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Uncoupled Cardiac Nitric Oxide Synthase Mediates Diastolic Dysfunction

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Abstract

Background—Heart failure with preserved ejection fraction is one consequence of hypertension and 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 nitric oxide synthase (NOS) becomes uncoupled when oxidative depletion of its co-factor tetrahydrobiopterin (BH₄) occurs. Similar events may occur in the heart leading 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 to sham-operated animals, male mice with unilateral nephrectomy, with subcutaneous implantation of a controlled release deoxycorticosterone acetate (DOCA) 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 biopterins, NOS-dependent superoxide production, reduced NO production, and phosphorylated phospholamban. Feeding hypertensive mice BH₄ (5 mg/day), but not treating with hydralazine or tetrahydrobiopterin, improved cardiac BH₄ stores, phosphorylated phospholamban levels, and diastolic dysfunction. Isolated cardiomyocyte experiments revealed impaired relaxation that was normalized with acute

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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, independent of vascular changes, can lead to uncoupled cardiac NOS and diastolic dysfunction. BH₄ may represent a possible treatment for diastolic dysfunction.

Keywords

diastole; heart failure; nitric oxide synthase

Introduction

Heart failure with preserved ejection fraction as a result of diastolic dysfunction accounts for significant mortality and healthcare expenditures, especially amongst hypertensive individuals.^{1,2} The incidence of this type of heart failure is increasing¹.

There are no specific treatments for diastolic dysfunction, partly because of a relative lack of the mechanistic understanding of this disorder³. There is a strong epidemiological association between hypertension and diastolic dysfunction.^{2,4,5} In the vasculature, hypertension and activation of the renin-angiotensin system (RAS) leads to reduced vascular nitric oxide (NO), in part because nitric oxide synthase (NOS) becomes uncoupled when oxidative depletion of its co-factor tetrahydrobiopterin (BH₄) occurs, leading to production of superoxide (O₂^{•-}) instead of NO.⁶ NO and NOS have been identified recently as having a role in the modulation of cardiac relaxation.⁷ Therefore, we hypothesized that hypertension and or activation of the RAS in the absence of an increase in blood pressure may lead to NOS uncoupling in the heart and to diastolic dysfunction.

Methods

All 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 Supplement).^{2,4,5,8,8} Previously, we have shown that this model leads to vascular oxidative stress, BH₄ depletion, and NOS uncoupling⁶. A subset of hypertensive mice were randomly selected on post-operative day one to receive 5 mg/day of either BH₄ (AXXORA, San Diego, CA) or tetrahydroneopterin (H₄N), an enzymatically inactive analog of BH₄ with equivalent antioxidant properties (Schrieks Laboratories, Jona, Switzerland).^{9,10} 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 BH₄ feeding as described above or treatment with hydralazine (25–30 mg/kg/d in 1% saline; Sigma-Aldrich, St. Louis, MO).

Echocardiography was performed on a subset of mice 24–48 h prior to terminal procedures. These 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 (see Supplement).

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 (see Supplement).¹¹ These mice

have increased cardiac ACE, minimal extra-cardiac ACE expression, and preserved ejection fraction.¹¹

Noninvasive assessment of diastolic dysfunction

Mice were studied by pulsed-wave tissue Doppler from the apical four-chamber view.¹² Results correlated with invasive measures (see Supplement).

Invasive assessment of diastolic dysfunction

(see Supplement).¹³

Cardiac biopterin content

Cardiac biopterins were measured as previously reported (see Supplement).^{6,14–16}

Measurement of cardiac superoxide

Cardiac $O_2^{\bullet-}$ was measured using a dihydroethidium based HPLC assay as previously described.¹⁷ Tissue was either kept in plain buffer, treated with 1 mM of the non-selective NOS inhibitor N_{ω} -Nitro-L-arginine methyl ester hydrochloride (L-NAME; Sigma) or 10 μ M of the neuronal NOS (nNOS) inhibitor 7-nitroimidazole (7N) (AXXORA, San Diego, CA; see Supplement).¹⁸

NOS activity and expression

NOS activity was determined by measuring the conversion of [¹⁴C] labeled arginine to citrulline in myocardial homogenates (Calbiochem, San Diego, CA). Neuronal NOS (nNOS) and endothelial NOS (eNOS) monomers were assayed using cold SDS-PAGE western blot analysis under reducing conditions (see Supplement).

Phospholamban (PLB) expression

Frozen samples were homogenized in protease and phosphatase inhibitors (Sigma). SDS-PAGE was performed using a 15% acrylamide gel (see Supplement).

Myocyte isolation and cell shortening

Cardiac ventricular myocytes were isolated and analyzed from the hearts of DOCA-salt mice 11–14 days post-operatively and of age matched controls using a previously described protocol (see Supplement).^{19,20}

Histology

Heart tissue was stained with hematoxylin and eosin or Masson's trichrome to determine myocyte cross-sectional diameter and interstitial fibrosis (see Supplement).

Statistical Analysis

Data are presented as mean \pm SE unless otherwise specified. Comparison between two groups was done with a two-tailed Student's t-test. Multiple groups were compared with a one-way ANOVA and a post-hoc test (Student–Newman–Keuls or Bonferroni).

The authors had full access to the data and take responsibility for its integrity. All 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 as compared to age matched controls (Table 1).

Hypertensive mice had echocardiographic evidence of diastolic dysfunction^{21,22} (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 ventricle (LV) inflow propagation velocity (V_p) compared to 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 dysfunction²³. Hypertensive mice had a higher E/E' compared to 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 LVEF(%), fractional shortening (FS, %), and septal annulus systolic velocity (S_m) were 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 as compared to controls (108 ± 3 vs. 95 ± 2 mmHg, $p=0.002$; 7.2 ± 0.7 vs. 4.5 ± 0.4 mmHg, $p=0.004$; Figure 2, a–c), respectively. Compared to controls, hypertensive mice had prolonged time constants for isovolumic relaxation calculated by two standard methods, τ_{Weiss} (10.3 ± 0.08 vs. 8.1 ± 0.03 ms, $p = 0.02$) and τ_{Glantz} (5.9 ± 0.02 vs. 4.9 ± 0.02 ms, $p=0.03$; Figure 2, d).²⁵ The best fit for the end-diastolic pressure volume-relation (EDPVR) was described by the linear function $\text{Pressure}_{\text{end diastole}} = \text{EDPVR} * \text{Volume}_{\text{end diastole}} + \text{intercept}$ (median r value 0.99, range 0.91–0.99, for both groups combined; Figure 2, e & f). Hypertensive DOCA-salt mice had a steeper EDPVR compared to controls (1.3 ± 0.1 vs. 0.67 ± 0.1 mmHg/ μL , $p=0.0004$; Figure 2, e).

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 to controls based on multiple invasive indices including: the slope of the end-systolic pressure-volume relation (ESPVR; 6.7 ± 0.6 vs. 5.3 ± 0.5 mmHg/ μL , $p=NS$; Figure 2, f and g) and its volume axis intercept (V_0 ; -2.6 ± 1.3 vs. -1.6 ± 2.1 mmHg/ μL , $p=NS$), LV ejection fraction (52 ± 2.0 vs. $45 \pm 1.1\%$, $p=NS$), stroke volume (16.0 ± 0.4 vs. 14.4 ± 0.4 μL , $p=0.009$), and peak rate of pressure rise (dp/dt_{max} ; 10690 ± 459 vs. 11680 ± 470 mmHg/s, $p=NS$). Body weight was similar between the two groups (23.1 ± 0.2 vs. 23.3 ± 0.2 g, $p=NS$). These changes were unexplained by differences in heart rate (553 ± 17 vs. 547 ± 10 beats/min, $p=NS$; Figure 2, h) and arterial elastance, a measure of vascular stiffness that is calculated by dividing the end-systolic pressure by stroke volume, was similar between hypertensive mice and controls (6.64 ± 0.2 vs. 6.67 ± 0.3 mmHg/ μL , $p=NS$; Figure 2, i).²⁶

At a cellular level, LV tissue from mildly hypertensive mice did not show an increase in collagen staining with Mason's trichrome (Supplemental Figure 2). Myocytes from DOCA-salt mice had similar cell diameters and length measurements in comparison to control cells ($P=NS$; Table 1). Fractional shortening % in isolated myocytes was similar between groups ($P=NS$; Supplemental Figure 3).

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 to controls (103.3 ± 3 vs. 99.5 ± 3 mmHg, $p=NS$). There were no significant differences in ventricular relaxation in these mice compared to controls: E' (5.0 ± 0.6 vs. 5.6 ± 0.1 cm/s), V_p (47.3 ± 3.3 vs. 50.8 ± 1.9 cm/sec), and E/E' (18.2 ± 1.9 vs. 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 two-fold increase in $O_2^{\bullet-}$ as measured by 2-oxyethidium (2HO-ET) levels (0.09 ± 0.01 vs. 0.18 ± 0.02 μ M/mg, $p=0.02$). Administering BH_4 to hypertensive mice on postoperative days 1–14 significantly blunted the increase in cardiac 2HO-ET compared to that observed in the untreated DOCA-salt group (0.12 ± 0.01 vs. 0.18 ± 0.02 μ M/mg, $p=0.02$; Figure 3, a–b).

$O_2^{\bullet-}$ 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 $O_2^{\bullet-}$ production from uncoupled NOS.⁶ Both eNOS and neuronal nNOS have been reported in heart.^{10,27} In order to examine whether uncoupled NOS enzymes were contributing to cardiac $O_2^{\bullet-}$ production in hypertensive mice, LV tissue was incubated with the non-selective NOS inhibitor, L-NAME, or the selective nNOS inhibitor, 7N.¹⁸ The percent reduction in $O_2^{\bullet-}$ production between control and hypertensive animals was $65 \pm 22\%$ for L-NAME- and $100 \pm 34\%$ for 7N-treated tissue ($p=NS$), suggesting that nNOS uncoupling was responsible for most of the increase in $O_2^{\bullet-}$ production in hypertensive mice (Figure 3, c).

Cardiac oxidation was associated with less cardiac BH_4 and reduced NO

BH_4 is an essential cofactor for NOS²⁸. When BH_4 is oxidized, NOS becomes uncoupled.^{6,10} Cardiac bipterins were quantified in LV tissue obtained on postoperative day 14. Consistent with the increase in $O_2^{\bullet-}$ and evidence of NOS uncoupling, oxidized pterins were increased in hypertensive mice compared to controls (0.97 ± 0.1 vs. 0.46 ± 0.3 pmol/mg protein, $p=0.04$). Total bipterins (1.8 ± 0.2 vs. 1.9 ± 0.3 pmol/mg protein) and reduced BH_4 (1.4 ± 0.2 vs. 0.94 ± 0.2 pmol/mg protein) were unchanged between the groups ($p=NS$). 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 to controls (1.2 ± 0.4 vs. 5.0 ± 1.2 , $p=0.02$). Feeding BH_4 to DOCA-salt mice ($n=3$; days 1–14) increased cardiac BH_4 and total bipterins ($p<0.01$ vs. DOCA-salt and controls), 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 as compared to hypertensive mice without BH_4 feeding ($p=NS$; Figure 3, d). NO production by NOS was reduced to similar degrees in DOCA-salt and in L-NAME treated control hearts when compared to untreated controls (1.1 ± 0.02 vs. 1.0 ± 0.03 vs. 1.3 ± 0.03 counts/min respectively, $p=0.0005$; Figure 3, e) without changes in NOS isoform levels ($p=NS$; Supplemental Figure 4).

BH_4 prevented or reversed diastolic dysfunction in hypertensive mice

We examined whether BH_4 could prevent diastolic function in mice treated with DOCA-salt. BH_4 administration prevented the hypertension associated with DOCA-salt treatment (Figure 4, a). Assessment of diastolic function by echocardiography showed preserved LV relaxation with BH_4 based on all indices (Figure 4, b–d). BH_4 prevented the increase in LV end diastolic pressure seen with hypertensive DOCA-salt mice (6.3 ± 0.9 mmHg; $p=NS$ compared to controls). The slope of the EDPVR was significantly lower in BH_4 prevention mice compared to DOCA-salt mice and was statistically indistinguishable from controls (Figure 4, e–f). Measures of systolic function including the ESPVR and its volume axis intercept (V_0) 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 (Supplemental Table 1). Moreover, BH₄ did not affect heart rate. The mean heart rate was 550–576 beats/min during non-invasive and invasive studies for all 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 hypertensive mice had evidence of diastolic dysfunction, with a 12–14 day course of BH₄. Mean systolic blood pressure (96 ± 2 and 99 ± 3 mmHg in control; p=NS) and the invasively measured LV end systolic pressure (LVESP) were normalized in BH₄ treated mice. LV end diastolic pressure (LVEDP) did not increase compared to controls (6.2 ± 0.9 mmHg; p=NS). Echocardiographic indices of diastolic function in the BH₄ treatment group mirrored those observed in the BH₄ prevention group and controls (Figure 4, b–d). The EDPVR was significantly improved in the BH₄ treated mice compared to DOCA mice (Figure 4, e–f). Systolic function, arterial elastance, and heart rate were similar in BH₄ treated mice as compared to all other groups (Supplemental Table 1). The lack of improvement in blood pressure or lusitropy after treatment of DOCA-salt mice with tetrahydroneopterin, a free radical scavenger without NOS catalytic activity, implied the effects were specific to BH₄ (Figure 4, b–d).^{6,10}

Since 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 improvement in NOS coupling. Hydralazine, an antihypertensive agent that lowers blood pressure without re-coupling NOS, was used to evaluate these possibilities.²⁹ Mice treated with hydralazine had a similar reduction in blood pressure 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 (Figure 4, a–d).

A possible mechanism for the BH₄ effect

In order to determine if 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 1 and Supplemental Figure 3), but the relaxation constant τ was prolonged in myocytes from hypertensive hearts compared to controls (137.3 ± 8.5 vs. 79.3 ± 4.9 ms, $p < 0.0001$; Figure 4, g). Incubating myocytes from hypertensive mice with $10 \mu\text{M}$ BH₄ for 15 min normalized τ (86.0 ± 7.6 ms, $p = \text{NS}$ vs. control and $p < 0.0001$ vs. DOCA; Figure 4, h). Addition of BH₄ to control myocytes had no effect on τ or fractional shortening (p=NS; Figure 4, g and Supplemental Figure 3).

Recently nNOS-mediated oxidation changes to the phosphorylation state of phospholamban (PLB), a key protein controlling sarcoplasmic reticulum Ca²⁺ pump activity and cytosolic Ca²⁺ levels, have been described.^{30,31} The ratio of phosphorylated-PLB to total PLB was quantified in DOCA-salt, control, BH₄ prevention, and BH₄ treatment groups (Figure 4, h). DOCA-salt mice had a marked reduction in the phosphorylated-PLB to total PLB ratio compared to all other groups (0.09 ± 0.04 vs. 0.76 ± 0.2 vs. 1.2 ± 0.1 vs. 1.0 ± 0.2 ; $p = 0.003$), respectively. Feeding BH₄ to control mice had no effect on the phosphorylated-PLB to total PLB ratio (Supplemental Figure 7).

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 Since cardiac

AngII levels are increased in ACE 1/8 mice and AngII causes increase 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 indices of diastolic dysfunction when compared to controls (Figure 5, a–c). $O_2^{\bullet-}$ levels were higher in ACE 1/8 mice compared to control littermates (0.24 ± 0.03 vs. 0.14 ± 0.3 $\mu\text{M}/\text{mg}$ tissue, $p=0.04$; Figure 5, d). $O_2^{\bullet-}$ production was suppressed by $60 \pm 17\%$ in ACE 1/8 mice by L-NAME. NOS inhibition had no effect on $O_2^{\bullet-}$ production in control littermates, suggesting that uncoupled NOS was involved in the cardiac oxidation seen in ACE 1/8 mice (Figure 5, e).

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.^{4,34} Above, 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. Further suggesting uncoupled NOS was the increase in oxidized cardiac pterins and concomitant decrease in reduced cardiac BH_4 . Prevention or reversal of diastolic dysfunction by BH_4 treatment but not HN_4 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 acutely treating these cells with BH_4 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 BH_4 leading to myocyte uncoupled NOS.

Our results are consistent with those of Takimoto and Moens et al.^{10,35} These investigators showed that transaortic constriction with resultant systolic dysfunction was associated with cardiac BH_4 depletion and NOS uncoupling. Exogenous BH_4 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.

Based on inhibitor experiments, nNOS was the largest contributor to cardiac $O_2^{\bullet-}$ production in diastolic dysfunction. eNOS and nNOS isoforms are known to be present in heart, and BH_4 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 nitroso-redox balance and function.^{38–41} For example, Casadei and her co-workers have shown that nNOS modulates cardiac relaxation via effects on phospholamban phosphorylation.³¹ On the other hand, interpretation that nNOS generates most of the increased oxidative stress in hypertensive mice may be misleading if 7N is less specific than reported.¹⁸

Our data suggest that reduced NO leads to PLB changes that are consistent with increased cytosolic Ca^{2+} and diastolic dysfunction. The beneficial effects of BH_4 correlate with the phosphorylation state of PLB, consistent with work demonstrating NO is a mediator of cardiac lusitropy and the finding that Ca^{2+} handling and the phosphorylation state of PLB are subject to oxidative regulation.^{7,30,31,35,42} Other possible targets of oxidative modification that were not examined in the current study include the SR Ca^{2+} release channel, SR Ca^{2+} pump, or the sarcolemmal L-type Ca^{2+} channel. The troponin T- I79N mutation leads to diastolic dysfunction as a result of enhanced calcium myofilament sensitivity,⁴³ suggesting 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 BH₄ to normalize relaxation in these cells support the concept that diastolic dysfunction in this model is in large measure myocyte dependent. The ability of the RAS 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 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 a debate about the proper methodology to assess diastolic dysfunction, we chose to use both noninvasive and invasive means.^{25,45} Diastolic function was assessed using the combination of three echocardiographic modalities.⁴⁶ Two of these modalities, tissue Doppler imaging and color M-mode assessment of left ventricular inflow propagation velocity, are relatively independent of cardiac loading conditions.^{21,47} Moreover, the invasively-derived EDPVR was used, generally thought to be the standard for lusitropic assessment.²⁵ The strong correlation observed between E', Vp, and E/E' and the invasively-derived EDPVR suggests that these techniques assess similar myocardial properties. Measures indicated perturbations in active and passive stiffness. The abnormal phosphorylation state of PLB observed in hypertensive mice would be expected to impact active relaxation predominately. Since increased collagen deposition and hypertrophy were not observed, the increased passive stiffness may have resulted from enhanced myofilament Ca²⁺ sensitivity, changes in titin isoform expression, or changes in the composition of collagen.

Oral BH₄, but not HN₄, was able to raise cardiac BH₄ levels and prevent or reverse the diastolic parameter changes seen in hypertensive mice. This suggests that the BH₄ effect was not the result of nonspecific radical scavenging. Nevertheless, BH₄ lowered blood pressure, in accordance with previously published data from human and animal studies, and this might prevent or ameliorate diastolic dysfunction,^{29,48} experiments with hydralazine, a direct vasodilator that does not re-couple NOS, suggest otherwise.²⁹ In this case, hydralazine lowered blood pressure identically to BH₄ but did not prevent diastolic dysfunction.

In summary, this study demonstrates that cardiac oxidation can lead to BH₄ depletion, NOS uncoupling, and diastolic dysfunction. This dysfunction can be ameliorated by BH₄ treatment. Diastolic dysfunction can occur in the absence of changes in the vasculature. Finally, these results suggest that BH₄ may be of value in the treatment of diastolic dysfunction.

Clinical Summary

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 (RAS) leads to reduced vascular nitric oxide (NO), in part because nitric oxide synthase (NOS) becomes uncoupled. This dysfunctional state of NOS is characterized by oxidative depletion of its co-factor 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 RAS leads 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 RAS 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Control

Hypertension

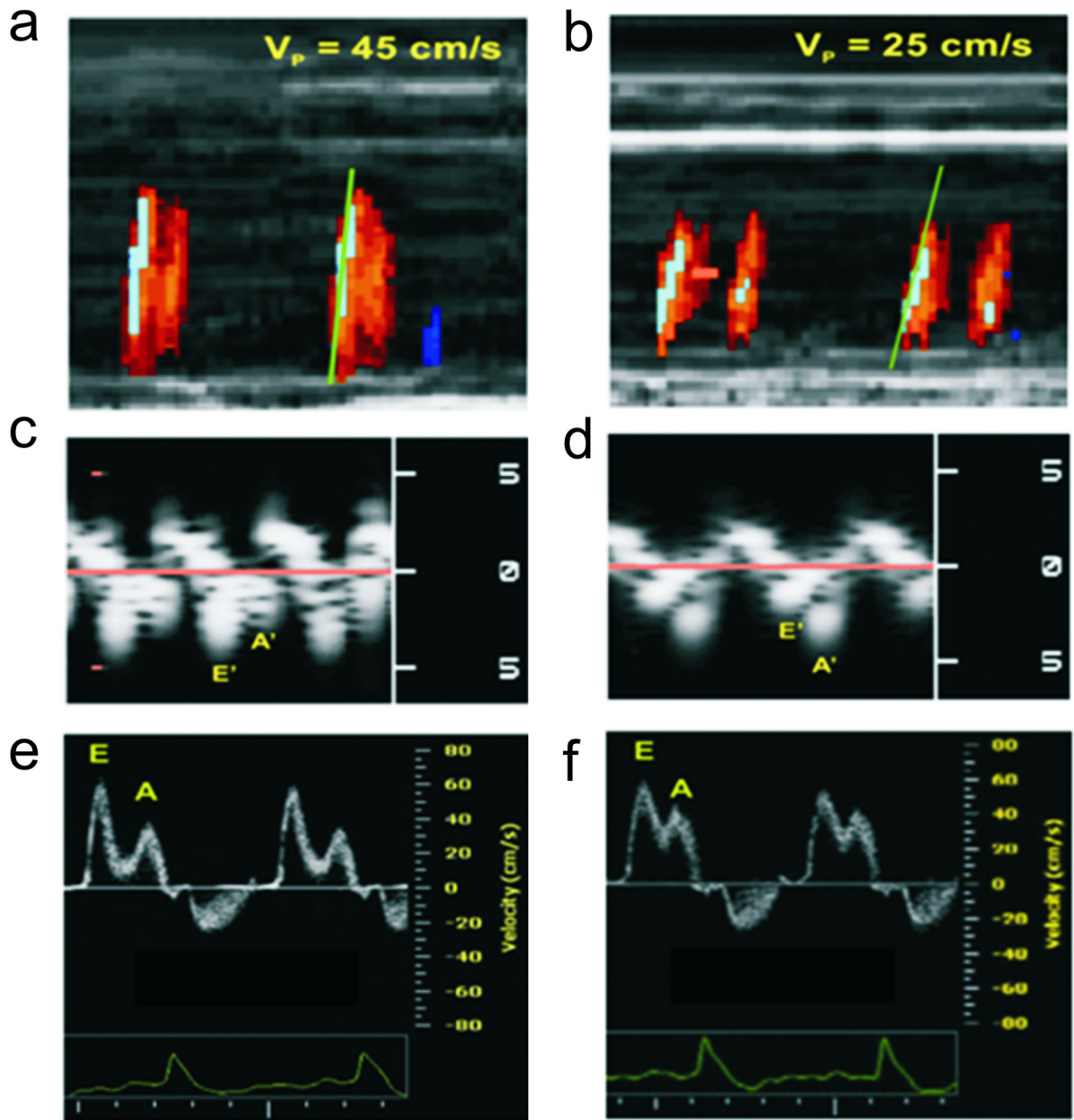


Figure 1.

Representative echocardiographic assessments of LV diastolic function. *Panels a and b:* Left ventricular inflow propagation velocity (V_p) interrogated with color M-mode Doppler. A control mouse shows a steeper isovelocity line slope, corresponding to a higher V_p compared with a hypertensive DOCA mouse. *Panels c and d:* Septal mitral annulus velocities interrogated with tissue Doppler imaging (TDI). The control animal has a higher E' (early diastolic velocity), and lower A' (late diastolic velocity) than the hypertensive animal. *Panels e and f:* Conventional pulsed wave Doppler shows a normal E/A (early to late diastolic filling velocity ratio) of >1 and <2 for both the control and DOCA mice, a "pseudonormal" pattern.

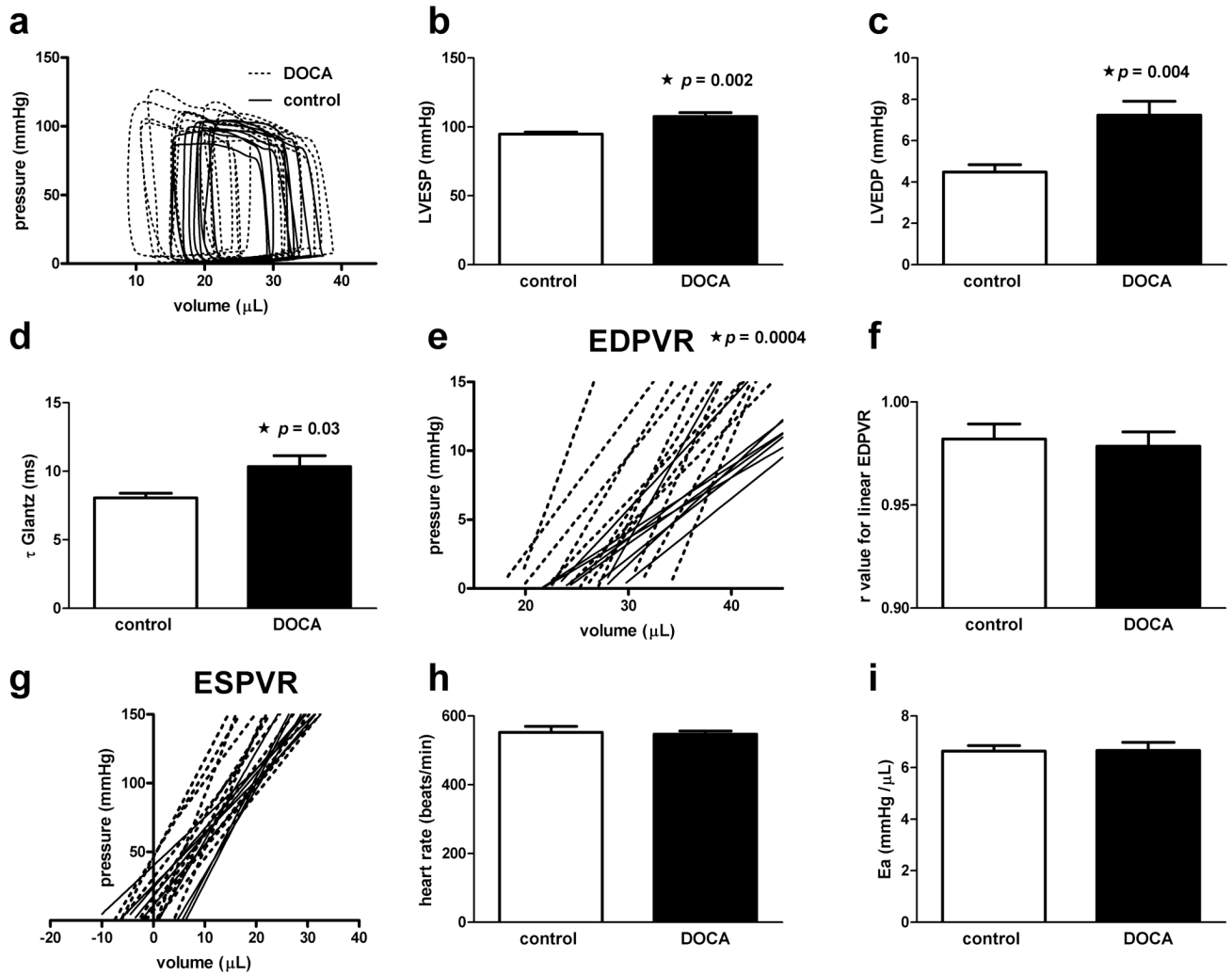


Figure 2. Invasive hemodynamic assessment of LV diastolic dysfunction. *Panel a:* Baseline pressure-volume loops for hypertensive and control animals. *Panel b:* Comparison of LVESP for hypertensive and control animals ($p=0.002$). *Panel c:* Comparison of LVEDP for hypertensive and control animals (7.2 ± 0.7 vs. 4.5 ± 0.4 mmHg, $p=0.004$). *Panel d:* The time constant for isovolemic relaxation (τ_{Glantz}) is increased in hypertensive mice compared to controls ($p=0.03$). *Panel e:* The end-diastolic pressure-volume relation (EDPVR) slope is steeper in hypertensive mice as compared to controls ($p=0.0004$). *Panel f:* Pearson correlation coefficients for linear fitting of the EDPVR. *Panel g:* LV contractility assessed by the end-systolic pressure-volume relation (ESPVR) slope ($p=\text{NS}$) and the volume axis intercept V_0 ($p=\text{NS}$) are similar between DOCA and control groups. *Panel h:* Mean heart rate between groups ($p=\text{NS}$). *Panel i:* Arterial elastance (E_a) a measure of vascular stiffness is similar between hypertensive and control mice ($p=\text{NS}$).

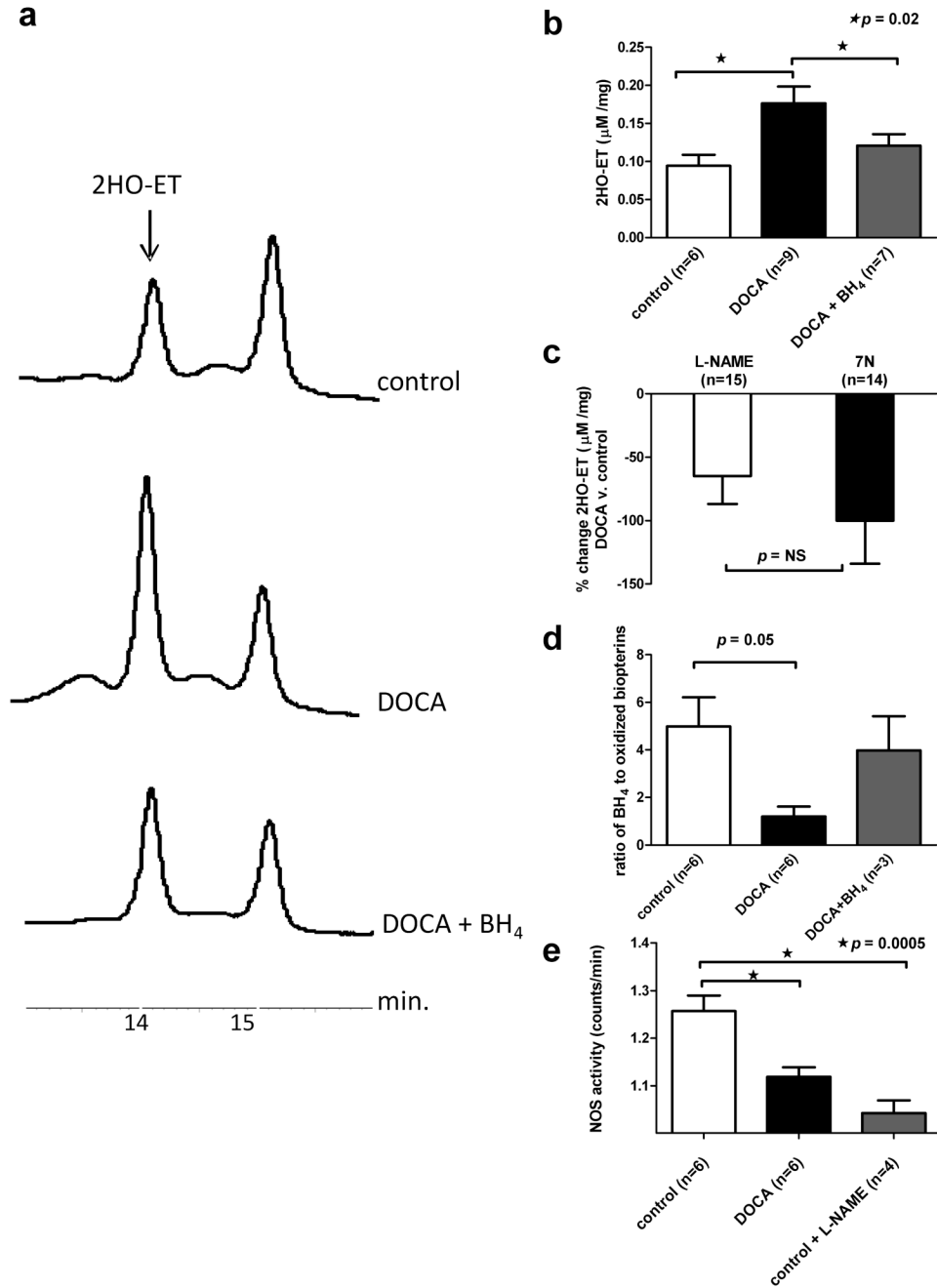


Figure 3. Hypertensive mice show cardiac oxidation and reduced BH₄. *Panel a:* Typical HPLC spectra obtained from a control (top), DOCA (middle), BH₄ treated DOCA mice (bottom) show a larger oxyethidium (2HO-ET) peak in the DOCA animal. *Panel b:* Mean 2HO-ET levels are significantly higher in DOCA mice as compared to control and BH₄ treated mice (days 1–14). *Panel c:* The percent O₂^{•-} reduction between control and DOCA LV tissue treated with the non-selective NOS inhibitor, L-NAME, and the selective neuronal NOS inhibitor, 7-nitroimidazole (7N). *Panel d:* BH₄ and oxidized bipterins are quantified using the differential oxidation method and HPLC. The ratio of reduced BH₄ to oxidized pterins in the heart is lower in DOCA mice versus controls. Feeding BH₄ to hypertensive mice (days 1–14) increases the

cardiac reduced BH₄ to oxidized pterins ratio. *Panel e*: Total NOS activity is reduced to a similar degree in DOCA and L-NAME treated control hearts.

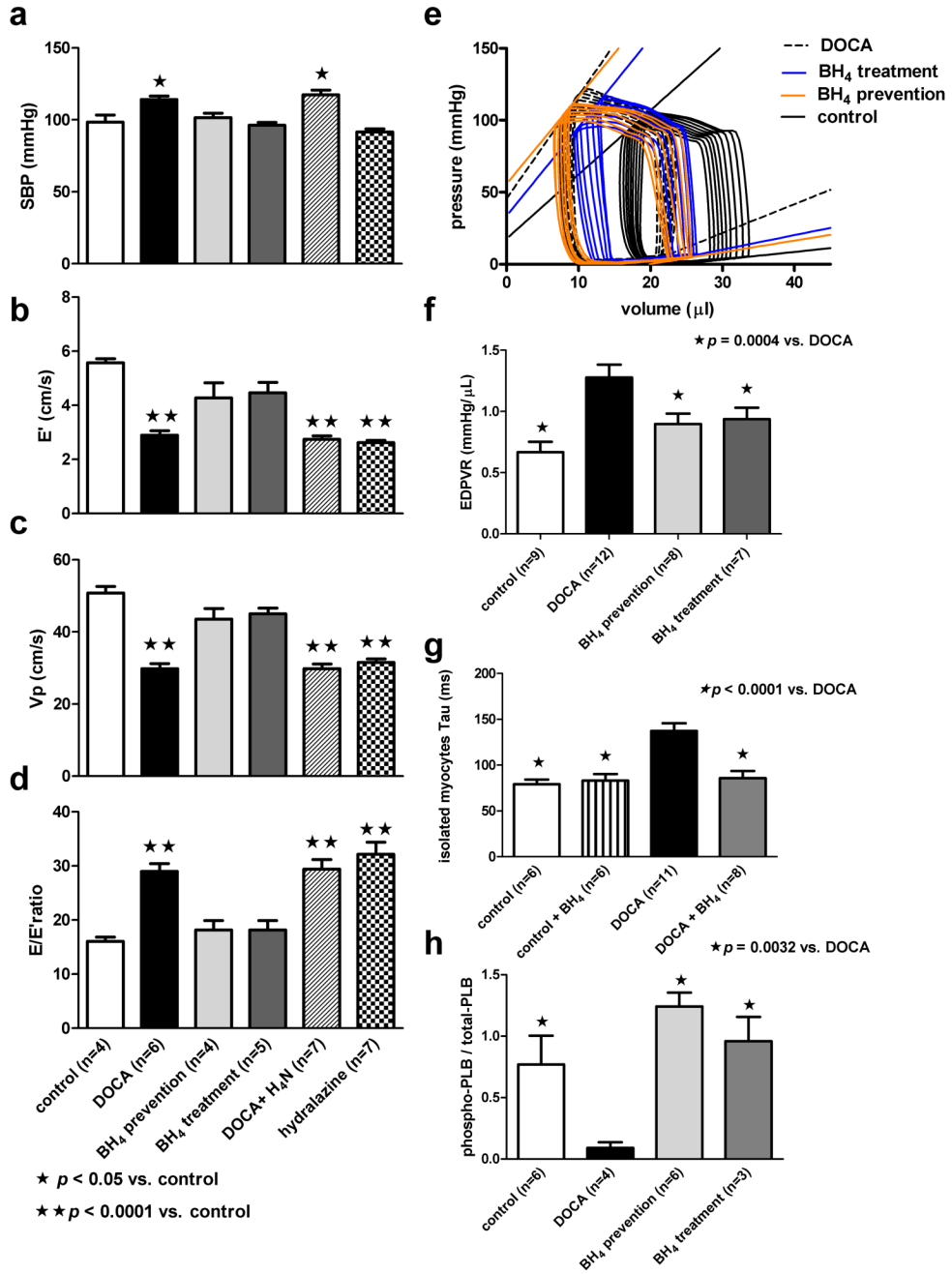


Figure 4. Tetrahydrobiopterin (BH₄) prevents or reverses diastolic dysfunction. *Panel a:* DOCA mice treated with BH₄ or hydralazine (days 12–24) show a reduction in systolic blood pressure (SBP) to levels observed in control animals. Treatment with tetrahydrobiopterin (H₄N; days 12–24), an enzymatically inactive analog of BH₄ with equivalent antioxidant properties, has no effect on blood pressure in hypertensive DOCA mice. *Panels b, c, and d:* Assessment of diastolic function using echocardiography: E' (early septal mitral annulus velocity measured with tissue Doppler imaging), Vp (left ventricular inflow propagation velocity interrogated with color M-mode Doppler) and E/E' ratio show BH₄, but not H₄N or hydralazine, prevents or reverses diastolic dysfunction in hypertensive DOCA mice. *Panel e:* Sample pressure-volume loops

obtained during inferior vena cava occlusion demonstrate the EDPVR and ESPVR in control-, DOCA-, and BH₄-treated mice. *Panel f*: The mean EDPVR is steeper in DOCA mice versus control, BH₄ prevention, and BH₄ treatment groups. *Panel g*: Isolated myocytes from DOCA mice have a prolonged relaxation constant (τ) compared to control animals. The addition of exogenous BH₄ to isolated DOCA myocytes normalizes relaxation kinetics. *Panel g*: DOCA mice have a reduced phosphorylated to total PLB ratio compared to control, BH₄ prevention, and BH₄ treatment groups.

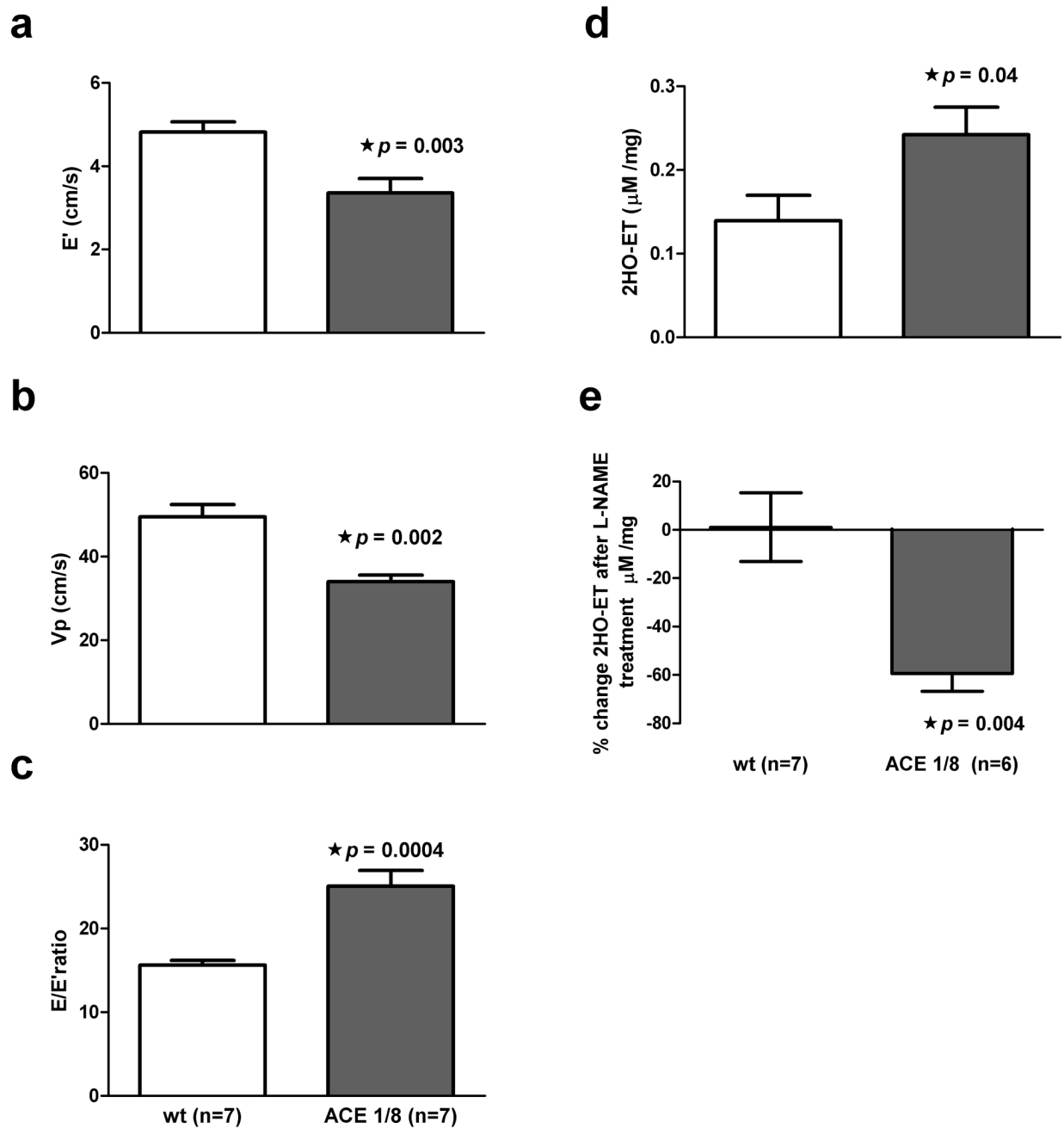


Figure 5.

Cardiac-specific ACE overexpression results in increased oxidation and diastolic dysfunction. *Panels a, b, and c:* ACE 1/8 mice have reduced E', lower Vp, and an increased E'/E' ratio when compared to wild-type (wt) controls. *Panel d:* Mean oxyethidium (2HO-ET) levels are significantly higher in ACE 1/8 mice versus controls. *Panel e:* L-NAME suppresses $\text{O}_2^{\bullet-}$ production in ACE 1/8 mice ($p=0.004$).

Table 1

Comparison of control and hypertensive mice.

	control	DOCA	p value
	(n=10)	(n=9)	
Systolic	98 ± 5	114 ± 2	0.01
HR (beats/min)	564 ± 15	609 ± 14	0.03
Diastolic	(n=4)	(n=6)	
E' (cm/s)	5.6 ± 0.2	2.9 ± 0.2	<0.001
E'/A'	1.5 ± 0.1	0.7 ± 0.1	<0.001
Vp (cm/s)	50.8 ± 1.9	29.8 ± 1.4	<0.001
E/E'	16.1 ± 0.8	29.0 ± 1.5	<0.001
E/A	1.4 ± 0.4	1.5 ± 0.1	NS
Systolic	(n=6)	(n=5)	
%FS (%)	29 ± 1.9	33 ± 2.0	NS
%EF (%)	56 ± 2.9	60 ± 0.2	NS
Sm (cm/s)	3.3 ± 0.4	2.9 ± 0.1	NS
LV dimensions	(n=6)	(n=5)	
LVPWd (mm)	0.74 ± 0.01	0.76 ± 0.01	NS
LVEDd (mm)	3.8 ± 0.1	3.7 ± 0.2	NS
Myocyte	(n=9)	(n=9)	
Diameter (µm)	16.9 ± 0.5	16.8 ± 0.3	NS
	(n=6)	(n=6)	
Length (µm)	123.2 ± 6.1	111.3 ± 12.9	NS

BP, blood pressure; HR, heart rate; E', early septal mitral annulus velocity measured by tissue Doppler imaging (TDI); A', late diastolic septal mitral annulus velocity (TDI); Sm, systolic septal mitral annulus velocity (TDI); Vp, left ventricular inflow propagation velocity by color M-mode Doppler; E, early diastolic filling velocity measured by conventional Doppler; A, late diastolic filling velocity measured by conventional Doppler; %FS, percent fractional shortening; %LVEF, percent ejection fraction; LV, left ventricle; LVPWd, LV posterior wall thickness; LVEDd, LV end diastolic dimension.