Effects of Fixed-Dose Isosorbide Dinitrate/Hydralazine on Diastolic Function and Exercise Capacity in Hypertension-Induced Diastolic Heart Failure

Richard M. Wilson, Deepa S. De Silva, Kaori Sato, Yasuhiro Izumiya, Flora Sam

Abstract—Hypertension-induced diastolic heart failure accounts for a large proportion of all heart failure presentations. Hypertension also induces left ventricular (LV) hypertrophy. Fixed-dose isosorbide dinitrate/hydralazine (HISDN) decreased mortality in human systolic heart failure but it is unknown whether it improves maladaptive myocardial remodeling. We sought to test the hypothesis that chronic HISDN modulates LV hypertrophy and myocardial remodeling in hypertension-induced diastolic heart failure. FVB mice underwent either saline (n=18) or aldosterone (n=28) infusion. All underwent uninephrectomy and drank 1% salt water for 4 weeks. Mice were randomized after surgery to regular chow or chow containing HISDN (isosorbide dinitrate: 26 mg/kg per day; hydralazine: 50 mg/kg per day) for 4 weeks. Aldosterone infusion increased tail-cuff blood pressure (161±3 mm Hg) versus saline-infused mice (129±2 mm Hg). Aldosterone induced LV hypertrophy versus saline-infused mice (LV:body weight ratio: 4.2±0.1 versus 3.6±0.1 mg/g). HISDN attenuated the aldosterone-induced increased in systolic blood pressure (137±5 mm Hg) and also lowered blood pressure in saline-infused mice (114±2 mm Hg). However, HISDN did not cause LV hypertension regression in aldosterone-infused mice. Aldosterone increased LV end-diastolic dimensions that were not attenuated by HISDN. Similarly, neither aldosterone infusion nor HISDN affected LV end-systolic dimensions. LV ejection fraction and wet:dry lung ratio were not different between aldosterone-untreated and aldosterone-HISDN mice. However, mitral Doppler E/A ratio (a measure of diastolic function), exercise capacity, and plasma soluble vascular cell adhesion molecule 1 levels were improved in aldosterone-HISDN hearts. In conclusion, fixed-dose HISDN improved hypertension, diastolic function, and exercise capacity and reduced soluble vascular cell adhesion molecule 1 levels. There were no reductions in LV hypertrophy, cardiac fibrosis, or pulmonary congestion. These functional improvements are likely related to extracardiac effects, such as effects on the vasculature.

Key Words: diastolic heart failure ■ hydralazine ■ nitrates ■ hypertension ■ exercise capacity

Hypertension induces left ventricular (LV) hypertrophy (LVH) and is a major cause of both diastolic and systolic heart failures (HF).1 Diastolic HF accounts for up to 50% of all HF presentations2 and is associated with increasing morbidity and mortality.3 Diastolic HF refers to the clinical syndrome of pulmonary congestion in the presence of a normal LV ejection fraction, whereas diastolic dysfunction denotes an abnormality of mechanical properties that exist during LV relaxation and filling.4,5 Diastolic dysfunction may be an intermediary between hypertension and HF.6 In the setting of hypertension, diastolic HF represents a diverse clinical syndrome with various associated comorbidities (eg, age and sex) and manifests a spectrum of symptoms ranging from exercise intolerance to acute pulmonary edema. In addition to LVH, cardiac fibrosis, altered myocyte calcium handling,7 and ventricular-vascular stiffening plays a significant role in the pathophysiology of diastolic HF.7

In the African-American Heart Failure Trial (AHeFT), fixed-dose isosorbide dinitrate and hydralazine (HISDN) reduced mortality in black patients with advanced systolic HF.8 The predominant cause of HF in AHeFT was hypertension.9 Benefits of HISDN are partially derived from nitric oxide (NO),10 which results in vasorelaxation, inhibition of cardiac hypertrophy,11 and improved cardiac remodeling.11-13 Unfortunately the use of NO donors is limited by the development of tolerance. With HISDN, the component of hydralazine (a potent antioxidant) is thought to diminish NO consumption by reactive oxygen species14,15 and reactive nitrogen species.16 The survival benefits seen in AHeFT were reported to be attributed to myocardial effects, because LV ejection fraction improved, and LV mass was reduced.17 However, there are no experimental studies thus far that test this hypothesis. We, therefore, sought to test the hypothesis that chronic fixed-dose HISDN modulates LVH and adverse myocardial remodeling in hypertension-induced diastolic HF.
and LV end-systolic dimensions were unaltered by HISDN (Figure 1A and 1B). LV fractional shortening was unchanged and not different between aldosterone-untreated and aldosterone-HISDN hearts (Figure 1C). Consistent with LVH and cardiomyocyte hypertrophy, total wall thickness was increased with aldosterone and unaffected by HISDN (Figure 1D and Table 1).

### HISDN Improved Diastolic Dysfunction
Aldosterone infusion induced diastolic HF (normal LV systolic function and pulmonary congestion; Table 2).\(^{19}\) Mitral valve inflow velocity was determined by Doppler echocardiography. Measurements were standardized at a lower and comparable heart rate in all of the groups to control for loading.\(^{19,20}\) The normal physiological heart rate in mice is rapid (>600 bpm) and results in the loss of the A velocity.\(^{19,20}\) Aldosterone increased the mitral E velocity (early diastolic filling velocity) with minimal change in late diastolic filling (mitral A wave). The resultant E/A ratio (diastolic dysfunction) was, therefore, increased, indicating reduced LV compliance (increased stiffness).

**HISDN Had No Effect on Cardiac Fibrosis and Cytokine Expression**
To determine whether the improvement in diastolic dysfunction was associated with a reduction in cardiac fibrosis, both interstitial and perivascular fibrosis were measured. Total fibrosis (interstitial and perivascular fibrosis) was increased in both groups of aldosterone-infused mice but was not different between aldosterone-untreated and aldosterone-HISDN hearts by quantitative analysis (Figure 2A and 2B).

Aldosterone also induces a proinflammatory response.\(^{21}\) Macrophage infiltration by ED-1 staining was measured and shown to be present in the interstitial areas of the LV. HISDN did not alter ED-1 staining (Figure 3A). Myocardial cytokine mRNA levels and quantitative RT-PCR were performed with specific primer sets with normalization to GAPDH. Aldosterone infusion increased LV interferon-γ expression 2.6-fold (\(P<0.05\)), interleukin (IL) 6 expression 2.8-fold (\(P<0.05\)), and tumor necrosis factor (TNF)-α expression 1.8-fold (\(P<0.01\)) compared with saline infusion (Figure 3B through 3E). HISDN decreased interferon-γ and IL-6 transcripts (\(P<0.05\) versus aldosterone untreated) with no effect on TNF-α expression. LV IL-1β expression was negligibly altered by aldosterone infusion or therapy with HISDN (Figure 3E).

### HISDN and Fetal Gene Re-Expression
Aldosterone infusion increased myocardial atrial natriuretic peptide (ANP) mRNA expression by \(\approx2.0\)-fold (\(P<0.05\)

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**Table 1. Characteristics of Untreated and HISDN-Treated Mice 4 Weeks After Saline or Aldosterone Infusion**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Untreated</th>
<th>HISDN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Aldosterone</td>
</tr>
<tr>
<td>No.</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>28.1±0.9</td>
<td>28.4±0.7</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>129±2</td>
<td>161±3††</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>729±5</td>
<td>690±9</td>
</tr>
<tr>
<td>LV/BW, mg/g</td>
<td>3.6±0.1</td>
<td>4.2±0.1††</td>
</tr>
<tr>
<td>Lung, wet: dry ratio</td>
<td>4.3±0.2</td>
<td>5.4±0.5§§</td>
</tr>
<tr>
<td>Myocyte cross-sectional area, (\mu m^2)</td>
<td>180±24</td>
<td>380±38*</td>
</tr>
</tbody>
</table>

Data are represented as mean±SEM.

\(^*P<0.01\) vs saline-untreated.

\(^†P<0.0001\) vs saline-untreated.

\(^‡P<0.01\) vs saline-HISDN.

\(^§P<0.05\) vs saline-untreated.

\(\|P<0.001\) vs aldosterone-untreated.

\(^††P<0.05\) vs aldosterone-untreated.

\(^#P<0.0001\) vs saline-HISDN.

\(^**P<0.05\) vs saline-untreated.

\(^†††P<0.001\) vs saline-untreated.
versus saline infusion) and had negligible effects on SERCA2 mRNA expression. Both ANP and sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2) transcripts were decreased significantly in aldosterone-HISDN hearts (P<0.05 and P<0.001, respectively, versus aldosterone untreated; Figure 4A through 4B).

**HISDN Improved Exercise Capacity**

Exercise limitation occurs in human diastolic HF, and “quality-of-life” scores were improved in AHeFT with HISDN therapy. We sought to determine whether diastolic dysfunction improvement was associated with improved functional outcome by measuring exercise capacity in these mice. Exercise capacity (determined by running distance) was measured in a blinded manner. There were no differences between the saline-untreated and saline-HISDN groups (Figure 5A). Exercise capacity was impaired in aldosterone-untreated mice (P<0.05 versus saline infusion). HISDN improved exercise capacity by \( \approx 40\% \) in aldosterone-infused mice (P<0.05 versus aldosterone untreated). Running time was also increased in aldosterone-HISDN (999±68 seconds) versus aldosterone-untreated (776±47 seconds; P<0.05) mice.

**HISDN Improved Vascular Inflammation**

Plasma soluble vascular cell adhesion molecule (sVCAM-1; a marker of vascular inflammation) was increased in hypertensive, untreated mice with diastolic HF (P<0.01 versus saline

![Figure 1. In vivo transthoracic echocardiography measurements with or without fixed-dose HISDN. Two- and 4-week measurements. A, LV end-diastolic diameter (LVEDD); (B) LV end-systolic diameter (LVESD); (C) fractional shortening (FS); and (D) total wall thickness (TWT). Saline-untreated (●), aldosterone-untreated (■), saline-HISDN (□), and aldosterone-HISDN (■) mice. (P<0.05 vs wild-type saline; †P<0.01 vs wild-type saline). Data are mean±SEM; n=5 to 9 per group. *P<0.05 vs saline-HISDN at 4 weeks; ‡P<0.001 vs saline-untreated at 4 weeks; †P<0.001 vs saline-HISDN at 2 weeks; ‡P<0.01 vs saline-HISDN at 4 weeks. E, Representative mitral Doppler inflow pattern: an aldosterone-infused mouse on no HISDN and an aldosterone-infused mouse on HISDN. E indicates early ventricular filling; A, late filling caused by atrial contraction; IVRT, isovolumetric contraction time; DT, deceleration time.
Table 2. Measurements of Diastolic Function by Mitral Doppler Imaging in Untreated and HISDN-Treated Mice After 4 Weeks of Saline- or Aldosterone Infusion

<table>
<thead>
<tr>
<th>Groups</th>
<th>Saline (N=5)</th>
<th>Aldosterone (N=7)</th>
<th>Saline (N=5)</th>
<th>Aldosterone (N=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>5</td>
<td>7</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Mitral E velocity, mm/s</td>
<td>623±29</td>
<td>1026±44**</td>
<td>545±25</td>
<td>768±77†*</td>
</tr>
<tr>
<td>Mitral A velocity, mm/s</td>
<td>400±20</td>
<td>456±20</td>
<td>374±12</td>
<td>553±35†*</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.6±0.1</td>
<td>2.3±0.1**</td>
<td>1.5±0.1</td>
<td>1.4±0.1†</td>
</tr>
<tr>
<td>IVRT, ms</td>
<td>20±0.9</td>
<td>28±1.9**</td>
<td>20±1.1</td>
<td>13.5±0.8§‡</td>
</tr>
<tr>
<td>DT, ms</td>
<td>23±1</td>
<td>21±0.5</td>
<td>22±1</td>
<td>15.5±1.1¶</td>
</tr>
</tbody>
</table>

Data are represented as mean±SEM.
*P<0.01 vs aldosterone-untreated.
†P<0.05 vs saline-HISDN.
‡P<0.0001 vs aldosterone-untreated.
§P<0.001 vs saline-HISDN.
¶P<0.01 vs saline-HISDN.
*P<0.01 vs aldosterone-untreated.
**P<0.001 vs saline-untreated.

Exercise intolerance is seen in diastolic HF; therefore, exercise capacity was determined in the mice. The improved exercise capacity seen in our study may be because of the following: (1) a reduction in SBP despite the presence of LVH; (2) an improvement in diastolic dysfunction; and (3) a decrease in vascular inflammation/stiffness. Although not directly addressed in our study, the effect of HISDN was not limited to the myocardium. Vascular-ventricular stiffening during exercise (or other stressors) may lead to an exaggerated hypertensive response and further load-dependent diastolic dysfunction. Therefore, it is possible that HISDN enhanced vascular and ventricular interactions (that affect LV filling and ejection) and, thus, improved diastolic dysfunction and exercise capacity.

HISDN and Diastolic Dysfunction

Diastolic dysfunction is an independent risk factor for HF and cardiovascular death. If diastolic dysfunction is considered a “preclinical” diagnosis, its early recognition may represent an important way to reduce the incidence of congestive HF. Conversely, diastolic dysfunction may also be an early marker of cardiac end-organ damage in hypertension that precedes the development of LVH.

The presence of hypertension also increases afterload and decreases early diastolic filling and myocardial lengthening. Increased preload and afterload affect the LV relaxation rate, and these become more pronounced during neurohormonal stimulation (eg, aldosterone infusion or during exercise), which then further exacerbates diastolic dysfunction. In our study, SBP reduction improved diastolic dysfunction and exercise capacity. In humans, diastolic dysfunction need not be present to meet the definition of diastolic HF. Similarly, diastolic dysfunction may herald the development of diastolic HF or systolic HF. We cannot exclude the possibility that, if mice were followed for a longer period, improvement in diastolic dysfunction may precede potential decreases in pulmonary congestion (ie, diastolic HF) with HISDN therapy. Thus, recognition of the beneficial effects of HISDN on Doppler diastolic indices and exercise capacity may potentially translate into relief of pulmonary congestion because of vasodilatory effects of HISDN on the vasculature.

Discussion

In this study, aldosterone-induced hypertension resulted in LVH, diastolic dysfunction, and diastolic HF. Fixed-dose HISDN lowered SBP and improved diastolic dysfunction and exercise capacity. It also decreased plasma sVCAM-1 (a marker of vascular inflammation) and myocardial interferon-γ and IL-6 expression. HISDN did not ameliorate LVH or cardiomyocyte size. Similarly pulmonary congestion and cardiac fibrosis remained unaffected.

HISDN and Exercise

Exercise intolerance is seen in diastolic HF; therefore, exercise capacity was determined in the mice. The improved
Characterizing LV diastolic properties in humans is usually done by measuring pressure and volume with high-fidelity micromanometers. In our study, the Vevo 770 High-Resolution In Vivo Imaging System provided the measures of murine diastolic function. Mitral Doppler measurements are good measures of diastolic function in mice. In a subset of mice, increased LVEDP correlated with an increased E/A ratio (data not shown). SBP control and LVH regression improve LV diastolic filling; however, LVH regression was not seen in our study. Once again, it is possible that, if mice were followed for an extended period, SBP control with HISDN and improved diastolic dysfunction may precede potential reductions in cardiac mass. Equally plausible is that a delay in measurable change in structure may allow functional changes to precede structural changes.

HISDN and Cardiac Remodeling
Controversy surrounds the roles of LVH and HF. LVH regression, an intermediary between hypertension and HF, may prevent both systolic and diastolic HFs. Conversely, although LVH increases the risk of HF, hypertensive subjects without LVH may also develop HF, suggesting that targeting LVH may be unnecessary to prevent HF. The effect of LVH regression on diastolic dysfunction, one of the earliest changes in hypertension, is unclear. Even in the absence of LVH, SBP control improves diastolic filling irrespective of the regimen used. It is unclear why LVH regression was not seen in the present study, but this indicates that LVH and hypertension may not have a causal relationship in this model. Furthermore, study duration may have been insufficient.

Figure 3. Myocardial inflammation. A, Representative ED-1 staining for macrophages in cross-sections of the LV at 4 weeks. Photomicrographs of LV sections from the 4 groups: saline-untreated, saline HISDN, aldosterone-untreated, and aldosterone-HISDN. Black arrows indicate the presence of immunostaining specific for the ED-1 antibody in both groups of aldosterone-infused hearts (magnification: ×200). Increased myocardial cytokine expression in aldosterone-untreated hearts: (B) interferon-γ, (C) IL-6, and (D) TNF-α expression. E, IL-1β expression was not significantly increased. HISDN decreased interferon-γ and IL-6 expression but not TNF-α expression in aldosterone-infused mice. *P<0.05 vs saline-untreated; **P<0.01 vs saline-untreated; †P<0.05 vs saline-HISDN; n=4 to 5 per group.

Figure 4. Molecular markers of cardiac remodeling. A, ANP gene expression by RT-PCR is significantly increased in untreated aldosterone-infused hearts. Chronic therapy with HISDN reduced myocardial ANP expression; *P<0.05 vs saline-untreated. B, SERCA2 expression in the hearts was not significantly altered in untreated aldosterone-infused hearts. SERCA2 expression was decreased with HISDN; P<0.001. Data are mean±SEM; n=4 to 5 per group.
Myocardial Fibrosis and Extracellular Matrix Remodeling

Myocardial fibrosis is closely related to diastolic stiffness. Aldosterone-induced fibrosis was unaltered by HISDN, but collagen and matrix metalloproteinases were not measured. It has been suggested that total collagen is unaltered in diastolic dysfunction. Posttranslational modification of myocardial collagen occurs, and local formation of advanced glycation end products-collagen links correlate with LV stiffness. Myocardial fibrosis may contribute equally to LVH in diastolic HF. In the present study, HISDN had negligible effects on the extracellular “milieu” of the aldosterone-infused myocardium despite improvements in the E/A ratio. Thus, neither a reduction in myocardial fibrosis nor a decrease in LVH contributed to the improved diastolic dysfunction seen in aldosterone-induced diastolic HF, suggesting possible “extramyocardial” effects such as a reduction in vascular inflammation.

HISDN and Cytokines

Myocardial interferon-γ, IL-6, and TNF-α expressions were increased in hypertension-induced diastolic HF. HISDN only decreased interferon-γ and IL-6 expression. Chronic NO (with HISDN) may directly reduce interferon-γ and IL-6 expression, or these decreased cytokines may simply reflect improved diastolic function. IL-6 has been shown to be prohypertrophic. It is interesting that TNF-α expression remains unaffected by HISDN. It may be that concurrent TNF-α expression and the presence of myocardial NO may foster self-sustaining positive autocrine/paracrine feedback inflammatory circuits in diastolic HF.

HISDN and Vascular Inflammation

Vascular inflammation, as reflected by sVCAM-1 levels, was elevated in hypertensive, aldosterone-infused mice. sVCAM levels were decreased with HISDN. In humans, sVCAM-1 is significantly associated with asymmetrical dimethylarginine, an important endogenous NO synthase inhibitor. Similarly, NO synthesis inhibition is associated with an increase in endothelial adhesion molecule expression. Thus, HISDN likely exerts beneficial effects on the vasculature.

Limitations

First, previous experimental studies using combination NO and hydralazine measured only acute hemodynamic effects. There are no human or animal studies that address...
the effect of combined HISDN (which is used clinically) on diastolic dysfunction and diastolic HF either acutely or chronically. Second, few animal models can successfully replicate human diastolic disease, and a single-cell study is limited in its inability to mimic diastolic disease given the importance of the vasculature (in preload and afterload), heart rate, chamber geometry, and extracellular matrix in diastology. Therefore, this model of hypertension-induced diastolic HF is a clinically relevant model. It encompasses the myocardium, potential effects of the vasculature, and exercise impairment. Third, although Millar catheterization was not performed in all of the mice, LVEDP correlated with the E/A ratio. Echocardiography allowed us to follow the mice longitudinally. Fourth, this study was performed exclusively in male mice. The prevalence of diastolic HF is greatest in elderly female mice. A future study proposes to study aged, female mice. Finally, although not directly addressed in the present study, our findings suggest that HISDN effects were not limited exclusively to the myocardium, and the improvements seen were likely attributed to effects on the vasculature and interactions of vascular and ventricular stiffness. Our findings represent not so much a change in diastolic function as a change in myocardial load induced by alterations in vascular inflammation and/or stiffness because of HISDN.

In conclusion, HISDN did not prevent the progression to LVH or diastolic HF. LVH regression is unnecessary for the improvement in exercise capacity and diastolic dysfunction in hypertension. It is possible that improvement in ventricular inflammation/stiffness results in enhanced diastolic function and will require further study.

Perspectives
Diastolic dysfunction may precede the development of LVH in hypertension and is the connection between hypertension and diastolic HF. Although a lack of therapies continues to plague diastolic HF patients, understanding mechanisms primarily responsible for this clinical syndrome and its relationship to hypertension and diastolic dysfunction is important. Unlike human systolic HF studies, our findings indicate that HISDN does not exert direct myocardial effects but rather influences ventricular-vascular interactions in diastolic HF. Additional studies are warranted to determine whether LVH regression is beneficial in hypertension-mediated diastolic dysfunction and to determine causality in diastolic dysfunction and cardiac remodeling.

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Disclosures
None.

References


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Expanded Materials And Methods

Aldosterone infusion. Uninephrectomized mice (25-27g) received osmotic minipumps (Alzet, Durect Corporation) that delivered a continuous infusion of saline or \(d\)-aldosterone (0.15 \(\mu\)g/hour) (Sigma-Aldrich Co.) for 4 weeks\(^1\). All mice were maintained on 1% NaCl drinking water.

Treatment. Forty-six mice were randomly assigned on the same day as the surgery to either regular chow or chow containing hydralazine: 50mg/kg/day and isosorbide dinitrate (ISDN): 26mg/kg/day, the combination known as HISDN, for 4 weeks. The dose of hydralazine and ISDN has been used by others\(^2-4\) and shown to be non-toxic. This combination maintains the same dose ratio that was used in A-HeFT\(^5\) i.e., the ISDN:hydralazine HCl ratio was 20mg:37.5mg. The 4 groups studied were: a) saline-untreated, n=9; b) saline-HISDN, n=9; c) aldosterone-untreated, n=14 and d) aldosterone-HISDN, n=14.

Physiological measurements. Heart rate (HR) and tail cuff blood pressure (SBP) were determined non-invasively (BP-2000, VisiTech) as previously described\(^1,6\). Transthoracic echocardiography was performed in conscious mice biweekly after surgery using the Acuson Sequoia C-256 echocardiograph machine with a 15-MHz probe, as previously described\(^1\). Total wall thickness (TWT) was derived from an average of the interventricular septum and posterior wall thickness.

Doppler echocardiography. Mitral Doppler flow study was performed using the Vevo 770 High-Resolution In Vivo Imaging System (VisualSonics,Toronto). Images were acquired using a high-resolution (30 MHz) transducer. Mice were anesthetized using isoflurane (0.5-1.5%) and titrated to achieve a HR around 350 beats/min since diastolic function measurement are sensitive to HR and loading conditions. The maximum 1.5% of isoflurane has minimal effects on diastolic function\(^7\). Images were recorded for 30–40 cardiac cycles and measurements were made from 3–5 representative cycles. The apical four-chamber view was used to record the mitral Doppler flow spectrum. Peak early (E) and late (A) mitral inflow velocities, deceleration time of early filling (DT) and isovolumetric relaxation time (IVRT) were measured as previously described\(^8\).

In a subset of mice (n=5), hemodynamic measurements were performed 4 weeks after aldosterone infusion using a 1.4F catheter tip micromanometer (ARIA, Millar Instruments). Aldosterone infusion resulted in an increased LV end-diastolic pressure (EDP). Increased LVEDP correlated with the increased E/A ratio, \(R=0.95\) (\(P<0.05\); data not shown).

Exercise Treadmill analysis. Exercise capacity was measured on a rodent motor-driven treadmill (Columbus Instruments) as previously described\(^9\). Mice were acclimated for at least 3 days (15% incline, speed of 15 m/min for 10 minutes and subsequently increased 2 m/min each 2 minutes, ending after 20 minutes). Total exercise time was recorded as the elapsed time to exhaustion and then converted to distance. Exhaustion was determined by an observer blinded to treatment groups and was defined as the point at which the animals could not keep pace with the treadmill and no longer avoided the electrical stimulus.

Organ weight, tissue and blood analysis. After 4-weeks mice were sacrificed, at which time blood was obtained to determine plasma soluble vascular cell adhesion molecule (VCAM-1) levels (R&D Systems). Body weight (BW), heart weight (HW) and LV weights were also determined. Hearts were
either: (a) arrested in diastole by KCl (30mmol/l), weighed, perfused with 10% buffered formalin and sliced horizontally for histology or (b) snap-frozen in liquid nitrogen. Trichrome-stained sections (5μm) were visualized by light microscopy to measure fibrosis and the entire section was quantified using Bioquant Image analysis software. The wet-to-dry lung weight ratios were determined as an index of pulmonary congestion.

**Determination of mRNA.** Total RNA from 4 groups of mice hearts were extracted with PureLink™ Micro-to-Midi Total RNA purification system (Invitrogen). cDNA synthesis from total RNA was performed using a Thermoscript™ RT-PCR system (Invitrogen) according to the manufacturer’s instructions. Transcript expression levels of ANP, SERCA-2, IL-1β, IL6, INF-γ, TNF-α and GAPDH were quantified by iCycler iQ Real-Time PCR Detection Systems (BIO-RAD) using FastStart Universal SYBR Green Master Mix (Roche, IN, USA). Transcript levels were adjusted relative to the expression of GAPDH. Sequences of PCR primers are as listed.

**Primer sequence for mouse** are as follows: **ANP** (F): 5'-GAG AGA CGG CAG TGC TTC TAG GC-3', ANP (R): 5'-CGT GAC ACA CCA CAA GGC CTT AGG-3'; **SERCA2** (F): 5'-TAC TGA CCC TGT CCC TGA CC-3'; (R): 5'-CAC CAC CAC TCC CAT AGC TT-3'; **IL-1β** (F): 5'-AGA CAC AGA TTC CAT GGT GAA GT-3', IL-1β (R): 5'-TCT CAG CTT CAA TGA AAG ACC TC-3'; **IL-6** (F): CCC AAT TTC CAA TGC TCT CCT-3, IL-6 (R): TAA CGC ACT AGG TTT GCC GAG; **INFγ** (F): 5'-CAT GGC TGT TTC TGG CTG TTA C- 3', IFNγ (R): 5'-CCA GTT CCT CCA GAT ATC CAA GA-3'; **TNF-α** (F): 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3', (R): 5'-TGG GAG TAG ACA AGG TAC AAC CC-3', **GAPDH** (F): 5'-TCA CCA CCA TGG AGA AGG-3', GAPDH (R): 5'-GCT AAG CAG TTG GTG GTG CA-3'.

**Immunohistochemistry.** To visualize macrophages, ED-1 staining was used as previously described. Sections were deparaffinized, rehydrated, and treated with 20μg proteinase K per mL of Tris-HCl (pH 8.5) for 25min at room temperature to recover antigenicity. Sections were then stained with a 1:25 dilution of rat anti-mouse cd68 primary antibody (Serotec, Raleigh, NC) in PBS with 1% BSA overnight at 4°C. Nonspecific binding was blocked by incubation with 10% horse serum in PBS (pH 7.4) for 30min before incubation with the antibody. A biotinylated anti-rat antibody (Vector) was used as secondary antibody and Vector Red alkaline phosphatase substrate (Vector) was applied. Sections were visualized under bright-field microscopy and images were recorded using an Optronics camera with Bioquant hardware and software.

**Statistical analysis.** Results are presented as mean±SEM. Statistical analyses of the data were carried out using the Student’s t test (2-sided). When necessary, 1- or 2-way ANOVA (followed by Student-Newman-Keuls post-hoc tests when appropriate) was applied. A value of *P*<0.05 was considered statistically significant.
Reference for Online Methods


