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**A mouse model of heart failure with preserved ejection fraction due to chronic infusion of a low subpressor dose of angiotensin II**

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Heart failure (HF) with preserved ejection fraction (HFpEF) is a clinical syndrome of symptoms of HF, such as breathlessness and exercise intolerance, associated with impaired left ventricular (LV) diastolic function in the presence of a normal LV ejection fraction (LVEF > 50%) (2). HFpEF carries significant morbidity and mortality burdens, and the prevalence of the disease has increased over the past 30 yr (2, 17). To date, the mechanisms underlying diastolic dysfunction and the progression of HFpEF are poorly understood. The pathophysiology involves impaired LV relaxation and contractile reserve, increased LV stiffness, as well as abnormal renal sodium handling, arterial stiffness, and aberrant ventriculo-arterial coupling (2).

The limited availability of animal models of HFpEF has potentially represented a major limitation in conducting mechanistic studies in the field (11). The animal models of HFpEF have hitherto involved mostly studies of increased afterload and LV hypertrophy (i.e., aortic banding or systemic arterial hypertension), models of increased preload (i.e., aorto-caval fistulas), or models of altered metabolism (i.e., obesity, diabetes, hyperlipidemia), making it difficult to distinguish between the intrinsic mechanism(s) of diastolic dysfunction vs. the mechanism(s) causing dysfunction (11). Moreover, aortic banding and aorto-caval fistulas do not reflect clinically existing conditions, and the majority of patients with HFpEF continue to have HF symptoms, even when they have controlled blood pressure (BP). In fact, HFpEF patients have LV hypertrophy in <50% of cases and often show no evidence of increased preload or LV dilatation, thus making the value of the current preclinical models questionable (1, 2, 11).

Angiotensin II (ATII) occupies a central role in homeostasis, hypertension, and HF, regulating afterload, preload, cardiac hypertrophy, and fibrosis (4). Chronic infusion of ATII has been used as a model of chronic hypertension, while low-dose infusion of ATII affects the cardiovascular system without inducing systemic arterial hypertension (4).

We hypothesized that low-dose ATII would reproduce the cardiac phenotype of HFpEF in the mouse in the absence of elevations in systemic arterial BP or LV hypertrophy, and thus...
serve as a preclinical model of HFrEF free of confounding factors.

METHODS

Ethical aspects. The experiments were conducted under the guidelines of the “Guide for the Care and Use of Laboratory Animals”, published by the National Institutes of Health (revised 2011). The study protocol was approved by the Virginia Commonwealth Institutional Animal Care and Use Committee.

Experimental model. Eight-week-old outbred male CD1 mice were supplied by Harlan (Indianapolis, IN). Mice were sedated with pentobarbital (50–70 mg/kg), and osmotic mini-pumps (DURECT, Cupertino, CA) were steriley implanted in the intrascapular space to allow subcutaneous infusion of low-dose ATII (0.2 mg·kg⁻¹·day⁻¹) or vehicle sterile water (at the same flow rate), for 28 days. Mice were randomly assigned to different groups: sham-operated mice (no implantation of any pump; N = 8); mice implanted with an infusion pump that infused vehicle for 28 days (N = 8); and mice implanted with an infusion pump with ATII for 28 days (N = 8). The infusion of vehicle had no significant effects on any of the measured parameters and was indistinguishable from the sham-operated mice, and, therefore, throughout the rest of the paper, only data of vehicle are shown compared with ATII pump, while data on the sham-operated mice are not presented.

Noninvasive arterial BP measurement. We measured systolic and diastolic arterial BP at baseline and at 28 days using a noninvasive tail-cuff BP analyzer (CODA System, Kent Scientific, Torrington, CT). Ten cycle measurements were collected through a dedicated software, and the mean was calculated for both the systolic and the diastolic BP (7).

Echocardiography. All mice underwent transthoracic echocardiography at baseline (before surgery) and at 28 days, under light anesthesia (50 mg/kg pentobarbital sodium). Echocardiography was performed with the Vevo770 imaging system (VisualSonics, Toronto, Ontario, Canada) and a 30-MHz probe (3). The heart was visualized in B-mode from parasternal short-axis and apical views. We measured the LV end-diastolic diameter, LV end-systolic diameter, LV anterior wall diastolic thickness, and LV posterior wall diastolic thickness at M-mode, as previously described and according to the American Society of Echocardiography recommendations (3). LV fractional shortening, LVEF, and LV mass were calculated from the measurements of wall thickness and chamber diameters. Right ventricular (RV) systolic function was estimated using M-mode and measuring the tricuspid annular plane systolic excursion (24). The transmitral LV outflow tract Doppler spectra (E, A, ET) were recorded from an apical four-chamber view, and the myocardial performance index (MPI) was calculated as the ratio of isovolumetric contraction time and isovolumetric relaxation time (IRT) divided by the ejection time (21). Tissue Doppler was used to measure the lateral E’ and calculate the E-to-E’ ratio (E/E’). Two-dimensional video loops were reviewed for abnormalities in the pericardial structure (effusion or thickening), RV dilatation, or interventricular dependence. The investigators performing and reading the echocardiograms were blinded to the treatment allocation.

LV catheterization. Mice were anesthetized (70 mg/kg pentobarbital sodium), and a pressure probe catheter (AD Instruments, Colorado Springs, CO) was retrogradely inserted in the LV from the right carotid artery. LV end-diastolic pressures (LVEDP), and the pressure-volume loops were recorded and measured using LabChart Pro 5 (AD Instruments). The end-diastolic pressure-volume relationship (ED-PVR) and end-systolic pressure-volume relationship (ESPVR) were calculated by pressure-volume measurements by concomitant Millar catheter and transthoracic echocardiography (29). Changes in preload were studied in a closed chest system, applying a resistance to preload by inflating the lungs artificially using a standard tidal volume (7.5 μm/g body wt) and rate (300 breaths/min). To determine changes in preload, the ventilator was turned off, allowing for a progressive increase of venous return and recording several consecutive beats. The EDVPR and ESPVR were then calculated according to the changes in pressure-volume loops by interpolating the points of end-diastolic pressure and end-systolic pressure with and without preload. The IRT constant, t, was measured using the formula P = P0e⁻t/μ, where P represents the diastolic pressure, P0 is the pressure at the moment of maximum –dp/dt, and μ is the IRT.

Sample collection. Immediately following LV catheterization, hearts were rapidly excised and placed in 10% formalin and fixed for a minimum of 48 h and then embedded in paraffin. The lungs and the pericardium were inspected for gross anatomical abnormalities. Embedded heart tissues were sectioned into 5-μm slides and stained with Masson’s trichrome (Sigma-Aldrich) to detect collagen fibers. The area of myocardial fibrosis was measured as percentage of collagen area on total myocardial tissue area. The cross-sectional area (CSA) of cardiomyocytes was measured to determine cardiomyocyte hypertrophy. Computer morphometry was performed using Image J software.

Statistical analysis. All statistical analyses were performed using SPSS 21.0 package for Windows (Chicago, IL). Continuous variables are expressed as mean and SE, and one-way ANOVA to compare between three or more groups at any individual time point, followed by Bonferroni-corrected T-test for unpaired data was used. For interval changes over time, comparing ATII and vehicle infusion, an ANOVA for repeated measures was used, assessing the time×group interaction. P values <0.05 were considered statistically significant.

RESULTS

Procedural data. Sixteen mice underwent infusion pump implantation without any acute or late mortality (100% survival). Morphometric data are reported in Table 1.

Systemic arterial BP measurement. Systolic and diastolic arterial BP values, measured noninvasively with a tail cuff, were within the normal range before implantation of the pump and remained unchanged after 28-day infusion of ATII (N = 4, all P >0.81, Fig. 1).

Echocardiography. LV mass, LV end-diastolic diameter, LVEF, and tricuspid annular plane systolic excursion values were unchanged between baseline and after 28 days of infusion of ATII or vehicle (all P >0.22, Fig. 2). No changes in E/E’ were observed between the groups.

LV catheterization. Representative images of the pulsed wave Doppler spectra are shown in Fig. 3. Table 2 reports Doppler data for each group. ATII infusion induced a significant impairment in LV diastolic function, as measured by a significant increase of the IRT and of the MPI vs. baseline values (P = 0.018 and P <0.001, respectively), whereas vehicle infusion had no significant effects on IRT or MPI (all P >0.23) (Fig. 3). No changes in E/E’ were observed between the groups.

LVEDP. Representative images of the pressure changes over time are reported in Fig. 4. ATII-treated mice showed significantly increased LVEDP, τ, and EDPVR compared with vehicle-treated mice (P = 0.002, P <0.001, and P = 0.040, respectively). The EDVPR and ESPVR were then calculated according to the changes in pressure-volume loops by interpolating the points of end-diastolic pressure and end-systolic pressure with and without preload. The IRT constant, t, was measured using the formula P = P0e⁻t/μ, where P represents the diastolic pressure, P0 is the pressure at the moment of maximum –dp/dt, and μ is the IRT.

Table 1. Morphological parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, g</th>
<th>LVM, mg</th>
<th>LVEDD, mm</th>
<th>LVESD, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATII</td>
<td>38.4 ± 0.8</td>
<td>108 ± 7</td>
<td>3.96 ± 0.10</td>
<td>2.58 ± 0.13</td>
</tr>
<tr>
<td>Vehicle</td>
<td>39.0 ± 0.9</td>
<td>96 ± 5</td>
<td>4.19 ± 0.06</td>
<td>2.63 ± 0.13</td>
</tr>
</tbody>
</table>

Values are group means ± SE; N = 8 animals/group. ATII, angiotensin II; BW, body weight; LVM, left ventricular mass; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end systolic diameter. All P >0.15.

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respectively, Fig. 4), reflecting increased LV elastance. The ESPVR was unchanged, reflecting similar systolic performance between the control vehicle and the ATII-treated mice.

**Cardiomyocyte hypertrophy and interstitial myocardial fibrosis.** Histological analysis revealed that AT II led to a significantly increased cardiomyocyte CSA vs. vehicle ($P < 0.01$, Fig. 5), reflecting cardiomyocyte hypertrophy. ATII treatment also led to increased interstitial myocardial fibrosis compared with the vehicle-treated group ($P = 0.036$, Fig. 5). The associations between the cardiomyocyte CSA or the interstitial fibrosis and IRT, MPI, and LVEDP are shown in Fig. 6. We found no statistically significant correlation between cardiomyocytes hypertrophy and fibrosis ($R = 0.38$, $P = 0.28$), or between these parameters with IRT, MPI, and LVEDP within the individual animals (Fig. 5).

**DISCUSSION**

Chronic infusion of low-dose ATII recapitulates the phenotype of HfPEF in the mouse, independent of systemic arterial hypertension and/or LV hypertrophy. HfPEF is a clinical syndrome of breathlessness, fatigue, and exercise intolerance, despite preserved LV systolic function (LVEF >50%), and direct or indirect evidence of diastolic dysfunction and/or elevated LV filling pressures, in the absence of other structural or functional abnormalities that may explain such symptoms (2, 19). HfPEF is a clinically heterogeneous syndrome, and, unlike other cardiac diseases, the incidence of HfPEF is increasing (2). Therapeutic strategies that benefit patients with HF with reduced ejection fraction (EF) (or systolic HF) have proven to be marginally effective or ineffective in patients with HfPEF (2). Despite the disease burden of HfPEF and associ-
ated morbidity and mortality, the pathophysiology remains unclear (2). Understanding the mechanisms involved in the progression of HFpEF is further complicated by the many comorbidities and risk factors affecting HFpEF patients, including age, obesity, hypertension, and diabetes. Therefore, there is a need for additional and more accurate preclinical models to understand the pathogenesis of HFpEF and potentially develop targeted therapies.

In this regard, an animal model of HFpEF that explores diastolic dysfunction independent of LV hypertrophy and/or pressure overload and of metabolic changes is lacking, although several animal models have been proposed to study HFpEF (11). The mouse model of transaortic constriction is a pressure-overload model where a band is placed around the aorta, and, as the mouse grows, the band becomes obstructive by preventing sufficient enlargement of the aortic CSA, thus increasing afterload (5, 15, 23). This model is subject to procedural variability based on the size of the banding, the age of mice at time of surgery, and the length of follow up, but most importantly the diastolic dysfunction is secondary to pressure overload and dependent on the severity and rapidity of onset of the constriction. As such, this model represents more closely a model of progressive aortic valve stenosis rather than a model of HFpEF. This model also develops decompensated eccentric LV hypertrophy associated with LV systolic dysfunction, which is an uncommon feature in HFpEF, as the evolution to HF with reduced EF is uncommon in patients with HFpEF (2). Of note, in some experimental settings, the banding was applied to induce a relatively rapid pressure overload (18). However, the clinical translational value of these models is difficult to understand, since, with the exception of acute prosthetic aortic valve thrombosis, or perhaps an acute hypertensive crisis in a patient without hypertension, a clinical scenario of acutely increased afterload is not observed.

Other commonly used models of diastolic dysfunction are related to systemic hypertension in the rat (16). The spontaneous hypertensive rat and the salt-sensitive rat models are models of cardiac dysfunction in the setting of large increases

Table 2. Wave Doppler and tissue Doppler parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>E (cm/s)</th>
<th>A (cm/s)</th>
<th>E/A</th>
<th>S' (cm/s)</th>
<th>E' (cm/s)</th>
<th>A' (cm/s)</th>
<th>E/E'</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATII</td>
<td>703 ± 45</td>
<td>266 ± 35</td>
<td>2.52 ± 0.27</td>
<td>15.9 ± 0.7</td>
<td>15.0 ± 1.4</td>
<td>11.6 ± 0.8</td>
<td>47 ± 5</td>
</tr>
<tr>
<td>Vehicle</td>
<td>668 ± 13</td>
<td>300 ± 51</td>
<td>2.42 ± 0.39</td>
<td>15.5 ± 0.6</td>
<td>12.4 ± 1.2</td>
<td>9.3 ± 1.4</td>
<td>57 ± 5</td>
</tr>
</tbody>
</table>

Values are group mean ± SE; N = 8 animals/group. All P > 0.13.
in systemic arterial BP (27). These models are useful in studying hypertensive cardiomyopathy, which also evolves through concentric LV hypertrophy with preserved EF to eccentric LV hypertrophy with reduced EF (11). While many patients with HFpEF have arterial hypertension, less than one-half have LV hypertrophy, and eccentric LV hypertrophy with reduced EF is not a common feature of HFpEF (2).

The rodent model of aorto-caval fistula is a model of volume overload characterized by biventricular hypertrophy, LV enlargement, and increased systolic and diastolic pressure (1). This model is used to study congestive HF, and, therefore, the prominent systolic nature of ventricular dysfunction represents a limitation of the use of this model to study diastolic dysfunction (26, 28).

The db/db leptin receptor-deficient morbidly obese mouse has been used as a model of HFpEF; however, diastolic dysfunction is associated with morbid obesity and severe hyperglycemia secondary to diabetes (22). The db/db model has an inherent value in exploring the contribution of obesity and metabolic syndrome, often seen in patients with HFpEF, but may not be representative of all patients with HFpEF. The ZSF1 obese rat model is a model of metabolic dysfunction in which HFpEF is associated with obesity and diabetes (9). The rat develops HF in association with hyperglycemia and insulin resistance, and, therefore, while it may represent a good model for HFpEF with metabolic alterations, it is unlikely to be representative of all patients with HFpEF. Genetic modifications of titin and cardiac myosin binding protein C have also been proposed as models of HFpEF (12, 25). These genetic models allow for the intrinsic changes to develop progressively as the mouse grows and ages, but do not reflect the clinical HFpEF syndrome.

Unlike other models of ATII infusion at higher doses, which induce significant systemic hypertension, we used a subpressor dose of ATII that recapitulates the enhanced activation of the renin-angiotensin-aldosterone system (RAAS) in patients with HFpEF, devoid of systemic arterial hypertension (17, 28). The proposed model, therefore, differs from prior models by the

Fig. 5. Measurement of cardiomyocyte hypertrophy and quantification of the interstitial fibrosis. A and B: Masson’s Trichrome stain on heart sections were used to visualize the cross-sectional areas of cardiomyocytes (×20 magnification) of mice infused with control vehicle or ATII, respectively. C and D: areas of magnification of A and B, respectively. E: quantification of the cardiomyocyte cross-sectional areas of the control vehicle and ATII infused mice. F and G: representative pictures of Masson’s Trichrome staining to quantify the fibrosis in tissue sections of hearts of mice infused with control vehicle or ATII, respectively (×20 magnification). H and I: areas of magnification of F and G, respectively. J: quantification of the interstitial fibrosis reported as percentage (%) of the area of fibrotic tissue on the total area. Values are group means ± SE; N = 8 animals/group. *P < 0.05.
absence of increased afterload, hypertrophy, dilatation, or metabolic abnormalities, and as such it is likely to provide information that is not available from the others, yet it is complementary to the already available models. This model shows that, in the mouse, ATII signaling is sufficient to induce diastolic dysfunction and HFpEF. This may appear at odds with clinical trials, failing to clearly show significant benefits in survival with blockers of the RAAS (2). It should be considered, however, that RAAS blockers in HFpEF have been mostly tested on top of other vasodilators and allowed for crossover to open-label treatments, thus biasing toward the null hypothesis (8, 13). Moreover, failure of RAAS blocker to improve outcomes in HFpEF should not be viewed as proof of a pathogenic role of ATII in HFpEF, as it may simply reflect the inability of RAAS blockers to reverse the disease process once fully established.

We describe that infusion of subpressor doses of ATII in the mouse leads to increased IRT, MPI, EDPVR, and LVEDP, indicative of impaired myocardial relaxation, increased elastance, wall stress, and diastolic dysfunction. The inability to assess for symptoms of HF is an obvious limitation to any animal study. Nevertheless, the increase in LVEDP is considered a reliable sign of HF. The increase in IRT and MPI and the upward shift in EDPVR in absence of changes in the pericardium or RV suggest that the LVEDP is indeed due to abnormal LV relaxation and/or elastance. We did not find a change in E/E’ in this study; while this appears to be at odds with the reported finding of increased E/E’ in patients with HFpEF, it should be considered that elevation in the E/E’ may only occur when LVEDP is markedly increase (i.e., >20 mmHg in patients with HFpEF) and, therefore, be a less sensitive marker of HFpEF (14). We measured changes in LVEDP and EDPVR in a closed chest/abdomen model, representing a potential way to reduce confounders related to surgery, bleeding, pain, and changes in temperature in the chest. Pentobarbital sodium has become the preferred sedative in our experiments, as it does not interfere with physiological or pharmacological preconditioning, and, although it likely reduced heart rate, contractility, and lusitropy in all groups, we expect the effects to be equal in all groups and thus not interfere with the comparisons between groups.

The histological analysis revealed also mild, but significant, increase in cardiomyocyte size (hypertrophy) and interstitial fibrosis, indicative of increased cardiac elastance. These changes developed in the absence of hypertension, systolic dysfunction, or LV remodeling. This model of chronic low-dose ATII infusion may, therefore, be a novel and useful tool to provide in-depth mechanistic insight for understanding the development and contribution of diastolic dysfunction in HFpEF, independent of genetic modifications, metabolic abnormalities, or pressure and volume overload.

This model, however, is not without limitations. First, we only studied young adult male CD1 mice. In the future, additional studies should initiate low-dose ATII infusion in aging mice to understand how the disease progression may be altered with age. HFpEF is more prevalent in women; therefore, it will be crucial to examine this model in female mice, at various stages of their life (i.e., pre- and post-ovarian senes-
ence or ovarioctomized mice). The characteristic hypertrophic response in females may be protective in some instances but deleterious in others, such as in HfPfEF (20). The infusion may also be extended to determine whether disease severity increases with a longer infusion period, and to see if mice will eventually develop systemic arterial hypertension, LV hypertrophy, and/or LV systolic dysfunction. The mechanisms by which AT1R induces diastolic dysfunction in this HfPfEF model are also not explored in this study. Impaired relaxation may be a dynamic effect on calcium handling, or dependent on the structural changes seen in interstitial fibrosis and cardiomyocyte hypertrophy (10). An additional limitation is the lack of data on isolated cardiomyocyte stiffness, shown to be abnormal in HfPfEF (6). We also did not measure changes in titin isoform expression or phosphorylation status, which appear to regulate the cardiomyocyte stiffness (9). The effects of AT1R on cardiomyocyte stiffness and titin will require further focused investigations.

In conclusion, a 28-day infusion of suprressor dose of AT1R recapitulates the HfPfEF features of impaired LV relaxation and increased LV elastance in the absence of pressure overload, LV systolic dysfunction, LV dilatation or hypertrophy, and metabolic abnormalities and may, therefore, be considered as a novel tool for mechanistic preclinical studies in HfPfEF with immense translational potential.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES

