Dual Endothelin-A/Endothelin-B Receptor Blockade and Cardiac Remodeling in Heart Failure With Preserved Ejection Fraction

Maria Valero-Munoz, PhD*; Shanpeng Li, BS*; Richard M. Wilson, BS; Batbold Boldbaatar, BS; Marc Iglarz, PharmD, PhD; Flora Sam, MD

Background—Despite the increasing prevalence of heart failure with preserved ejection fraction (HFpEF) in humans, there remains no evidence-based therapies for HFpEF. Endothelin-1 (ET-1) antagonists are a possibility because elevated ET-1 levels are associated with adverse cardiovascular effects, such as arterial and pulmonary vasoconstriction, impaired left ventricular (LV) relaxation, and stimulation of LV hypertrophy. LV hypertrophy is a common phenotype in HFpEF, particularly when associated with hypertension.

Methods and Results—In the present study, we found that ET-1 levels were significantly elevated in patients with chronic stable HFpEF. We then sought to investigate the effects of chronic macitentan, a dual ET-A/ET-B receptor antagonist, on cardiomyocyte function and ET-1 expression in mice with HFpEF..macitentan caused LV hypertrophy regression independent of blood pressure changes in HFpEF. Although macitentan did not modulate diastolic dysfunction in HFpEF, it significantly reduced wall thickness and relative wall thickness after 2 weeks of therapy. In vitro studies showed that macitentan decreased the aldosterone-induced cardiomyocyte hypertrophy. These changes were mediated by a reduction in the expression of cardiac myocyte enhancer factor 2a. Moreover, macitentan improved adverse cardiac remodeling, by reducing the stiffer cardiac collagen I and titin n2b expression in the left ventricle of mice with HFpEF.

Conclusions—These findings indicate that dual ET-A/ET-B receptor inhibition improves HFpEF by abrogating adverse cardiac remodeling via antihypertrophic mechanisms and by reducing stiffness. Additional studies are needed to explore the role of dual ET-1 receptor antagonists in patients with HFpEF. (Circ Heart Fail. 2016;9:e003381. DOI: 10.1161/CIRCHEARTFAILURE.116.003381.)

Key Words: endothelin-1 ■ endothelin receptor antagonists ■ heart failure, diastolic ■ hypertrophy, left ventricular ■ myocytes, cardiac

Heart failure (HF) with preserved ejection fraction (HFpEF) is a clinical syndrome and a disease characterized by signs and symptoms of HF, a preserved left ventricular (LV) EF (≥50%) and, is often, accompanied by abnormalities in diastolic function.1 Despite similarities in presenting symptoms, fundamental differences exist between HFpEF and HF with reduced EF (HFrEF), the most significant of which is the lack of approved evidence-based therapies for HFpEF. Furthermore, the prevalence of HFpEF continues to increase, relative to HFrEF.2

Inadequately treated hypertension leads to adverse cardiac remodeling, diastolic dysfunction and the development of HF, particularly HFpEF. Hypertension is the most important risk factor for HFpEF, with a high prevalence seen in large controlled trials, epidemiological studies, and HF registries.3 The prevalence of hypertension in addition to other comorbidities commonly seen in HFpEF, such as obesity, diabetes mellitus, and atrial fibrillation, is projected to also increase.4 Yet therapies directed only at comorbidities remain inadequate in preventing and treating HFpEF. Therefore, there is a pressing need to identify new pathogenic mechanisms in HFpEF that might provide new pathways for which to target drug therapies.

Mechanisms purported to play a role in HFpEF include vascular dysfunction,2 alterations in extracellular matrix composition,5 and modification of the intrinsic contractile properties of cardiomyocytes.6 Moreover, maladaptive LV hypertrophy (LVH) and diastolic dysfunction (DD), both frequently seen in HFpEF, although not mutually exclusive, may also provide mechanistic insights. An improvement
in DD is not necessarily associated with a reduction of LVH and conversely a reduction in LVH may not result in an improvement of DD. Differences in the molecular signals that modulate maladaptive LVH may explain the disparate responses to the approved evidence-based therapies in HFrEF and HfP EF. Thus, elucidating the mechanisms of maladaptive LVH regression could provide insights into developing new targets for treatment.

One such possibility is endothelin-1 (ET-1) antagonists. Although ET-1 levels are elevated and predict mortality in patients with HFrEF, ET-1 antagonists have not demonstrated beneficial outcomes in humans with HFrEF. ET-1, as well as both its receptors ET-A and ET-B, are synthesized and secreted by cardiac myocytes and other cells of the heart. ET-1 synthesis in cardiac myocytes is increased during the hypertrophic response. Likewise, the contribution of ET-1 to abnormalities in endothelial function, vascular compliance, pulmonary hypertension, impaired diastolic relaxation and myocardial fibrosis, implicates ET-1 in the pathophysiology of HfP EF. Therefore, ET-1 inhibition could have therapeutic applications in HfP EF.

The present study demonstrates that circulating levels of ET-1 are increased in patients with chronic, stable HfP EF. Therefore, we hypothesized that ET-1 blockade modulates structural, functional, and molecular alterations in HfP EF. The aim of this study was to evaluate the effect of macitentan, a dual ET-A/ET-B receptor antagonist, in a murine model of HfP EF and to investigate the mechanisms implicated in those effects.

Methods

Detailed methods are available in the Data Supplement.

Human Population

Chronic, stable patients with HfP EF (n=30) were enrolled from an outpatient HF Clinic at Boston Medical Center. Patients with HfP EF were included if they were previously admitted with a HfP EF exacerbation to the HF service within the previous year and had a LVEF >50% as measured by echocardiogram within 6 months before enrollment. Healthy, control subjects without HF or known cardiac disease were also enrolled (n=10). Controls had normal blood pressure (BP) and were not taking cardiovascular medications. Written informed consent was obtained from all participants before the collection of clinical samples. The study was approved by the Boston University Medical Center Institutional Review Board and conducted according to Declaration of Helsinki principles. Inclusion and exclusion criteria are included in the Data Supplement.

Serum ET-1 Measurements

Total circulating levels of ET-1 were measured in serum samples by ELISA according to the manufacturer’s instructions (R&D systems, Inc., Minneapolis, MN). Standards, controls, and samples were run in triplicate and averaged. The protein concentrations were calculated using a standard curve generated with recombinant standards provided by the manufacturer.

Animal Study

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The Institutional Animal Care and Use Committee at Boston University School of Medicine approved all study procedures related to the handling and surgery of the mice.

Animal Model of HfP EF

This murine model of HfP EF recapitulates the following aspects of human HfP EF: pulmonary congestion, preserved LVEF, DD, LVH, moderate hypertension, and exercise intolerance. Importantly, aldosterone levels are not supratherapeutic and compares well to acute human HF. Eight-week-old C57BL/6 male mice (Jackson Laboratories) were anesthetized with 80 to 100 mg/kg ketamine and 5 to 10 mg/kg xylazine intraperitoneally. All mice (20–25 g) underwent uninephrectomy and then received either a continuous infusion of saline (Sham) or aldosterone (0.3 μg/h; Sigma-Aldrich, St. Louis, MO; HfP EF) for 4 weeks via osmotic minipumps (Alzet, Durect Corp., Cupertino, CA). All mice were maintained on 1.0% sodium chloride drinking water. Two weeks post surgery, mice were randomized to receive either normal chow or chow-containing macitentan, a dual ET-A/ET-B receptor antagonist (MACI; 30 mg/kg per day; Figure I in the Data Supplement).

Chronic aldosterone for 4 weeks duration weeks induced the HfP EF phenotype. Mice were then euthanized at the end of 4 weeks. The 4 groups studied were as follows: (1) Sham: normal chow; (2) HfP EF: normal chow; (3) Sham-MACI: chow-containing MACI; and (4) HfP EF-MACI: chow-containing MACI (n=10–16 per group).

In a dose–response protocol, mice (n=3–6 per group) were fed chow with 2 different macitentan doses (10 and 30 mg/kg per day) 2 weeks after surgery, once hypertension was apparent. The dose of 30 mg/kg per day was selected as there were changes in cardiac hypertrophy that were independent of changes in body weight and systolic BP (Table I in the Data Supplement).

Physiological Measurements

Systolic and diastolic BP and heart rate were measured weekly using a noninvasive tail-cuff BP analyzer (BP-2000 Blood Pressure Analysis System; Visitech Systems, Inc., Apex, NC). Transthoracic echocardiography was performed at the end of 4 weeks to assess structure and diastolic function. See Data Supplement for further details. Mice were euthanized after these measurements. The ratio of wet: dry lung weight was determined as an indicator of pulmonary congestion.

In Vitro Studies

Isolation and Treatment of Adult Rat Cardiac Myocytes

Primary cultures of isolated adult rat ventricular cardiac myocytes (ARVM) were prepared as previously described. ARVM (90%–95% purity) were harvested from adult male Sprague–Dawley rats (200–220 g) and plated in a nonconfluent manner on laminin-coated (1 μg/cm²; Invitrogen, Carlsbad, CA) plastic culture dishes (Fisher Scientific, Pittsburgh, PA) at a density of 30 to 50 cells/mm². Cardiac myocytes were maintained at 37°C before treatment in Dulbecco Modified Eagle Medium (Invitrogen) containing 2 mg/mL BSA, 2 mmol/L l-carnitine, 5 mmol/L creatinine, 5 mmol/L taurine (Sigma–Aldrich), 100 IU/mL penicillin, and 10 g/mL streptomycin (Invitrogen).

ARVM were pretreated with or without macitentan (MACI; 10 μmol/L) for 30 minutes and were then stimulated with 1 μmol/L aldosterone (Sigma–Aldrich, St. Louis, MO; n=6 experiments for all conditions).

Statistical Analysis

Normality of distributions was verified by D’Agostino & Pearson omnibus normality test. Differences between 2 groups were analyzed by 2-tailed unpaired Student t tests or Mann–Whitney U test as parametric and nonparametric tests, respectively. Specific differences among at least 3 groups were analyzed using 1-way ANOVA and the Newman–Keuls post hoc test for normal distribution, and Kruskall–Wallis test followed by Dunn test for non-normal distribution variables. Spearman test was used to determine the correlation between ET-1 levels and echocardiographic measurements of PA pressures in patients with HfP EF. Pearson test was used to determine the correlation between titin N2B and collagen 1 mRNA expression.
in the experimental model. \( P \leq 0.05 \) was considered statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Results

ET-1 Serum Levels Were Elevated in Humans With Chronic, Stable HFpEF

Patients were recruited from an ambulatory HF clinic and had chronic, stable HFpEF (Table II in the Data Supplement). Mean New York Heart Association functional class was 2.1±0.7 at the time of enrollment. The HFpEF cohort was predominantly black (66%), women (62%), and mean age was 64±7 years. Comorbidities included hypertension (87%), type 2 diabetes mellitus (62%), and obesity (90%) and mean body mass index was 38.75±8.2 kg/m². Mean LVEF and LV mass were 63.9±6.3% and 200.2±54.4 g (normal <162 g), respectively. DD was present in 72% of patients with HFpEF and is comparable to other community HFpEF cohorts. Additional evidence of cardiac remodeling was seen with increased relative wall thickness (0.52±0.19) and left atrial volume index (37.8±4.2 mL/m²). Mean circulating levels of brain natriuretic peptide were also elevated (25±287 pg/mL).

Circulating Levels of ET-1

As shown in Figure 1, HFpEF patients had higher serum levels of ET-1 than controls (2.61±0.81 versus 1.74±0.52 pg/mL; \( P<0.001 \)). Elevated pulmonary artery systolic pressure is often seen in HFpEF and was evident in this cohort of HFpEF patients. Pulmonary artery systolic pressure was 41.6±10.8 mm Hg (range, 24–59 mm Hg) and showed a positive correlation with the ET-1 levels (\( R=0.627; P<0.01; 95\% \) confidence interval, 2.744–13.34). A subgroup of patients from this cohort with only hypertension-associated HFpEF (n=26) was selected to more closely resemble the hypertension-induced HFpEF murine model. These hypertension-associated HFpEF patients also demonstrated elevated ET-1 levels (2.62±0.79 pg/mL) versus controls (1.74±0.52 pg/mL; \( P<0.001 \); Figure 1B).

ET-1 Antagonism Reduced Cardiac Hypertrophy in Mice With HFpEF

Diastolic Function and LV Structure in Macitentan-Treated Mice With HFpEF

We examined the impact of dual ET-A/ET-B receptor blockade with macitentan on LV structure and diastolic function in mice with HFpEF (Table). As expected, chronic aldosterone caused DD in HFpEF mice versus Sham. HFpEF mice had a higher peak E velocity to peak A velocity ratio (E/A) and an increase in the isovolumetric relaxation time. These parameters were unaffected by macitentan treatment (E/A in HFpEF versus HFpEF-MACI, 1.85±0.09 versus 1.74±0.16; \( P=NS \)). Moreover, early filling deceleration time was similar in all the groups and LVEF was preserved. As previously shown, LV end-systolic dimensions were significantly decreased in both groups of HFpEF mice versus respective Shams (1.15±0.13 versus 1.57±0.11 mm; in HFpEF) and (1.64±0.12 versus 1.81±0.15 mm; in HFpEF-MACI) but were not different between HFpEF and HFpEF-MACI. Both groups of HFpEF mice also had a trend to decreased LV end-diastolic dimensions but were not significantly different between respective Shams and treatment group. Total wall thickness, relative wall thickness, and posterior wall thickness were increased by 1.40-, 1.47-, and 1.31-fold, respectively, in HFpEF mice compared with Sham (\( P<0.001 \)), but these parameters were significantly decreased with macitentan treatment (0.88-, 0.81-, and 0.85-fold, respectively, in HFpEF-MACI versus HFpEF; \( P<0.05 \)). Noticeably, these changes were independent of alterations in body weight, BP, and heart rate (Table). As expected, at 4 weeks, mice with HFpEF had moderate hypertension (138.6±11 versus 118.8±2.8 mm Hg; \( P<0.05 \) versus Sham); however, systolic BP in HFpEF-MACI mice was no different from HFpEF alone (135.4±2.0 mm Hg). Fulton index, as an indicator of right ventricular hypertrophy, was no different between all groups (Table). Lung congestion was significantly decreased in HFpEF-MACI mice versus HFpEF alone (\( P<0.05 \), Table). There were no deaths during the 4 weeks of aldosterone infusion in Sham or HFpEF mice with/witout treatment with macitentan.

Morphological and Molecular LV Myocardial Changes in HFpEF Mice After Treatment With Macitentan

As previously described, HFpEF mice had cardiac hypertrophy, as measured by heart weight/body weight ratio (5.4±0.25 versus 4.3±0.05; \( P<0.001 \) versus Sham; Figure 2A and 2B). Cardiomyocytes were also increased in size (314±24 versus 196±19 \( \mu \)m²; \( P<0.01 \) versus Sham; Figure 2A and 2C), with increased LV mRNA expression of atrial and brain natriuretic peptides in HFpEF hearts versus Sham (\( P<0.05 \) for both; Figure 2C through 2E). Treatment with macitentan reduced cardiomyocyte size (235±19 \( \mu \)m²; \( P<0.01 \); Figure 2A and 2C).

Figure 1. Serum endothelin (ET)-1 levels in patients with heart failure with preserved ejection fraction (HFpEF). A, ET-1 levels are increased in patients with chronic, stable HFpEF (n=30) vs controls (n=10), and B, in a subcohort (n=26) of only those patients with hypertension-associated HFpEF (HTN-HFpEF).
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and decreased brain natriuretic peptide mRNA expression ($P<0.05$; Figure 2E).

**Mechanisms Implicated in the Antihypertrophic Effect of Macitentan**

To investigate the mechanism of ET-1 receptor blockade in the observed antihypertrophic effects, we performed in vitro studies using ARVM stimulated with aldosterone ($1\mu$mol/L) and pretreated with or without macitentan ($10\mu$mol/L). Aldosterone stimulation induced prepro–ET-1 mRNA expression in cardiac myocytes, as previously described.18 Not unexpected, macitentan pretreatment did not affect prepro–ET-1 mRNA expression (Figure II in the Data Supplement). More importantly, as seen in vivo, pretreatment with macitentan reduced aldosterone-induced cardiomyocyte hypertrophy by $28\pm1.0\%$ ($P<0.01$ versus aldosterone alone; Figure 3A). To investigate the molecular underpinnings responsible of this effect, key signaling molecules involved in cardiomyocyte hypertrophy were measured, such as GATA binding protein 4 (gata4), myocardin, myocyte enhancer factor 2a (mef2a), and 2c (mef2c), and nuclear factor of activated T cells (nfat), in aldosterone-stimulated ARVM. The transcript encoding mef2a expression was increased by 1.95-fold in aldosterone-stimulated ARVM compared with control ($P<0.05$). Pretreatment with dual ET-A/ET-B receptor antagonist, macitentan, abrogated this mef2a expression ($P<0.05$). Neither aldosterone nor macitentan had an effect on gata4, myocardin, mef2c, or nfat expression (Figure 3B). Based on these in vitro findings, we conclude that aldosterone induces cardiomyocyte hypertrophy by activating ET-1 autocrine/paracrine signaling via mef2a. Thus, ET-1 receptor antagonism with macitentan decreased cardiac hypertrophy by inhibiting mef2a (Figure 3C).

**ET-1 Antagonism Improves Molecular Markers of Cardiac Remodeling in HFpEF Mice**

We next examined the effect of dual ET-A/ET-B receptor antagonism on HFpEF-induced cardiac fibrosis. Cardiac collagen content, increased in HFpEF mice compared with Sham (6.63±0.52 versus 4.39±0.51%; $P<0.05$) and decreased in HFpEF-MACI mice (5.06±0.62%; $P<0.05$; Figure 4A). This was associated with the following molecular changes. Transforming growth factor-$\beta$ and collagen type-I mRNA levels in the LV of HFpEF mice were significantly increased, and macitentan normalized the relative expression of both markers ($P<0.05$ for all; Figure 4B and 4C). There were no changes in collagen type-III expression (Figure 4D). Therefore in HFpEF, the stiffer collagen I was increased over the more compliant collagen III. The
resultant collagen I:collagen III ratio was increased in HFpEF mice (P<0.05 versus Sham). Noticeably, macitentan decreased the stiffer collagen I without any effect on the more compliant collagen III (P<0.05 versus HFpEF; Figure 4E).

Titin, a large, multifunctional filament, traverses half of the cardiomyocyte sarcomere. Two titin isoforms exist, the more compliant (N2BA) and the stiffer isoform (N2B), and both play a pivotal role in diastolic stiffness and in HFpEF.5,6 Thus, we measured the expression of the titin transcript variants n2ba and n2b from the LV of HFpEF mice treated with or without macitentan. Both titin isoforms, n2ba and n2b, were significantly increased 1.56- and 1.68-fold, respectively, in HFpEF (P<0.05; versus Sham). Macitentan treatment only reduced the expression of the stiffer titin isoform, n2b, by 38±10% versus HFpEF (P<0.05, Figure 5A and 5B). Moreover, these changes in n2b expression showed a positive correlation with the changes observed in collagen I expression (R=0.648; P<0.001; Figure III in the Data Supplement).

**Discussion**

ET-1 levels are elevated and predict mortality in HFrEF8; yet, human studies with ET-1 inhibition showed no clinical benefit. Recently, ET-1 levels were also shown to be elevated in HFpEF patients with diabetes mellitus10 and selective inhibition of the ET-A receptor with sitaxsentan in HFpEF improved only exercise tolerance with negligible effects on LV structure and impaired diastolic function.20 In the present study, ET-1 levels are elevated in patients with stable, chronic HFpEF. In addition, these patients demonstrated increased cardiac hypertrophy. Noticeably, circulating ET-1 levels were also elevated in a subgroup that included only patients with hypertension-associated HFpEF patients, thus supporting the relevance of the experimental findings in this specific HFpEF murine phenotype.

Chronic treatment with macitentan, a dual ET-A/ET-B receptor antagonist, reduced cardiac hypertrophy in experimental HFpEF. These novel findings were independent of changes in BP. Chronic macitentan therapy (1) reduced wall thickness and decreased heart weight in HFpEF and (2) reduced cardiomyocyte size and transcript expression of myocardial brain natriuretic peptide. In vitro findings showed that this reduction in cardiac hypertrophy is associated with an inhibition of mef2a expression. Macitentan also decreased LV collagen content and the expression of the titin transcript variant n2b in mice with HFpEF. These data indicate that modulation of cardiac hypertrophy and remodeling by a dual ET-A/ET-B receptor antagonist may thus have a beneficial effect on the HFpEF phenotype.

The finding that selective inhibition of the ET-A receptor with sitaxsentan in HFpEF did not modify LV structure or improve impaired diastolic function20 was unexpected given that contractile dysfunction and the prohypertrophic effects of ET-1 are believed to be mediated principally through the ET-A receptor.21 Our findings suggest that blockade of both ET-A
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Figure 3. Pretreatment with macitentan (MACI 10 μmol/L) prevented hypertrophy in cardiac myocytes via myocyte enhancer factor 2 (MEF2). A, Adult rat ventricular cardiac myocyte (ARVM) relative size and representative microscopic images (×10). B, Relative mRNA expression of hypertrophic signaling pathway markers: gata4, mef2a, mef2c, myocardin, and nfat. Histogram bars represent the mean±SEM of n=4 to 6 experiments. C, Simplified diagram of the proposed mechanism of action of MACI on the regulation of cardiomyocyte hypertrophy. ALDO indicates aldosterone (1 μmol/L); and CT, control.

Cardiomyocytes stimulated with aldosterone increased the expression of prepro–ET-1 mRNA, which was unaffected by pretreatment with macitentan. However, macitentan prevented cardiomyocyte hypertrophy, indicating that, at least partially, dual ET-A/ET-B receptor antagonism inhibited aldosterone-induced hypertrophy. Thus, it is likely that there is cross talk between ET-1 and aldosterone because the activation/inhibition of one system seems to modify levels of the other. This is also supported by clinical evidence, showing that hyperaldosteronism is associated with increased ET-1 production and treatment in patients with HF with a dual ET-1 receptor antagonist decreased circulating aldosterone concentrations.

Reprogramming of the gene expression profile is responsible for many of the phenotypic changes observed in hypertrophied cardiomyocytes, and it is perceived to be critical for disease-associated impairment of cardiac myocyte function. Thus, we sought to investigate the potential mechanisms implicated in the reduction of hypertrophy by macitentan in cardiomyocytes. In our study, aldosterone stimulation of cardiomyocytes increased mef2a expression, which in turn was inhibited by pretreatment with macitentan. This suggests that mef2a plays a pivotal role in aldosterone-induced cardiomyocyte hypertrophy. Interestingly there were no changes in gata4, myocardin, mef2c, or nfat expression.

Cardiomyocyte hypertrophy in HFP EF is often accompanied by increased fibrosis in the myocardium that leads to alterations...
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in myocardial stiffness. The extracellular matrix, namely the collagen network, and cardiomyocytes, in which titin plays a key regulatory role, regulate myocardial stiffness. Collagen I (the stiffer isoform) and cross-linked collagen affect collagen solubility and content, extracellular matrix deposition, and thus myocardial stiffness. In cardiomyocytes, titin operates as a bidirectional spring that gives stability to the other myofilaments and is able to modulate cardiomyocyte-based stiffness, by switching isoforms, and undergoes post-translational modification by phosphorylation and oxidation. In this study, collagen synthesis and extracellular matrix deposition were increased in HFP EF hearts. This was also accompanied by changes in the expression of the titin variant isoforms n2ba and n2b. Macitentan treatment reduced cardiac collagen I and extracellular matrix deposition in mice with HFP EF. This is consistent with previous findings where elevated levels of ET-1 caused fibroblasts to proliferate.

Figure 4. Pharmacological inhibition of endothelin (ET)-1 reduces cardiac fibrosis and stiffness in heart failure with preserved ejection fraction (HFP EF) mice. Fibrosis quantification of picrosirius red staining and representative microscopic images (×20; A), and relative mRNA expression of tgfβ (B), collagen 1 (C) and collagen 3 (D), and collagen 1:collagen 3 ratio (E) in left ventricular tissue of Sham and HFP EF mice fed a normal chow (NC) or treated with macitentan (MC; 30 mg/kg per day) for 2 weeks. n=5 to 10 mice per group. Data shown as mean±SEM. MACI indicates macitentan; n.s., not significant; and ROI, regions of interest.

Figure 5. Effect of pharmacological inhibition of endothelin (ET)-1 in heart failure with preserved ejection fraction (HFP EF) mice. Relative mRNA expression of titin transcription variant n2ba (A) and n2b (B) in the left ventricle of Sham and HFP EF mice fed a normal chow (NC) or treated with macitentan (MC; 30 mg/kg per day) for 2 weeks. n=5 to 10 mice per group. Data shown as mean±SEM. n.s. indicates not significant.
and increase the secretion of extracellular matrix proteins. In human fibroblasts, dual ET-1 receptor blockade prevented the induction by TGFβ transcripts involved in tissue remodeling. Similarly, our results showed a reduction of TGFβ expression in the LV of HFP EF mice treated with macitentan, indicating that the modulation of fibrosis is through a TGFβ-depending mechanism. Moreover, although both titin isoforms were increased in the LV of HFP EF mice, only the variant isoform n2b (the stiffer isoform) was diminished in HFP EF mice treated with macitentan. Collagen content and titin isoforms have been shown to inversely proportional to preserve relative stiffness contributions, with lower sarcomere length (titin) present at higher length (collagen). In our study, collagen I mRNA expression and titin variant isoform n2b showed a positive correlation. Thus, given the generally coordinated expression of the stiffer titin isoform with fibrosis, it is likely that changes in remodeling and stiffness after macitentan treatment were temporally associated. However, the mechanisms leading to the observed changes were not investigated, limiting the interpretation of these results.

**Limitations**

The reduction in lung congestion suggests an improvement in DD. However, only E/A, deceleration time, and isovolumetric relaxation time were measured and were no different between HFP EF and HFP EF-MACI. e′ velocity or E/e′ ratio was not determined in this study. It is also possible that if therapy with macitentan was given for a longer duration, the reduction in LVH might precede changes in DD. However, other studies have shown that LVH reduction is not always accompanied by improvement in parameters of DD, and conversely, improvement in DD parameters is not consistently accompanied by LVH regression. The underlying pathogenic mechanism(s) that may link LVH to DD and HFP EF are not completely understood and is unlikely to be simply temporally related and will require further study.

Control subjects in this study were matched by age and gender and not comorbidities because of the small sample size. However, serum ET-1 levels were elevated both in the group of chronic stable HFP EF patients (similar to other HFP EF cohorts), as well as in the subgroup that had specifically hypertension-associated HFP EF.

In conclusion, given the lack of therapies that continues to plague patients with HFP EF, understanding the mechanisms primarily responsible for this clinical syndrome is important. Our findings indicate that dual ET-A/ET-B receptor blockade modulates morphological and cellular changes associated with cardiac hypertrophy and the giant myocyte protein, titin, in HFP EF. Additional studies are warranted to determine whether regression of cardiac hypertrophy and improvement in cardiac stiffness observed in a murine model of HFP EF is likely to be beneficial in humans with HFP EF.

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**Disclosures**

Dr Iglarz is an employee of Actelion Pharmaceuticals Ltd., Allschwil, Switzerland. The other authors report no conflicts.

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**CLINICAL PERSPECTIVE**

Despite the increasing prevalence of heart failure with preserved ejection fraction (HFpEF) and the escalating burden on healthcare costs, therapy for HFpEF has been plagued by continued negative/neural clinical trials. Therefore, discerning the mechanistic underpinnings, which play a role in the pathogenesis of HFpEF, is a priority. The present study provides new insights into the mechanisms of cardiac hypertrophy and adverse cardiac remodeling in HFpEF and suggests a therapeutic target for pathological cardiac hypertrophy in HFpEF. In this study, circulating levels of endothelin-1 (ET-1) were elevated in patients with HFpEF. In a murine model of HFpEF, which features most of the characteristics of human HFpEF, dual ET-A/ET-B receptor inhibition modulated cardiac hypertrophy and adverse cardiac remodeling, independent of changes in blood pressure. In concert with these structural alterations, there was a reduction in cardiomyocyte size and decreased brain natriuretic peptide expression in the heart, potentially via the regulation of mef2a. ET-A/ET-B receptor blockade also decreased collagen content and the expression of the stiffer titin transcript variant, n2b, in the left ventricle. Given the lack of therapy to reduce morbidity or mortality in patients with HFpEF, these data provide novel insights. Additional studies are warranted to determine whether combined ET-A/ET-B receptor blockade will have a favorable outcome in clinical HFpEF particularly since ET-inhibition has had no role in patients with HF with reduced EF.
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**SUPPLEMENTAL MATERIAL**

**Supplemental methods**

**Study population**

*Inclusion/Exclusion criteria:* Patients were included if they were previously admitted with heart failure (HF) to a heart failure service and had a left ventricular ejection fraction (LVEF) >50% as measured by echocardiogram within 6 months prior to enrollment. Patients with infiltrative disease (e.g. amyloid), a genetic cardiomyopathy (e.g., HOCM); or a family history of sudden cardiac death and restrictive disease (e.g. constrictive pericarditis) were excluded. HFpEF etiology was defined as: 1) ischemic: prior history of myocardial infarction (electrocardiogram/positive troponin), results of a positive non-invasive stress test, or cardiac catheterization; 2) hypertensive: documented history of pharmacologically treated hypertension; and 3) Unknown: having no identifiable cause of the cardiomyopathy.

*Sampling:* Serum samples were obtained on an outpatient basis from hemodynamically stable patients who had no evidence of acute decompensation or acute renal failure. NYHA classification, a functional assessment of HF symptoms, was determined. Echocardiographic evaluation was performed on all patients for determination LVEF, structure and chamber dimensions. After informed consent, blood samples were obtained using the routine venipuncture procedure. Samples were centrifuged at 2000g for 15 minutes within 1 hour from collection. Serum samples were then aliquotted and stored at -80°C

*Echocardiography:* Two-dimensional and Doppler echocardiography was performed as previously described using the Vingmed Vivid Five System (GE Healthcare, Milwaukee, WI) with a 2.5-Mhz phased-array transducer. Echocardiograms were performed and analyzed in a
blinded manner. Measurements of systolic and diastolic chamber dimensions and wall thickness were obtained from 2D imaging according to the recommendations of the American Association of Echocardiography\textsuperscript{2}. The standard cube formula was utilized in order to calculate LV mass. Doppler echocardiography was used to estimate the pulmonary artery systolic pressure (PASP). The maximum tricuspid regurgitant jet velocity is recorded. PASP is then calculated using the formula: 

\[(4 \times [TRV]^2) + RAP\]

where TRV is the maximum tricuspid regurgitant jet velocity and RAP is the right atrial pressure estimated from the size and respiratory variation of flow in the inferior vena cava.

\textit{Laboratory values:} Levels of brain natriuretic peptide (BNP), creatinine, glomerular filtration rate, glucose, hemoglobin, sodium and cardiac troponin I (TnI) were measured as part of routine laboratory testing using the ADVIA Centaur assay (Siemens Healthcare Diagnostics, Deerfield, IL).

\textbf{Animal study}

\textit{Echocardiography:} Transthoracic echocardiography was performed at the end of 4 weeks using the Vevo 770 High-Resolution In Vivo Micro-Imaging System and a Real-Time Micro Visualization 707B Scanhead (VisualSonics Inc, Toronto, Ontario, Canada). Interventricular septum wall thickness (IVST), LV posterior wall thickness (LVPWT), LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and LVEF were obtained. Total wall thickness (TWT) was derived from an average of the IVST and LVPWT. Relative wall thickness (RWT) was calculated as \((2 \times \text{LVPWT})/\text{LVEDD}\). LV mass was calculated using the formula \(\text{LV mass} = 1.05 \times [(\text{LVEDD} + \text{IVST} + \text{PWT})^3 - (\text{LVEDD})^3]\) as described by Kiatchoosakun et al.\textsuperscript{3}. To assess
diastolic function, mice were anesthetized with isoflurane (0.5% for induction followed by 0.5 to 1.5% for maintenance) and maintained at a HR of ~350 beats per minute (bpm) since diastolic function is sensitive to HR and loading conditions\textsuperscript{4, 5}. 1.5% of isoflurane had minimal effects on diastolic function\textsuperscript{4, 5}. Pulse wave measurements were recorded and blinded analyzed.

**Right Ventricular Weight Measurement and Determination of Fulton's Index:** After the heart was excised, the right ventricular wall was dissected, and the remaining left ventricular wall and ventricular septum were weighed. Fulton's Index was calculated as the ratio of right ventricular weight/ (left ventricular + septum weight)\textsuperscript{6}.

**Histopathological Analysis:** Paraffin-embedded sections of the mid-ventricle (5μm) were stained with hematoxylin and eosin (H&E, Sigma-Aldrich, St. Louis, MO) and Pricosirius red staining (Sigma-Aldrich, St. Louis, MO) to assess LV cardiac myocyte cross-sectional (C/S) and myocardial fibrosis respectively. These were analyzed blinded to group identity.

**Gene expression analysis by qRT-PCR:** RNA was obtained using Trizol reagent (Life Technologies Corporation, Carlsbad, CA) and RNeasy kit (QIAGEN, Valencia, CA). 2 μg of RNA were retro-transcribed using qScript XLT cDNA Supermix synthesis kit (Quanta Biosciences, Gaithersburg, MD). qRT-PCR was performed with PerfeCta SYBR® Green FastMix (Quanta Biosciences, Gaithersburg, MD) in a ViiA7 PCR system (Life Technologies Corporation, Carlsbad, CA). Primers sequences are shown in **Supplemental Table 3**. Results were analyzed with the ΔΔ Ct method using gapdh expression as reference for normalization.
**Supplemental Tables**

Supplemental Table 1: Dose response

<table>
<thead>
<tr>
<th>(Dose)</th>
<th>Sham</th>
<th>Sham-MACI (10)</th>
<th>Sham-MACI (30)</th>
<th>HFpEF</th>
<th>HFpEF-MACI (10)</th>
<th>HFpEF-MACI (30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>24.80±1.01</td>
<td>25.71±0.85</td>
<td>25.38±0.53</td>
<td>24.88±0.77</td>
<td>25.02±0.59</td>
<td>24.86±0.29</td>
</tr>
<tr>
<td>LV/BW</td>
<td>3.37±0.07</td>
<td>3.04±0.07</td>
<td>3.29±0.01</td>
<td>3.77±0.05*</td>
<td>3.60±0.13*</td>
<td>3.43±0.06†</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>111.2±3.6</td>
<td>116.5±4.6</td>
<td>112.9±9.2</td>
<td>137.7±6.9*</td>
<td>133.6±3.1*</td>
<td>131.1±4.4*</td>
</tr>
</tbody>
</table>

Body weight (BW), left ventricle weight to body weight ratio (LV/BW) and systolic blood pressure (SBP) in Sham and HFpEF mice untreated or treated with macitentan (MACI (10): 10mg/kg/day; MACI (30): 30mg/kg/day) for two weeks. Data are means ± SEM. *P≤0.05 vs. respective Sham; †P<0.05 vs. HFpEF. (N=3-6 mice/group).
Supplemental Table 2: Characteristics of Chronic, Stable Patients with HFpEF

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Values</th>
<th>Normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>64±7</td>
<td></td>
</tr>
<tr>
<td>Gender: Male/Female (%)</td>
<td>38/62</td>
<td></td>
</tr>
<tr>
<td>Race:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>31%</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>Other (Asian, Hispanic, Unknown)</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>Etiology of HFpEF:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>Hypertensive/ Non-ischemic/Unknown</td>
<td>97%</td>
<td></td>
</tr>
<tr>
<td>Comorbidities:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obesity: Body Mass Index &gt;30 (kg/m²)</td>
<td>90%</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>87%</td>
<td></td>
</tr>
<tr>
<td>Type 2 Diabetes Mellitus</td>
<td>62%</td>
<td></td>
</tr>
<tr>
<td>Atrial fibrillation/flutter</td>
<td>31%</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>133±25</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>77±9</td>
<td></td>
</tr>
<tr>
<td>Heart Rate (bpm)</td>
<td>75±13</td>
<td></td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>38.75±8.2</td>
<td></td>
</tr>
<tr>
<td>QRS duration (ms)</td>
<td>104±24</td>
<td></td>
</tr>
<tr>
<td>NYHA Functional Class</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>20%</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>43%</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>37%</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Echocardiography:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV EF (%)</td>
<td>63.9±6.3</td>
<td></td>
</tr>
<tr>
<td>Parameter</td>
<td>Value</td>
<td>Normal Range</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>12.1±2.9</td>
<td>7-11</td>
</tr>
<tr>
<td>PW (mm)</td>
<td>11.4±3.1</td>
<td>6-11</td>
</tr>
<tr>
<td>LV EDD (mm)</td>
<td>45.2±6</td>
<td>&lt;57</td>
</tr>
<tr>
<td>LV ESD (mm)</td>
<td>29.6±7</td>
<td>21-40</td>
</tr>
<tr>
<td>RWT</td>
<td>0.52±0.19</td>
<td>0.22–0.42</td>
</tr>
<tr>
<td>LV mass (g)</td>
<td>200.2±54.4</td>
<td>67–162</td>
</tr>
<tr>
<td>PA pressures (mmHg)</td>
<td>41.6±10.8</td>
<td>15-25</td>
</tr>
<tr>
<td>Normal diastolic function (%)</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Grade I diastolic dysfunction (%)</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Grade 2 diastolic dysfunction (%)</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Grade 3 diastolic dysfunction (%)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Indeterminate diastolic function (%)</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

**Medications**

- Diuretics (loop/ thiazide): 100%
- Angiotensin-converting enzyme inhibitors / Angiotensin receptor blockers: 66%
- β-blockers: 73%
- Calcium channel blockers: 43%
- Nitrates: 17%
- Statin: 80%
- Aspirin: 63%

**Laboratory values:**

- cardiac TnI (mg/mL): 0.028±0.035 <0.033
- BNP (pg/mL)*: 156 (66-284)* <100
- Creatinine (mg/dL): 1.46±0.78 0.7-1.3
- MDRD eGFR (mL/min/1.73 m²): 51.5±14 >60
- Sodium (mmol/L): 140±3.5 135-145
- Hemoglobin (g/dL): 12.1±2.4 11.8-16.0
- Glucose (mg/dL): 138±89 70-100
Data are expressed as mean±SD for continuous variables or numbers or percent (%) for
categorical variables with the exception of BNP* which is listed as median (25th-75th
percentile). LVEF: Left Ventricular Ejection Fraction; LV: Left ventricular; IVS: Intraventricular
septal thickness; LVEDD: LV End Diastolic Diameter; LVESD: LV End Systolic Diameter; PW:
Posterior Wall Thickness; RWT: Relative Wall Thickness; NYHA, New York Heart
Association; MDRD eGFR, glomerular filtration rate by Modification of Diet in Renal Disease
equation; BNP, brain natriuretic peptide; TnI, troponin I. Grade I diastolic dysfunction: impaired
relaxation; Grade 2 diastolic dysfunction: pseudonormal filling pattern; Grade 3 diastolic
dysfunction: reversible restrictive filling pattern.
**Supplemental Table 3: Primer sequences for qRT-PCR**

<table>
<thead>
<tr>
<th>MOUSE PRIMERS</th>
<th>forward</th>
<th>reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse-anp</td>
<td>TTCCTCGTCTTGCCCTTTTG</td>
<td>CCTCATCTCTTACCCGGCATC</td>
</tr>
<tr>
<td>mouse-bnp</td>
<td>GTCCAGCAGAGACCTCAAAA</td>
<td>AGGCAGAGTCAGAAACTGGA</td>
</tr>
<tr>
<td>mouse-collagen I</td>
<td>CAGAAGATGTTAGGAGTGCGAG</td>
<td>GGACCCAAGGGAGACCCCTGG</td>
</tr>
<tr>
<td>mouse-collagen III</td>
<td>GTGGACTGCTTGACCTCCA</td>
<td>GGTATCAAAGGCCCAGCTGG</td>
</tr>
<tr>
<td>mouse-tgfβ</td>
<td>CAGAAGTTGGGCATGGTAGCC</td>
<td>TGCTTCAGCTCCACAGAGAA</td>
</tr>
<tr>
<td>mouse-titin variant n2ba</td>
<td>GAGACATTGCTCCTGCTTTTC</td>
<td>GATCTCCAAGAGGCTGTC</td>
</tr>
<tr>
<td>mouse-titin variant n2b</td>
<td>ACAGTGGGAAAGCAGAGACATC</td>
<td>AGGTGGCCCAGAGCTACTT</td>
</tr>
<tr>
<td>mouse-gapdh</td>
<td>CCAAGGTCATCCATGACAACACT</td>
<td>GGGCCATCCACAGTCTTCT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RAT PRIMERS</th>
<th>forward</th>
<th>reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat-gata4</td>
<td>CTGTCATCTCCTATGGGCAC</td>
<td>CCAAGTCCGGAGCAGGAATTG</td>
</tr>
<tr>
<td>rat-mef2a</td>
<td>ACAACCTCTTGCCACGCCC</td>
<td>AGGGGAGCGCCCCATTTCT</td>
</tr>
<tr>
<td>rat-mef2c</td>
<td>GATGGGCATGACAGACAGAGAG</td>
<td>GCCAATGACTGGGCCAGCGACTG</td>
</tr>
<tr>
<td>rat-myocardin</td>
<td>GTTCAGCTACCCCTGGGATGCACCAA</td>
<td>GGCCTGTTTGAGAGAAGAACCAC</td>
</tr>
<tr>
<td>rat-nfat</td>
<td>ACCAGCTCTGTATGGTG</td>
<td>GAGACTTGATAGGGACACC</td>
</tr>
<tr>
<td>rat-pre-pro-et1</td>
<td>GCCAAGCAGACAAAAGAACCTCAGAG</td>
<td>GCTCTGTAGTCAATGCTCGGTT</td>
</tr>
<tr>
<td>rat-gapdh</td>
<td>CTGCACCACCAACTGCTTAG</td>
<td>CTTCTGAGTGGCCAGTGATG</td>
</tr>
</tbody>
</table>


Experimental design: Uninephrectomized C57BL/6J wild-type mice received a continuous infusion of either d-aldosterone (0.3μg/h; HFpEF) or saline (Sham) and were given 1% NaCl water for 4 weeks. Two weeks post-surgery, mice were randomized to receive normal chow or chow containing macitentan (MACI; 30mg/Kg/day). Arrows represent start of the treatment.
Supplemental Figure 2.

Pre-pro-endothelin 1 (pre-pro-et1) mRNA expression in adult rats ventricular myocytes pre-treated with macitentan (MACI; 10μM) and stimulated with aldosterone (ALDO; 1μM). Histogram bars represent the mean±SEM of 5 experiments.
Positive correlation between titin transcript variant N2B and collagen I mRNA expression in Sham and HFpEF mice fed a normal chow or treated with macitentan (MACI: 30mg/Kg/day) for 2 weeks. N=5-8 mice per group.
Supplemental References


