High-intensity interval training attenuates endothelial dysfunction in a Dahl salt-sensitive rat model of heart failure with preserved ejection fraction

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APPROXIMATELY 14 MILLION EUROPEANS are diagnosed with heart failure (HF), 50% characterized as having a preserved left ventricular ejection fraction (HFpEF) with hypertension as the main risk factor (32). The high morbidity and mortality commonly observed in HFpEF is compounded by recent large-trial pharmacological interventions showing only limited benefits (9, 13, 43, 51). Exercise training (ET) might be an alternative treatment option, since it was recently demonstrated to have beneficial clinical outcomes in patients with HFpEF (14). Indeed, exercise training is now considered to form a key component in the treatment of patients that have HF with a reduced ejection fraction (HFrEF) (37). These findings are likely underpinned by the main symptom associated with HF being exercise intolerance (i.e., muscle fatigue and dyspnea). Yet, the benefits of exercise training with respect to HFpEF patients remain unclear, with only a few randomized studies completed (2, 14, 17, 24, 25, 42) and some meta-analyses performed (12, 16, 33). Initial findings have suggested that high-intensity interval training (HIT) is optimal (3). In contrast to HFrEF, therefore, the underlying mechanisms of both exercise intolerance and the benefits associated with exercise training in HFpEF still remain largely unclear.

It has been proposed recently (35) that HFpEF (and its subsequent sequelae) may be initiated by a cascade of events related to endothelial dysfunction. Indeed, it is now well established that endothelial dysfunction is a key risk factor in patients with HFrEF, which closely relates to exercise intolerance, disease severity, and clinical outcome (15, 39). In contrast, current noninvasive data on this issue in HFpEF patients remain controversial with some (1, 8), but not all (21, 23), studies reporting an impaired endothelial response in HFpEF. This controversy has been further exacerbated by the existence of only a few animal studies of which none investigated endothelial function, limiting any direct measurement of functional and molecular alterations associated with HFpEF. Of the few animal studies performed, it was reported in a porcine model of HFpEF that low-intensity exercise training attenuated diastolic impairments by lowering fibrosis and normalizing metalloproteinase (MMP)-2 expression (29).
The present study, therefore, used an established hypertensive rat model of HFpEF (27) to directly assess molecular and functional alterations in the aortic endothelium, while further assessing the effects of exercise training (i.e., HIT). We hypothesized that the development of HFpEF would initiate a cascade of molecular alterations in both endothelial and vascular smooth muscle cells leading to consequent endothelial dysfunction. However, we reasoned exercise training would act to attenuate endothelial impairments mediated by HFpEF, as supported by empirical evidence from HFpEF studies (reviewed in Ref. 18). For the molecular analyses, we focused on enzymes influencing the bioavailability of nitric oxide in the endothelium [expression of endothelial nitric oxide synthase (eNOS) and the gp91phox subunit of the NAD(P)H oxidase] and also indirect markers of vascular stiffness [advanced glycation end product (AGE)-modified proteins, MMP activity].

**METHODS**

**Animals and study design.** Female Dahl salt-sensitive rats were randomized at the age of 7 wk into the following groups: 1) control: fed with a chow diet containing 0.3% NaCl; 2) HFpEF: fed with a chow diet containing 8% NaCl; and 3) HFpEF-high intensity interval training (HFpEF + HIT): fed with a chow diet containing 8% NaCl in combination with HIT. Rats were exposed to identical conditions in a 12-h light-dark cycle, with food and water provided ad libitum. After 28 wk, echocardiography and invasive hemodynamic measurements were performed to elucidate the degree of diastolic dysfunction. Rats were subsequently killed and the aorta was removed for the following: 1) functional measurements in vitro; 2) fixation in formalin for histological analysis; and 3) being snap frozen in liquid nitrogen for molecular analysis. All procedures and experiments were approved by the Norwegian Council for Animal Research, which was in accordance with Use of Laboratory Animals by the European Commission Directive 86/609/EEC.

**Training intervention.** Rats performed HIT three times per week over 28 wk on a treadmill at an inclination of 25°, with VO2peak assessed as previously described (50) and measured every third week to adjust exercise intensity. HIT consisted of four intervals for 4 min corresponding to 90% VO2peak, with 3 min of active rest at 60% VO2peak-separating intervals. Exercise was preceded and followed by 10 min of running at 40-50% VO2peak.

**Heart function.** Transthoracic echocardiography (Vevo 2100: VisualSonics, Ontario, Canada) was performed in the supine position on lightly anesthetized (1.5 - 2% isoflurane) but spontaneously breathing rats using a 24-MHz transducer. Left ventricular (LV) structural parameters were measured from the short-axis view in M-mode to calculate left ventricular ejection fraction (LVEF), whereas the early diastolic filling velocity-to-early septal mitral annulus velocity (E/E′) ratio was measured using Doppler recordings from the apical four chamber view. All measurements were performed excluding the respiration peaks and obtained in triplicate. All calculated parameters were automatically computed by the Vevo 2100 standard measurement package.

Invasive hemodynamic pressure measurements were also measured as the terminal procedure. After induction of anesthesia (2% isoflurane), the right carotid artery was cannulated with a conductance catheter (1.4 Fr.; Transonic Science, London, Canada) and a pressure transducer penetrated the aortic valve into the LV. The LV end-diastolic pressure (LVEDP) and phasic and mean arterial pressures were measured (after withdrawal of the catheter into the aorta). Data were recorded on LabChart 7 software (ADInstruments).

**Measurement of endothelial function.** Endothelial function of aortic rings was analyzed in vitro, which provided a standardized environment not affected by external factors (i.e., inflammatory cytokines or oxidative stress). For the measurement of endothelial function, the aorta was rapidly excised and aortic rings (0.5 cm) mounted between a hook and a force transducer in a buffer-filled organ bath (in mmol/l: 118 NaCl, 25 NaHCO3, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 2.5 CaCl2, and 5.5 glucose). Following an equilibration period of 30 min, the maximal constriction was achieved by adding potassium chloride (KCl, final concentration 100 mmol/l) to the buffer. After the ring was carefully rinsed several times, the aortic ring was preconstricted to ~70% of maximal KCl constriction by adding increasing concentrations of phenylephrine (10-9 to 10-3 mol/l; Sigma, Taufkirchen, Germany). Relaxations to increasing concentrations to acetylcholine (ACH; 10-9 to 10-4 mol/l) and sodium nitroprusside (SNP; 10-10 to 10-4 mol/l; Sigma) were recorded until no further changes were observed.

**Histology and immunohistochemical staining.** The aorta was fixed in 4% formalin (buffered with phosphate buffer solution), embedded into paraffin, and cut into sections (3 μm) stained with hematoxylin and eosin. The thickness of the vessel wall (an index of aortic hypertrophy) was then quantified using imaging software (Analysis Five; Olympus, Münster, Germany). Endothelial cell coverage was determined by staining sections with an antibody incubated over night at 4°C against endothelial nitric oxide synthase (eNOS; 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), with bound antibodies detected using the CSA-II kit (Dako, Hamburg, Germany). Endothelial cell coverage (%) of the aorta was then calculated in relation to overall circumference (Analysis Five; Olympus), which provided an index for an intact endothelial cell layer. Localization of advanced glycation end products (AGE) was similarly determined, but sections were stained with an anti-AGE antibody (1:5,000; Abcam, Cambridge, UK).

**Protein expression.** Western blot was used to quantify protein expression. Frozen aortic rings were homogenized in lysis buffer (50 mmol/l Tris, 150 mmol/l sodium chloride, 1 mmol/l EDTA, 1% NP-40, 0.25% sodium-deoxycholate, 0.1% SDS, and 0.1% Triton X-100 pH 7.4), which contained a protease inhibitor mix (Inhibitor mix M; Serva, Heidelberg, Germany). Samples were then sonicated and centrifuged at 16,000 g for 5 min, with the supernatant isolated and protein content determined (BCA assay; Pierce, Bonn, Germany). Protein homogenates (10 μg) were separated on polyacrylamide gels by electrophoresis, transferred to polyvinylidene fluoride membranes, and blocked with 5% fat-free milk. Membranes were then incubated overnight at 4°C with antibodies, which included: anti-eNOS (1:200; Santa Cruz Biotechnology), anti-gp91phox (1:1,000; Abcam), and anti-AGEs (1:5,000; Abcam). Membranes were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody and specific bands visualized by enzymatic chemiluminescence (Super Signal West Pico; Thermo Fisher Scientific, Bonn, Germany) and densitometry quantified by densitometry (Scalanitics, Rockville, MD). Samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; HyTest, Turku, Finland), which served as the loading control. Protein expression is therefore presented as arbitrary units.

**MMP activity.** MMP-2 and MMP-9 activity was measured by gelatin zymography. Aortic rings were incubated overnight in RIPA buffer (20-fold of wet weight), which contained a protease inhibitor (Inhibitor mix M; Serva). Samples were subsequently centrifuged at 16,000 g for 5 min, with the supernatant isolated and protein content determined (BCA assay; Pierce). Equal amounts of protein (10 μg) were mixed with sample buffer (250 mmol/l Tris pH 7.4, 10% sodium dodecylsulfate, 20% glycerol, and 0.005% bromphenolblue) under nondenaturating conditions. After electrophoresis (10% polyacrylamide gel containing 1% gelatin), gels were washed for 1.5 h in renaturation buffer (2.5% Triton X-100) and incubated in incubation buffer (in mmol/l: 50 Tris pH 7.6, 10 CaCl2, 50 NaCl, and 0.05% Brij-35) at 37°C for 72 h. Gels were then stained with 0.25% Comassie Brilliant Blue R-250, and MMP activity was observed as cleared unstained regions. Densitometry was used to quantify activity (Scalanitics).

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EC50 for SNP was greater in HFpEF compared with control largely prevented by exercise training (Fig. 1) significantly impaired endothelium-independent vasodilation in the HFpEF group compared with control and HFpEF + HIT rats (79.5 ± 1.4 vs. 89.0 ± 1.4 and 84.6 ± 1.0%, respectively; Fig. 1B). No correlation was evident between EC50 Ach and EC50 SNP (r = 0.3, P = 0.13).

Vessel wall hypertrophy. Compared with controls, aortic wall thickness was found to be increased in both HFpEF and HFpEF + HIT animals (132 ± 6 vs. 180 ± 3 and 181 ± 4 μm; P < 0.001), indicating aortic wall hypertrophy was present. Expression of eNOS and NADP(H) oxidase. Compared with controls, the expression of eNOS in the HFpEF group was reduced by 46% (P < 0.05; Fig. 2A). However, exercise training normalized eNOS expression (Fig. 2A). In addition, a significant positive correlation was observed between the expression of eNOS and the maximal ACh-induced vasodilation (r = 0.59; P < 0.001). Nevertheless, the three groups did not differ with regard to endothelial cell coverage, indicating that endothelial cell destruction did not occur in HFpEF (Fig. 2B).

Similarly, the expression of NAD(P)H oxidase subunit gp91phox in groups was also not different (Fig. 3A).

Table 1. Animal characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>HFpEF (n = 11)</th>
<th>HFpEF + HIT (n = 11)</th>
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<tbody>
<tr>
<td>Weight, g</td>
<td>292 ± 5</td>
<td>283 ± 5</td>
<td>279 ± 6</td>
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<tr>
<td>Heart weight, mg</td>
<td>940 ± 10</td>
<td>1,250 ± 20†</td>
<td>1,370 ± 70†</td>
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<tr>
<td>Left ventricle, mg</td>
<td>738 ± 13</td>
<td>1,027 ± 22†</td>
<td>1,110 ± 62†</td>
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<tr>
<td>Systolic BP, mmHg</td>
<td>149 ± 5</td>
<td>213 ± 5†</td>
<td>203 ± 8†</td>
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<tr>
<td>Diastolic BP, mmHg</td>
<td>106 ± 4</td>
<td>153 ± 7†</td>
<td>143 ± 6†</td>
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<tr>
<td>Serum NT-proBNP, pg/ml</td>
<td>19.7 ± 5.1</td>
<td>646 ± 14.6†</td>
<td>852 ± 10.4†</td>
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<tr>
<td>Echocardiography</td>
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<tr>
<td>LVEF, %</td>
<td>82.7 ± 1.1</td>
<td>69.2 ± 1.8*</td>
<td>61.1 ± 3.4†</td>
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<tr>
<td>E/E’ ratio</td>
<td>10.6 ± 1.5</td>
<td>19.4 ± 1.1*</td>
<td>24.5 ± 3.2†</td>
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<tr>
<td>Invasive hemodynamic</td>
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<td>LVEDP, mmHg</td>
<td>5.6 ± 0.9</td>
<td>13.0 ± 2.6*</td>
<td>12.7 ± 1.8*</td>
</tr>
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</table>

Values are means ± SE. Blood pressure; LVEF, left ventricular ejection fraction; E’, early diastolic filling velocity; E/E’, early septal mitral annulus velocity; LVEDP, left ventricular end-diastolic pressure; HFpEF, heart failure preserved ejection fraction; HIT, high-intensity interval training, NT-proBNP, NH2-terminal prohormone of brain natriuretic peptide. *P < 0.05 vs. control; †P < 0.01 vs. control.

Statistical analyses. Data are presented as means ± SE. Between-group differences were assessed by independent ANOVA, while endothelial-dependent and -independent vasodilation were assessed by two-way repeated measures ANOVA using SPSS version 22 (SPSS, Chicago, IL). Statistical significance was accepted as P < 0.05.

RESULTS

Animal characteristics. Baseline characteristics of the three groups are presented in Table 1. These data demonstrate that animals on the high-salt diet (both without or with HIT) developed HFpEF compared with controls on low-salt diet, as supported by significantly increased E/E’, LV mass, LVEDP, blood pressure, and NH2-terminal prohormone of brain natriuretic peptide (NT-proBNP) despite a preserved LVEF. Mortality rates for the control, HFpEF, and HFpEF + ET groups were 5, 57, and 40%, respectively, and measured blood pressure values are in line with previous measurements in Dahl salt-sensitive rats (10).

Endothelial-dependent and -independent vasodilation. The addition of 100 mmol/l KCl resulted in a 21% lower contraction of the aortic rings in the HFpEF group compared with the control group (HFpEF: 1.51 ± 0.08 vs. control: 1.91 ± 0.04 g; P < 0.05). This reduction was attenuated in the HFpEF + HIT group (1.99 ± 0.16 g). Stimulation of aortic rings with increasing concentrations of SNP revealed that HFpEF is associated with impaired endothelial-dependent vasodilation. This was attenuated by exercise training (Fig. 1A). As such, maximal dilatation was significantly reduced in the HFpEF group compared with the control and HFpEF + HIT rats (49.9 ± 3.5 vs. 66.6 ± 3.4 and 61.5 ± 2.6%, respectively; Fig. 1A). Hence, a significantly greater concentration of Ach was required in the HFpEF group to induce a 50% relaxation response (EC50) in aortic rings compared with control and HFpEF + HIT rats (1.164 ± 302 vs. 230 ± 44 and 255 ± 44 mmol/l, respectively; P < 0.05). In addition, the HFpEF group also demonstrated significantly impaired endothelial-dependent vasodilation in response to SNP compared with controls, but this was largely prevented by exercise training (Fig. 1B). Therefore, the EC50 for SNP was greater in HFpEF compared with control and HFpEF + HIT rats (388 ± 65 vs. 205 ± 31 and 216 ± 44 mmol/l, respectively; P < 0.05). In addition, maximal SNP-induced dilation in the HFpEF group was significantly lower compared with control and HFpEF + HIT rats (79.5 ± 1.4 vs. 89.0 ± 1.4 and 84.6 ± 1.0%, respectively; Fig. 1B). No correlation was evident between EC50 Ach and EC50 SNP (r = 0.3, P = 0.13).

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AGE-modified protein expression. The measurement of AGE-modified proteins, which provides an indirect measurement of protein cross-linking and therefore vessel stiffness, revealed an increase in AGE-modified proteins by 126% in the HFpEF animals compared with controls (P<0.05; Fig. 3B). However, this was prevented by exercise training, with AGE-modified proteins in HFpEF/HIT rats having similar values to those of controls (P>0.05; Fig. 3B). Figure 3C specifically demonstrates where AGE-related proteins were localized in the aorta.

MMP activity. MMP activity provides an indirect measure of extracellular matrix degradation and therefore vessel wall modulation. Compared with controls, activity in HFpEF animals was increased by 183 and 68% for MMP-2 and MMP-9, respectively (P<0.05; Fig. 4, A and B). The increase in MMP-9 activity was blunted (P<0.05) by exercise training, whereas the MMP-2 activity was significantly reduced by HIT, but the remaining activity was still significantly above the control level (Fig. 4, A and B).

DISCUSSION

The HFpEF paradigm (35) that low endothelial NO impairs myocardial contractility, which then drives the development of the disease via endothelial-dysfunction, may have important implications for future treatment strategies in patients. As such, in the present study we utilized a hypertensive animal model that resembles HFpEF to assess functional and molecular alterations in the aortic endothelium associated with HFpEF, while further assessing the effects of exercise training. The key findings that emerged from this animal model of HFpEF were 1) HFpEF caused endothelial dysfunction via an endothelium-dependent and independent-mechanism; 2) impaired endothelial function of HFpEF was associated with numerous molecular alterations, including a reduced expression of eNOS, an increase in AGE-modified proteins, and elevated MMP-2 and MMP-9 activity; and 3) HIT attenuated both endothelium-dependent and -independent dysfunction concomitant with normalized measurements of eNOS, AGE-modified proteins,

Fig. 2. Protein Expression of endothelial nitric oxide synthase (eNOS) was quantified in the aorta of control (con), HFpEF (HFpEF), and HFpEF + exercise training (HFpEF + HIT) animals by Western blot (A). The endothelial coverage of the aortic lumen was evaluated by immunohistochemical staining of endothelial cells using an antibody directed against eNOS. The %coverage of the aorta was calculated by measuring the area covered by endothelial cells in relation to overall circumference (B). Representative examples of Western blots are depicted at top. *P<0.05 vs. control.

Fig. 3. Protein Expression of NAD(P)H oxidase subunit gp91phox (A) and advanced glycation end product (AGE)-modified proteins (B) was quantified in the aorta of control (con), HFpEF (HFpEF), and HFpEF + exercise training (HFpEF + HIT) animals by Western blot. To localize the AGE-modified proteins in the aorta, immunohistochemistry on paraffin sections was performed using an anti-AGE specific antibody followed by DAB staining (brown color) (C). *P<0.05 vs. control. §P<0.05 vs. HFpEF.
and MMPs. Collectively, therefore, these data provide novel data related to endothelial dysfunction in HFpEF and identify HIT as a potential treatment.

**Endothelial dysfunction in HFpEF.** In HFrEF, the involvement of endothelial dysfunction in disease development and progression is clearly documented (36, 39). An impaired vasodilation, due to endothelial dysfunction, limits oxygen delivery to the skeletal muscles and other organs, which directly contributes to exercise intolerance. The situation is less clear in HFpEF, partly consequent to the contradictory data that exist in the literature. For example, some reports describe endothelial dysfunction in HFpEF patients compared with healthy age-matched controls (1, 8), whereas others report none (21, 23). For example, Haykowsky et al. (21) measured brachial artery flow-mediated dilation (FMD) in HFpEF patients and age-matched healthy controls. They reported no difference in FMD between both groups. This result was confirmed by Hundley et al. (23), who reported also no difference in FMD measured in the femoral artery between HFpEF patients and controls. On the other hand, a recently performed cohort study in 321 HFpEF patients and 173 patients without heart failure detected a highly significant difference in endothelial function, measured as reactive hyperemic index (RHI) between both cohorts (1). Furthermore, the authors even identified endothelial dysfunction as an independent predictor for future cardiovascular events. The reason for these discrepancies is still unclear and awaits clarification, but either the different techniques used to measure endothelial function (FMD vs. RHI) or as recently documented (36, 39). An impaired vasodilation, due to endothelial dysfunction, limits oxygen delivery to the skeletal muscles and other organs, which directly contributes to exercise intolerance. The latter implies that not only is the endothelial cell layer affected by HFpEF but that these alterations occur in the intima, media or adventitia (discussed below). Importantly, the impact of HFpEF on endothelium-independent vasodilation is strikingly different to what is described for HFrEF patients and animals, in which SNP-induced vasodilation is preserved (40, 46). We suggest, therefore, that this may represent a novel and different mechanism contributing to exercise intolerance in HFpEF, which is not observed in HFrEF.

**Molecular mechanisms of endothelial dysfunction in this model of HFpEF.** The molecular mechanisms underpinning altered endothelial function in HFpEF remain poorly investigated, which is likely a consequence of the paucity in experimental animal studies. However, here we provide novel evidence of likely contributors to endothelial dysfunction in HFpEF, which include a reduction of eNOS expression and an increase in AGE-modified proteins and in the activity of MMP-9 and MMP-2. Based on clinical studies and animal models of HFrEF, we know that NO plays a pivotal role in regulation of vascular tone by its generation via eNOS (4, 47). In addition, our findings of a 46% decrease in eNOS expression are also in line with an earlier report documenting Dahl salt-sensitive animals fed a high-salt diet had a 40% reduction of eNOS expression (53). In summary, we suggest the reduction of eNOS expression plays a central role for the impaired endothelium-dependent vasodilation observed HFpEF. This is further supported by the positive correlation between the expression of eNOS and the maximal ACh-induced vasodilation.

Surprisingly, we also found endothelium-independent vasodilation was impaired in HFpEF. It is well documented that ventricular stiffening and diastolic dysfunction in HFpEF are consequences of titin hypophosphorylation (7), endothelial inflammation (35), reduced NO bioavailability (35), and a declining PKG activity (48). An increased carotid arterial stiffness is also documented in HFpEF (26), but the molecular basis remains unknown. In the present study, we found an increased modification of proteins by AGEs in HFpEF aorta. AGE levels in the aorta are significantly elevated with age and diabetes (31, 41), and correlate directly with vascular stiffness (41). AGEs also contribute to a variety of micro- and macro-

![Fig. 4. Activity of matrix metalloproteinase-2 (MMP-2; A) and MMP-9 (B) was quantified in the aorta of control (con), HFpEF (HFpEF), and HFpEF + exercise training (HFpEF + HIT) animals by zymography and followed by densitometry. *P < 0.05, **P < 0.01 vs. control. §P < 0.05 vs. HFpEF.](http://jap.physiology.org/Downloadedfrom/http://www.jappl.org)
vascular complications, via the formation of cross links between molecules in the extracellular matrix such as elastin, collagen, and reduced eNOS activity (19). Interestingly, we found our HFpEF rats not only to have increased levels of AGEs in the endothelial cell layer but also in the intima and adventitia. Together with the increased activity of MMP-9 and MMP-2, this finding may partly explain why endothelium-independent vasodilation is also impaired in this model of HFpEF. The suggested increased aortic stiffness in our HFpEF animals is further supported by the reduced contraction towards KCl. In addition, the increase in aortic wall thickness, as already documented in HFpEF patients (22), may further aggravate endothelial independent vasodilation. Further research is therefore required to help elucidate the mechanisms responsible for the endothelium-dependent and -independent vasodilatation in HFpEF.

Effects of exercise training in HFpEF. Exercise training is a well-established treatment in patients with HFrEF (38). A modest number of studies also showed exercise training improves exercise capacity and quality of life in HFpEF patients (2, 3, 14, 17, 24, 25, 42). However, the results of the two studies that measured endothelial function showed that exercise training, including one that tested HIT, has no benefit for endothelial function (3, 24). This is in clear contrast to the results presented in our study, where exercise training clearly attenuated both endothelial-dependent and -independent vasodilation dysfunction. The reason for this discrepancy is not completely clear, but is likely explained by the different measurement techniques and vessel type. The two clinical studies investigated the noninvasive FMD of the brachial artery, whereas we directly measured ACh and SNP-induced dilation capacity of the aorta. Further research is clearly warranted on the effects of exercise training on endothelial function in HFpEF, with an ongoing multicenter randomized trial likely providing more insight (45).

Our findings that exercise training was able to preserve endothelial function in this model of HFpEF rats, which was associated with normal molecular measures to those of controls, help to provide new insight into endothelial dysfunction in HFpEF. We found that exercise training attenuated the reduction in eNOS expression in HFpEF and blunted the increase in MMP activity and AGE-modified proteins. Indeed, the modulation of eNOS, MMPs, and AGEs by exercise training has already been documented in other diseases such as hypertension, coronary artery disease, and diabetes (18). All these training-induced modifications may lead (alone or in combination) to the correction of endothelial function in HFpEF. Overall, however, our initial findings suggest HIT in patients with HFpEF may be a viable treatment strategy by targeting endothelial-related dysfunction among many other benefits.

Study limitations. Given the substantial heterogeneity that exists between patients with HFpEF, it is likely that our animal model only resembles a certain proportion of HFpEF patients. Indeed, the complexity of what defines HFpEF is supported by the different recommendations in management and diagnosis of HFpEF from the American (52) and European (30) cardiology societies. In the American Heart Association/American College of Cardiology statement on management of HFpEF, neither LV hypertrophy nor diastolic dysfunction is required for diagnosis, whereas relevant structural and/or diastolic dysfunction is a prerequisite in the European guidelines. Further data also suggest multiple noncardiac comorbidities also likely play a key role in the pathogenesis of HFpEF (6). Thus our model may overestimate the contribution of LV hypertrophy and/or diastolic dysfunction and underestimate the influence of comorbidities associated with the syndrome of HFpEF, with more studies still needed to confirm the translation of animal models to patients. What is apparent, however, is that HFpEF is a highly heterogeneous syndrome, with patients showing a wide variation in pathophysiology. Nevertheless, despite such potential limitations we feel our data provide evidence, drawn from both clinical evidence and clinical recommendations (34), that this animal model of HFpEF provides one of the best currently available. Our high-salt rats demonstrated many clinical features to those observed in a large proportion of patients (e.g., increased E/E′, increased LVEDP, increased NT-proBNP, increased LV mass, and preserved ejection fraction). Although additional measures such as tissue velocities and strain rates would have been useful to further improve the characterization of our HFpEF rats, these were not assessed. Furthermore, the present animal model also used hypertension to induce HFpEF, which is one of the key risk factors for HFpEF development. Although all pharmaceutical studies have found lowering blood pressure in HFpEF patients does not improve clinical outcomes (5), it still remains unclear whether a reduction in blood pressure is expected to improve outcomes if the syndrome of HFpEF has since ensued.

In addition, even though peripheral arterial measurements in vivo are regularly performed in human subjects, in the present study (as in most murine experiments) the vast majority of experiments assess endothelial function in vitro from the central artery of the aorta due to technical limitations in accessing peripheral arteries. However, evidence is now available demonstrating endothelial function measured in the aorta resembles that in peripheral arteries (20), with a strong correlation between in vivo and in vitro measures (11), hence supporting a generalized systemic effect. Furthermore, our functional analysis of the aorta also failed to directly assess the influence of NO availability on vasodilation by incorporating an eNOS inhibitor substance [e.g., nitro-L-arginine methyl ester (L-NAME)]. Given the extended duration of the in vitro assessments that can impact vessel function, we instead decided to focus on the endothelium-independent response to SNP, which allowed us a more broad assessment of the vessel function in HFpEF. However, our suggestion of reduced NO as inferred from the decrease in ACh-induced vasodilation in combination with the decrease in eNOS protein expression is supported by recent experiments (44). This study in the aorta from 5/6 nephrectomized rats (another HFpEF model) showed a lower ACh-mediated dilation compared with sham-treated animals, but preincubating aortic rings with L-NAME (100 μmol/l) for 15 min (before ACh induced vasodilation) confirmed that ACh-induced aortic relaxation was totally dependent on eNOS function. Finally, in the present study we also assessed the generation of reactive oxygen species by quantifying the protein expression of NADPH oxidase (gp91phox subunit) using Western blot. NADPH oxidase is only one potential source of reactive oxygen species in addition to the mitochondria and eNOS uncoupling. Although we failed to assess the latter, a previous study using the Dahl salt-sensitive rat model suggests significant eNOS uncoupling would have been likely, as an


