

# Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury

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Nitrite has emerged as an endogenous signaling molecule with potential therapeutic implications for cardiovascular disease. Steady-state levels of nitrite are derived in part from dietary sources; therefore, we investigated the effects of dietary nitrite and nitrate supplementation and deficiency on NO homeostasis and on the severity of myocardial ischemia-reperfusion (MI/R) injury. Mice fed a standard diet with supplementation of nitrite (50 mg/liter) in their drinking water for 7 days exhibited significantly higher plasma levels of nitrite, exhibited significantly higher myocardial levels of nitrite, nitroso, and nitrosyl-heme, and displayed a 48% reduction in infarct size (Inf) after MI/R. Supplemental nitrate (1 g/liter) in the drinking water for 7 days also increased blood and tissue NO products and significantly reduced Inf. A time course of ischemia-reperfusion revealed that nitrite was consumed during the ischemic phase, with an increase in nitroso/nitrosyl products in the heart. Mice fed a diet deficient in nitrite and nitrate for 7 days exhibited significantly diminished plasma and heart levels of nitrite and NO metabolites and a 59% increase in Inf after MI/R. Supplementation of nitrite in the drinking water for 7 days reversed the effects of nitrite deficiency. These data demonstrate the significant influence of dietary nitrite and nitrate intake on the maintenance of steady-state tissue nitrite/nitroso levels and illustrate the consequences of nitrite deficiency on the pathophysiology of MI/R injury. Therefore, nitrite and nitrate may serve as essential nutrients for optimal cardiovascular health and may provide a treatment modality for cardiovascular disease.

dietary supplementation | myocardial infarction | nitric oxide

The loss of nitric oxide (NO) generation as a result of a dysfunctional vascular endothelium is an often cited correlate of heart disease (1). Continuous generation of NO is essential for the integrity of the cardiovascular system, and a decreased production and/or bioavailability of NO is central to the development of cardiovascular disorders (2, 3). NO is a highly reactive and diffusible gas formed by three NO synthase (NOS) isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). NO has been extensively studied in the setting of ischemia-reperfusion (I/R) injury. Previous studies clearly demonstrate that the deficiency of eNOS exacerbates myocardial I/R (MI/R) injury (4), whereas the overexpression of eNOS (5, 6), NO donor (7, 8), or inhaled NO gas (9) therapy significantly protect the myocardium (10). NO possesses a number of physiological properties that make it a potent cardioprotective-signaling molecule. These include vasodilation and the inhibition of oxidative stress, platelet aggregation, leukocyte chemotaxis, and apoptosis (11–13). NO synthesis is influenced critically by various cofactors, such as tetrahydrobiopterin, flavin mononucleotide, and flavin adenine dinucleotide, as well as the presence of reduced thiols, the endogenous NOS inhibitor asymmetric dimethylarginine, and substrate and oxygen availability. Without an adequate delivery of substrate and/or cofactors (i.e., ischemia), NOS function is compromised and NO production limited. Therefore, alternate means to produce NO in ischemic tissues is an attractive mechanism to limit I/R injury.

Nitrite is an oxidative breakdown product of NO that has been shown to serve as an acute marker of NO flux/formation (14). Nitrite recently has moved to the forefront of NO biology (15)

because it represents a major storage form of NO in blood and tissues (16). In addition to the oxidation of NO, nitrite is also derived from reduction of salivary nitrate by commensal bacteria in the mouth and gastrointestinal tract (17, 18), as well as from dietary sources such as meat, vegetables, and drinking water. Much of the recent focus on nitrite physiology is attributable to its ability to be reduced to NO during ischemic or hypoxic events (16, 19–21). Nitrite reductase activity in mammalian tissues has been linked to the mitochondrial electron transport system (22), protonation (20), deoxyhemoglobin (23), and xanthine oxidase (24–25). Nitrite can also transiently form nitrosothiols (RSNOs) under both normoxic and hypoxic conditions (19), and a recent study by Bryan *et al.* (26) demonstrates that steady-state concentrations of tissue nitrite and nitroso are affected by changes in dietary NOx (nitrite and nitrate) intake. Previous studies have shown that nitrite therapy before reperfusion protects against hepatic and MI/R injury (25, 27). Additionally, experiments in primates have revealed a beneficial effect of long-term administration of nitrite on cerebral vasospasm (28). Oral nitrite has also been shown to reverse  $N^G$ -nitro-L-arginine methyl ester-induced hypertension and serve as an alternate source of NO *in vivo* (29).

A recent report by Kleinbongard *et al.* (30) demonstrates that plasma nitrite levels progressively decrease with increasing cardiovascular risk. Although a correlation exists in the plasma, it is not known whether the situation is mirrored in the heart and other tissues. If so, tissue nitrite may serve as an index of risk and restoring tissue nitrite may protect organs from ischemic injury. Because a substantial portion of steady-state nitrite concentrations in blood and tissue are derived from dietary sources (26), modulation of nitrite and/or nitrate intake may provide a first-line defense against ischemic heart disease. However, at present, there is no experimental evidence indicating the consequences of dietary nitrite or nitrate supplementation or deficiency on NO homeostasis or severity of I/R injury. We, therefore, investigated dietary nitrite and nitrate supplementation and insufficiency in mice, the effects of this manipulation on blood and heart nitrite/NO levels, and the effects of the manipulation on the severity of MI/R injury.

## Results

**Nitrite Supplementation Protects Against MI/R Injury by Increasing Plasma and Heart NO Stores.** It is known that nitrite given intravenously immediately before reperfusion can protect from I/R injury

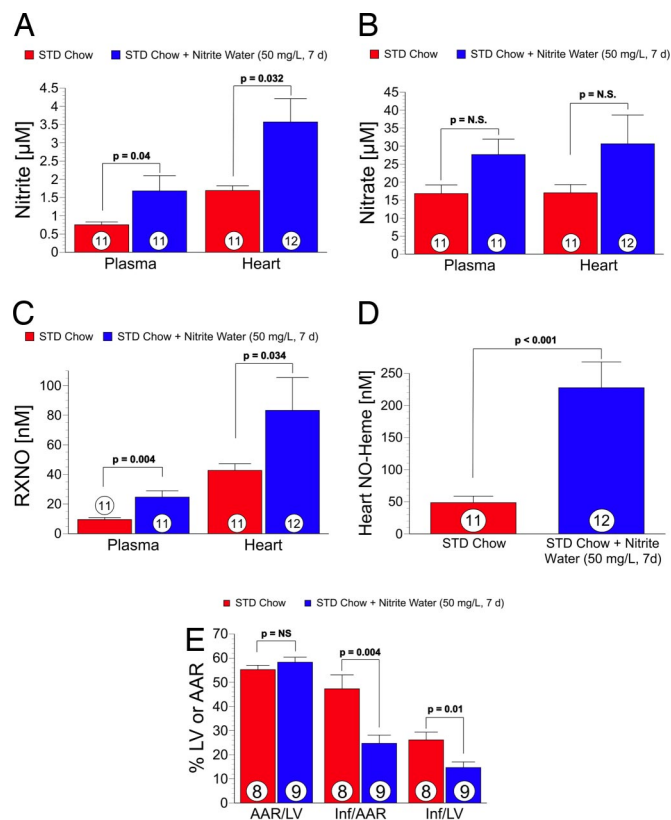
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**Fig. 1.** Steady-state plasma and heart NO<sub>x</sub> and nitrosylation levels. (A–D) Mice were fed a STD rodent chow with or without 50 mg/liter nitrite supplementation for 7 days, at which time steady-state levels of plasma and heart nitrite (A), nitrate (B), nitroso (RXNO) (C), and heart nitrosyl–heme (NO–Heme) (D) were measured. (E) Mice fed STD rodent chow supplemented with nitrite in their drinking water exhibited significantly higher plasma levels of nitrite, as well as significantly higher heart levels of nitrite, nitroso, and nitrosyl–heme. Nitrite supplementation significantly attenuated myocardial infarct size (Inf/AAR) by 44%. The numbers inside bars indicate the number of animals per group. LV, left ventricle.

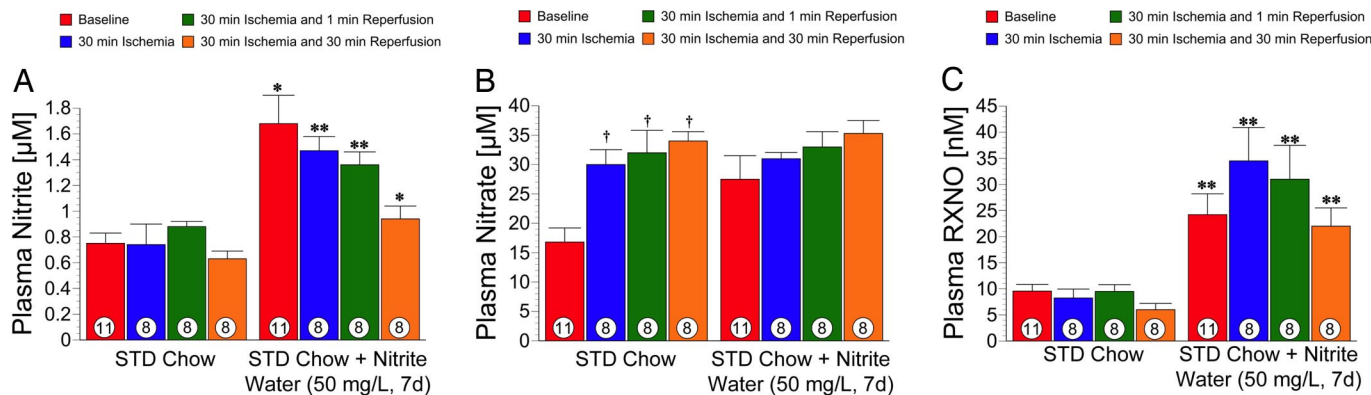
(27). Because of the relatively short circulating half-life of nitrite in blood (110 seconds) (31), it is not clear whether nitrite administered subchronically in the drinking water can affect blood and tissue nitrite concentrations and therefore affect the outcome from an I/R insult. To test this notion, nitrite (50 mg/liter) was administered in the drinking water of mice on a standard (STD) rodent chow (Purina 5001; Purina) for 7 days. As shown in Fig. 1, there was a significant increase in steady-state concentrations of plasma nitrite (Fig. 1A) and nitroso products (RXNO) (*S*-nitrosothiols and *N*-nitrosoproteins) (Fig. 1C), as well as a significant increase in steady-state concentrations of heart nitrite (Fig. 1A), nitroso products (Fig. 1C), and nitrosyl–heme products (Fig. 1D). Plasma and heart nitrate (Fig. 2B) levels trended toward an increase, although no statistical significance was observed. To test whether nitrite supplementation and the biochemical changes resulted in protection against I/R injury, mice were subjected on the seventh day to 30 min of ischemia followed by 24 h of reperfusion. The area at risk (AAR) per left ventricle was similar for the mice maintained on a STD rodent diet ( $55.2 \pm 1.8\%$ ) and those supplemented with nitrite ( $58.3 \pm 2.1\%$ ) (Fig. 1E). The mice supplemented with nitrite water for 7 days displayed a 48% reduction in infarct size relative to the AAR ( $47.3 \pm 5.8\%$  vs.  $24.7 \pm 3.4\%$ ) and the infarct size relative to the entire left ventricle by 44% ( $26.1 \pm 3.2\%$  vs.  $14.7 \pm 2.3\%$ ). These data reveal that increasing nitrite dietary intake affects steady-state concentrations of cardiac nitrite, nitroso modified

proteins, and nitrosyl–heme products and provides significant cardioprotection against I/R injury.

**Nitrite Consumption During Myocardial Ischemia Leads to Increased Levels of Nitroso and NO–Heme Products in the Heart.** Exogenous administration of nitrite has been shown to be protective in both the heart and the liver after I/R (25, 27). It has been speculated that nitrite is reduced to NO under ischemic conditions to provide an alternate source of NO when NOS is inactive because of decreased oxygen saturation and substrate delivery. Recent data also demonstrate that RSNOs are cytoprotective in I/R, possibly through NO-independent transnitrosation reactions (32). To better understand the fate of nitrite, we conducted a time course of nitrite metabolism after MI/R. As shown in Fig. 2, plasma nitrite (Fig. 2A) and nitroso (Fig. 2C, RXNO) are unchanged in control mice fed STD chow during I/R. Interestingly, plasma nitrate levels (Fig. 2B) increased by 44% ( $P < 0.01$  vs. baseline) at the end of ischemia and remained at elevated levels for up to 30 min of reperfusion. The plasma nitrite (Fig. 2A) and nitroso (Fig. 2C) levels in the mice supplemented with nitrite water for 7 days were significantly higher than those levels measured in the mice fed a STD diet before the onset of ischemia. These levels remained higher after I/R. The plasma nitrite levels in these mice tended to decline (44% decrease by 30 min of reperfusion) during I/R, and there was a concomitant increase in plasma RXNO during the ischemic phase (Fig. 2C), which decayed during reperfusion.

The heart revealed a similar profile, but the changes were much more dramatic (Fig. 3). Mice on STD chow without nitrite supplementation revealed a trend for nitrite consumption during ischemia (Fig. 3A). Although the difference between the values at baseline and the end of ischemia was not significant, a 28% decrease in nitrite levels was observed. These levels remained decreased at 1 min of reperfusion and had almost rebounded to baseline values by 30 min of reperfusion. The mice supplemented with nitrite displayed significantly higher cardiac nitrite levels at baseline (Fig. 3A). The same trend was observed with nitrite levels, which decreased by 24% at the end of ischemia. However, the nitrite levels in these mice remained lower for up to 30 min of reperfusion, suggesting that nitrite is consumed during I/R. No significant changes in cardiac nitrate (Fig. 3B) were observed in either group at any time point evaluated. In sham-operated animals, the baseline levels of nitrite remained constant over a period of 60 min, suggesting that the nitrite levels were stable in our model system for at least 60 min (equivalent to 30 min ischemia plus 30 min of reperfusion). Furthermore, this observation suggests that the reduction of nitrite levels during ischemia is likely attributable to consumption and bioconversion to nitric oxide during ischemia. Along with a decrease in nitrite, there was a substantial and significant increase in both nitroso (Fig. 3C) and nitrosyl–heme products (Fig. 3D), both in the mice on a STD diet and the mice supplemented with nitrite, with slightly more in the supplemented group. During reperfusion, cardiac nitroso and nitrosyl–heme products decayed over time to reach near starting concentrations by 30 min of reperfusion.

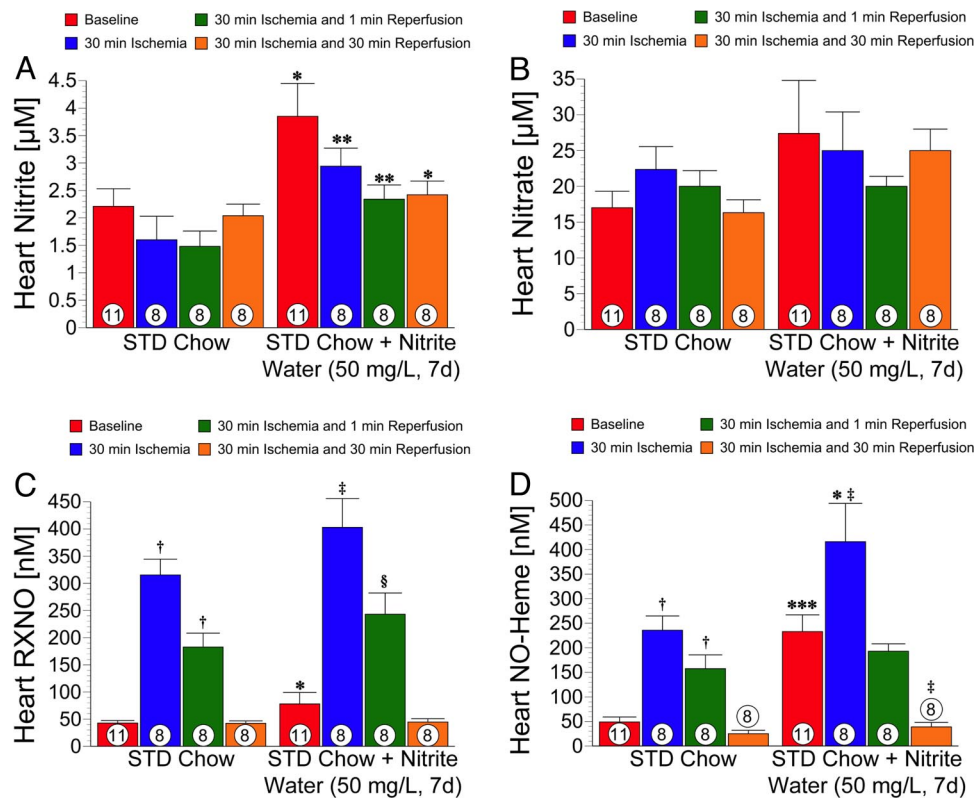
**Dietary Nitrite Deficiency Decreases Steady-State Levels of Nitrite and NO Metabolites and Exacerbates MI/R Injury.** There is a growing appreciation that nitrite therapy may provide benefit from I/R injury (33). However, there are no data on the effects of nitrite insufficiency in the setting of I/R injury. To reveal the biochemical and physiological effects of dietary nitrite insufficiency, mice were fed a STD rodent chow (Purina 5001) for 9 wk and then switched to a purified amino acid diet low in nitrite and nitrate (TD 99366; Harlan) for 7 days. (Control mice were fed Purina 5001 for 10 wk) Consistent with an earlier report (26), the low NO<sub>x</sub> diet significantly decreased plasma and heart steady-state nitrite and nitrate concentrations, which could be restored by the addition of 50 mg/liter nitrite in the drinking water for 1 wk (Fig. 4A and B). Blood and tissue nitroso products have been shown to preserve NO bioactivity



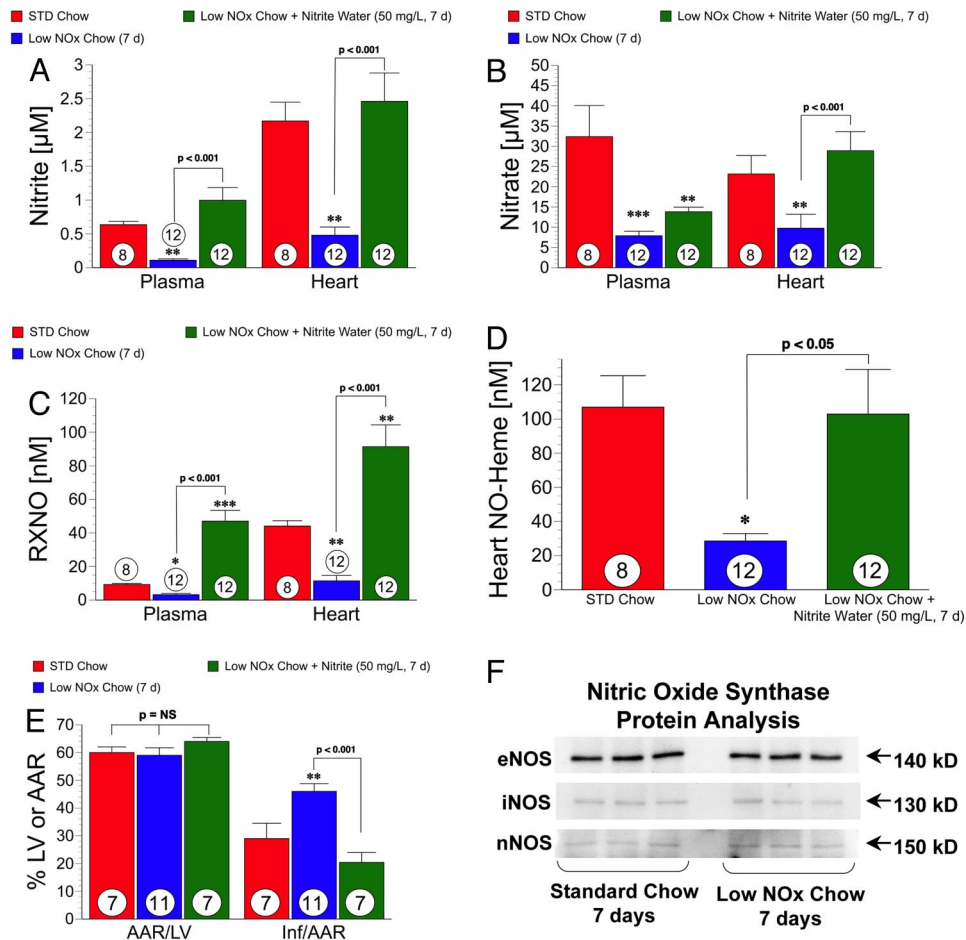
**Fig. 2.** Changes in plasma NOx and nitroso levels after MI/R. Mice fed a STD rodent chow with or without 50 mg/liter nitrite supplementation for 7 days were subjected to 30 min of left coronary artery ischemia followed by either 1 or 30 min of reperfusion. Plasma levels of nitrite (A), nitrate (B), and nitroso (C) were measured before ischemia (baseline), at the end of ischemia, at 1 min of reperfusion, and at 30 min of reperfusion. In the mice maintained on STD rodent chow, nitrite and nitroso levels remained unchanged during I/R, whereas nitrate levels increased during ischemia and remained at this elevated state during reperfusion. Mice supplemented with nitrite had higher baseline levels of nitrite, nitrate, and nitroso. The nitrite in these animals slowly declined over the course of I/R, whereas nitroso levels rose during ischemia and declined during reperfusion. Nitrate levels remained unchanged in these animals. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. corresponding STD chow time point; †,  $P < 0.01$  vs. STD chow baseline.

(34), and protein nitrosation modification confer cGMP-independent NO signaling events (35). We have previously shown that changes in dietary nitrite consumption affect cellular signaling events (26). Mice fed a low NOx diet for 1 wk demonstrated a significant reduction in plasma and heart nitroso levels compared

with mice fed STD chow, which could be replenished and increased with 50 mg/liter nitrite in the drinking water for 1 wk (Fig. 4C). Nitrosyl-heme products (Fig. 4D) were also reduced in the mice fed a low NOx diet and replenished by nitrite supplementation in the drinking water. These data reveal that changes in dietary nitrite



**Fig. 3.** Changes in heart NOx, nitroso, and nitrosyl-heme levels after MI/R. Mice fed a STD rodent chow with or without 50 mg/liter nitrite supplementation for 7 days were subjected to 30 min of left coronary artery ischemia followed by 1 or 30 min of reperfusion. Myocardial levels of nitrite (A), nitrate (B), nitroso (C), and nitrosyl-heme (D) were measured before ischemia (baseline), at the end of ischemia, at 1 min of reperfusion, and at 30 min of reperfusion. Mice supplemented with nitrite had higher baseline levels of nitrite, nitrate, nitroso, and nitrosyl-heme. Nitrite was consumed during the ischemic phase in both groups of mice with a concomitant increase in nitroso and nitrosyl products. During reperfusion, nitroso and nitrosyl products declined to near baseline levels by 30 min of reperfusion. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  vs. corresponding STD chow time point; †,  $P < 0.01$  vs. STD chow baseline; §,  $P < 0.05$ ; ‡,  $P < 0.01$  vs. STD chow plus nitrite water baseline.



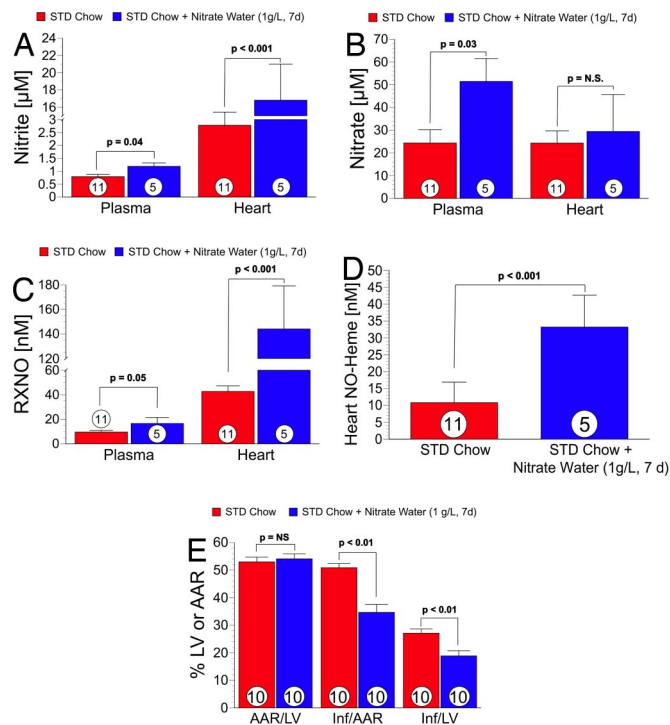
**Fig. 4.** Steady-state plasma and heart NOx and nitrosylation levels in mice after nitrite insufficiency and supplementation. (A–D) Mice were fed a STD rodent chow or a low NOx chow with or without 50 mg/liter nitrite supplementation for 7 days, at which time steady-state levels of plasma and heart nitrite (A), nitrate (B), nitroso (C), and heart nitrosyl–heme (D) were measured. Consumption of low NOx chow resulted in a significant reduction in plasma and heart nitrite, nitrate, and nitroso, as well as heart nitrosyl–heme. The steady-state concentrations could be restored by supplementation of nitrite in the drinking water for 7 days. (E) Mice fed a low NOx diet for 7 days exhibited an exacerbated injury after MI/R. This increase in injury was reversed, however, in those animals fed a low NOx diet supplemented with nitrite. (F) Representative immunoblots of eNOS, iNOS, and nNOS from myocardial tissue homogenates of mice on STD chow and low NOx diet for 7 days. Animals on low NOx chow for 7 days did not show any changes in the protein expression of any of the isoforms of NOS. The numbers inside the bars indicate the number of animals per group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  vs. STD chow.

and/or nitrate consumption can affect steady-state concentrations of blood and tissue NO products/metabolites commonly used to assess NO production.

We next sought to determine whether dietary restriction of nitrite affected the severity of cardiac I/R injury. The decrease in steady-state nitrite concentrations in blood and heart was found to significantly exacerbate myocardial injury (Fig. 4E). The mice fed a low NOx diet displayed a 59% increase in myocardial infarct size relative to the AAR compared with mice fed a STD chow ( $29.0 \pm 5.5\%$  vs.  $46 \pm 2.8\%$ ). To ensure the observed effect depended on NOx intake, and not attributable to an alteration in the nutritional value of the low NOx diet, a subset of mice on the low NOx diet were given 50 mg/liter sodium nitrite ad libitum in the drinking water to restore steady-state concentrations of blood and tissue nitrite. Nitrite supplementation in animals on the low NOx diet reversed the previously observed increase in myocardial infarct size by 57% ( $46.0 \pm 2.8$  vs.  $20.4 \pm 3.6$ ). Additionally, mice fed the low NOx diet displayed a higher mortality rate (58% survival) 24 h postmyocardial infarction than mice on the STD rodent chow (71% survival). Likewise, survival improved in mice on the low NOx diet with nitrite-supplemented drinking water to 77%. Because nitrite is derived both from diet and oxidation of enzymatic NO production from NOS, we investigated potential compensatory changes in NOS

expression after 1 wk on low NOx diet. Western blot analysis of myocardial tissue lysate revealed no significant alterations in NOS expression (eNOS, nNOS, and iNOS) (Fig. 4F). These data suggest that the increased injury is attributable to changes in steady-state concentrations of plasma and heart nitrite as a result of decreased dietary NOx consumption and not from changes in enzymatic NO production.

**Nitrate Supplementation Protects Against MI/R Injury by Increasing Steady-State Plasma and Heart Nitrite Levels.** Because  $\approx 25\%$  of plasma nitrate is actively taken up by the salivary glands and secreted (36) and  $\approx 20\%$  of this nitrate is reduced to nitrite by commensal bacteria in the mouth (37–39), we investigated whether oral nitrate supplementation would increase steady-state plasma and tissue levels of nitrite. To test this notion, nitrate (1 g/liter) was administered in the drinking water of mice on a STD rodent chow (Purina 5001) for 7 days. As shown in Fig. 5, there was a significant increase in steady-state concentrations of plasma nitrite (Fig. 5A) and nitroso products (Fig. 5C), as well as a significant increase in steady-state concentrations of heart nitrite (Fig. 5A), nitroso products (Fig. 5C), and nitrosyl–heme products (Fig. 5D). Plasma nitrate levels increased in the nitrate-supplemented group (Fig. 5B). Heart nitrate levels trended toward an increase, although no



**Fig. 5.** Steady-state plasma and heart NOx and nitrosylation levels. (A–D) Mice were fed a STD rodent chow with or without 1 g/liter nitrate supplementation for 7 days, at which time steady-state levels of plasma and heart nitrite (A), nitrate (B), nitroso (C), and heart nitrosyl-heme (D) were measured. (E) Mice fed STD rodent chow supplemented with nitrate (1 g/liter) in their drinking water exhibited significantly higher plasma levels of nitrite, as well as significantly higher heart levels of nitrite, nitroso, and nitrosyl-heme. Nitrate supplementation significantly attenuated myocardial infarct size (Inf/AAR) by 33%. The numbers inside the bars indicate the number of animals per group.

statistical significance was observed. The observed increases in plasma and heart nitrite and nitroso/nitrosyl levels afforded significant protection (Fig. 5E) against MI/R injury.

## Discussion

The results of the present study demonstrate that modest changes in dietary nitrite and nitrate intake significantly alter steady-state concentrations of nitrite, nitroso modified proteins, and nitrosyl-heme products and that these biochemical changes have a profound outcome on the severity of acute myocardial infarction. It has long been appreciated that our diet exerts important long-term effects on vital body functions and impacts overall cardiovascular health and disease. There has been a well known connection between increased risk for cardiovascular disease (CVD) and poor diet, often involving high intake of saturated fat and limited fruit and vegetable intake, characteristic of a contemporary Western diet. However, dietary considerations usually focus on only fat and caloric intake with regard to preserving cardiovascular health but should now consider NOx intake as a dietary parameter for assessing cardiovascular risk. The data from this investigation reveal that mice are afforded significant cardioprotection from consuming 0.25 mg of nitrite per day and 5 mg of nitrate per day (assuming 5 ml of water consumption per day with 50 mg/liter nitrite and 1 g/liter nitrate). Interestingly, a report from the National Academy of Sciences (40) estimated, based on food consumption tables, that the average total nitrite and nitrate intake in the United States was 0.77 mg and 76 mg, respectively. Therefore, the cardioprotective levels reported in this current study can be achieved easily through increasing consumption of nitrite-/nitrate-rich foods. In fact, earlier

reports by Lundberg and Govoni have revealed that high intake of nitrate results in increased systemic nitrite levels (38), and, most recently, it has been reported that dietary nitrate reduces blood pressure in healthy volunteers (41). The present findings suggest the possibility that nitrite-/nitrate-rich foods may provide protection against cardiovascular conditions characterized by ischemia. An optimal diet may then consist of a sufficient supply of nitrite or nitrate for health and protection from I/R injury. Regular intake of nitrite-containing food, such as green leafy vegetables, may ensure that blood and tissue levels of nitrite and NO pools are maintained at a level sufficient to compensate for any disturbances in endogenous NO synthesis (42). Because low levels of supplemental nitrite have been shown to enhance blood flow (23), dietary sources of NO metabolites could therefore improve circulation and oxygen delivery. This dietary pathway, therefore, may provide not only essential nutrients for NO production but also a rescue or protective pathway for people at risk for CVD (16). Moreover, any intervention that increases blood and tissue concentrations of nitrite may also provide protection against I/R injury. The paramount question then becomes whether some humans at risk for CVD are nitrite deficient? Indeed, a recent study suggests that plasma nitrite levels progressively decrease with increasing cardiovascular risk (30). Therefore, modest changes in the diet to include NOx rich foods may offer benefit and protection from those at risk for a myocardial infarction.

We have previously shown that steady-state RSNOs are linked to steady-state nitrite concentrations under normoxic conditions (26). Nitrite can also be reduced to NO under anaerobic conditions (21). Because both NO and RSNOs have now been shown to be protective in the setting of I/R (27, 32), nitrite now becomes a critical molecule in that it can form both NO and RSNOs. We propose that nitrite serves two functions in the setting of I/R. First, it serves as a NOS-independent source of NO by which nitrite is reduced to NO during ischemia when NOS is inactive. Second, nitrite reacts with critical thiols to form RSNOs. This nitroso modification may act as a reversible protective shield that prevents irreversible oxidation of proteins and lipids during the oxidative burst of reperfusion, or it may alter protein or enzymatic function and thereby modulate protective signaling pathways. Aside from thiol modification, we propose that the nitroso products can also release NO or the  $\text{NO}^+$  moiety during the reperfusion phase and act as a redox-sensitive NO donor (43). Incidentally, the release of  $\text{NO}^+$  will result in the instantaneous reaction of  $\text{NO}^+$  with water to regenerate nitrite. Our biochemical data support this notion by the increase in nitroso at the expense of nitrite, followed by the decay of nitroso over time during reperfusion. Therefore, adding supplemental nitrite provides protection during I/R by not only increasing plasma and tissue levels of nitrite but also increasing steady-state levels of nitroso. On the contrary, nitrite insufficiency leads to increased injury because there are not enough nitrite or nitroso products stored in blood or tissue to perform these actions.

With one in every three men and one in every 10 women in the United States expected to develop CVD before reaching the age of 60 years (44), it is critical to examine preventive measures that promote cardiovascular health. NOx have generally been regarded as harmful substances because of their propensity to form N-nitrosamines, some of which are known carcinogens, but a causative link between nitrite or nitrate exposure and cancer is still missing (45). These data demonstrating protective effects of dietary NOx, along with numerous other reports suggesting the cardioprotective nature of dietary NOx (41, 42), warrant a paradigm shift on the nature of nitrite and nitrate in physiology and the food technologies industry. In summary, the findings of this current study demonstrate the influence of dietary NOx intake on plasma and myocardial levels of nitrite and NO and illustrate the cytoprotective effects of dietary NOx supplementation and consequences of NOx deficiency on the pathophysiology of MI/R injury. Furthermore, these data

