Effects of Adiponectin on Calcium-Handling Proteins in Heart Failure With Preserved Ejection Fraction

Komei Tanaka, MD, PhD; Richard M. Wilson, BA; Eric E. Essick, PhD; Jennifer L. Duffen, PhD; Philipp E. Scherer, PhD; Noriyuki Ouchi, MD, PhD; Flora Sam, MD

Background—Despite the increasing prevalence of heart failure with preserved ejection fraction (HFpEF) in humans, there remains no therapeutic options for HFpEF. Adiponectin, an adipocyte-derived cytokine, exerts cardioprotective actions, and its deficiency is implicated in the development of hypertension and HF with reduced ejection fraction. Similarly, adiponectin deficiency in HFpEF exacerbates left ventricular hypertrophy, diastolic dysfunction, and HF. However, the therapeutic effects of adiponectin in HFpEF remain unknown. We sought to test the hypothesis that chronic adiponectin overexpression protects against the progression of HF in a murine model of HFpEF.

Methods and Results—Adiponectin transgenic and wild-type mice underwent uninephrectomy, a continuous saline or aldosterone infusion and given 1.0% sodium chloride drinking water for 4 weeks. Aldosterone-infused wild-type mice developed HFpEF with hypertension, left ventricular hypertrophy, and diastolic dysfunction. Aldosterone infusion increased myocardial oxidative stress and decreased sarcoplasmic reticulum Ca2+-ATPase protein expression in HFpEF. Although total phospholamban protein expression was unchanged, there was a decreased expression of protein kinase A–dependent phospholamban phosphorylation at Ser16 and CaMKII (Ca2+/calmodulin-dependent protein kinase II)-dependent phospholamban phosphorylation at Thr17. Adiponectin overexpression in aldosterone-infused mice ameliorated left ventricular hypertrophy, diastolic dysfunction, lung congestion, and myocardial oxidative stress without affecting blood pressure and left ventricular EF. This improvement in diastolic dysfunction parameters in aldosterone-infused adiponectin transgenic mice was accompanied by the preserved protein expression of protein kinase A–dependent phosphorylation of phospholamban at Ser16. Adiponectin replacement prevented the progression of aldosterone-induced HFpEF, independent of blood pressure, by improving diastolic dysfunction and by modulating cardiac hypertrophy.

Conclusions—These findings suggest that adiponectin may have therapeutic effects in patients with HFpEF. (Circ Heart Fail. 2014;7:976-985.)

Key Words: adiponectin, calcium-binding proteins, heart failure, diastolic, hypertrophy, left ventricular, oxidative stress

Heart failure with preserved ejection fraction (HFpEF), also known as diastolic HF, is a clinical syndrome characterized by signs and symptoms of HF with preservation of left ventricular EF (LVEF).1 HFpEF accounts for up to 50% of all patients presenting with HF;2 yet there remains no therapies for HFpEF. In addition to associated comorbidities, there are likely many divergent pathophysiological mechanisms similar to HF with reduced EF. HFpEF is highly prevalent in obese individuals,3,4 and hypertension remains the major cause of HFpEF.5-5 Emerging evidence also indicates that factors secreted by adipocytes play a role in hypertension-related diseases,6,8 such as hypertension, coronary artery disease, obesity, and insulin resistance.9-12 As such in experimental models, adiponectin deficiency exacerbates the development of obesity-related hypertension,13 adverse cardiac remodeling14,15 in ischemia-reperfusion injury,16 and myocardial infarction.17 Recently, we showed that lack of adiponectin in a murine model of HFpEF/diastolic HF increased the propensity to develop diastolic HF and diastolic dysfunction.18 Although hypoadiponectinemia in aldosterone-induced HFpEF exacerbates hypertension, LVM, diastolic dysfunction, and HF,19 the pathophysiological role and therapeutic effects of adiponectin repletion in HFpEF are unknown. We thus sought to test the hypothesis that chronic adiponectin overexpression protects against the progression of HFpEF and sought to investigate the proposed mechanism.
Methods

An expanded Materials and Methods are available in the Data Supplement. 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. Adiponectin transgenic (APNTG) and wild-type (WT) mice in a C57BL/6J background were generated as previously described.19

Experimental Model

Twelve-week-old APNTG mice and WT littermates were anesthetized with pentobarbital sodium (50 mg/kg IP). They underwent uninephrectomy and intraperitoneal implantation of osmotic mini-pumps (Durect Corp, Cupertino, CA) that delivered a continuous infusion of either saline or 0.15 μg/h D-aldosterone (Sigma-Aldrich Co, St. Louis, MO) for 4 weeks (see Data Supplement for mice groups).

Physiological Measurements

Heart rate and blood pressure (BP) were measured weekly using a noninvasive tail-cuff BP analyzer, BP-2000 Blood Pressure Analysis System (Visitech Systems, Inc, Apex, NC).18,20

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 (Visual Sonic Inc, Toronto, Ontario, Canada).18,20 For details of LV structure, function, and Doppler measurements see Data Supplement.

Mice were euthanized 4 weeks after saline or aldosterone infusion, and biomarker, organ weight, tissue analysis, and cardiomyocyte sizes were determined. Myocardial oxidative stress by 3-nitrotyrosine staining and immunoblotting of calcium-handling proteins and signaling pathway were also measured. Quantitative reverse transcriptase-polymerase chain reaction for atrial natriuretic peptide mRNA expression was also determined (see Data Supplement for all details).

Statistical Analysis

Data are expressed as mean±SEM. For comparisons of multiple groups, Kruskal–Wallis 1-way ANOVA was performed with a post hoc Dunn test for multiple comparisons. Paired data were evaluated by Mann–Whitney test. P<0.05 values were considered significant.

Table 1. Characteristics of WT/APNTG Mice 4 Weeks After Saline/Aldosterone Infusion

<table>
<thead>
<tr>
<th>Groups</th>
<th>WT-Saline (n=7)</th>
<th>APNTG-Saline (n=7)</th>
<th>WT-Aldo (n=15)</th>
<th>APNTG-Aldo (n=15)</th>
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</thead>
<tbody>
<tr>
<td>General</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>21.9±1.2</td>
<td>22.5±2.4</td>
<td>22.5±0.7</td>
<td>24.2±1.0</td>
</tr>
<tr>
<td>Heart rate, beats per min</td>
<td>676±13</td>
<td>679±18</td>
<td>654±13</td>
<td>675±12</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>105±2</td>
<td>100±2</td>
<td>127±3*</td>
<td>125±4†</td>
</tr>
<tr>
<td>Heart weight/body weight, mg/g</td>
<td>4.35±0.78</td>
<td>4.42±0.21</td>
<td>5.69±0.15*</td>
<td>5.14±0.07††</td>
</tr>
<tr>
<td>Wet-lung weight/dry-lung weight</td>
<td>3.97±0.06</td>
<td>3.99±0.11</td>
<td>4.48±0.05*</td>
<td>4.25±0.06§</td>
</tr>
<tr>
<td>Fibrosis area, %</td>
<td>1.0±0.1</td>
<td>0.9±0.2</td>
<td>6.7±0.7*</td>
<td>4.3±0.8§</td>
</tr>
<tr>
<td>Blood chemical analysis</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Serum aldosterone levels, pg/mL</td>
<td>626.0±66.4</td>
<td>810.7±85.9</td>
<td>6542.6±324.2*</td>
<td>5317.4±551.7†</td>
</tr>
<tr>
<td>Serum adiponectin levels, μg/mL</td>
<td>14.5±1.3</td>
<td>27.0±1.0*</td>
<td>14.0±0.9</td>
<td>29.2±0.3††</td>
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</tbody>
</table>

Data are expressed as mean±SEM. Aldo indicates aldosterone; APNTG, adiponectin transgenic; and WT, wild-type. *P<0.01 vs WT-saline, †P<0.01 vs APNTG-saline, ‡P<0.05 vs WT-aldo, §P<0.05 vs APNTG-saline.

Table 2. Echocardiographic Parameters of WT/APNTG Mice 4 Weeks After Saline/Aldosterone Infusion

<table>
<thead>
<tr>
<th>Group</th>
<th>WT-Saline (n=7)</th>
<th>APNTG-Saline (n=7)</th>
<th>WT-Aldo (n=15)</th>
<th>APNTG-Aldo (n=15)</th>
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</thead>
<tbody>
<tr>
<td>LV structure and systolic function</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TWT, mm</td>
<td>0.75±0.01</td>
<td>0.73±0.01</td>
<td>1.04±0.02*</td>
<td>0.93±0.02†‡</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.42±0.06</td>
<td>3.44±0.08</td>
<td>3.49±0.08</td>
<td>3.34±0.23</td>
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<tr>
<td>LVESD, mm</td>
<td>1.95±0.06</td>
<td>2.04±0.12</td>
<td>2.03±0.07</td>
<td>1.95±0.09</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>75.12±1.59</td>
<td>73.83±1.65</td>
<td>73.96±0.93</td>
<td>75.69±1.62</td>
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<tr>
<td>LV mass, mg</td>
<td>83.40±2.26</td>
<td>81.38±3.72</td>
<td>140.4±6.52*</td>
<td>110.9±12.69§‖</td>
</tr>
<tr>
<td>Diastolic function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak E, mm/s</td>
<td>654.83±13.79</td>
<td>677.60±14.50</td>
<td>1008.73±27.19*</td>
<td>873.75±20.96†‡</td>
</tr>
<tr>
<td>Peak A, mm/s</td>
<td>454.04±9.37</td>
<td>449.61±15.34</td>
<td>490.50±40.89</td>
<td>591.24±34.94§</td>
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<tr>
<td>E/A</td>
<td>1.45±0.04</td>
<td>1.51±0.04</td>
<td>2.17±0.17*</td>
<td>1.50±0.09†‡</td>
</tr>
<tr>
<td>DT, ms</td>
<td>22.08±1.00</td>
<td>21.88±0.63</td>
<td>15.56±1.00*</td>
<td>20.42±0.77†‡</td>
</tr>
<tr>
<td>IVRT, ms</td>
<td>19.58±0.77</td>
<td>20.63±1.88</td>
<td>19.72±0.77</td>
<td>20.00±1.12</td>
</tr>
<tr>
<td>e’, mm/s</td>
<td>27.26±1.25</td>
<td>28.26±1.56</td>
<td>17.81±0.33*</td>
<td>20.55±0.69†‡</td>
</tr>
<tr>
<td>E/e’</td>
<td>24.17±0.72</td>
<td>24.14±1.02</td>
<td>56.84±1.96*</td>
<td>42.67±1.28†‡</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM. Indicates late; APNTG, adiponectin transgenic; DT, early filling deceleration time; E, early; e’, peak early diastolic myocardial velocity; IVRT, isovolumic relaxation time; LVEF, left ventricular ejection fraction; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; TWT, total wall thickness; and WT, wild-type. *P<0.01 vs WT-saline, †P<0.01 vs APNTG-saline, ‡P<0.01 vs WT-aldo, §P<0.05 vs APNTG-saline, ‖P<0.05 vs WT-aldo.
All statistical analyses were performed using Graph Pad Prism (GraphPad Software, Inc, La Jolla, CA).

**Results**

**General Characteristics**

There were no deaths in the mice during the 4-week period. Characteristics of WT and APNTG mice 4 weeks after saline or aldosterone infusion are summarized in Table 1. Body weights were comparable between WT and APNTG mice regardless of saline or aldosterone infusion.

**Hemodynamic Parameters**

Heart rates were comparable between WT and APNTG mice regardless of saline or aldosterone infusion (Table 1). Systolic BP was measured weekly (Figure I in the Data Supplement) and was significantly increased by 4 weeks of aldosterone infusion in WT-aldosterone (127±3 versus 105±2 mmHg; P<0.01) and APNTG-aldosterone mice (125±4 versus 100±2 mmHg; P<0.01) when compared with their respective saline-infused controls. There was no difference in systolic BP between the aldosterone-infused WT and APNTG mice.

**Serum Aldosterone Levels**

Serum aldosterone levels in WT-aldosterone (6542.6±324.2 versus 626.0±66.4 pg/mL; P<0.01) and APNTG-aldosterone mice (5317.4±551.7 versus 810.7±85.9 pg/mL; P<0.01) were significantly elevated when compared with respective saline-infused mice. There was no difference in serum aldosterone levels between WT and APNTG mice regardless of saline or aldosterone infusion (Table 1).

**Serum Adiponectin Levels**

Serum adiponectin levels in APNTG-saline mice (27.0±1.0 μg/mL) were approximately 1.9-fold higher than in WT-saline mice (14.5±1.3 μg/mL; P<0.01). Serum adiponectin levels were significantly elevated in APNTG-aldosterone mice (27.0±1.0 μg/mL) compared to saline-infused controls (14.5±1.3 μg/mL; P<0.01). There was no difference in serum adiponectin levels between WT and APNTG mice regardless of saline or aldosterone infusion (Table 1).

**Figure 1.** Representative pulse wave and tissue Doppler images in wild-type (WT) and adiponectin transgenic (APNTG) mice 4 weeks after saline or aldosterone infusion. A, WT-saline; B, WT-saline, APNTG-saline, WT-aldosterone, APNTG-aldosterone. A indicates peak late transmitral flow velocity; DT, early filling deceleration time; E, peak early transmitral flow velocity; e', peak early diastolic myocardial velocity; and IVRT, isovolumetric relaxation time.
elevated in APNTG-aldosterone mice (29.2±0.3 μg/mL) versus APNTG-saline mice (27.0±1.0 μg/mL;  *P* <0.05). There was no difference in adiponectin levels between WT-saline and WT-aldosterone mice (Table 1).

**LV Structure and Systolic Function**

Echocardiographic parameters for LV structure and systolic function are summarized in Table 2. Aldosterone infusion significantly increased total wall thickness and LV mass in WT-aldosterone (1.04±0.02 mm and 140.4±6.5 mg) and APNTG-aldosterone mice (0.93±0.02 mm and 110.9±12.7 mg). Consistent with these findings, the heart weight to body weight ratio was also significantly increased in the WT-aldosterone (5.69±0.15) and APNTG-aldosterone mice (5.14±0.07; Table 1). There were no significant differences in cardiac hypertrophy between WT-saline and APNTG-saline mice (Table 1 and 2). However, cardiac hypertrophy in APNTG-aldosterone mice was significantly less versus WT-aldosterone mice ( *P* <0.01 for total wall thickness;  *P* <0.05 for LV mass and  *P* <0.05 for heart weight to body weight). There was no difference in LV chamber size and LVEF between the WT and APNTG mice regardless of saline or aldosterone infusion (Table 2).

**Diastolic Function**

Echocardiographic parameters (mitral and tissue Doppler) for LV diastolic function in the mice are summarized in Table 2 and Figure 1A and 1B.

**Mitral Doppler**

Aldosterone infusion significantly increased peak E velocity in the WT-aldosterone versus WT-saline mice and in APNTG-aldosterone versus APNTG-saline mice (Table 2; Figure 1B). The peak E velocity in APNTG-aldosterone mice was, however, significantly lower than in WT-aldosterone mice ( *P* <0.01). Aldosterone infusion significantly increased peak A velocity in APNTG-aldosterone versus APNTG-saline mice but not in the WT-aldosterone versus WT-saline mice ( *P* =NS). The resultant ratio of peak E velocity to peak A velocity (E/A) was significantly higher in WT-aldosterone (2.17±0.17) versus WT-saline mice (1.45±0.04;  *P* <0.01). This increase in E/A ratio in WT-aldosterone mice, indicating
impaired LV compliance, was attenuated in APNTG-aldosterone mice (1.50±0.09; P<0.01). Deceleration time was significantly shortened in WT-aldosterone versus WT-saline mice (P<0.01). The shortened of deceleration time in WT-aldosterone mice indicates abnormal LV relaxation and was attenuated in APNTG-aldosterone mice (P<0.01). There was no difference in isovolumic relaxation time between WT and APNTG mice regardless of saline or aldosterone infusion.

**Tissue Doppler**
Aldosterone infusion significantly decreased peak e’ velocity in WT-aldosterone versus WT-saline mice (P<0.01) and in APNTG-aldosterone mice versus APNTG-saline mice (P<0.01). However, the peak e’ velocity was significantly higher in APNTG-aldosterone mice than in WT-aldosterone mice (P<0.01). The resultant ratio of peak E velocity to peak e’ velocity (E/e’) was significantly higher in WT-aldosterone mice when compared with that in WT-saline mice (P<0.01). The increase in E/e’ in WT-aldosterone mice indicates elevated diastolic filling pressure and was significantly attenuated in APNTG-aldosterone mice (P<0.01; Table 2).

APNTG-aldosterone mice showed a reduction in the ratio of peak E velocity to peak A velocity when compared with WT-aldosterone mice. However relative to the saline-infused mice, this ratio of peak E velocity to peak A velocity was elevated. The reduction in E/A ratio in APNTG-aldosterone mice is because of a decrease in peak E velocity demonstrating an improvement in early transmitral flow (E wave) in the restrictive filling pattern. Despite a reduction in E/A ratio in APNTG mice, the E/e’ remained elevated suggesting elevated filling pressures or restrictive filling (Table 2).

**Lung Congestion**
Aldosterone infusion significantly increased wet/dry lung weight ratio, an indicator of pulmonary congestion, in WT-aldosterone versus WT-saline mice (P<0.01) and in APNTG-aldosterone versus APNTG-saline mice (P<0.05). There was no difference in wet/dry lung weight ratio, between saline-infused WT and APNTG mice. However, the wet/dry lung weight ratio was significantly lower in APNTG-aldosterone mice when compared with that in WT-aldosterone mice, indicating less pulmonary congestion (P<0.05).

**LV Cardiomyocyte Hypertrophy**
Aldosterone infusion significantly increased LV cardiomyocyte C/S area in the WT-aldosterone and APNTG-aldosterone mice when compared with respective saline-infused mice (P<0.01 for both; Figure 2A and 2B). Consistent with these findings, atrial natriuretic peptide mRNA expression, a molecular marker of cardiomyocyte hypertrophy, was increased in the LV of WT-aldosterone versus WT-saline mice (P<0.01) and in APNTG-aldosterone versus APNTG-saline mice (P<0.05; Figure 2C). There was no difference in the LV cardiomyocyte C/S area and atrial natriuretic peptide mRNA expression in saline-infused WT and APNTG mice. However, both LV cardiomyocyte C/S area and atrial natriuretic peptide mRNA expression were significantly decreased in APNTG-aldosterone mice versus WT-aldosterone mice (P<0.01 and P<0.05, respectively; Figure 2).

**Myocardial Fibrosis**
Aldosterone infusion significantly increased the area of myocardial fibrosis in WT-aldosterone versus WT-saline mice (P<0.01) and in APNTG-aldosterone versus APNTG-saline mice (P<0.05; Figure 3). Myocardial fibrosis was significantly less in the APNTG-aldosterone mice than in the WT-aldosterone mice (P<0.05).

**Myocardial Oxidative Stress**
Myocardial oxidative stress, assessed by 3-nitrotyrosine staining, was markedly increased in WT-aldosterone mice versus WT-saline mice. The increase in nitrotyrosine staining in WT-aldosterone mice was attenuated in APNTG-aldosterone mice (Figure 4A and 4B). There was a 54% reduction in nitrotyrosine staining in APNTG-aldosterone mice (P<0.05 versus WT-aldosterone mice).

**Calcium-Handling Proteins: Sarcoplasmic Reticulum Ca²⁺-ATPase and Phospholamban**
Four weeks of aldosterone infusion significantly decreased sarcoplasmic reticulum Ca²⁺-ATPase (SERCA2a) protein expression in WT-aldosterone versus WT-saline mice (0.71-fold; P<0.01) and in APNTG-aldosterone versus APNTG-saline mice (0.66-fold; P<0.05). The decrease in SERCA2a protein expression was comparable between WT-aldosterone mice and APNTG-aldosterone mice.

**Figure 4.** Myocardial oxidative stress in wild-type (WT) and adiponectin transgenic (APNTG) mice 4 weeks after saline or aldosterone infusion. A, Representative 3-nitrotyrosine staining. Original magnification ×400. B, Semiquantitative analysis of nitrotyrosine staining of C/S of murine myocardium. Nitrotyrosine staining was scored using an arbitrary grade from 1 to 4 (n=4–9 per group). †P<0.01 vs WT-saline, *P<0.05 vs WT-aldosterone (WT-aldo).
and APNTG-aldosterone mice (Figure 5A). There was no difference in phospholamban protein expression between WT and APNTG mice regardless of saline or aldosterone infusion (Figure 5B). Four weeks of aldosterone infusion significantly decreased phosphorylation of phospholamban at Ser16 in WT-aldosterone versus WT-saline mice (0.55-fold; P<0.01; Figure 5C). However, this decrease in phospholamban phosphorylation at Ser16 in WT-aldosterone mice was significantly attenuated in APNTG-aldosterone mice (P<0.05; Figure 5C). Four weeks of aldosterone infusion significantly decreased phosphorylation of phospholamban at Thr17 in WT-aldosterone versus WT-saline mice (0.73-fold; P<0.01) and in APNTG-aldosterone versus APNTG-saline mice (0.68-fold; P<0.01; Figure 5D). Phospholamban phosphorylation at Thr17 was similar between WT-aldosterone and APNTG-aldosterone mice (Figure 5D).

**Protein Kinase A Expression**

Four weeks of aldosterone infusion significantly decreased protein kinase A (PKA) C-α protein expression in WT-aldosterone versus WT-saline mice (0.78-fold; P<0.01). This decrease of PKA C-α protein expression in WT-aldosterone mice was significantly attenuated in APNTG-aldosterone mice (P<0.05; Figure 6).

**Ca²⁺/Calmodulin-Dependent Protein Kinase II Expression**

There was no difference in Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) protein expression between WT and...
APNTG mice regardless of saline or aldosterone infusion (Figure 7A). Four weeks of aldosterone infusion significantly decreased phosphorylation of CaMKII in WT-aldosterone versus WT-saline mice (0.65-fold; \( P<0.01 \)) and in APNTG-aldosterone versus APNTG-saline mice (0.66-fold; \( P<0.05 \); Figure 7B). There was no difference in phosphorylation of CaMKII between WT-aldosterone and APNTG-aldosterone mice.

**Adiponectin Supplementation**

To examine whether adenovirus-adiponectin (Ad-APN) supplementation ameliorated aldosterone-induced diastolic dysfunction in vivo, both WT-saline and WT-aldosterone mice were treated with either Ad-APN or Ad-\( \beta \)-galactosidase (Ad-\( \beta \)gal). Ad-APN or Ad-\( \beta \)gal were injected into the jugular vein of mice 14 days after surgery. This dose of Ad-APN raises adiponectin levels in the physiological range and contains the 3 isoforms that are present in mice with the hexamer form being dominant. Neither Ad-APN nor Ad-\( \beta \)gal had an effect on systolic BP in WT-saline and in WT-aldosterone mice (data not shown).

Ad-APN attenuated the aldosterone-induced changes in diastolic dysfunction: E/A ratio WT-aldosterone treated with Ad-APN (2.4) versus WT-aldosterone mice treated with Ad-\( \beta \)gal (1.75; \( P<0.01 \)). Similarly, E′/decreased 34% versus WT-aldosterone mice treated with Ad-\( \beta \)gal (\( P<0.01 \); Figure 8A and 8B).

**Discussion**

In this study, aldosterone-infused WT mice developed hypertension, LVM, diastolic dysfunction, and increased lung congestion, while maintaining a preserved LVEF, thus resulting in HFpEF. Aldosterone infusion also increased myocardial oxidative stress, decreased SERCA2a protein expression, decreased PKA-dependent phospholamban phosphorylation at Ser16, and decreased CaMKII-dependent phosphorylation of phospholamban at Thr17. Chronic hyperadiponectinemia ameliorated LVH, diastolic dysfunction, and lung congestion without effects on BP or LVEF in HFpEF mice. Chronic adiponectin overexpression also decreased myocardial nitrotyrosine staining, a measure of oxidative stress. The improvement of diastolic dysfunction parameters was associated with preserved PKA-dependent phospholamban phosphorylation at Ser16. In addition, adiponectin supplementation with Ad-APN improved measures of diastolic dysfunction in HFpEF mice infused with aldosterone.

We previously showed that adiponectin deficiency in aldosterone-induced HFpEF mice exacerbated hypertension and LVH. Although it has been reported that hyperadiponectinemia is a risk factor for hypertension, the therapeutic effect of adiponectin on hypertension is largely unknown. Ohashi et al reported that Ad-mediated overexpression of adiponectin ameliorated obesity-related hypertension in mice. However, in our study, transgenic mice with chronic adiponectin overexpression and Ad-APN supplementation of WT mice did not affect BP. This difference may be because of pathophysiological differences of targeted experimental models and less likely because of the difference of adiponectin levels in each experimental model. Adiponectin levels in our APNTG mice were 2-fold higher than those in WT mice, and although we did not measure adiponectin levels in the adiponectin supplementation experiments, it was likely similar to Ad-mediated adiponectin levels in the study of Ohashi et al where adiponectin levels were 6-fold higher than those at baseline. Additional studies are needed to determine the therapeutic effect of adiponectin in hypertension, but the importance of our study is that chronic adiponectin overexpression ameliorates the progression of LVH, independent of alterations in BP. Aldosterone-induced LVH, which is composed of LV cardiomyocyte hypertrophy and myocardial fibrosis, is accompanied by oxidative stress via mineralocorticoid receptor activation. Thus, suppression of oxidative stress might be an important therapeutic target in HFpEF. Several studies have recently shown that adiponectin exerts its cardioprotective effect by inhibiting oxidative stress. Consistent with these findings, we showed that adiponectin overexpression diminished aldosterone-induced myocardial 3-nitrotyrosine production, a marker of oxidative stress. Adiponectin likely mitigates aldosterone-induced adverse cardiac remodeling partly by suppressing oxidative stress. In a high-fat experimental model, adiponectin prevented platelet aggregation by attenuating oxidative and nitrosative stress and modulated reactive oxygen species metabolite levels and increased antioxidant levels in an ischemia/reperfusion porcine model. Mitochondrial-targeted antioxidant peptide, SS-31, may prove to be a therapeutic option in patients with HFpEF because it targets mitochondrial reactive oxygen species and modulates LVH, fibrosis, and LV diastolic dysfunction in an experimental model.

Both cardiomyocyte hypertrophy and myocardial fibrosis contribute to impaired active relaxation and increased passive stiffness of the LV and subsequently leads to diastolic dysfunction and clinical HF. In our study, hyperadiponectinemia decreased cardiac hypertrophy and improved some measures of diastolic dysfunction, independent of BP. Thus, chronic hyperadiponectinemia may improve diastolic
dysfunction and HF by ameliorating LVH.\textsuperscript{18} In human studies, it has been reported that low plasma adiponectin is associated with diastolic dysfunction.\textsuperscript{36}

Diastolic intracellular calcium handling is a major determinant of LV relaxation.\textsuperscript{35,37} Dephosphorylated phospholamban is an inhibitor of SERCA2a, but PKA-catalyzed (or CaMKII) phosphorylation of phospholamban results in the dissociation of phospholamban from SERCA2a, thus activating this Ca\textsuperscript{2+} pump and augmenting SERCA2a activity. In our study, chronic aldosterone infusion decreased SERCA2a protein expression and phospholamban phosphorylation at both Ser16 and Thr17 but did not alter phospholamban protein expression. In addition, PKA C-\ensuremath{\alpha}, an active catalytic subunit of PKA, and protein expression and phosphorylation of CaMKII, an indicator of CaMKII activity, were both decreased by chronic aldosterone infusion. Accumulating evidence indicates that β-adrenergic receptor stimulation regulates PKA and CaMKII activity.\textsuperscript{38–40} In addition, it has also been shown that Ser16 phosphorylation is mainly affected by PKA activity, whereas Thr17 phosphorylation is affected by CaMKII activity.\textsuperscript{38,39,41} Although β-adrenergic signaling affected by chronic aldosterone infusion remains to be clarified in this study, decreased PKA and CaMKII activity suggests that β-adrenergic signaling is downregulated by chronic aldosterone infusion. Collectively, our data indicate that chronic aldosterone infusion decreased PKA-dependent phosphorylation of phospholamban at Ser16 and CaMKII-dependent phosphorylation of phospholamban at Thr17, presumably followed by β-adrenergic signaling downregulation and subsequently suppressed SERCA2a protein expression. We did not measure intracellular Ca\textsuperscript{2+} concentrations. However, evidence indicates that diastolic intracellular calcium handling is mainly regulated by SERCA2a and its modulator phospholamban.\textsuperscript{35,37,41} Thus, changes in SERCA2a protein expression and phosphorylation of phospholamban in our study may be associated with abnormal intracellular diastolic calcium handling and subsequent diastolic dysfunction, as demonstrated by impaired LV compliance (increased E/A), abnormal LV relaxation (shortened deceleration time), and elevated diastolic filling pressure (increased E/e’). Nonetheless, in our study hyperadiponectinemia and chronic adiponectin overexpression ameliorated these measures of diastolic dysfunction in HFpEF, indicating that the improvement in these measures might be because of alterations in calcium-handling protein signaling.

Figure 7. Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) protein expression and phosphorylation of CaMKII in wild-type (WT) and adiponectin transgenic (APNTG) mice 4 weeks after saline or aldosterone infusion. A, Top, Representative blots of CaMKII and GAPDH. Bottom, Quantitative analysis of CaMKII protein expression. B, Top, Representative blots of phosphorylation of CaMKII and GAPDH. Bottom, Quantitative analysis of phosphorylation of CaMKII (n=3–8 per group). ††P<0.01 vs WT-saline, ‡P<0.05 vs APNTG-saline.

Figure 8. Adenovirus-adiponectin (Ad-APN) supplementation modulates diastolic dysfunction in aldosterone-infused wild-type (WT). A, Ad-APN attenuated the E/A ratio by 37% in WT-aldosterone (WT-ald) mice treated with Ad-APN vs WT-aldosterone mice treated with Ad-β-galactosidase (Ad-βgal; **P<0.01). B, Ad-APN decreased E/e’ by 34% in WT-aldosterone mice vs WT-aldosterone mice treated with Ad-βgal (††P<0.01).
At cellular and molecular levels, adiponectin induces Ca²⁺ influx via AdipoR1 and subsequently activates CaMKK, AMPK, and SIRT1 in skeletal muscle. However in the myocardium, SERCA2a protein expression in chronic aldosterone infusion was not affected by chronic adiponectin overexpression. Likewise, CaMKII activity and CaMKII-dependent phosphorylation of phospholamban at Thr17 were both decreased and to comparable levels. Yet chronic adiponectin overexpression ameliorated the decrease of phospholamban phosphorylation at Ser16, followed by preserved PKA activity. SERCA2a function is determined not only by SERCA2a protein expression but also by the phosphorylation status of phospholamban. In our study, chronic adiponectin overexpression did not affect the SERCA2a protein expression but improved diastolic dysfunction. These findings may be partly explained by improvement of SERCA2a function through phosphorylation of phospholamban. In addition, several studies have shown that adiponectin is associated with cAMP-dependent PKA signaling. Thus, our finding suggests that chronic adiponectin overexpression preserves phosphorylation of phospholamban at Ser16 through PKA activation and improves SERCA2a dysfunction.

Finally, the potential beneficial effects of adiponectin may extend to the downregulation of inflammatory cytokines or the upregulation of anti-inflammatory cytokines, which may affect cardiac hypertrophy, the extracellular matrix, diastolic dysfunction, and HFpEF. Proinflammatory cytokines, such as tumor necrosis factor-α that is prohypertrophic, are increased in diastolic dysfunction and HFpEF[48] (Figure II in Data Supplement). We recently showed that interferon-γ, a proinflammatory cytokine, attenuated cardiac hypertrophy and is a regulator of cytokine, attenuated cardiac hypertrophy and is a regulator of adiponectin and improved insulin sensitivity. 49

In conclusion, chronic adiponectin overexpression and supplementation prevented the progression of aldosterone-induced HFpEF, independent of BP. The beneficial effect of adiponectin was associated with reduced myocardial oxidative stress and modulation of intracellular calcium-handling regulatory proteins. Our findings indicate that adiponectin and its signaling pathway may be a therapeutic target for patients with HFpEF.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

There is a pressing need to develop therapies targeting patients with heart failure and preserved ejection fraction (HFpEF) because large clinical trials for HFP EF have all been negative to date. Future trials will likely benefit from characterizing subsets of patients with HFpEF that are phenotypically more homogenous because the pathogenesis of HFP EF remains incompletely understood. Preclinical studies are important not only to test drug therapies but also to understand the pathogenesis and disease pathway for the development of future drug therapies. Adiponectin is an adipocyte-derived cytokine that exerts cardioprotective actions, and its deficiency is implicated in the development of hypertension and HF with reduced ejection fraction. Similarly, adiponectin deficiency in HFP EF exacerbates left ventricular hypertrophy, diastolic dysfunction, and HF. However, the therapeutic effects of adiponectin in HFP EF remain unknown. In this murine model of hypertension-induced HFP EF, the improvement in diastolic dysfunction parameters and the regression of cardiac hypertrophy in HFP EF with adiponectin replacement is independent of changes in blood pressure and may be because of alterations in calcium-handling proteins. Specifically, adiponectin preserves protein expression of PKA-dependent phosphorylation of phospholamban at Ser16. Although this requires proof of concept in humans, these findings suggest that adiponectin may have therapeutic benefits in patients with HFP EF.
Effects of Adiponectin on Calcium-Handling Proteins in Heart Failure With Preserved Ejection Fraction
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Experimental mice groups. Mice were maintained on standard rodent chow and 1.0% sodium chloride drinking water for 4 weeks\textsuperscript{1,2}. The 4 groups studied were: (a) WT mice infused with saline (WT-saline, \textit{n} = 7); (b) APNTG mice infused with saline (APNTG-saline, \textit{n} = 7); (c) WT mice infused with \textit{d}-aldosterone (WT-aldosterone, \textit{n} = 15); (d) APNTG mice infused with \textit{d}-aldosterone (APNTG-aldosterone, \textit{n} = 15).

Another group of WT mice underwent the same surgical procedures as outlined above; however 14 days after surgery, mice were treated with adenoviral vectors expressing either APN (Ad-APN) or \(\beta\)-galactosidase (Ad-\(\beta\)gal) as a control after aldosterone or saline infusion. 2X10\textsuperscript{8} plaque-forming units (pfu) of Ad-APN or Ad-\(\beta\)gal were injected into the jugular vein of WT mice. (a) WT-saline plus Ad-\(\beta\)gal, \textit{n}=3; (b) WT-aldosterone plus Ad-\(\beta\)gal, \textit{n}=4; (c) WT-saline plus Ad-APN, \textit{n}=3; (d) WT-aldosterone plus Ad-APN, \textit{n}=4.

Physiological Measurements. Heart rate and blood pressure were measured weekly using a noninvasive tail-cuff blood pressure analyzer, BP-2000 Blood Pressure Analysis System (Visitech Systems, Inc., Apex, NC)\textsuperscript{1,2}.

Echocardiography. Diastolic function measurements: 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 heart rate (HR) of \textasciitilde350 beats per minute (bpm) since diastolic function is sensitive to HR and loading conditions. The maximum dose of isoflurane 1.5% has minimal effects on diastolic function\textsuperscript{2,3}. Pulse wave and tissue Doppler measurements were recorded.

LV structure and function: Interventricular septum wall thickness (IVST), LV posterior wall thickness (LVPWT), LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and LV ejection fraction (LVEF) were obtained. Total wall thickness (TWT) was derived from an average of the IVST and LVPWT. LV mass was calculated using the formula LV mass= 1.05[(LV EDD + IVST + PWT)\textsuperscript{3} - (LV EDD)\textsuperscript{3}] as described by Kiatchoosakun S et al\textsuperscript{4}.

Biomarker, organ weight and tissue analysis. After 4 weeks mice were sacrificed, and blood was obtained to determine serum adiponectin (B-Bridge International, Inc., Cupertino, CA) and aldosterone levels (Alpha Diagnostic Intl. Inc., San Antonio, TX). Body weights and heart weights were determined. Hearts were either arrested in diastole by KCl (30mmol/l), weighed, perfused with 10% buffered formalin and sliced horizontally for histology, or snap-frozen in liquid nitrogen. To measure fibrosis, Masson trichrome-stained sections (5\(\mu\)m) were visualized by using Olympus BX41 Clinical Microscope (Olympus America Inc., Center Valley, PA). The ratio of the fibrotic area to the entire heart area was calculated using ImageJ (National Institutes of Health, Bethesda, MD).
LV cardiomyocyte cross-sectional (C/S) area was assessed. For each section, 100 cardiomyocytes, showing a central nucleus, were randomly selected and C/S areas were measured \((\text{Area} = \pi r^2)\) using ImageJ (National Institutes of Health, Bethesda, MD). The wet-to-dry lung ratio, as an indicator of pulmonary congestion and HF was determined\(^2\)\(^5\).

**Assessment of myocardial oxidative stress.** Myocardial specimens were stained with 3-nitrotyrosine staining as described previously\(^6\). Briefly, sections were treated with 10 mmol/L citric acid (pH 6.0) and heated with a microwave (2 minutes, 3 times at 700W) to recover antigenicity. Nonspecific binding was blocked with 10% normal goat serum in phosphate-buffered saline (PBS) (pH 7.4) for 30 minutes before incubation with polyclonal anti-3-nitrotyrosine antibody (1 μg/mL) (Millipore, Billerica, MA) in PBS with 1% bovine serum albumin overnight at 4°C. Tissue sections were then incubated for 30 minutes at room temperature with a biotinylated anti-rabbit IgG (1:800) secondary antibody by using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). Vector Red alkaline phosphatase substrate (Vector Laboratories, Inc., Burlingame, CA) was used to visualize 3-nitrotyrosine. Semiquantitative analysis of tissue immunoreactivity for nitrotyrosine was done by estimating the degree of staining with the use of an arbitrary grading system from 1 to 4 as described previously\(^7\).

**Western Blot Analysis.** Protein kinase A (PKA) C-α, Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII), phospho-CaMKII at Thr286, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a), phospholamban (PLN), phospho-PLN at Ser16, and phospho-PLN at Thr17 protein expression in the heart were determined by western blot analysis. Aliquots of cardiac tissue lysates (5-30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, England). The membranes were immunoblotted with the following primary antibodies: anti-PKA C-α (Cell Signaling Technology, Inc., Danvers, MA), anti-CaMKII (Cell Signaling Technology, Inc., Danvers, MA), anti-phospho-CaMKII at Thr286 (Cell Signaling Technology, Inc., Danvers, MA), anti-SERCA2a (Thermo Fisher Scientific Inc., Waltham, MA), anti-PLN (Thermo Fisher Scientific Inc., Waltham, MA), anti-phospho-PLN at Ser16 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-PLN at Thr17 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anit-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam plc., Cambridge, MA), followed by the horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoblots were detected by ECL or ECL plus Western Blotting Detection Reagents (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, England). The chemiluminescence intensities were quantified by ImageJ (National Institutes of Health, Bethesda, MD) and normalized to those of GAPDH or Coomassie Brilliant Blue (Sigma-Aldrich Co., St. Louis, MO) staining of the PVDF membranes.

**Quantitative real-time polymerase chain reaction (qRT-PCR).** Atrial natriuretic peptide (ANP) mRNA expression was determined by qRT-PCR. Total RNA was extracted by using RNeasy Fibrous Tissue Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer’s protocol. Complementary DNA (cDNA) from 1000 ng of
total RNA was synthesized by using a ThermoScript™ Reverse Transcriptase (RT)-PCR System (Life Technologies Corporation, Carlsbad, CA) according to the manufacturer’s protocol. qRT-PCR was performed on the StepOne™ Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA) using SYBR Green PCR Master Mix (Life Technologies Corporation, Carlsbad, CA). The primer sequences were as follows: 5’-ATCTGCCCTCTTGAAGCA-3’ and 5’-AAGCTGTGCAGCCTAGTCC-3’ for mouse ANP; 5’-CCAAGGTCATCCGACAACCT-3’ and 5’-GGGCCCAGTCGAGCAGTCTTCT -3’ for mouse GAPDH. The expression levels of examined transcripts were compared to those of GAPDH and normalized to the mean value of controls.
Supplemental Figure 1

The graph presents the systolic blood pressure (mm Hg) over weeks. Different groups are represented by distinct markers: **WT-aldol**, **APNTG-aldol**, **WT-saline**, and **APNTG-saline**. The x-axis represents weeks, ranging from 1 to 4, and the y-axis represents systolic blood pressure, ranging from 0 to 140 mm Hg. The graph shows a trend of increasing blood pressure over time for all groups, with the **APNTG-aldol** group showing the highest systolic blood pressure. The NS (not significant) text indicates that there is no statistical difference between the groups.

Key
- **WT-aldol**: Black circles
- **APNTG-aldol**: Black squares
- **WT-saline**: Open circles
- **APNTG-saline**: Open squares

Significance levels:
- †: p < 0.05
- ††: p < 0.01
- †††: p < 0.001

Legend:
- N.S. (not significant)
Supplemental
Figure 2

**A**

TNF-α mRNA expression (Fold change rel. to WT-saline)

- WT-saline
- APNTG-saline
- WT-aldosterone
- APNTG-aldosterone

**B**

MCP-1 mRNA expression (Fold change rel. to WT-saline)

- WT-saline
- APNTG-saline
- WT-aldosterone
- APNTG-aldosterone

NS

¶
**Supplemental Figure Legends**

**Supplemental Figure 1.** Tail cuff systolic blood pressure in WT and APNTG mice. There was a significant and progressive rise in tail-cuff blood pressure in APNTG-aldosterone. †P<0.01 vs. WT-saline, ††P<0.01 vs. WT-saline, ‡P<0.05 vs. APNTG-saline, ‡‡P<0.01 vs. APNTG-saline

**Supplemental Figure 2.** (A) Aldosterone infusion significantly TNF-α mRNA expression in the hearts of WT-aldosterone vs. WT-saline mice (¶ P<0.05) but not in APNTG-aldosterone (P=NS vs. APNTG-saline mice and WT-aldosterone mice). (B) There was no significant difference in myocardial MCP-1 mRNA expression between saline and aldosterone-infused WT and APNTG mice.
Supplemental References


