Uncoupled Cardiac Nitric Oxide Synthase Mediates Diastolic Dysfunction

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Background—Heart failure with preserved ejection fraction is 1 consequence of hypertension and is caused by impaired cardiac diastolic relaxation. Nitric oxide (NO) is a known modulator of cardiac relaxation. Hypertension can lead to a reduction in vascular NO, in part because NO synthase (NOS) becomes uncoupled when oxidative depletion of its cofactor tetrahydrobiopterin (BH4) occurs. Similar events may occur in the heart that lead to uncoupled NOS and diastolic dysfunction.

Methods and Results—In a hypertensive mouse model, diastolic dysfunction was accompanied by cardiac oxidation, a reduction in cardiac BH4, and uncoupled NOS. Compared with sham-operated animals, male mice with unilateral nephrectomy, with subcutaneous implantation of a controlled-release deoxycorticosterone acetate pellet, and given 1% saline to drink were mildly hypertensive and had diastolic dysfunction in the absence of systolic dysfunction or cardiac hypertrophy. The hypertensive mouse hearts showed increased oxidized biopertins, NOS-dependent superoxide production, reduced NO production, and dephosphorylated phospholamban. Feeding hypertensive mice BH4 (5 mg/d), but not treating with hydralazine or tetrahydroneopterin, improved cardiac BH4 stores, phosphorylated phospholamban levels, and diastolic dysfunction. Isolated cardiomyocyte experiments revealed impaired relaxation that was normalized with short-term BH4 treatment. Targeted cardiac overexpression of angiotensin-converting enzyme also resulted in cardiac oxidation, NOS uncoupling, and diastolic dysfunction in the absence of hypertension.

Conclusions—Cardiac oxidation, independently of vascular changes, can lead to uncoupled cardiac NOS and diastolic dysfunction. BH4 may represent a possible treatment for diastolic dysfunction. (Circulation. 2010;121:519-528.)

Key Words: diastole ■ heart failure ■ nitric oxide ■ nitric oxide synthase

Heart failure with preserved ejection fraction as a result of diastolic dysfunction accounts for significant mortality and healthcare expenditures, especially among hypertensive individuals.1,2 The incidence of this type of heart failure is increasing.1

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There are no specific treatments for diastolic dysfunction, partly because of a relative lack of a mechanistic understanding of this disorder.3 A strong epidemiological association exists between hypertension and diastolic dysfunction.2,4,5 In the vasculature, hypertension and activation of the renin-angiotensin system lead to reduced vascular nitric oxide (NO), in part because NO synthase (NOS) becomes uncoupled when oxidative depletion of its cofactor tetrahydrobiopterin (BH4) occurs, leading to production of superoxide (O2·−) instead of NO.6 NO and NOS have recently been identified as having a role in the modulation of cardiac relaxation.7 Therefore, we hypothesized that hypertension and/or activation of the renin-angiotensin system in the absence of an increase in blood pressure may lead to NOS uncoupling in the heart and to diastolic dysfunction.

Methods

All of the experiments were approved by the appropriate institutional animal care and use committees.

Hypertensive Mouse Model

We used a mouse model of hypertension to mimic the most common human risk factor for diastolic dysfunction (see the online-only Data Supplement).
Supplement). We have shown that this model leads to vascular oxidative stress, BH4 depletion, and NOS uncoupling. A subset of hypertensive mice were randomly selected on postoperative day 1 to receive 5 mg/d of either BH4 (AXXORA, San Diego, CA) or tetrahydropterin (H4N), an enzymatically inactive analog of BH4 with equivalent antioxidant properties (Schricks Laboratories, Jona, Switzerland). A cohort of hypertensive deoxycorticosterone acetate (DOCA)–salt mice were randomized on postoperative day 11, a time when all DOCA-salt mice have evidence of diastolic dysfunction, to either 12 to 14 days of BH4 feeding as described above or treatment with hydralazine (25 to 30 mg·kg⁻¹·d⁻¹ in 1% saline; Sigma-Aldrich, St. Louis, Mo).

Echocardiography was performed on a subset of mice 24 to 48 hours before terminal procedures. These procedures were done on postoperative day 14 for hypertensive DOCA-salt, BH4 prevention, H4N, and control mice and on postoperative days 22 to 24 for mice in the BH4 or hydralazine treatment groups (see the online-only Data Supplement).

Angiotensin-Converting Enzyme 1/8 Mouse Model
Male compound heterozygous angiotensin-converting enzyme (ACE) 1/8 mice and age-matched littermate controls were used to test whether cardiac oxidation could lead to diastolic dysfunction (see the online-only Data Supplement). These mice have no hypertension, increased cardiac ACE, minimal extracellular ACE expression, and preserved ejection fraction.

Noninvasive Assessment of Diastolic Dysfunction
Mice were studied by pulsed-wave tissue Doppler from the apical 4-chamber view. Results correlated with invasive measures (see the online-only Data Supplement).

Invasive Assessment of Diastolic Dysfunction
The online-only Data Supplement provides details on the invasive assessment of diastolic dysfunction.

Cardiac Biopterin Content
Cardiac biopterins were measured as previously reported (see the online-only Data Supplement).

Measurement of Cardiac Superoxide
Cardiac O₂⁻ was measured with a dihydroethidium-based high-performance liquid chromatography assay as previously described. Tissue either was kept in plain buffer or was treated with 1 mmol/L of the nonselective NOS inhibitor Nω-nitro-L-arginine methyl ester hydrochloride (t-NNAME; Sigma) or 10 μmol/L of the neuronal NOS (nNOS) inhibitor 7-nitroindazole (7N; AXXORA, San Diego, Calif; see the online-only Data Supplement).

NOS Activity and Expression
NOS activity was determined by measuring the conversion of [¹⁴C]-labeled arginine to citrulline in myocardial homogenates (Calbiochem, San Diego, Calif). nNOS and endothelial NOS monomers were assayed with cold SDS-PAGE Western blot analysis under reducing conditions (see the online-only Data Supplement).

Phospholamban Expression
Frozen samples were homogenized in protease and phosphatase inhibitors (Sigma). SDS-PAGE was performed with a 15% acrylamide gel (see the online-only Data Supplement).

Myocyte Isolation and Cell Shortening
Cardiac ventricular myocytes were isolated and analyzed from the hearts of DOCA-salt mice 11 to 14 days postoperatively and of age-matched controls with a previously described protocol (see the online-only Data Supplement).

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BP indicates blood pressure; HR, heart rate; Sm, systolic septal mitral annulus velocity measure by tissue Doppler imaging; FS, fractional shortening; LVEF, LV ejection fraction; LVPWd, LV posterior wall thickness; LVEDd, LV end-diastolic dimension.

Histology
Heart tissue was stained with hematoxylin and eosin or Masson trichrome to determine myocyte cross-sectional diameter and interstitial fibrosis (see the online-only Data Supplement).

Statistical Analysis
Data are presented as mean±SE unless otherwise specified. Comparisons between 2 groups were done with a 2-tailed Student t test. Multiple groups were compared by use of a 1-way ANOVA and a posthoc test (Student-Newman-Keuls or Bonferroni).

The authors had full access to and take full responsibility for the integrity of the data. All of the authors have read and agree to the manuscript as written.

Results
Hypertensive Mice Show Diastolic Dysfunction
Tail-cuff blood pressure measurements and echocardiograms were obtained on postoperative days 11 to 13 in DOCA-salt mice. Mean systolic blood pressure and heart rate were mildly elevated in conscious, acclimated hypertensive mice compared with age-matched controls (Table).

Hypertensive mice had echocardiographic evidence of diastolic dysfunction (Figure 1). They had significant reduction in tissue mitral annulus early longitudinal (E’) velocities, ratio of tissue early to atria (E’/A’) velocities, and left ventricular (LV) inflow propagation velocity (Vp) compared with controls. The ratio of early diastolic filling velocity to the early diastolic mitral annulus velocity (E/E’).
has been reported to have the highest correlation with invasive hemodynamic measures of diastolic dysfunction.\(^{23}\) Hypertensive mice had a higher E/E\(^{′}\) compared with controls, consistent with impaired relaxation. The mitral inflow velocities, E and A, were similar between groups. The normal E/A ratio in conjunction with abnormal tissue Doppler imaging and color M-mode measurements implied that this value was "pseudonormal," a pattern associated with advanced diastolic dysfunction.\(^{24}\) The changes in relaxation parameters occurred in the absence of valvular regurgitation, LV wall motion abnormalities, or hypertrophy. Systolic function, including LV ejection fraction (percent), fractional shortening (percent), and septal annulus systolic velocity, was statistically indistinguishable between groups.

Invasive hemodynamic evaluation confirmed the echocardiographic findings (Figure 2). As expected, LV end-systolic pressure and LV end-diastolic pressure were mildly elevated in hypertensive mice compared with controls (108±3 versus 95±2 mm Hg, \(P=0.002\); 7.2±0.7 versus 4.5±0.4 mm Hg, \(P=0.004\); Figures 2A through 2C), respectively. Compared with controls, hypertensive mice had prolonged time constants for isovolumetric relaxation calculated by 2 standard methods (\(\tau_{\text{Weiss}}\), 10.3±0.08 versus 8.1±0.03 ms, \(P=0.02\); \(\tau_{\text{Glanz}}\), 5.9±0.02 versus 4.9±0.02 ms, \(P=0.03\); Figure 2D).\(^{25}\) The best fit for the end-diastolic pressure-volume relation (EDPVR) was described by the following linear function: 

\[
\text{Pressure}_{\text{end diastole}} = \text{EDPVR} \times \text{volume}_{\text{end diastole}} + \text{intercept}
\]

both groups combined: median \(r=0.99\); range, 0.91 to 0.99; Figures 2E and 2F). Hypertensive DOCA-salt mice had a steeper EDPVR compared with controls (1.3±0.1 versus 0.67±0.1 mm Hg/\(\mu\)L; \(P=0.0004\); Figure 2E).

Diastolic dysfunction did not appear to be the result of changes in myocardial systolic contractile properties. LV systolic function was preserved in hypertensive mice compared with controls as indicated by multiple invasive indexes, including the slope of the end-systolic pressure-volume relation (6.7±0.6 versus 5.3±0.5 mm Hg/\(\mu\)L; \(P=NS\); Figure 2G) and its volume axis intercept (−2.6±1.3 versus −1.6±2.1 mm Hg/\(\mu\)L; \(P=NS\)), LV ejection fraction (52±2.0% versus 45±1.1%; \(P=NS\)), stroke volume (16.0±0.4 versus 14.4±0.4 \(\mu\)L; \(P=0.009\)), and peak rate of pressure rise (dP/dtmax: 10 690±459 versus 11 680±470 mm Hg/s; \(P=NS\)). Body weight was similar between the 2 groups (23.1±0.2 versus 23.3±0.2 g; \(P=NS\)). These changes were unexplained by differences in heart rate (553±17 versus 547±10 bpm; \(P=NS\); Figure 2H), and arterial elastance, a measure of vascular stiffness calculated by dividing the end-systolic pressure by stroke volume, was similar between hypertensive mice and controls (6.64±0.2 versus 6.67±0.3 mm Hg/\(\mu\)L; \(P=NS\); Figure 2I).\(^{26}\)

At a cellular level, LV tissue from mildly hypertensive mice did not show an increase in collagen staining with Masson trichrome (Figure II of the online-only Data Supplement). Myocytes from DOCA-salt mice had similar cell diameters and length measurements compared with control cells (\(P=NS\); Table). Fractional shortening percent in isolated myocytes was similar between groups (\(P=NS\); Figure III of the online-only Data Supplement).

In the DOCA-salt model, the changes in diastolic properties were dependent on the presence of mild hypertension. Mice implanted with a DOCA pellet in the absence of unilateral nephrectomy and salt supplementation had similar mean systolic blood pressure compared with controls (103.3±3 versus 99.5±3 mm Hg; \(P=NS\)). There were no significant differences in ventricular relaxation in these mice compared with controls (\(E’, 5.0±0.6 versus 5.6±0.1\) cm/s; \(V_p, 47.3±3.3 versus 50.8±1.9\) cm/s; and \(E/E’; 18.2±1.9 versus 16.1±0.8; n=5\) per group).

**Diastolic Dysfunction Is Associated With Cardiac Oxidation**

LV tissue obtained from hypertensive mice on postoperative day 14 showed a 2-fold increase in \(O_2^-\) as measured by 2-oxyethidium (2HO-ET) levels (0.09±0.01 versus 0.18±0.02 \(\mu\)mol·L\(^{-1}·mg^{-1}; P=0.02\)). Administering BH4 to hypertensive mice on postoperative days 1 to 14 significantly blunted the increase in cardiac 2HO-ET compared with that observed in the untreated DOCA-salt group (0.12±0.01 versus 0.18±0.02 \(\mu\)mol·L\(^{-1}·mg^{-1}; P=0.02\); Figures 3A and 3B).

**\(O_2^-\) Production in Diastolic Dysfunction Could Be Suppressed by NOS Inhibitors**

Hypertension is known to cause increased oxidative stress in the vasculature, in part as a result of \(O_2^-\) production from uncoupled NOS.\(^{27}\) Both endothelial NOS and nNOS have been reported in heart.\(^{10}\) To examine whether uncoupled NOS en-
zymes were contributing to cardiac $O_2^-\text{ production in hypertensive mice, LV tissue was incubated with the nonselective NOS inhibitor, l-NAME, or the selective nNOS inhibitor, 7N.}^{18}$ The percent reduction in $O_2^-\text{ production between control and hypertensive animals was } 65\pm22\%\text{ for l-NAME–treated tissue and } 100\pm34\%\text{ for 7N-treated tissue (}P=0.004\text{).} E, \text{ The EDPVR slope is steeper in hypertensive mice vs controls (}P=0.0004\text{).} F, \text{ Pearson correlation coefficients for linear fitting of the EDPVR. G, LV contractility assessed by the end-systolic pressure-volume relation (ESPVR) slope (}P=NS\text{) and the volume axis intercept (Vo; }P=NS\text{) are similar between the DOCA and control groups. H, Mean heart rate between groups (}P=NS\text{). I, Arterial elastance (Ea), a measure of vascular stiffness, is similar between hypertensive and control mice (}P=NS\text{).}

Cardiac Oxidation Was Associated With Less Cardiac BH$_4$ and Reduced NO

BH$_4$ is an essential cofactor for NOS.$^{28}$ When BH$_4$ is oxidized, NOS becomes uncoupled.$^{4,10}$ Cardiac bioperins were quantified in LV tissue obtained on postoperative day 14. Consistent with the increase in $O_2^-\text{ and evidence of NOS uncoupling, oxidized pterins were increased in hypertensive mice compared with controls (0.97±0.1 versus 0.46±0.3 pmol/mg proteine; }P=0.04\text{). Total bioperins (1.8±0.2 versus 1.9±0.3 pmol/mg protein) and reduced BH$_4$ (1.4±0.2 versus 0.94±0.2 pmol/mg protein) were unchanged between groups (}P=NS\text{). Therefore, the ratio of reduced BH$_4$ to oxidized pterins, which reflects the amount of BH$_4$ available for NOS, was significantly lower in hypertensive mice compared with controls (1.2±0.4 versus 5.0±1.2; }P=0.02\text{). Feeding BH$_4$ to DOCA-salt mice (n=3; days 1 to 14) increased cardiac BH$_4$ and total bioperins (P<0.01 versus DOCA-salt and control mice), demonstrating that oral administration could deliver reduced BH$_4$ to the myocardium. A 3.3-fold increase in the ratio of reduced BH$_4$ to oxidized pterins was seen in BH$_4$-fed DOCA-salt animals compared with hypertensive mice without BH$_4$ feeding (P=NS; Figure 3D). NO production by NOS was reduced to similar degrees in DOCA-salt and in l-NAME–treated control hearts compared with untreated controls (1.1±0.02 versus 1.0±0.03 versus 1.3±0.03 counts per minute, respectively; }P=0.0005\text{; Figure 3E) without changes
in NOS isoform levels (P=NS; Figure IV of the online-only Data Supplement).

**BH₄ Prevented or Reversed Diastolic Dysfunction in Hypertensive Mice**

We examined whether BH₄ could prevent diastolic function in mice treated with DOCA-salt. BH₄ administration prevented the hypertension associated with DOCA-salt treatment (Figure 4A). Assessment of diastolic function by echocardiography showed preserved LV relaxation with BH₄ on the basis of all indexes (Figures 4B through 4D). BH₄ prevented the increase in LV end-diastolic pressure seen with hypertensive DOCA-salt mice (6.3±0.9 mm Hg; P=NS versus controls). The slope of the EDPVR was significantly lower in

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**Figure 3.** Hypertensive mice show cardiac oxidation and reduced BH₄. A, Typical high-performance liquid chromatography spectra obtained from a control (top), DOCA (middle), BH₄-treated DOCA mice (bottom) show a larger 2HO-ET peak in the DOCA animal. B, Mean 2HO-ET levels are significantly higher in DOCA mice vs control and BH₄-treated mice (days 1 to 14). C, The percent O₂ reduction between control and DOCA LV tissue treated with the nonselective NOS inhibitor, L-NAME, and the selective neuronal NOS inhibitor, 7N. D, BH₄ and oxidized biopterins are quantified with the differential oxidation method and high-performance liquid chromatography. The ratio of reduced BH₄ to oxidized pterins in the heart is lower in DOCA mice vs controls. Feeding BH₄ to hypertensive mice (days 1 to 14) increases the cardiac ratio of reduced BH₄ to oxidized pterins. E, Total NOS activity is reduced to a similar degree in DOCA and L-NAME–treated control hearts.
BH₄ prevention mice compared with DOCA-salt mice and was statistically indistinguishable from controls (Figures 4E and 4F). Measures of systolic function, including the end-systolic pressure-volume relation and its volume axis intercept, were statistically equivalent between groups, and arterial elastance was unchanged, suggesting that neither arterial nor systolic function alterations explained the prevention of diastolic dysfunction seen with BH₄ (Table I of the online-only Data Supplement). Moreover, BH₄ did not affect heart rate. The mean heart rate was 550 to 576 bpm during noninvasive and invasive studies in all of the groups (P=NS).

BH₄ could also reverse diastolic dysfunction once established. Hypertensive DOCA-salt mice were treated on postoperative day 11, a time when all of the hypertensive mice had evidence of diastolic dysfunction, with a 12- to 14-day course of BH₄. Mean systolic blood pressure (96±2 and

Figure 4. BH₄ prevents or reverses diastolic dysfunction. A, DOCA mice treated with BH₄ or hydralazine (days 12 to 24) show a reduction in systolic blood pressure (SBP) to levels observed in control animals. Treatment with H₄N (days 12 to 24), an enzymatically inactive analog of BH₄ with equivalent antioxidant properties, has no effect on blood pressure in hypertensive DOCA mice. B through D, Assessment of diastolic function with echocardiography; E', Vp, and E'/E' ratio show that BH₄ but not H₄N or hydralazine prevents or reverses diastolic dysfunction in hypertensive DOCA mice. E, Sample pressure-volume loops obtained during inferior vena cava occlusion demonstrate the EDPVR and end-systolic pressure-volume relation in the control, DOCA, and BH₄-treated mice. F, The mean EDPVR is steeper in DOCA mice vs control, BH₄ prevention, and BH₄ treatment groups. G, Isolated myocytes from DOCA mice have a prolonged r vs control animals. The addition of exogenous BH₄ to isolated DOCA myocytes normalizes relaxation kinetics. H, DOCA mice have a reduced ratio of phosphorylated to total phospholamban (PLB) vs the control, BH₄ prevention, and BH₄ treatment groups.
99±3 mm Hg in controls; P=NS) and the invasively measured LV end-systolic pressure were normalized in BH4-treated mice. LV end-diasstolic pressure did not increase compared with controls (6.2±0.9 mm Hg; P=NS). Echocardiographic indexes of diastolic function in the BH4 treatment group mirrored those observed in the BH4 prevention group and controls (Figures 4B through 4D). The EDVPR was significantly improved in the BH4-treated mice compared with the DOCA mice (Figures 4E and 4F). Systolic function, arterial elastance, and heart rate were similar in BH4-treated mice compared with all other groups (Table I of the online-only Data Supplement). The lack of improvement in blood pressure or lusitropy after treatment of DOCA-salt mice with H2N, a free radical scavenger without NOS catalytic activity, implied that the effects were specific to BH4 (Figures 4B through 4D).

Because lower blood pressures were observed in BH4-treated mice, it was unclear whether the improvements observed in diastolic function were a result of reduced blood pressure or an improvement in NOS coupling. Hydralazine, an antihypertensive agent that lowers blood pressure without recoupling NOS, was used to evaluate these possibilities.29 Mice treated with hydralazine had a reduction in blood pressure similar to that observed with BH4. Nevertheless, diastolic dysfunction did not improve in hydralazine-treated mice, suggesting that the effect of BH4 on DOCA-salt mice was independent of blood pressure lowering (Figures 4A through 4D).

A Possible Mechanism for the BH4 Effect
To determine whether abnormalities in diastolic function or the beneficial lusitropic effects seen with BH4 in hypertensive mice were mediated at the myocyte level, cell shortening was measured in isolated myocytes. Cell length and fractional shortening were similar between groups (P=NS; Table, and Figure III of the online-only Data Supplement), but the relaxation constant (τ) was prolonged in myocytes from hypertensive hearts compared with controls (137.3±8.5 versus 79.3±4.9 ms; P=0.0001; Figure 4G). Incubating myocytes from hypertensive mice with 10 μmol/L BH4 for 15 minutes normalized τ (86.0±7.6 ms; P=NS versus control and P<0.0001 versus DOCA; Figure 4H). The addition of BH4 to control myocytes had no effect on τ or fractional shortening (P=NS; Figure 4G, and Figure III of the online-only Data Supplement).

Recently, nNOS-mediated oxidation changes in the phosphorylation state of phospholamban, a key protein controlling sarcoplasmic reticulum Ca2+ pump activity and cytosolic Ca2+ levels, have been described.30,31 The ratio of phosphorylated phospholamban to total phospholamban was quantified in DOCA-salt, control, BH4 prevention, and BH4 treatment groups (Figure 4H). DOCA-salt mice had a marked reduction in the ratio of phosphorylated phospholamban to total phospholamban compared with all of the other groups (0.09±0.04 versus 0.76±0.2 versus 1.2±0.1 versus 1.0±0.2, respectively; P=0.003). Feeding BH4 to control mice had no effect on the ratio of phosphorylated phospholamban to total phospholamban (Figure VII of the online-only Data Supplement).

Cardiac Oxidation and Diastolic Dysfunction in ACE 1/8 Mice
We investigated the role of cardiac oxidation in the pathogenesis of diastolic dysfunction using ACE 1/8 mice. Because these mice express ACE only in the heart, their blood pressure is unchanged from controls. Moreover, these mice have normal systolic function.11 Because cardiac angiotensin II levels are increased in ACE 1/8 mice and angiotensin II causes increased oxidative stress, we evaluated whether cardiac oxidation in the absence of hypertension resulted in diastolic dysfunction.32,33 Ten-week-old ACE 1/8 mice showed multiple indexes of diastolic dysfunction compared with controls (Figures 5A through 5C). O2− levels were higher in ACE 1/8 mice compared with control littersmates (0.24±0.03 versus 0.14±0.3 μmol·L−1·mg−1 tissue; P=0.04; Figure 5D). O2− production was suppressed by 60±17% in ACE 1/8 mice by L-NAME. NOS inhibition had no effect on O2− production in control littersmates, suggesting that uncoupled NOS was involved in the cardiac oxidation seen in ACE 1/8 mice (Figure 5E).

Discussion
Heart failure with preserved systolic function is growing in prevalence and has no specific therapies.1,2 One of the most common associated risk factors for diastolic dysfunction is hypertension.5,34 Here, we showed that mild hypertension resulted in diastolic dysfunction. This relaxation defect was associated with cardiac oxidation and reduced NOS NO production. NOS inhibitors suggested that the oxidative stress was in large part the result of uncoupled NOS. The increase in oxidized cardiac pterins and the concomitant decrease in reduced cardiac BH4 further suggested uncoupled NOS. Prevention or reversal of diastolic dysfunction by BH4, but not HN4, treatment implied that NOS uncoupling played a pathogenic role in the diastolic changes observed. The impaired relaxation observed in isolated myocytes from hypertensive animals, coupled with the beneficial lusitropic effect observed by short-term treatment of these cells with BH4, supports the role of myocyte-specific uncoupled NOS as a mediator of diastolic dysfunction. Therefore, it appears that hypertension can cause diastolic dysfunction by oxidizing cardiac BH4, leading to myocyte-uncoupled NOS.

Our results are consistent with those of Takimoto et al10 and Moens et al.35 These investigators showed that transaortic constriction with resultant systolic dysfunction was associated with cardiac BH4 depletion and NOS uncoupling. Exogenous BH4 treatment halted the progression of ventricular remodeling, reversed fibrosis, and improved calcium handling in this model. In our case, the lusitropic role for NOS was suggested in the absence of systolic dysfunction, marked ventricular remodeling, or increased arterial elastance.

Inhibitor experiments showed that nNOS was the largest contributor to cardiac O2− production in diastolic dysfunction. The endothelial NOS and nNOS isoforms are known to be present in heart, and BH4 depletion appears to result in uncoupling of either form.7,10,36,37 A growing body of work points to nNOS as an important modulator of cardiac nitrooxidredox balance and function.38–41 For example, Zhang and coworkers31 have shown that nNOS modulates cardiac relax-
Our data suggest that reduced NO leads to phospholamban changes that are consistent with increased cytosolic Ca\(^{2+}\) and diastolic dysfunction. The beneficial effects of BH\(_4\) correlate with the phosphorylation state of phospholamban, consistent with work demonstrating that NO is a mediator of cardiac lusitropy and the finding that Ca\(^{2+}\) handling and the phosphorylation state of phospholamban are subject to oxidative regulation.\(^7\)\(^,\)\(^30\)\(^,\)\(^31\)\(^,\)\(^35\)\(^,\)\(^42\) Other possible targets of oxidative modification that were not examined in the present study include the sarcoplasmic reticulum Ca\(^{2+}\) release channel, sarcoplasmic reticulum Ca\(^{2+}\) pump, or the sarcosomal L-type Ca\(^{2+}\) channel. The troponin T–I79N mutation leads to diastolic dysfunction as a result of enhanced calcium myofilament sensitivity,\(^43\) suggesting that oxidative stress–dependent changes in myofilament Ca\(^{2+}\) sensitivity may be another possible mechanism.

Previously, it has been difficult to resolve the contributions of changes in the vasculature versus changes in the myocardium in the pathogenesis of diastolic dysfunction.\(^44\) We attempted to address this quandary with isolated myocyte experiments and gene-targeted animals. The abnormal relaxation pattern observed in cardiomyocytes isolated from hypertensive mice, combined with the ability of BH\(_4\) to normalize relaxation in these cells, supports the concept that diastolic dysfunction in this model is in large measure myocyte dependent. The ability of the renin-angiotensin system to lead to vascular oxidation and uncoupling of NOS is well documented.\(^6\) Using a gene-targeted mouse that overexpressed ACE in the heart while simultaneously eliminating ACE elsewhere, we created a mouse that has increased cardiac oxidation and diastolic dysfunction in the absence of hypertension, suggesting that cardiac oxidation is sufficient to cause diastolic dysfunction. This notion is further supported by the lack of change in vascular elastance in the mildly hypertensive mice with diastolic dysfunction.

Because there is debate about the proper methodology to assess diastolic dysfunction, we chose to use both noninvasive and invasive means.\(^25\)\(^,\)\(^45\) Diastolic function was assessed with the combination of 3 echocardiographic modalities.\(^46\) Two of these modalities, tissue Doppler imaging and color M-mode assessment of Vp, are relatively independent of cardiac loading conditions.\(^21\)\(^,\)\(^47\) Moreover, the inversely derived EDPVR, generally thought to be the standard for lusitropic assessment, was used.\(^23\) The strong correlation observed between E′, Vp, and E/E′ and the inversely derived EDPVR suggests that these techniques assess similar myocardial properties. Measures indicated perturbations in active and passive stiffness. The abnormal phosphorylation state of phospholamban observed in hypertensive mice would be expected to affect active relaxation predominantly. Be-
cause increased collagen deposition and hypertrophy were not observed, the increased passive stiffness may have resulted from enhanced myofilament Ca\(^{2+}\) sensitivity, changes in titin isoform expression, or changes in the composition of collagen.

Oral BH\(_4\), but not HN\(_4\), was able to raise cardiac BH\(_4\) levels and prevent or reverse the diastolic parameter changes seen in hypertensive mice. This suggests that the BH\(_4\) effect was not the result of nonspecific radical scavenging. Nevertheless, BH\(_4\) lowered blood pressure, in accordance with previously published data from human and animal studies, which may prevent or ameliorate diastolic dysfunction.\(^{29,48}\) Experiments with hydralazine, a direct vasodilator that does not recouple NOS, suggest otherwise.\(^{29}\) In this case, hydralazine lowered blood pressure as much as BH\(_4\) but did not prevent diastolic dysfunction.

**Conclusions**

This study demonstrates that cardiac oxidation can lead to BH\(_4\) depletion, NOS uncoupling, and diastolic dysfunction. This dysfunction can be ameliorated by BH\(_4\) treatment. Diastolic dysfunction can occur in the absence of changes in the vasculature. Finally, these results suggest that BH\(_4\) may be of value in the treatment of diastolic dysfunction.

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**Disclosures**

Drs Dudley, Fan, and Harrison hold patent 60/840,368, Methods and Compositions for Treating Diastolic Dysfunction. The other authors report no conflicts.

**References**


**CLINICAL PERSPECTIVE**

Heart failure with preserved ejection fraction as a result of diastolic dysfunction accounts for significant morbidity, mortality, and healthcare expenditures. Although many proven therapies exist for heart failure patients with reduced ejection fraction, specific treatments for diastolic dysfunction are lacking. In the vasculature, hypertension and activation of the renin-angiotensin system lead to reduced vascular nitric oxide (NO), in part because NO synthase (NOS) becomes uncoupled. This dysfunctional state of NOS is characterized by oxidative depletion of its cofactor tetrahydrobiopterin (BH₄), which leads to production of superoxide instead of NO. NO and NOS are also modulators of cardiac relaxation. We hypothesized that hypertension or activation of the renin-angiotensin system leads to cardiac NOS uncoupling and diastolic dysfunction. Using a mouse model of mild hypertension to mimic the most common risk factor for diastolic dysfunction in humans, we showed that isolated impaired cardiac relaxation is associated with uncoupled NOS. Local cardiac activation of the renin-angiotensin system demonstrated that cardiac oxidation was sufficient to cause diastolic dysfunction without hypertension. Diastolic dysfunction could be treated or prevented by oral administration of BH₄, which recoupled cardiac NOS. These results suggest that BH₄ may be of value in the treatment of diastolic dysfunction.
Uncoupled Cardiac Nitric Oxide Synthase Mediates Diastolic Dysfunction

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Methods:

_Hypertensive mouse model:_ We used a mouse model of mild hypertension to mimic the most common human risk factor for diastolic dysfunction.\(^1-3\) Previously, we have shown that this model leads to vascular oxidative stress, BH\(_4\) depletion, and NOS uncoupling\(^4\). Mild elevation in blood pressure was induced in seven to eight week old, male, C57BL/6 mice (22-25 g; Charles River, Wilmington, MA) by unilateral nephrectomy, subcutaneous implantation of a controlled release deoxycorticosterone acetate (DOCA) pellet (0.7 mg/d; Innovative Research of America, Sarasota, FL), and substituting drinking water with 1% saline.\(^4\) Control animals underwent a sham operation, had placebo pellet implantation, and received water without salt.

A subset of hypertensive mice were randomly selected on post-operative day one to receive 5 mg/day of either BH\(_4\) (AXXORA, San Diego, CA) or tetrahydroneopterin (H\(_4\)N), an enzymatically inactive analog of BH\(_4\) with equivalent antioxidant properties (Schricks Laboratories, Jona, Switzerland).\(^5,6\) BH\(_4\) and H\(_4\)N were pressed into pellets of standard rodent chow (Purina Laboratory Rodent Diet 5001).\(^4,7\) Pellets containing pterins were stored at -20°C until the day of use. Under these conditions, we and others have shown that the pterins retain biological activity.\(^4,6\)

A cohort of hypertensive DOCA-salt mice was randomized on post-operative day 11, a time when all DOCA-salt mice have evidence of diastolic dysfunction, to either 12-14 d of BH\(_4\) feeding as described above or treatment with hydralazine (25-30 mg/kg/d in 1% saline; Sigma-Aldrich, St. Louis, MO).

Blood pressure and heart rate were measured in acclimated conscious mice by tail-cuff plethysmography (Columbus Instruments, Columbus, Ohio). As described
below, echocardiography was performed on a subset of mice 24-48 h prior to terminal procedures. Invasive hemodynamic studies and/or tissue harvest for biochemical analysis were done on postoperative day 14 for hypertensive DOCA-salt, BH₄ prevention, H₄N, and control mice and on postoperative days 22-24 for mice in BH₄ or hydralazine treatment groups.

**Angiotensin converting enzyme (ACE) 1/8 mouse model:** Male compound heterozygous ACE 1/8 mice and age matched littermate controls were used to test if cardiac oxidation could lead to diastolic dysfunction. Briefly, selective cardiac overexpression of the ACE gene was achieved by using gene-targeted substitution of the somatic ACE promoter with the αMHC promoter in a single allele and ablation of the other ACE allele. These ACE 1/8 mice have increased cardiac ACE, increased cardiac angiotensin II, minimal extra-cardiac ACE expression, no hypertension, and preserved ejection fraction. Echocardiography and tissue harvest for biochemical analysis was performed at ten weeks of age.

**Noninvasive assessment of diastolic dysfunction:** Mice were anesthetized with 1% isoflurane via nose cone at 0.5 L/min. Electrocardiogram (ECG), respiration, and rectal temperature were continuously monitored with an integrated physiology platform. Temperature was kept at 37°C with a heated platform and ceramic heating lamp. The Vevo 770 (VisualSonics, Toronto, Canada) or a SONOS 5500 (Philips Medical Systems, Bothell, WA,) ultrasound units were used. M-mode images in the parasternal long axis and the left ventricle (LV) short-axis views at the mid-papillary level were used for the measurement of LV end systolic dimensions (LVESd), LV end diastolic dimensions (LVEDd), and LV posterior wall thickness (LVPWd). Measurements were
averaged from three consecutive beats during expiration. Percent fractional shortening (%FS) was calculated as \(100 \times \frac{(LVEDd) - (LVESd)}{(LVEDd)}\) and percent LV ejection fraction (%EF) was calculated as \(100 \times \frac{\left[(\frac{7}{2.4}+LVEDd)xLEDd^3\right] - \left[(\frac{7}{2.4}+LVESd)xLVESd^3\right]}{\left[(\frac{7}{2.4}+LVEDd)xLEDd^3\right]}\). LV inflow velocities (E and A wave velocities) were interrogated by conventional pulsed-wave Doppler from the apical four-chamber view with the sample volume placed at the tip of the mitral valve leaflets. LV inflow propagation velocity (Vp) was measured off-line as the slope of the first aliasing line from the mitral valve tips to the LV apical region\(^9,10\). The mitral annulus longitudinal velocities (Sm, E', and A') were determined by pulsed-wave tissue Doppler from the apical four-chamber view with the sample volume placed at the septal side of the mitral annulus\(^11\). Interpretation was done by a single investigator blinded to the treatment groups. Results were comparable between machines and correlated with invasive measures.

**Invasive assessment of diastolic dysfunction:** The Mikro-Tip® (1.4 Fr) pressure-volume (PV) catheter and the MPVS-300 system (Millar Instruments, Houston, TX, USA) were used to assess in vivo LV function. Mice were anesthetized with 1-2% isoflurane and ventilated via tracheostomy (Inspira ASVP, Harvard Apparatus, Boston, MA). A weight based algorithm for selection of tidal volume (6-7 µL/g) and respiratory rate (134-148 breaths/min) was used to ensure a minute ventilation rate of 1.0-1.1 mL/min/g with an FIO\(_2\) of 1.0. An initial 150 µL of bovine albumin 12.5% (Sigma-Aldrich, St. Louis, MO) was given over five min via a left internal jugular catheter and was followed by infusion of 5 µL/min for the duration of the study. A single dose of pancuronium (0.12 mg/kg IV) was given. Body temperature was maintained at 37°C.
using a rectal thermometer probe and a DC temperature control module (FHC, New Brunswick, ME). The PV catheter was inserted into the right common carotid artery and advanced through the aortic valve into the LV. Inferior vena cava occlusion was performed via a midline abdominal incision. Volume and parallel conductance calibration were performed as previously described\(^{12}\). The time constant for isovolumic relaxation was calculated by regression of the log(pressure) versus time method (\(\tau_{\text{Weiss}}\)) and by the regression of dP/dt versus pressure method (\(\tau_{\text{Glantz}}\)). Arterial elastance was calculated by dividing end-systolic pressure by stroke volume.

**NOS expression:** Neuronal NOS (nNOS) and endothelial NOS (eNOS) monomers were assayed using cold SDS-PAGE western blot analysis under reducing conditions. Samples were boiled for 5 min and loaded on 7% SDS polyacrylamide gel and run for 3 h, then transferred to nitrocellulose membranes (Amersham Biosciences) and blocked for 1 h at room temperature. Immunoblotting was performed with nNOS monoclonal antibody (1:500; Cell Signaling, Danvers, MA) and eNOS (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). GAPDH (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) was used to control for protein loading. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce, Rockford, IL). Protein levels were assessed by densitometric analysis using the ImageJ software (NIH).

**Phospholamban (PLB) expression:** Frozen samples were homogenized in protease and phosphatase inhibitors (Sigma, St. Louis, MO). SDS-PAGE was performed using a 15% acrylamide gel. Proteins were transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and incubated with anti-PLB (1:2000; Millipore, Temecula, CA), anti-phospho-PLB Ser16 (1:5000; Millipore, Temecula, CA),
and anti-GAPDH (1:5000; Santa Cruz Biotechnologies, Santa Cruz, CA). Following incubation membranes were washed and incubated with horseradish peroxidase-coupled secondary antibodies (1:5000 anti-phospho-PLB and 1:10,000 anti-PLB, Amersham, Piscataway, NJ) followed by ECL plus detection (Pierce, Rockford, IL). Protein levels were assessed by densitometric analysis and normalized to GAPDH expression to control for protein loading.

Measurement of cardiac superoxide: Cardiac \( \text{O}_2^\cdot \) was measured using a dihydroethidium based HPLC assay as previously described.\(^{13}\) Mice were anesthetized with isoflurane (4-5%) and hearts were rapidly excised and placed in a modified and chilled Krebs-HEPES buffer (composition in mM: 99.01 NaCl, 4.69 KCl, 2.50 CaCl\(_2\), 1.20, MgSO\(_4\), 25 NaHCO\(_3\), 1.03 K\(_2\)HPO\(_4\), 20 Na-HEPES, and 5.6 D-glucose, pH 7.40 at 6°C). A punch biopsy tool (0.2 mm OD; API, Indianapolis, IN) was used to obtain three to five sections (8-10 mg each) from the anterior, lateral, and posterior walls of the LV. Tissue was weighed and examined to confirm full thickness sections with intact epicardial and endocardial surfaces were used for analysis. A standard 24-well plate containing 1 mL of chilled Krebs-HEPES/well was placed on ice. Individual tissue samples were placed in each well and washed three times with 1 mL of chilled buffer. Tissue was either kept in plain buffer, treated with 1 mM of the non-selective NOS inhibitor \( \text{N}_n\text{-Nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma-Aldrich, St. Louis, MO) or 10 µM of the neuronal NOS (nNOS) inhibitor 7-nitroinidazole (7N) (AXXORA, San Diego, CA).}^{14} \) Plates were placed at 4°C for 10 min. Dihydroethidium (50 µM; Molecular Probes, Eugene, OR) was added to each well in low light conditions. Plates were incubated in the dark for 30 min at 37°C. Tissue and 100 µL of media were
removed and placed into separate vials containing 300 µL of chilled methanol (Sigma Aldrich, St Louis, MO) and stored at -80°C. Immediately prior to HPLC analysis, tissue was homogenized and all samples were filtered (0.22 µm). Separation of oxyethidium (2HO-ET), ethidium, and dihydroethidium was done using HPLC with a C-18 reverse phase column (Nucleosil 250, 4.5mm; Sigma-Aldrich, St. Louis, MO). 13 2HO-ET, the specific product of the reaction between O2•− and dihydroethidium, was measured and used to determine cardiac O2•− content, expressed as µM/mg tissue.

**Cardiac biopterin content:** Cardiac biopterins were measured as previously reported. 4,15-17 Hearts were rapidly excised and stored at -80°C. High performance liquid chromatography (HPLC) analysis (System GOLD, Beckman Coulter, Fullerton, CA) using a differential oxidation method described previously was used to measure BH4, BH2, and biopterin in homogenized heart samples.

**Myocyte isolation and cell shortening:** As we have previously reported, Cardiac ventricular myocytes were isolated and analyzed from the hearts of DOCA-salt mice 11-14 days post-operatively and from age matched controls using a modified enzymatic digestion protocol (Alliance for Cellular Signaling). 18,19 Mice were anesthetized by intraperitoneal injection of ketamine, and the hearts were rapidly excised, cannulated with a 22-guage gavage feeding needle, connected to a Langendorff perfusion apparatus, and perfused for 7 min at 3 mL/min with a perfusion buffer containing (in mM): 113 NaCl, 12 NaHCO3, 4.7 KCl, 0.6 Na2HPO4, 0.032 phenol red, 10 KHCO3, 10 HEPES, 30 taurine, 0.6 KH2PO4, 1.2 MgSO4 ·7H2O, 5.5 glucose, and 10 butanedione monoxime (BDM), pH 7.4 at 37°C. After 7 min, a digestion buffer consisting of the perfusion buffer with the addition of 12.5 µM CaCl2, 0.14 mg/mL trypsin (Invitrogen,
Carlsbad, CA), and 0.1mg/mL Liberase Blendzyme 4 (Roche Diagnostics, Indianapolis, IN) was used to perfuse hearts for 5 min at a rate of 3 mL/min. Then, the heart was cut from the cannula below the atria and placed in a dish containing 2.5 mL of digestion buffer. The tissue was gently titrated into small pieces with fine forceps and gently dissociated. Then, 7.5 mL of myocyte stopping buffer (perfusion buffer with 10% bovine calf serum and 12.5 µM CaCl$_2$) was added to the cell suspension to inactivate the digestion buffer. After additional dissociation using a sterile plastic transfer pipette, the solution was filtered through a 70 µM nylon filter and allowed to settle to the bottom of the tube for 10 min. The supernatant was discarded, and the pellet was resuspended in 10 mL of a second myocyte stopping buffer containing 5% bovine calf serum and 50 µM CaCl$_2$. The cells were allowed to settle to the bottom of the tube, and the supernatant was discarded. The pellet was then washed in a modified Krebs buffer with 1mg/mL bovine serum albumin to remove BDM and CaCl$_2$ was increased in a step wise manner in 4 min increments to a final concentration of 1.2 mM CaCl$_2$.

Cell shortening was recorded by illumination of the myocytes with red transmitted light (>600 nm). The cell image was collected through the microscope with a 40x objective lens and transmitted to the multi-image module, where it was separated from the fluorescence signal by a 580-nm dichroic mirror. Output from the camera was split and sent to a chart recorder and to a video-edge detector (Crescent Electronics, Sandy, UT). The cell length was recorded on an acquisition computer for later analysis offline. Cell were perfused in a Tyrode’s solution with 1.5 mM calcium at room temperature and stimulated at 0.5 Hz. Prior to recording, myocytes needed to demonstrate stable contractions with pacing. Baseline measurements were made for 5 min, then the cells
were treated with 10 µM BH₄ for 5 min and recorded for a subsequent 10 min. Clampfit was used to analyze the data. The time constant of relaxation ($\tau$) was calculated by the formula $a_0 + a_1 e^{t/t}$.  

**Histology:** Hearts were arrested in diastole using KCL (10%), excised, pressure perfused, and fixed in 10% formalin. Tissue was embedded in paraffin and stained with hematoxylin and eosin or Masson’s trichrome to determine myocyte cross-sectional diameter and interstitial fibrosis. Myocyte diameter was determined by examining 4 hearts per group, 3 slides per heart, and counting 30 myocytes per slide.
References


(8) Xiao HD, Fuchs S, Bernstein EA, Li P, Campbell DJ, Bernstein KE. Mice expressing ACE only in the heart show that increased cardiac angiotensin II is not associated with cardiac hypertrophy. *Am J Physiol Heart Circ Physiol* 2008;294:H659-H667.


Supplemental Figure 1. Echocardiographic and invasively indices of diastolic dysfunction correlate. Linear regression analysis of echocardiographic data obtained 36-48 hr prior to invasive hemodynamic studies on ten mice (DOCA 0.4mg/day, n=4; DOCA 0.7mg/day, n=3; DOCA + BH₄ treatment, n=1; control, n=3) show Pearson correlation coefficients of 0.71, 0.65, and 0.66 between the E’ (early septal mitral annulus velocity measured with tissue Doppler imaging; Panel a), Vp (left ventricular inflow propagation velocity interrogated with color M-mode Doppler; Panel b) and E/E’ (ratio of early diastolic filling velocity interrogated by conventional Doppler to E’; Panel c) and the invasive end diastolic pressure-volume relationship (EDPVR), respectively.

Supplemental Figure 2. Sample sections of left ventricular tissue (40x) stained with Mason’s trichrome show no evidence of increased collagen staining or hypertrophy in DOCA hearts (right panel) compared to controls (left panel; n=4 hearts per group).

Supplemental Figure 3. Myocytes isolated from DOCA and control animals have similar contractile properties as assessed by percent fractional shortening (p=NS). The addition of BH₄ to isolated myocytes had no appreciable effect on fractional shorting.

Supplemental Figure 4. Sample tracings of isolated myocyte relaxation experiments. Panel a) control; Panel b) control with BH₄; Panel c) DOCA; Panel d) DOCA with BH₄.
**Supplemental Figure 5.** Total eNOS and nNOS protein levels were similar in hypertensive DOCA and control animals (P=NS).

**Supplemental Figure 6.** Sample Western blot comparing total phospholamban (PLB) and phosphorylated-PLB (P-PLB) in control, DOCA, BH₄ treatment, and BH₄ prevention hearts.

**Supplemental Figure 7.** BH₄ fed control animals have a similar phosphorylated-PLB (P-PLB) to total PLB ratio when compared with control animals (0.73 ± 0.08 vs. 0.81 ± 0.03: n=6 per group; P=NS).
Supplemental Figure 1.

(a) $E'$ (cm/s)
- Control
- DOCA 0.4mg/day
- DOCA 0.7mg/day
- DOCA + BH$_4$

(b) $V_p$ (cm/s)

(c) $E/E'$

$p = 0.03$
$r = -0.71$

$p = 0.04$
$r = -0.65$

$p = 0.04$
$r = 0.66$
Supplemental Figure 2.
Supplemental Figure 3.
Supplemental Figure 4.

(a) control
(b) control + BH₄
(c) DOCA
(d) DOCA + BH₄

μm

0 5 10

time (s)
Supplemental Figure 5

![Graph showing mean intensity for eNOS and nNOS in control and DOCA groups with p = NS for both comparisons.](image-url)
Supplemental Figure 6

P-PLB

total-PLB

control  DOCA  BH₄ treatment  BH₄ prevention
Supplemental Figure 7

$p = \text{NS}$

- control (n=6)
- control + BH₄ (n=6)
## Supplemental Table 1.

<table>
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<th>control (n=9)</th>
<th>DOCA (n=12)</th>
<th>BH₄ prevention (n=8)</th>
<th>BH₄ treatment (n=7)</th>
<th>p value</th>
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<td>ESPVR (mmHg/µL)</td>
<td>5.3 ± 0.5</td>
<td>6.7 ± 0.6</td>
<td>6.8 ± 0.8</td>
<td>6.3 ± 0.8</td>
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<tr>
<td>V₀ (mmHg/µL)</td>
<td>-1.6 ± 2.1</td>
<td>-2.6 ± 1.3</td>
<td>-2.9 ± 2.0</td>
<td>-4.4 ± 2.7</td>
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<td>Arterial elastance (mmHg/µL)</td>
<td>6.6 ± 0.2</td>
<td>6.6 ± 0.3</td>
<td>6.8 ± 0.3</td>
<td>7.1 ± 0.4</td>
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