Nitrite Therapy Improves Left Ventricular Function During Heart Failure via Restoration of Nitric Oxide–Mediated Cytoprotective Signaling

Shashi Bhushan,* Kazuhsa Kondo,* David J. Polhemus, Hiroyuki Otsuka, Chad K. Nicholson, Ya-Xiong Tao, Hui Huang, Vasiliki V. Georgiopoulou, Toyoaki Murohara, John W. Calvert, Javed Butler, David J. Lefer

Rationale: Nitric oxide (NO) bioavailability is reduced in the setting of heart failure. Nitrite (NO₂) is a critically important NO intermediate that is metabolized to NO during pathological states. We have previously demonstrated that sodium nitrite ameliorates acute myocardial ischemia/reperfusion injury.

Objective: No evidence exists as to whether increasing NO bioavailability via nitrite therapy attenuates heart failure severity after pressure-overload–induced hypertrophy.

Methods and Results: Serum from patients with heart failure exhibited significantly decreased nitrosothiol and cGMP levels. Transverse aortic constriction was performed in mice at 10 to 12 weeks. Sodium nitrite (50 mg/L) or saline vehicle was administered daily in the drinking water postoperative from day 1 for 9 weeks. Echocardiography was performed at baseline and at 1, 3, 6, and 9 weeks after transverse aortic constriction to assess left ventricular dimensions and ejection fraction. We observed increased cardiac nitrite, nitrosothiol, and cGMP levels in mice treated with nitrite. Sodium nitrite preserved left ventricular ejection fraction and improved left ventricular dimensions at 9 weeks (P<0.001 versus vehicle). In addition, circulating and cardiac brain natriuretic peptide levels were attenuated in mice receiving nitrite (P<0.05 versus vehicle). Western blot analyses revealed upregulation of Akt-endothelial nitric oxide-nitric oxide-cGMP-GS3KB signaling early in the progression of hypertrophy and heart failure.

Conclusions: These results support the emerging concept that nitrite therapy may be a viable clinical option for increasing NO levels and may have a practical clinical use in the treatment of heart failure. (Circ Res. 2014;114:1281-1291.)

Key Words: cardiomyopathy, hypertrophic • cyclic GMP • heart failure • nitric oxide • nitric oxide synthase • ventricular function, left

Left ventricular (LV) hypertrophy is a maladaptive response to chronic pressure overload and an important risk factor for atrial fibrillation, diastolic heart failure, systolic heart failure, and sudden death in patients.1 The integrity of the cardiovascular system is dependent on the continuous generation of nitric oxide (NO). As such, reduction in the bioavailability of NO is central to the development of cardiovascular disorders, including heart failure.2 For instance, patients with class III heart failure have blunted endothelium-dependent flow-mediated dilatation in response to acetylcholine infusion indicative of diminished NO release. NO bioavailability is affected by the activity of the 3 NO synthases (NOS) and the presence of reactive oxygen species. In heart failure, the activity of endothelial NOS (eNOS) is reduced, and there is an increase in reactive oxygen species levels because of decreased antioxidant defenses.3 Further evidence that eNOS/NO provides protection against heart failure comes from animal studies in which genetic overexpression of eNOS protects against,4 whereas the genetic deficiency of eNOS enhances the development of heart failure.5 Pharmacological approaches to upregulate eNOS function have been shown to decrease the severity of heart failure.6 Therefore, therapeutic agents that enhance NO bioavailability, improve NO signaling, or provide a more favorable shift in redox balance may improve the outcome of heart failure.7 Nitrite is a promising therapeutic agent for the treatment of cardiovascular disease.8 Nitrite is a stable oxidative metabolic product of NO that is readily reduced back to NO in hypoxic and...
acidic environments.\textsuperscript{9} Previously it has been demonstrated that administration of nitrite attenuates the severity of ischemia/reperfusion injury in many organs.\textsuperscript{10,11} We have shown that nitrite reduces myocardial ischemia/reperfusion injury.\textsuperscript{10,12} However, no evidence exists as to whether increasing NO bioavailability through nitrite therapy attenuates chronic heart failure. The purpose of the current study was to investigate the effects of sustained sodium nitrite therapy after transverse aortic constriction (TAC).

Methods

Experimental Animals
Male C57BL/6J mice and CS-eNOS transgenic mice at 10 to 12 weeks (Jackson Laboratories, Bar Harbor, ME) were used. All animals were housed in a temperature-controlled animal facility with a 12-hour light/dark cycle, with water and rodent chow provided ad libitum. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the NIH (Publication No. 85-23, Revised 1996).

Serum Samples From Patients With Heart Failure: The Atlanta Cardiomyopathy Consortium (TACC) Study
Serum samples were obtained from patients enrolled in the TACC study. This prospective cohort study enrolls patients from the Emory University–affiliated teaching hospitals. All patients undergo detailed medical history surveys, ECG, standardized questionnaires, and blood and urine sample collection at baseline. All patients provide written informed consent before enrollment. The Emory University Institutional Review Board has approved this study.

TAC Protocol
To establish myocardial pressure overload and heart failure, TAC surgery was performed as described previously.\textsuperscript{13,13a} The complete experimental protocol for these studies is depicted in Figure 2A.

Echocardiography and Hemodynamic Assessment
One week before TAC procedure, baseline transthoracic echocardiogram was performed using 30-MHz probe on a Vevo 2100 (Visualsonics) under anesthesia with isoflurane (0.25%–0.50%) supplemented with 100% O\textsubscript{2}. After TAC procedure, echocardiography was also performed in the same manner for ≤9 weeks at fixed intervals, and pulsed-wave Doppler mode was used to measure hemodynamic assessment of aortic peak flow velocity at 1 week.

Western Blot Analysis
Western blot analysis was performed as described previously.\textsuperscript{14}

Myocardial Measurement of NO Metabolites
Nitrite (NO\textsubscript{-}) nitroso products (RXNO) analysis of cardiac tissue and blood plasma were performed as previously described.\textsuperscript{15}

Measurements of Serum and Myocardial Brain Natriuretic Peptide
Serum levels of brain natriuretic peptide (BNP; EIA kit; Phoenix Pharmaceuticals, Inc) were determined by ELISA at 6 weeks after TAC. Myocardial RNA levels of BNP were determined using polymerase chain reaction methods as described previously.\textsuperscript{16}

cGMP Radioimmunoassay
The cGMP radioimmunoassay was modified from the study by Steiner et al.\textsuperscript{17} Briefly, cGMP standards (Sigma) and samples were acetylated by adding 8 μL of 5 N KOH and 2 μL of acetic anhydride in a volume of 200 μL and incubated at room temperature for 30 minutes. Then tubes were placed on ice to stop the reaction. All determinations were performed in duplicate. Each tube contained 50-μL acetylated standards or samples, 50-μL iodinated cGMP (14,000–16,000 cpm), and 50-μL cGMP antibody (Sigma). The reaction mixture was incubated overnight at 4°C. Then cGMP–antibody complex were precipitated by 12% polyethylene glycol 8000.

Histology and Immunohistochemistry
Hearts were collected at the specific time points and fixed in 10% buffered formalin, embedded in paraffin stained with Masson trichrome and Picrosirius Red to detect fibrosis. Immunohistochemistry was performed to visualize vascular density with a commercially available kit (Blood Vessel staining kit; Millipore). In additional studies, mice were subjected to TAC for 6 weeks at which time lungs were harvested, fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin stain to determine the extent of pulmonary inflammation and edema.

Statistical Analysis
All data are expressed as mean±SEM. Student t test or a 1-way ANOVA with Tukey or Dunnett post hoc analysis was performed using Prism 5 (GraphPad Software). Values >2 SDs outside the mean were considered as outliers. A value of P<0.05 was considered as statistically significant.

Results

Serum Levels of NO Metabolites and cGMP Are Decreased in Heart Failure
We evaluated circulating levels of NO intermediates in patients with heart failure (n=127) when compared with age-matched control subjects (n=93), and these data are depicted in Figure 1A and 1C. We failed to observe any differences in serum nitrite levels (μmol/L) in heart failure when compared with age-matched controls (Figure 1A). However, total serum RXNO (nmol/L) were significantly (P<0.001) reduced in patients with heart failure (Figure 1B). Serum cGMP levels (pmol/mL) were also reduced in the patients with heart failure (P<0.01 versus control) when compared with the controls (Figure 1C).

Effects of Nitrite Therapy on NO Metabolites and cGMP Levels in Mice
To investigate the role of nitrite therapy in pressure-overload–induced hypertrophy and heart failure, we performed TAC surgery in mice (Figure 2). Oral nitrite therapy (50 mg/L) was initiated postoperative day 1 after TAC surgery and was provided in drinking water continuously for 9 weeks. This oral nitrite resulted in the delivery of 8 to 12 mg/kg per day to the mice (Figure 1 in the online-only Data Supplement). To evaluate the effects of nitrite therapy on NO bioavailability and signaling, we examined serum and cardiac nitrite, RXNO, and cGMP levels in mice subjected to TAC (Figure 2). Similar to our clinical data TAC-induced heart failure resulted in decreased levels of serum nitrite (P<0.05 versus sham), serum RXNO, serum cGMP, and cardiac RXNO. Nitrite therapy after TAC restores serum RXNO (P<0.001), serum cGMP...
Nitrite therapy significantly prevented cardiac dilatation (LV end-diastolic diameter at 9 weeks; 4.8±0.2 mm in vehicle versus 3.5±0.1 mm in nitrite and LV end-systolic diameter at 9 weeks; 4.1±0.3 mm in vehicle versus 2.3±0.2 mm in nitrite; Figure 3D and 3E). Nitrite also attenuated LV dysfunction (Figure 3F) as assessed by LV ejection fraction (33.3±4.3% in vehicle versus 66.1±4.1% in nitrite; P<0.001 at 9 weeks).

Despite robust cardiac protection, we failed to observe any changes in survival with nitrite after TAC (Figure III in the online-only Data Supplement). Survival of mice receiving vehicle was 59% and in mice receiving nitrite was 65% (P=NS).

We also investigated the effects of oral nitrite started at 3 weeks after TAC (ie, delayed nitrite therapy) and evaluated cardiac function at baseline, 1, 3, 6, and 9 weeks after TAC (Figure 4). Nitrite treatment significantly prevented cardiac dilatation (LV end-diastolic diameter at 9 weeks; 4.38±0.1 mm in vehicle versus 3.4±0.1 mm in nitrite and LV end-systolic diameter at 9 weeks; 3.5±0.2 mm in vehicle versus 2.2±0.1 mm in nitrite; Figure 4A and 4B). Nitrite also attenuated LV dysfunction (Figure 4C) as assessed by LV ejection fraction (38.8±3.1% in vehicle versus 63.9±3.0% in nitrite; P<0.001 at 9 weeks).

### Hemodynamic Assessment of Aorta After TAC (Peak Velocity)

We measured aortic peak velocity after aortic banding in both the groups at 1 week after TAC. Peak velocity was elevated significantly in both the groups after TAC when compared with sham mice without TAC at each time (data not shown). There was no significant difference between the groups (3055.4±42 mm/s in vehicle versus 3265.3±67.1 mm/s in nitrite) at 1 week thus indicating a similar severity of TAC in both study groups.
Overexpression of eNOS Attenuates Cardiac Dysfunction After TAC

Cardiac-restricted overexpression of eNOS significantly increases nitrite and other NO metabolites in the heart and circulation. We examined whether overexpression of eNOS within the cardiac myocyte attenuates cardiac hypertrophy and dysfunction after TAC using CS-eNOS transgenic mice (Figure V in the online-only Data Supplement). There was no difference in the mortality between both groups (data not shown). CS-eNOS transgenic mice exhibited significantly less cardiac enlargement and pulmonary edema, as assessed by the ratio of heart and lung weights to tibia length (mg/cm) when compared with wild-type controls (Figure VA and VB in the online-only Data Supplement). CS-eNOS transgenic mice exhibited less cardiac dilatation (P<0.001 versus wild-type) and dysfunction (P<0.001 versus wild-type) from 3 to 9 weeks after TAC (Figure VC–VE in the online-only Data Supplement). Furthermore, although CS-eNOS transgenic mice exhibited a significant difference in interventricular septal width (during diastole) thickness when compared with wild-type mice at 1 week after TAC (P<0.01), this difference diminished after 3 to 9 weeks (Figure VF in the online-only Data Supplement).

Nitrite Attenuates Cardiac Fibrosis and Augments Vascular Density

Masson Trichome and Picrosirius Red staining at 6 weeks after TAC revealed extensive areas of intermuscular and perivascular fibrosis in hearts of vehicle TAC mice (P<0.05 versus sham) with significantly less fibrosis after nitrite therapy (Figure 5B and 5C). We also evaluated myocardial vascular density at 6 weeks after TAC surgery in vehicle and nitrite-treated hearts (Figure 5A and 5D). These data indicate significant vascular dropout after TAC that is partially restored with nitrite treatment (P<0.05 versus vehicle).

Nitrite Augments Akt-eNOS Signaling Acutely After TAC

The serine/threonine kinase Akt regulates cardiac growth, angiogenesis, and survival. We investigated whether nitrite altered...
Akt phosphorylation in the heart after TAC (Figure 6A–6C). Representative Western blots for Akt phosphorylation status in the heart at 1 and 6 weeks after TAC are shown in Figure 6A. Total Akt levels in the both vehicle and nitrite-treated mice significantly increased at 1 week after TAC and in nitrite-treated mice at 6 weeks. Nitrite treatment significantly increased the phosphorylated Akt at threonine residue 308 (Akt-PThr308; $P<0.001$) and serine residue 473 (Akt-PSer473; $P<0.001$) when compared with vehicle mice at 1 week after TAC (Figure 6B and 6C). Interestingly, both Akt-PThr308 ($P<0.05$) and Akt-PSer473 ($P<0.001$) were significantly attenuated by nitrite treatment when compared with vehicle mice at 6 weeks after TAC (Figure 6B and 6C).

Upregulation of Akt results in eNOS activation to generate NO that modulates vascular angiogenesis and to promote vascular and myocardial cytoprotection.20 To investigate the involvement of eNOS function after TAC, the expression and the phosphorylation status of eNOS at serine residue 1177 (eNOS-PSer1177) were assessed by Western blot analysis (Figure 6D–6F). There were no differences in total eNOS expression in the heart among all groups (Figure 6E). However, the eNOS activation site (eNOS-PSer1177) exhibited significantly greater phosphorylation (Figure 6F) in the nitrite group when compared with either sham ($P<0.01$) or vehicle mice ($P<0.01$) at 1 week. Similar to the activation of Akt, the increase in eNOS phosphorylation at Ser1177 was transient and not observed at 6 weeks after TAC.

Nitrite Augments GSK3β Signaling Inhibition in Acute Phase After TAC

Phosphorylation of protein kinase B/Akt has been linked with the inhibition of glycogen synthase kinase-3 beta (GSK3β) through phosphorylation at serine residue 9.21 Furthermore, we investigated whether nitrite treatment altered GSK3β phosphorylation in the heart after TAC (Figure 7). Representative Western blots for GSK3β phosphorylation status in the heart...

Figure 3. Nitrite therapy prevents cardiac dilatation and dysfunction following transverse aortic constriction (TAC) at 9 weeks.

A, Circulating brain natriuretic peptide (BNP) levels (ng/mL). B, The ratio of heart weight/tibia lengths. C, The ratio of lung weight/tibia lengths. D, Left ventricular end-diastolic diameter (LVEDD). E, left ventricular end-systolic diameter (LVESD), and F, left ventricular ejection fraction (LVEF %) from 1 to 9 weeks after TAC. Results are expressed as mean±SEM. NS indicates nonsignificant.

Figure 4. Delayed nitrite therapy prevents cardiac dilatation and dysfunction after transverse aortic constriction (TAC) at 9 weeks.

Mice were subjected to TAC surgery and nitrite therapy (50 mg/L) was initiated at 3 weeks after TAC. A, Left ventricular end-diastolic diameter (LVEDD). B, left ventricular end-systolic diameter (LVESD), and C, left ventricular ejection fraction (LVEF %) from 1 to 9 weeks after TAC. Results are expressed as mean±SEM. NS indicates nonsignificant.
at 1 and 6 weeks after TAC are shown in Figure 7A. Total GSK3β levels in the both vehicle and nitrite-treated mice remained the same at 1 and 6 weeks after TAC (Figure 7B). Nitrite treatment significantly increased the expression of phosphorylated-GSK3β at ser9 when compared with vehicle mice (P<0.05) at 1 week (Figure 7C). There were no differences in GSK3β phosphorylation between the nitrite and vehicle groups at 6 weeks after TAC as shown in Figure 7C.

Nitrite Augments ERK1/2 Signaling After TAC
Extracellular signal-regulated kinases (ERK1/2) are a known regulator of cardiac hypertrophy.22 We investigated whether nitrite treatment altered ERK phosphorylation in the heart after TAC (Figure 8A–8C). Representative Western blots for ERK phosphorylation status in the heart at 1 and 6 weeks after TAC are shown in Figure 8A. Total ERK levels (Figure 8B) in the both vehicle and nitrite-treated mice significantly increased at 1 and 6 weeks after TAC. Nitrite treatment significantly increased the expression of phosphorylated-ERK1/2 when compared with vehicle mice (P<0.001) at 1 and 6 weeks (P<0.05) after TAC (Figure 8C).

Nitrite Attenuates p38 Signaling After TAC
Previous studies have shown that activation of p38 leads to cardiac dysfunction by reducing LV function and increasing cardiac fibrosis.23,24 We investigated the phosphorylation status of p38 at 1 and 6 weeks after TAC (Figure 8D–8F). Representative Western blots for p38 phosphorylation status in the heart at 1 and 6 weeks after TAC are shown in Figure 8D. We found that nitrite treatment significantly attenuated p38 activation at 6 weeks when compared with the vehicle group, but failed to show any change at 1 week after TAC (Figure 8F).

Nitrite Attenuates Lung Inflammation and Edema
We also investigated the effect of oral nitrite on lung tissue after TAC in both the control and the nitrite-treated mice at 6 weeks. We found that in the nitrite treatment group, there is less inflammation and edema, as well less thicker alveolar walls, when compared with the vehicle group at 6 weeks. Representative pictures were taken at ×5 and ×20 magnification for both the groups (Figure IV in the online-only Data Supplement).

Discussion
Alterations in NO bioavailability play a prominent role in the development of heart failure.7,25 Studies have shown that enhancing NO levels through genetic manipulation leads to improved survival,4 decreased remodeling26 and improved cardiac function after ischemia-induced heart failure. Furthermore, eNOS-derived NO exerts antihypertrophic effects in the heart as evidenced by the findings that eNOS-deficient animals exhibit hypertension and cardiac hypertrophy.27,28 Moreover, cardiac-specific overexpression of eNOS attenuates isoproterenol-induced cardiac hypertrophy.29
The current study provides several lines of evidence to support these previous findings. Our study clearly demonstrates reductions in circulating levels of nitrosothiols and cGMP in patients with heart failure. We also demonstrate significant reductions in circulating and cardiac NO metabolites and cGMP in the setting of TAC-induced heart failure in mice. We also demonstrate that chronic oral nitrite therapy provides protection against the adverse remodeling and LV dysfunction associated with heart failure. Our findings suggest that increasing NO bioavailability in the myocardium protects against the development of pressure-induced heart failure.

The role of NO in the pathophysiology and treatment of chronic heart failure is not without controversy. Studies report that NO contributes to detrimental cardiac function in heart failure. In addition, Takimoto et al suggested that pressure overload results in eNOS-uncoupling resulting in increased myocardial oxidant production and exacerbated cardiac dysfunction. In sharp contrast to these findings, we found that the genetic overexpression of eNOS in the heart provided protection against the adverse remodeling and impaired LV function associated with TAC and not exacerbated cardiac pathology. In addition, the effect of NO on the mitochondria is recognized as one of its cardioprotective actions because this leads to a decrease of myocardial injury by extending the zone of adequate tissue cellular oxygenation away from vessels. Furthermore, physicians have been successfully using drugs that are able to activate eNOS (ie, ACE-I [angiotensin-converting enzyme-1] and ARB [angiotensin receptor blocker]) or increase NO bioavailability (ie, ISDN-hydralazine-Bidil) in the treatment of heart failure. Likewise, increasing the NO second messenger, cGMP, after sildenafil treatment has been shown to improve exercise capacity in patients with heart failure. Finally, statins that augment eNOS-NO signaling have been shown to prolong survival in a murine model of heart failure. Therefore, evidence is accumulating that under the appropriate conditions,
NO in general, and eNOS-derived NO in particular, impart cardioprotective effects in the failing heart. In an effort to provide insights into the mechanisms regulating the progression of heart failure, Haq et al. investigated the activity of several signaling cascades in heart samples taken from patients with compensated cardiac hypertrophy and advanced heart failure. Although they found a clear prohypertrophic activity profile in both patient populations, the signaling cascades activated in both were distinct. The results of the current study suggest that nitrite therapy prevents the development of heart failure in response to TAC. We evaluated the effects of nitrite therapy on multiple signal transduction pathways activated in response to heart failure to further elucidate the mechanisms by which nitrite protects the failing heart. We focused our analysis on 2 time points, representing an acute onset of hypertrophy (1 week) and heart failure (6 weeks).

The serine/threonine protein kinase Akt regulates cardiac growth, contractile function, and cell death and also is an important factor for VEGF-mediated angiogenesis. Our data suggest that nitrite therapy rapidly induces Akt activation, resulting in eNOS phosphorylation and inhibition of GSK3β at 1 week after the induction of aortic constriction. At 6 weeks of TAC, Akt phosphorylation remained elevated in the vehicle group but returned to baseline levels in the nitrite group. Previous studies suggest that short-term activation of Akt in cardiac muscle protects from contractile dysfunction in the failing heart, whereas long-term activation switches the heart from an adaptive, compensated state with preserved function to a pathological state with cardiac dysfunction. Our findings support the notion that Akt can exert a dual role in the development of heart failure after TAC. The activation of Akt in the failing heart is due to a number of stress-related stimuli. As such, the persistent activation of Akt observed in the vehicle-treated heart at 6 weeks after TAC could be indicative of still present stimuli. Likewise, the decrease in Akt activation observed in the nitrite-treated hearts could be because of a reduction in these stimuli at the 6-week time point. Akt signaling is well known to occur in cardiac hypertrophy and perhaps some of the mixed results could reflect a combination of survival signaling and cell growth stimulation. Further studies are required to determine the exact mechanism by which nitrite therapy modulates Akt activation during the development of heart failure.

GSK3β regulates a wide variety of cellular functions and serves as a master regulator of cell growth and death in cardiac myocytes in response to hypertrophic stimuli. The activity of GSK3β is negatively regulated by Akt and inhibiting GSK3β is critical to the antiapoptotic effects of Akt and to the hypertrophic response of cardiomyocytes. Our findings suggest that nitrite therapy prevents the transition from compensated to decompensated heart failure via the inhibition GSK3β.

We also demonstrate that nitrite therapy increases the activation of MAPK/ERK kinase (MEK1)–ERK1/2 signaling and attenuates p38 activation. Specifically, we found that nitrite therapy rapidly induces ERK1/2 activation at 1 week after the induction of aortic constriction. At 6 weeks of TAC, ERK1/2 phosphorylation levels remained higher in the nitrite-treated group when compared with the vehicle-treated group. However, the level of phosphorylation in the nitrite-treated group was similar to those found in sham animals. In regards to p38 activation, we found that nitrite therapy reduced the level of p38 phosphorylation at 6 weeks of TAC. It has been demonstrated that activation of MEK1–ERK1/2 signaling regulates hypertrophic response in vivo and promotes compensated...
cardiac hypertrophy and preservation of cardiac function. In addition to the reported hypertrophic effects of ERK1/2 signaling, the enhanced activation of this pathway has been shown to provide cardioprotection via robust antiapoptotic actions. Previous studies have indicated that p38 activation has a detrimental effect on cardiac function that leads to pathological hypertrophy rather than physiological compensation. The enhanced activation of a MEK1–ERK1/2 signaling pathway and inhibition of p38 in the nitrite-treated hearts is also responsible for preventing the transition from compensated to decompensated heart failure.

We also investigated its effect in an ischemic-induced heart failure model. Previous studies indicate that nitrite is both a storage form of NO in tissues and also a source of NO in ischemic conditions. Nitrite is cardioprotective during ischemia/reperfusion injury via increasing nitrosothiol levels. Our results demonstrate that chronic nitrite therapy leads to significantly higher plasma nitrosothiol levels and higher myocardial nitrite levels.

Although the current study demonstrates that chronic nitrite therapy improves LV function after heart failure, there are some limitations that need to be noted. Because a mouse model was used, these data may not accurately predict human disease. Future studies need to be conducted in large animal models that are more clinically relevant. Another limitation is that mice used in this study are without any other comorbid conditions such as hypertension, hypercholesterolemia, and diabetes mellitus. We also started nitrite therapy at 24 hours after TAC that attenuated the progression of heart failure. Future studies need to evaluate nitrite therapy at a time when heart failure is fully developed.
In summary, we have demonstrated a cardioprotective action for sodium nitrite therapy on the severity of heart failure after TAC. Specifically, the current study provides evidence that chronic oral administration of sodium nitrite increases NO bioavailability and signaling after TAC-induced cardiac hypertrophy and failure. These results support the emerging concept that nitrite therapy may be a viable clinical option for increasing NO levels and may have a practical clinical use in the treatment of heart failure.

Acknowledgments

We thank Bradley Schwartz, Nabil Sabbak, and Benjamin Hayes for their expert technical help during the course of these experimental studies. This article was sent to Elizabeth Murphy, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

Sources of Funding

This work was supported by grants from the National Heart, Lung, and Blood Institute (National Institutes of Health; 5R01HL092141, 5R01HL093579, 1U24HL094373, and 1P20HL113452 to Dr Lefer and 5R01HL098481 to Dr Calvert). This research was also supported by a research grant from the American Diabetes Association grant 1-12-BS212 to Dr Tao.

Disclosures

D.J. Lefer has served on the Scientific Advisory Board of Theravasc, Inc. Theravasc is currently developing novel nitrite formulations for the treatment of peripheral arterial disease and cardiovascular diseases.

References


• NO bioavailability is reduced in the myocardium and in circulation during heart failure in both mice and humans.

Novelty and Significance

What Is Known?

• In preclinical studies, nitrite therapy (ie, sodium nitrite) has been found to reduce ischemia/reperfusion injury in various organs by increasing the bioavailability of nitric oxide (NO) and augmenting NO signaling.

• In a recent clinical trial, chronic nitrite therapy has been shown to be safe and well tolerated in humans with peripheral artery disease (ie, SONIC [Safety, PK, & Tolerability of Sodium Nitrite in Patients With Peripheral Arterial Disease] Trial). Currently, there are 37 ongoing clinical trials (clinicaltrials.gov) to investigate the cytoprotective actions of sodium nitrite.

• Recent evidence suggests that sodium nitrite is not effective in reducing myocardial infarct size after myocardial ischemia and reperfusion. There was no reported reduction in myocardial infarct size in the National Institutes of Health CAESAR Preclinical Cardioprotection Consortium (mice, rabbit, and pig) and in the Nitrites in Acute Myocardial Infarction (NIAMI) human clinical trial.

What New Information Does This Article Contribute?

• In mice, chronic oral sodium nitrite therapy increases NO bioavailability and attenuates the severity of heart failure induced by chronic pressure overload.

• Sodium nitrite increased myocardial vascular growth while decreasing cardiac fibrosis in chronic heart failure.

In the present study, we demonstrate a cardioprotective action for sodium nitrite therapy on the severity of heart failure after transverse aortic constriction. The study provides evidence that chronic oral administration of sodium nitrite increases NO bioavailability and signaling after transverse aortic constriction–induced heart failure. These results support the emerging concept that nitrite therapy may be a viable therapeutic option for increasing NO levels and potentially for treating patients with heart failure.
Nitrite Therapy Improves Left Ventricular Function During Heart Failure via Restoration of Nitric Oxide–Mediated Cytoprotective Signaling

Shashi Bhushan, Kazuhisa Kondo, David J. Polhemus, Hiroyuki Otsuka, Chad K. Nicholson, Ya-Xiong Tao, Hui Huang, Vasiliki V. Georgioupolou, Toyoaki Murohara, John W. Calvert, Javed Butler and David J. Lefer

Circ Res. 2014;114:1281-1291; originally published online March 5, 2014; doi: 10.1161/CIRCRESAHA.114.301475

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2014 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circres.ahajournals.org/content/114/8/1281

Data Supplement (unedited) at:

http://circres.ahajournals.org/content/suppl/2014/03/05/CIRCRESAHA.114.301475.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:

http://circres.ahajournals.org/subscriptions/
Supplemental Material

Nitrite Therapy Improves Left Ventricular Function During Heart Failure via Restoration of Nitric Oxide (NO) Mediated Cytoprotective Signaling
Shashi Bhushan M.D.1,*, Kazuhisa Kondo M.D., Ph.D.2,*, David Polhemus, B.A.1, Hiroyuki Otsuka M.D., Ph.D.1, Chad Nicholson B.S.3, Ya-Xiong Tao Ph.D.4, Hui Huang, B.S.4, Vasiliki V. Georgiopoulou M.D.5, Toyoaki Murohara M.D., Ph.D.2, John W. Calvert Ph.D.2, Javed Butler, M.D.5, and David J. Lefer Ph.D.1

1LSU Cardiovascular Center of Excellence, LSU Health Sciences Center, New Orleans, Louisiana, 70112
2Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya, Japan 466-8550
3Department of Surgery, Division of Cardiothoracic Surgery, Emory University School of Medicine, Atlanta, Georgia, 30308 and the Carlyle Fraser Heart Center
4Department of Anatomy, Physiology, and Pharmacology, Auburn University College of Veterinary Medicine, Auburn, Alabama, 36849
5Department of Medicine, Division of Cardiology, Emory University School of Medicine, Atlanta, Georgia, 30308

Address for Correspondence:

David J. Lefer, Ph.D.
LSU Cardiovascular Center of Excellence
LSU Health Sciences Center
New Orleans, LA 70112
Phone: (504) 568-2109
FAX: (504) 568-2135
Email: dlefe1@lsuhsc.edu

*These authors contributed equally

Detailed Methods
Experimental Animals

Male C57BL/6J mice and CS-eNOS Tg mice at 10–12 weeks of age (Jackson Labs, Bar Harbor, ME) were used. All animals were housed in a temperature-controlled animal facility with a 12-hour light/dark cycle, with water and rodent chow provided ad libitum. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals published by the NIH (Publication No. 85-23, Revised 1996). The Emory University IACUC approved all animal procedures.

Serum Samples from Heart Failure Patients- TACC Study

Serum samples are derived from patients enrolled in the Atlanta Cardiomyopathy Consortium (TACC). This prospective cohort study is enrolling patients from the Emory University-affiliated teaching hospitals, the Emory University Hospital, Emory University Hospital Midtown, and Grady Memorial Hospital in Atlanta. All patients undergo detailed medical history surveys, electrocardiogram, 6-minute walk test, standardized questionnaires, and blood and urine sample collection at baseline. Every six months, the patients are contacted to assess outcomes including interim medication changes, procedures, new disease diagnoses, and hospitalizations. All patients provide written informed consent prior to enrollment. The Emory University Institutional Review Board has approved this study.

Transverse Aortic Constriction (TAC) Protocol

To establish myocardial pressure overload and heart failure transverse aortic constriction surgery was performed as described previously.1 The complete
experimental protocol for these studies is depicted in Figure 2A. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (8 mg/kg) and then orally intubated and placed on a rodent ventilator to maintain respiration during the surgical procedure. The core body temperature was maintained in normal range (36-37°C) throughout the surgical procedure. An incision was made in the second intercostal muscle to visualize the aortic arch. Following identification and dissection of the surrounding tissue of aortic arch, 7-0 silk suture was placed around the aortic arch between the brachiocephalic trunk and the left carotid artery and ligated with a 27G blunt needle. The needle was immediately removed after ligation. The chest was surgically closed under aseptic condition and mice were put in a recovery chamber with 100% oxygen along with a surgical warming pad to maintain core body temperature within normal limits. At day 1 following surgery mice were divided into control (water) group or sodium nitrite (NaNO₂, Sigma-Aldrich, St. Louis, MO) water (50 mg/L) group. During this period, nitrite water was changed twice a week. At the end of the experimental protocol (i.e. 1 or 6 weeks following TAC surgery) mice were euthanized and heart, lung and blood samples were collected.

**Echocardiography**

One week prior to TAC procedure, baseline transthoracic echocardiogram was performed using 30-MHz probe on a Vevo 2100 (Visualsonics) under anesthesia with isoflurane (0.25 to 0.50%) supplemented with 100% O₂. Following TAC procedure, echocardiography was also performed in the same manner for up to 9 weeks at fixed intervals. To determine cardiac structure and function, intraventricular septal end diastolic dimension (IVSd), LV end diastolic dimension (LVEDD), LV end systolic dimension (LVESD), and LV ejection fraction (LVEF) were analyzed from M-mode images.
images.

**Hemodynamic Assessment of Aortic Velocity**

Following TAC procedure, Pulsed-Wave (PW) Doppler was performed at 1 week to measure aortic peak velocity by using Vevo 2100 (Visualsonics) under anesthesia with isoflurane (0.25 to 0.50%) supplemented with 100% O₂.

**Western Blot Analysis**

Myocardial tissue samples (75 mg) taken from the left ventricle were homogenized and lysates were used for Western blot analysis. Protein concentrations were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were loaded into lanes of polyacrylamide-SDS gels. The gels were electrophoresed, followed by transfer of the protein to a PVDF membrane. The membrane was then blocked and probed with primary antibodies overnight at 4°C. The following primary antibodies were used: eNOS (1:5000, BD Biosciences); Phospho-eNOS Ser1177 (eNOS-PSer1177) (1:1000, Cell Signaling Technology); Phospho-eNOS Thr495 (eNOS-PThr495) (1:1000, Cell Signaling Technology); α-Tubulin (1:10,000, Cell Signaling Technology); iNOS (1:5000, BD Biosciences); nNOS (1:5000, BD Biosciences). Immunoblots were next processed with the appropriate secondary antibodies (Cell Signaling) for 1 h at room temperature. Immunoblots were then probed with a SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) to visualize signal, followed by exposure to X-ray film.²

**Analysis of Nitric Oxide Metabolites in Cardiac Tissue and Plasma**

Nitrite and nitrate concentrations were quantified by ion chromatography (ENO20 Analyzer, Eicom). Tissue nitroso compounds were quantified by using group-specific
reductive denitrosation by iodine-iodide with subsequent detection of the liberated NO by using gas-phase chemiluminescence. S-nitrosothiol levels were detected by pre-incubation with 2% mercuric chloride followed by acidified sulfanilamide. Nitroso levels were determined by the addition of acidified sulfanilamide alone. NO-heme levels were determined by parallel injection of replicate aliquots of tissue homogenates into a solution of 50 mmol/l ferricyanide in PBS at pH 7.5 and 37°C. This method employs one-electron oxidation rather than reduction to achieve denitrosation, with the liberated NO quantified by gas-phase chemiluminescence.  

Measurements of Serum and Myocardial Brain Natriuretic Peptide (BNP)

Serum levels of brain natriuretic peptide (BNP) (BNP EIA kit, Phoenix Pharmaceuticals, Inc.) were determined by ELISA at 6 weeks following TAC. For BNP PCR total RNA was extracted from whole hearts using TRizol (Invitrogen) and treated with Turbo DNase (Ambion, Austin, TX). RNA was reverse transcribed (MultiScribe Reverse Transcriptase, Applied Biosystems, Foster City, CA) and the resultant cDNA was used for Taqman RT-PCR reaction (7900HT Thermocycler, Applied Biosystems). The amount of target gene expressed was normalized to 18S levels. The PCR primers and probes were obtained from Applied Biosystems. The primers used are Nppa Assay ID: Mm01255747_g1 and Nppb Assay ID: Mm00435304_g1. 

cGMP RIA

The radioimmunoassay was modified from Briefly, cGMP standards (Sigma) and samples were acetylated by adding 8 µl of 5 N KOH and 2 µl of acetic anhydride in a volume of 200 µl, and were incubated at room temperature for 30 min. Then tubes were placed on ice to stop the reaction. All determinations were performed in duplicate. Each
tube contained 50 µl acetylated standards or samples, 50 µl iodinated cGMP (14000 to 16000 cpm), and 50 µl cGMP antibody (Sigma). The reaction mixture was incubated overnight at 4 °C. Then cGMP-antibody complex were precipitated by 12% polyethylene glycol 8000. Radioactivity was counted in a gamma counter.

**Histology and immunochemistry**

Hearts were collected at the specific time points and fixed in 10% buffered formalin, embedded in paraffin stained with Masson’s trichome and Picrosirius Red to detect fibrosis. Lung tissues were collected and stained with hematoxylin and eosin to look for edema and vascular congestion. Image J software was used to analyze digital pictures. Immunohistochemistry was performed to visualize vascular density with a commercially available kit (Blood Vessel staining kit, Millipore). Primary antibody against CD31 (Abcam; 1:50) was used. Digital images were obtained with a microscope at a magnification of 400X. CD31 positive vessels numbers were counted using Image J and vessels number per mm² was calculated to evaluate the number of vessels per field.

**Statistical analysis**

All data are expressed as mean ± SEM. Student’s t test or a one-way ANOVA with Tukey’s or Dunnett’s post-hoc analysis was performed using Prism 5 (GraphPad Software). Values greater than two Standard deviations outside the mean were considered as outliers. A value of p < 0.05 was considered as statistically significant.

**Figure Legends for Supplemental Figures**
**Supplemental Figure I.** Nitrite dosing (mg/kg/day) in sham mice and mice subjected to TAC. Nitrite (50 mg/L) was added to drinking water. Data are shown for n = 4-5 during the first week and second week following TAC surgery.

**Supplemental Figure II.** Exogenous nitrite therapy prevents heart failure in mice. (A) Representative heart pictures of sham, TAC + vehicle, and TAC + nitrite mice at 9 weeks of TAC. (B) Cardiac mRNA levels of BNP and (C) ANP in sham, vehicle and nitrite treated mice following TAC.

**Supplemental Figure III.** Kaplan-Meier survival curves for 60 days following transverse aortic constriction in vehicle (n = 22), and mice treated with nitrite (n = 20).

**Supplemental Figure IV.** Lung histology sections were stained with H&E stain and pictures were taken by using light microscopy at x5 and x20 magnification at 6 weeks following TAC. (A) Vehicle 5x. (B) Nitrite 5x. (C) Vehicle 20x. (D) Nitrite 20x.

**Supplemental Figure V.** Cardiac-specific endothelial nitric oxide synthase overexpression in mice (CS eNOS Tg) cardiac dilatation and dysfunction following TAC at 9 weeks as compared to wild-type. (A) The ratio of heart weight to tibia length. (B) The ratio of lung weight/tibia length. (C) Left ventricular ejection fraction (LVEF %), (D) Left ventricular end-diastolic diameter (LVEDD), (E) Left ventricular end-systolic diameter (LVESD), and (F) Interventricular septal diameter (IVSD) from 1 week to 9 weeks following TAC. Results are expressed as mean ± SEM.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>127</td>
</tr>
<tr>
<td>Age, y</td>
<td>56.6 ± 11.9</td>
</tr>
<tr>
<td>Male (%)</td>
<td>64.5</td>
</tr>
<tr>
<td>White (%)</td>
<td>55.2</td>
</tr>
<tr>
<td>Ischemic Etiology (%)</td>
<td>30.5</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>29.5 ± 15.2</td>
</tr>
<tr>
<td>Brain natriuretic peptide (ng/L)</td>
<td>509 ± 719</td>
</tr>
<tr>
<td>Defibrillator/Pacemaker (%)</td>
<td>64.5</td>
</tr>
</tbody>
</table>
References


Supplemental Figure I

Nitrite dose (mg/kg/day)

- Sham + Nitrite (50 mg/L)
- TAC + Nitrite (50 mg/L)

1st week 2nd week

0 5 10 15

p < 0.01  NS

5 5 4

5 5

1st week 2nd week
Supplemental Figure II

- Vehicle (n = 13/22)
- Nitrite 50 mg/L (n = 13/20)

p = 0.31
Supplemental Figure III

A. 

Sham 

TAC + Vehicle 

TAC + Nitrite 

B. 

Relative Cardiac BNP mRNA Expression 

Sham 

Vehicle 

Nitrite 

C. 

 Relative Cardiac ANP mRNA Expression 

Sham 

Vehicle 

Nitrite
Supplemental Figure IV
**Supplemental Figure V**

**A**
- Ratio of heart weights to tibia length (mg/cm) for Wild-Type and CS eNOS Tg.
- Wild-Type: 14
- CS eNOS Tg: 11

**B**
- Ratio of Lung weights to tibia length (mg/cm) for Wild-Type and CS eNOS Tg.

**C**
- EF (%) for Wild-Type and CS eNOS Tg.

**D**
- LVEDD (mm) for Wild-Type and CS eNOS Tg.

**E**
- LVESD (mm) for Wild-Type and CS eNOS Tg.

**F**
- IVSd (mm) for Wild-Type and CS eNOS Tg.

**Notes:**
- *p < 0.05
- **p < 0.01
- ***p < 0.001
- p = NS