and MAP were unaltered but triglyceride levels were reduced by nitrite. After 3 wk of HC feeding, plasma cholesterol levels were significantly elevated in the water-treated HC group compared with normocholesterolemic (ND) controls (Table 1). The addition of nitrite in the water at either 33 or 99 mg/l did not alter the diet-induced hypercholesterolemia, indicating that any protection observed was not due to normalization of the circulating cholesterol. There were also no differences in very-low-density lipoprotein, low-density lipoprotein, or high-density lipoprotein profiles between the groups (data not shown). The HC diet increased plasma triglycerides from 57 ± 21 to 75.33 ± 14.05 mg/dl. Nitrite treatment significantly reduced total triglycerides to below baseline levels (75.33 ± 14.05 to 47.25 ± 5.68 mg/dl, $P < 0.01$). Moreover, neither dose of nitrite affected MAP in HC-fed mice (Table 1).

Tissue nitrite levels are diminished by HC diet and enhanced by supplemental nitrite. We have previously shown that nitrite supplementation in drinking water (50 mg/l sodium nitrite, 1 wk) can replete NO homeostasis and biochemistry in eNOS knockout mice (10). Mice fed the HC diet had significantly lower cardiac nitrite and higher plasma nitrate. Tissue *S*-nitrosothiol (RSNO) levels were also elevated by hypercholesterolemia. Supplemental nitrite (33 mg/l) significantly increased tissue nitrite, nitrate, and RSNO concentrations well beyond the normal steady-state concentrations found in control mice and eNOS$^{-/-}$ mice on ND (Fig. 1) (10). This suggests an increased sensitivity to exogenous nitrite compared with control mice on ND or a change in its uptake and/or metabolism in the presence of the HC diet. Neither the HC diet nor the two doses of nitrite caused any measurable formation of methemoglobin (data not shown).

Three weeks of HC feeding promotes microvascular inflammation and endothelial dysfunction. Consistent with previous results in other models of acute hypercholesterolemia (62), 3 wk of feeding of the HC diet induced significant inflammation in C57 mice compared with age-matched controls on ND. Specifically, leukocyte adhesion in postcapillary venules and emigration into the interstitium of the cremaster muscle were increased by more than threefold in mice fed the HC diet (Fig. 2, *A* and *B*). Both doses of nitrite supplementation (33 and 99 mg/l) significantly inhibited the leukocyte recruitment and emigration induced by the cholesterol-enriched diet (Fig. 2, *A* and *B*). There were no significant differences in the protection conferred by the two doses of nitrite, indicating that 33 mg/l nitrite was sufficient to prevent inflammation with no additional benefit given by higher concentrations. Mice fed the HC diet also experienced significant impairment of endothelium-dependent vascular relaxation (Fig. 2*C*). In contrast, no such difference was noted in response to papaverine (data not shown), indicating that the microvascular dysfunction was at the level of the endothelium rather than due to an inability of the smooth muscle to relax. This is consistent with a reduced capacity of the endothelium to produce NO, as suggested by previous findings in models of hypercholesterolemia (63). In contrast, mice supplemented with nitrite alongside the HC diet did not develop the diet-induced arteriolar dysfunction; rather, they exhibited levels of vasorelaxation to ACh, comparable with normocholesterolemic controls. Both doses of nitrite were equally effective at preserving endothelial function with no difference in relaxation due to papaverine, suggesting a restoration of endothelial production of NO rather than a change in the ability of smooth muscle to dilate.

Anti-inflammatory effects of nitrite are not due to difference in the number of circulating leukocytes or WSR in venules. To determine if the reduction in adherent and emigrated leukocytes may be a result of decreased circulating leukocyte populations, we quantified the numbers of lymphocytes, monocytes, and neutrophils in whole blood and found no significant differences between the groups (total count shown in Table 1). These data suggest that nitrite affects the process of blood cell recruitment rather than reducing the numbers of cells available to adhere in postcapillary venules. Furthermore, WSRs were comparable between all groups, demonstrating not only that the leukocyte recruitment induced by hypercholesterolemia was independent of a reduction in WSR but that the anti-inflammatory effect of nitrite was not due to elevating the blood cell velocity within the venules (Table 1). This is also consistent with our findings that the concentrations of nitrite in this study had no effect on blood pressure.

Dietary nitrite supplementation affects BH_4 redox status. To gain an understanding on the effects of nitrite restoring endothelium-dependent NO production, we measured BH_4 levels in the liver. BH_4 availability is critical for endothelium-dependent

Table 1. *Plasma cholesterol and triglyceride levels, MAP, venular WSR, and circulating blood cell counts in mice maintained on the ND or HC diet for 3 wk*

Groups	Cholesterol Levels, mg/dl	Triglyceride Levels, mg/dl	MAP, mmHg	WSR, s^{-1}	Leukocyte Count, leukocytes/μl blood
ND + water	71±2.8	57.0±27	64±3.3	733±66.8	4,830±990.9
HC diet + water	116±4.2*	75.3±14.05	67±0.7	594±27.0	6,767±1238.2
HC diet + 50 mg/l nitrite	117±8.9*	47.3±5.68†	69±1.2	708±65.8	6,590±785.7
HC diet + 150 mg/l nitrite	123±7.7*	Not determined	69±2.7	756±44.8	6,080±761.3

Values are means ± SE. MAP, mean arterial pressure; WSR, wall shear rate; ND, normal diet; HC diet, high cholesterol diet. The drinking water of separate groups of HC diet-fed mice was supplemented with either 50 or 150 mg/ml sodium nitrite. *$P < 0.005$ vs. ND + water; †$P < 0.01$ vs. HC + water.

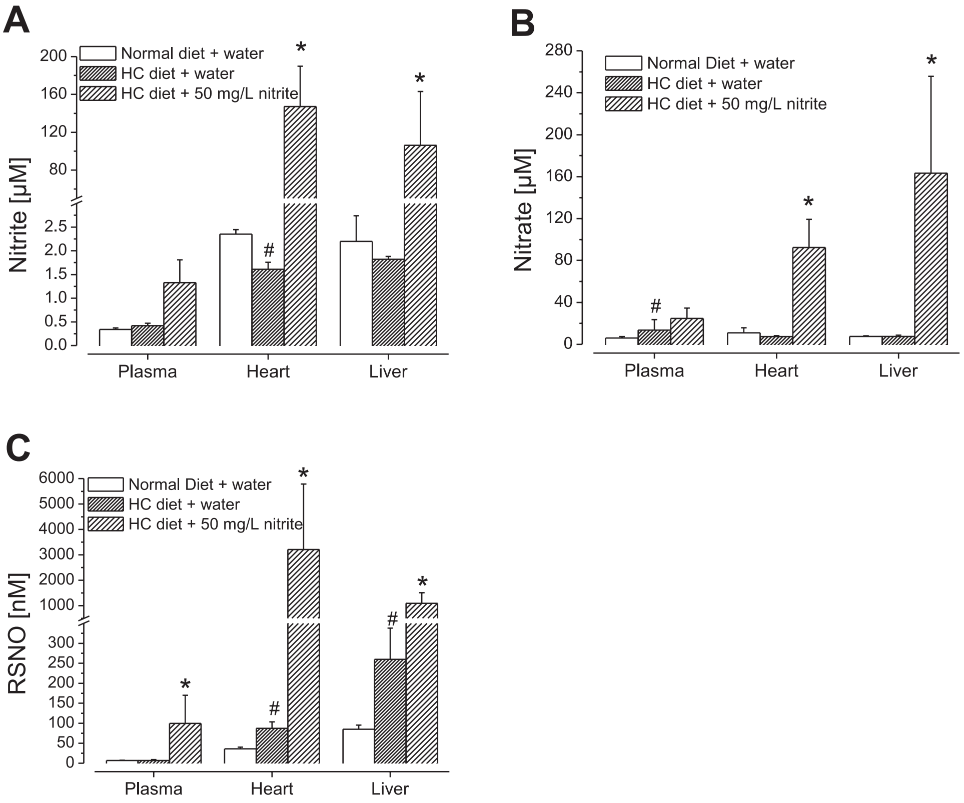

Fig. 1. High cholesterol (HC) diet causes a change in steady-state concentrations of nitric oxide (NO) products and metabolites. Supplementation with 50 mg/l sodium nitrite enhances nitrite (*A*), nitrate (*B*), and *S*-nitrosothiol (RSNO; *C*) in the plasma, heart, and liver. Data are means ± SE of $n = 3$ mice/group. #$P < 0.05$ vs. normal diet (ND) + nitrite-free water; *$P < 0.05$ vs. both diets + nitrite-free water.

NO production from L-arginine. As shown in Fig. 3*A*, hypercholesterolemia did not alter BH_4 levels in the liver. However, the BH_4-to-BH_2 ratio was reduced by almost 50%, although this did not reach significance (Fig. 3*B*). Mice coadministered nitrite with the HC diet had an almost twofold increase in hepatic BH_4 levels compared with animals fed either the ND or HC diet alone. Furthermore, the ratio of BH_4 to BH_2 was more than threefold higher in nitrite-treated HC-fed mice compared with mice administered the HC diet alone, suggesting a preservation of the reduced form. Taken together, these data support a role for nitrite administration in elevating the total hepatic production of BH_4 and preventing oxidation to BH_2. This reveals novel antioxidant properties of exogenous nitrite in preserving endothelial NO production and, furthermore, implicates these responses in the associated reversal of endothelial dysfunction caused by the HC diet.

Nitrite supplementation reduces the elevated CRP levels induced by hypercholesterolemia. CRP is an acute-phase protein elevated under conditions of inflammation and tissue damage (27). Since CRP has an established link with cardiovascular disease, linking the supplementation of metabolites to a reduction in CRP may provide more evidence of improved cardiovascular inflammation and function. As shown in Fig. 4, 3 wk of HC diet increased the circulating levels of CRP, indicating an ongoing inflammatory response. Nitrite supplementation reduced plasma CRP to control levels. The higher dose of nitrite (99 mg/l) appeared to attenuate CRP levels to a greater extent than the 33 mg/l nitrite dose.

DISCUSSION

It has been appreciated for many years now that restoring NO or endothelial function is key to slowing or preventing many diseases characterized by an inflammatory phenotype, including cardiovascular disease (1), as NO is a potent anti-inflammatory mediator. However, restoring NO production from L-arginine is a complex biochemical reaction requiring both a spatial and temporal arrangement of numerous cofactors and substrates and adequate blood flow and oxygen delivery. Therefore, providing an alternate NOS-independent source of NO is reasonable. Nitrite has been implicated as a reservoir of NO activity when the NOS pathway is inactive (8, 43). In fact, dietary nitrite has been shown to prevent injury after ischemia-reperfusion (9) and recapitulates NO homeostasis in eNOS knockout mice (10). The results from our study in a murine model of hypercholesterolemia-induced inflammation reveal novel beneficial properties of supplemental nitrite that include *1*) restoration and enhancement of NO biochemistry; *2*) inhibition of leukocyte adhesion to and emigration through the vasculature; *3*) reversal of endothelial dysfunction by an associated preservation of reduced BH_4; and *4*) reduction of circulating levels of CRP, a clinically useful acute-phase marker of systemic inflammatory status.

Under normal conditions, NO is considered to be an anti-inflammatory, antiadhesion molecule, and a reduction in NO bioavailablility during disease processes can switch the endothelial cell surface of vessels from a low-adhesion phenotype to a proadhesion phenotype. Activated endothelial cells express several types of adhesion molecules that support blood cell rolling on the vascular surface and subsequent adhesion at the site of activation (23). These blood cell-vessel wall interactions have been recognized as key early events in the development of atherosclerosis (30), in tissue injury after heart attack or stroke, in septic shock, and in certain cancers (4, 25, 33, 60). Therefore, targeting this early inflammatory response, perhaps by

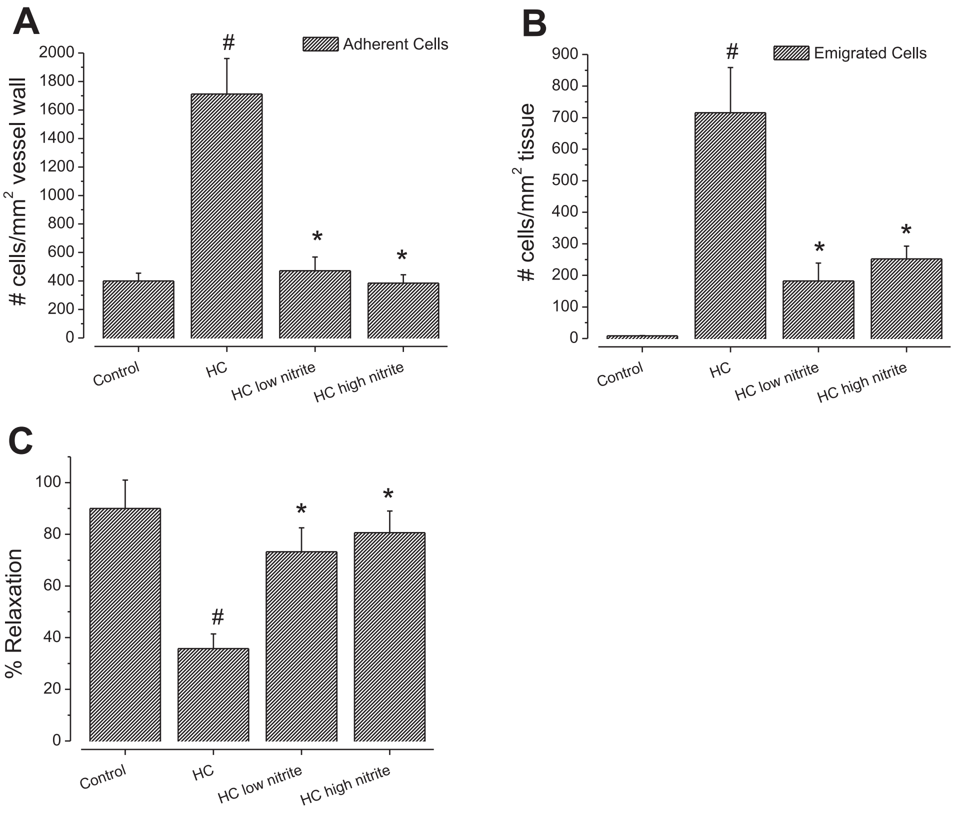

Fig. 2. HC diet induces microvascular inflammation and endothelial dysfunction that is reversed by nitrite treatment. Mice fed the HC diet for 3 wk exhibited significantly increased number of adherent (*A*) and emigrated leukocytes (*B*) compared with normocholesterolemic controls. These inflammatory indexes were normalized by nitrite (*A* and *B*). HC diet feeding also reduced endothelium-dependent vasodilation responses to ACh in arterioles compared with ND controls (*C*). The administration of nitrite in the drinking water restored endothelium-dependent relaxation to ACh. Data are means ± SE of $n = 5$–6 mice/group. ANOVA revealed a significant effect of treatment. #$P < 0.05$ vs. the ND + water group; *$P < 0.05$ vs. the HC diet + water group.

preventing a reduction in NO bioavailability, may inhibit the onset and progression of disease and minimize organ injury (22). Our findings that nitrite supplementation inhibited hypercholesterolemia-induced leukocyte adhesion and emigration in venules support such a scenario and may at least in part explain previous findings that nitrite protects against myocardial ischemia-reperfusion injury. Furthermore, endothelial dysfunction due to reduced NO bioavailability has been noted in arteries of patients with cardiovascular risk factors (29), which has important implications in their ability to perfuse tissues, in particular after an ischemic event. Here, we showed that nitrite prevented the impairment of arteriolar vasodilation to ACh during hypercholesterolemia, suggesting that nitrite may be acting by preserving vascular endothelial NO production, an effect that would also serve to reduce the venular inflammation. Thus, the findings from this study implicate a more diverse physiological role for nitrite than merely an alterative source of NO.

This possibility that nitrite may be acting on both the arteriolar and venular sides of the microvasculature by preventing the reduction of NO bioavailability that occurs during inflammation is supported by our results revealing that dietary nitrite preserves BH_4 bioavailability and actually conserves endothelium NO-dependent vasodilation. BH_4 is an essential cofactor for all three NOS isoforms (37, 45), and basal enzyme activity correlates with the amount of BH_4 bound tightly to the protein. BH_4 increases substrate affinity of NOS (37) and participates in the electron transfer process, being converted to the trihydrobiopterin radical during the NOS catalytic cycle and then restored to BH_4. When BH_4 bioavailability declines, NOS undergoes multiple changes. The dimer architecture is altered, possibly because of malrotation of the oxidase domains

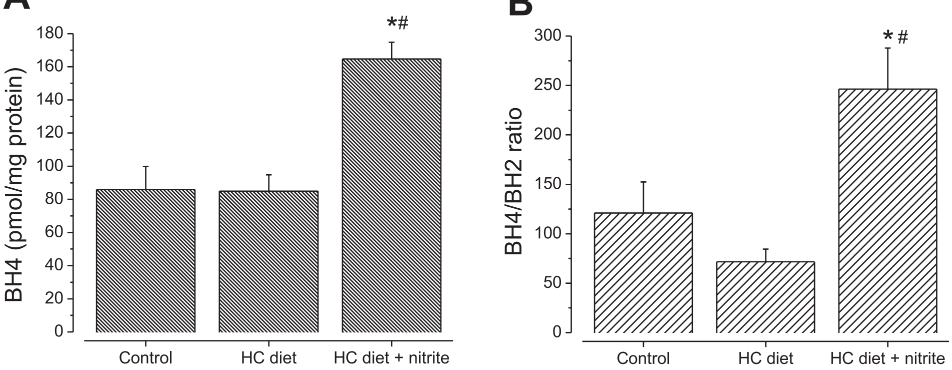

Fig. 3. Mice fed the HC diet have unaltered tetrahydrobiopterin (BH_4) levels in the liver, which was enhanced in nitrite-fed livers (*A*). The ratio of BH_4 to dihydrobiopterin (BH_2) (*B*) was enhanced in nitrite-fed mice. Total BH_4 and the ratio of BH_4 to BH_2 were determined from the livers of mice fed the ND or HC diet with or without 50 mg/l sodium nitrite. Data are means ± SE of $n = 6$–9 mice/group. ANOVA revealed a significant effect of treatment. Tukey's post hoc test revealed significant differences between the group treated with HC diet + nitrite compared with ND alone (*$P < 0.05$) and HC diet alone (#$P < 0.05$).

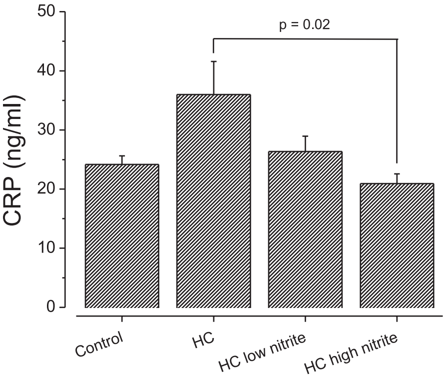

Fig. 4. C-reactive protein (CRP) is increased in the plasma of mice fed the HC diet. Nitrite supplementation of mice on a HC diet restored CRP levels to normal. Data are means ± SE of $n = 3–6$ mice/group. Low nitrite was 50 mg/l sodium nitrite; high nitrite was 150 mg/l sodium nitrite. ANOVA revealed a significant effect of treatment. Tukey's post hoc test indicated a significant difference between HC diet and HC diet + high nitrite treatment groups ($P = 0.02$).

to yield "molecular" uncoupling, and the catalytic activity becomes "functionally" uncoupled (48). In the latter situation, the stoichiometric coupling between the reductase domain and L-arginine at the active site is lost, resulting in the formation of superoxide and/or hydrogen peroxide. Therefore, it is thought that BH_4 availability is the major limitation for NOS activity. Nitrite's ability to preserve an essential cofactor for NO production from NOS is a novel and unexpected finding and may explain its potent anti-inflammatory properties. Furthermore, the enhancement of the BH_4-to-BH_2 ratio by relatively low doses of nitrite indicate that nitrite is actually antioxidant rather than prooxidant, and this property may have further implications in inflammatory diseases, including cardiovascular disease, where oxidative stress is an underlying cause of many of the pathophysiological events. Restoration of endothelial NO production by nitrite may also provide sufficient superoxide scavenging to prevent BH_4 oxidation.

There is substantial evidence that protein *S*-nitrosation provides a significant route through which NO-derived bioactivity is conveyed (26). Stamler and colleagues (32) have discovered that protein thiol modification by NO is a fundamental and principle mechanism of NO-based signaling that affects protein structure and function. We and others (2, 11, 13) have demonstrated that nitrite can form nitrosothiols. Although no attempt was made to identify specific nitrosated proteins, we cannot discount the fact that part of the observed effects may be due to a modulation of posttranslation modification by nitrogen oxides of adhesion molecules on blood and/or endothelial cells. Both β_2-integrins and ICAM-1 have been shown to be the primary adhesion glycoprotein complexes involved in leukocyte adhesion (3). Thom et al. (64) recently demonstrated that *S*-nitrosylation of actin inhibits β_2-integrin clustering and subsequent neutrophil adhesion. Further evidence provided by Prasad et al. (52) show that S-nitrosoglutathione inhibits monocyte adhesion to activated endothelial cells, which is mediated by downregulation of endothelial cell adhesion molecules. Due to the fact that the doses of nitrite administered in these studies had no effect on systemic blood pressure, it is likely that many of the observed effects may be due to posttranslation modification of proteins. A recent report by Bonini et al. (7) has revealed that the organic nitrate nitroglycerin modulates eNOS activity and NO output by phosphorylation. Since organic nitrates are known to produce much more nitrite than NO (24), the possibility that nitrite may be affecting eNOS protein phosphorylation cannot be dismissed.

High levels of triglycerides in the bloodstream have been linked to atherosclerosis and, by extension, the risk of heart disease and stroke (46). Elevated levels of triglycerides (and triglyceride-rich lipoproteins) are increasingly being recognized as treatment targets to lower cardiovascular risk in certain patient subgroups, including individuals receiving 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins). Our data that nitrite treatment reduces total triglyceride levels in the HC diet group indicates a novel pathway by which nitrite may be affecting fat metabolism or energy utilization; however, this remains to be elucidated.

Acute-phase proteins such as CRP (27) are elevated in the circulation during inflammation. With a half-life of 18 h, CRP is a very stable downstream marker of the inflammatory process (6), and most clinical studies have reported that CRP is an independent predictor of risk of atherosclerosis (40), cardiovascular events (6), and myocardial infarction (56). Interestingly, compared with other inflammatory markers (such as P-selectin, IL-6, IL-1, tumor necrosis factor-α, soluble ICAM-1, and fibrinogen), CRP has emerged as the most powerful inflammatory predictor of future cardiovascular risk (55). Our data reveal that the HC diet modestly increased circulating levels of CRP, which were restored to normal levels in hypercholesterolemic mice supplemented with nitrite. This may be a reflection of the reduced inflammation observed in these mice and suggests that the clinical benefit of nitrite treatment in a patient may be easily detected by measuring this clinically useful biomarker of systemic inflammation.

Scientific and medical data have shown for centuries that our diet is one of the main determinants of our health. As such, hypercholesterolemia as a result of a poor diet is the dominant risk factor for atherosclerosis in the United States and Europe. It has been appreciated for years that a diet rich in fruits and vegetables is generally regarded as healthful and beneficial. In fact, some have postulated that the beneficial effects of vegetables may be due to their nitrate content (41). We now know that nitrate can be used in our body to make nitrite and ultimately NO (for a review, see Ref. 44). Nitrite can also be derived from reduction of salivary nitrate by commensal bacteria in the mouth and gastrointestinal tract (47). About 25% of orally ingested available nitrate is actively secreted into the saliva. This nitrate is partially converted to nitrite by oral bacteria and then disproportionates with the formation of NO after entering the acidic environment of the stomach, helping to reduce gastrointestinal tract infection, increase mucous barrier thickness, and increase gastric blood flow (47). Humans, unlike prokaryotes, are thought to lack the enzymatic machinery to reduce nitrate back to nitrite. However, recent discoveries reveal a functional mammalian nitrate reductase (34). Commensal bacteria that reside within and on the human body can reduce nitrate, thereby supplying a large and alternative source of nitrite. Lundberg and Govoni (42) have demonstrated that plasma nitrite increases after the consumption of nitrate. Therefore, dietary and enzymatic sources of nitrate are potentially large sources of nitrite in the human body. The amounts of nitrite used in this study total ~0.1 mg/day for mice drinking the lower dose of supplemental nitrite, which modestly increases steady-state plasma nitrite. Therefore, any intervention that increases plasma nitrite will likely show benefit. A reasonable strategy then may be through the consumption of nitrate-rich vegetables.

Although the biomedical science community is aware of the emerging beneficial effects of nitrite, it is still regarded as an undesired food additive in cured and processed meats (70). However, studies (9, 10, 20) have revealed that dietary nitrite supplementation can restore NO biochemistry in eNOS$^{-/-}$ mice as well as prevent injury from ischemia-reperfusion insult, and we have shown here that nitrite attenuates inflammation and preserves endothelial function. Emerging evidence from animal models and human clinical studies has indicated that, independent of its role as a source of NO in tissues by reduction, nitrite exerts unique intracellular signaling properties that mediate physiological functions (8), which is supported by our novel findings that BH_4 levels are preserved by nitrite treatment. Because nitrite is a primary biologically active compound resulting from nitrate reduction in tissues, significant physiological benefits may be associated with the provision of nitrate from dietary sources. Despite the enormous effort over the past few decades to limit or even restrict dietary nitrite and nitrate consumption due to the potential to form carcinogenic *N*-nitrosamines, to date there are no conclusive data to indicate that dietary sources of nitrite and nitrate may be unsafe, especially at doses naturally occurring in foods. Since the early 1980s, there have been numerous reports on the association of *N*-nitrosamines and human cancers (16), but a causative link between nitrite exposure and cancer is still missing (66). In fact, a 2-yr study by the National Institutes of Health on the carcinogenicity of nitrite conclusively found that there was no evidence of carcinogenic activity by sodium nitrite in male or female rats or mice (51). Despite this, the negative connotations of nitrite and nitrate remain and have led the government to regulate and restrict levels in food and drinking water, particularly in cured and processed meats. However, this view of nitrite may be changing, as evidence is emerging for a protective role for nitrite against different cardiovascular-related disorders. One should not fear the nitrite contained in bacon or hot dogs. In fact, the nitrite in meats may provide vascular protection from the high fat and cholesterol content. It appears that we may have identified a critical component of our diet that many people are missing. In fact, the one compound we have been taught to fear and avoid may be saving our lives from inflammatory diseases.

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DISCLOSURES

N. S. Bryan serves on the scientific advisory board of Trivita Incorporated.

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