Circulation: Heart Failure

ORIGINAL ARTICLE

Musclin Counteracts Skeletal Muscle Dysfunction and Exercise Intolerance in Heart Failure With Preserved Ejection Fraction

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BACKGROUND: Exercise intolerance is a hallmark of heart failure with preserved ejection fraction (HFpEF) and is characterized by skeletal muscle (SkM) dysfunction with impaired oxidative capacity. To maintain oxidative capacity, the SkM secretes myokines such as musclin, which has been shown to potentiate NP (natriuretic peptide) signaling and induce PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1 alpha) signaling. We sought to investigate the role of musclin in SkM dysfunction in HFpEF. For this study, we selected the oxidative-predominant SkM soleus in HFpEF mice and vastus lateralis from patients with HFpEF.

METHODS: Using the SAUNA model, mice underwent HFpEF induction by uninephrectomy, d-aldosterone infusion, and 1% sodium chloride drinking water for 4 weeks. Exogenous musclin was given to HFpEF mice every 2 days during the last 2 weeks of HFpEF induction. Molecular analyses were conducted on blood samples and SkM from HFpEF mice and patients with HFpEF.

RESULTS: In HFpEF mice and patients with HFpEF, increased musclin expression was accompanied by decreased cyclic guanosine monophosphate levels and PGC-1α expression in SkM, suggesting impaired NP signaling. Exogenous administration of musclin in mice with HFpEF demonstrated augmented circulating musclin levels and potentiated NP signaling in SkM as shown by increased PKG1 (protein kinase G1) activity and PGC-1α expression. This was associated with a transition from type-2A to type-1 fiber (type-1 has more endurance) and increased succinate dehydrogenase activity, hindlimb blood flow, and capillary density in the soleus muscle. Exogenous musclin also mitigated cardiac hypertrophy without affecting blood pressure or diastolic function. Most importantly, HFpEF mice treated with musclin demonstrated improved functional and exercise capacity.

CONCLUSIONS: Musclin mediates beneficial effects in SkM and heart with improved exercise capacity likely by improving oxidative capacity in SkM. Future studies are warranted to address the therapeutic efficacy of exogenous musclin in humans with HFpEF.

Key Words: exercise tolerance ■ heart failure ■ muscle, skeletal ■ myokines ■ natriuretic peptides

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Recent positive studies and new treatment options for heart failure (HF) with preserved ejection fraction (HFpEF) are encouraging, but, despite this, the unmet need for HFpEF remains extraordinarily high as HFpEF incidence and prevalence continue to increase in the setting of ever-increasing comorbidities.[1,2] It is also

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WHAT IS NEW?

- NP (natriuretic peptide) signaling enhances oxidative capacity in skeletal muscle (SkM); however, NP resistance in heart failure with preserved ejection fraction (HFpEF) impairs and contributes to SkM dysfunction.
- Musclin is a myokine known to potentiate NP signaling by binding to the NPRC (NP clearance receptor) and, thus, preventing the degradation of circulating NPs.
- Our findings demonstrate that endogenous musclin is elevated in SkM and the circulation, but, despite this increase, it is ineffective in its attempt to compensate for impaired SkM NP signaling in HFpEF.
- Exogenous musclin administered to HFpEF mice counteracted impaired NP signaling, mitigating SkM dysfunction and cardiac hypertrophy. Functionally, exogenous musclin improved exercise capacity in HFpEF.

WHAT ARE THE CLINICAL IMPLICATIONS?

- There are limited evidence-based therapies for HFpEF, with no known therapies to specifically target SkM, so as to maintain physical activity in patients with HFpEF and ultimately to improve their quality of life.
- Our findings indicate that musclin could be a potential SkM-specific therapeutic target to improve oxidative capacity and function in HFpEF.
- Understanding noncardiac organs in HFpEF is needed because HFpEF is a multisystemic disease.

Nonstandard Abbreviations and Acronyms

ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
cGMP	cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
ERR	estrogen-related receptor
HF	heart failure
HFpEF	heart failure with preserved ejection fraction
LV	left ventricle
MHC	myosin heavy chain
NP	natriuretic peptide
NPR	natriuretic peptide receptor
NPRC	natriuretic peptide clearance receptor
NT-proBNP	N-terminal pro-B-type natriuretic peptide
PDE	phosphodiesterase
PGC-1α	peroxisome proliferator-activated receptor-γ coactivator-1 alpha
PKG1	protein kinase G1
SBP	systolic blood pressure
SDH	succinate dehydrogenase
SkM	skeletal muscle
VASP	vasodilator-stimulated phosphoprotein

increasingly recognized that dysfunction of noncardiac organs plays an important role in HFpEF pathogenesis.[3] Therefore, growing attention is being paid to noncardiac mechanisms that underlie effort intolerance in HFpEF.[4,5] Effort intolerance is fundamental to all patients with HFpEF, who may also present with dyspnea, edema, exertional fatigue, etc., and is characterized by reduced peak oxygen consumption.[6–8] Mounting evidence points to skeletal muscle (SkM) dysfunction and impaired peripheral oxygen extraction/utilization of SkM, which are key determinants of reduced peak oxygen consumption in patients with HFpEF.[6,7] SkM dysfunction in HFpEF also features muscle atrophy, reduced oxidative-type fibers (type-1 and type-2A fibers), increased glycolytic-type fibers (type-2B and type-2X fibers), decreased capillary density and mitochondrial dysfunction, and increased interstitial inflammation and fibrosis, all contributing to impaired oxidative capacity and decreased effort tolerance.[6–11]

As an endocrine organ, the SkM also secretes factors called myokines, which play a crucial role not only in regulating muscle function but also in maintaining whole-body homeostasis by intraorgan and interorgan crosstalk. Musclin, an exercise-responsive myokine,[12] potentiates NP (natriuretic peptide) signaling by binding competitively to the NPRC (NP clearance receptor) and, thereby, limiting NP degradation. This, in turn, results in an elevation of NPs, specifically ANP (atrial NP), BNP (brain NP), and CNP (C-type NP). Increased binding of ANP and BNP to the NPR (NP receptor) A and CNP to NPRB potentiates signaling.[12,13] In SkM, this NP signaling induces PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1 alpha) expression via the cyclic guanosine monophosphate (cGMP)/PKG1 (protein kinase G1) signaling cascade.[14] PGC-1α, a key transcriptional coactivator, plays an essential role in regulating SkM oxidative capacity.[14–17]

Earlier studies in severe HF demonstrated that despite higher endogenous ANP or BNP levels, these NPs were inadequate in preventing edema and vasoconstriction. Only exogenous ANP or BNP infusions improved these hemodynamic, renal, and endocrine abnormalities in HF.[18,19] In some chronic diseases, NP resistance is evident despite higher circulating NP levels (HF and renal disorders) or abnormally low NP levels (obesity and metabolic conditions).[20] Decreased expression and desensitization of NP receptors or defective intracellular signaling also occur in these dysregulated

NP states and likely contribute to disease progression.[21,22] We recently showed that SkM oxidative capacity is impaired in both mice and patients with HFpEF.[7,10] We, thus, postulated that NP resistance occurs in SkM in HFpEF despite increased endogenous NPs.[23,24] Although musclin enhances SkM oxidative capacity by activating NP signaling,[12] its role in response to NP resistance in SkM in HFpEF is unknown. In patients with acutely decompensated HF, acute intravenous infusion of nesiritide (a human BNP analog) for 24 to 48 hours improved dyspnea and decreased in-hospital mortality despite endogenous BNP levels already being elevated in these patients,[25,26] demonstrating a therapeutic strategy to overcome NP resistance. Here, musclin was administered intraperitoneally in HFpEF mice every other day for 14 days to explore its potential effect in overcoming NP resistance in SkM in HFpEF.

MATERIALS AND METHODS

Detailed experimental information is outlined in the Supplemental Material. All data are provided within the article and in the Supplemental Material. Tables S3 through S7 and Figures S9 and S10 are cited in the Supplemental Methods and Materials, as these data support the experimental design/methodology.

Ethical Approval

The Boston University Medical Campus and the University of Pennsylvania Institutional Review Board approved the use of human blood samples and vastus lateralis muscle biopsies, respectively. All subjects gave written consent before the collection of samples, and the use of samples conformed to the Declaration of Helsinki principles.

The Institutional Animal Care and Use Committee at Boston University School of Medicine approved all animal experiments, with all procedures conforming to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Although HFpEF is more common in elderly women, its prevalence is rising among younger men, who tend to have worse outcomes when it occurs.[27] Although female sex hormones are generally lower in postmenopausal elderly women, these hormones influence muscle mass and strength.[28] To minimize the impact of this confounding factor, male rodents were used in the present study.

Statistical Analysis

Analyses were performed using GraphPad Prism software. Data distribution was tested using the Shapiro-Wilk test, and outliers were determined by the Grubbs test. The unpaired *t* test or the Mann-Whitney *U* test as parametric and nonparametric tests, respectively, were used to test the difference between the 2 groups. One-way ANOVA with the Tukey comparison test or the Kruskal-Wallis test with the Dunn multiple comparison test was used to test the difference between ≥3 groups in normal and nonnormal distributed samples, respectively. Data were expressed as mean±SEM. $P<0.05$ was considered statistically significant.

RESULTS

Musclin and PGC-1α in HFpEF Mice and Rats

HFpEF SAUNA male mice recapitulated the cardiac and SkM phenotype seen in patients with HFpEF as previously described.[10,29–31] In our study, musclin and PGC-1α expressions were examined in the oxidative-predominant soleus muscle 4 weeks after HFpEF induction (Figure 1A). Musclin gene (*Ostn*) and protein expression were increased by 57% ($P=0.044$) and 33% ($P=0.001$), respectively, versus Sham mice (Figure 1B and 1C). Conversely, the PGC-1α gene (*Ppargc1a*) and protein expression were decreased by 34% ($P=0.049$) and 12% ($P=0.003$), respectively, in HFpEF mice versus Sham mice (Figure 1D and 1E).

To confirm that these findings in SAUNA mice were not model-specific, a cohort of obese, hypertensive ZSF1 male rats with HFpEF was included. *Ostn* gene expression was increased (110%; $P=0.013$) with a concomitant decrease in *Ppargc1a* gene expression (60%; $P=0.033$; Figure S1) in the soleus muscle of obese ZSF1 rats versus control lean rats. Obese ZSF1 rats demonstrate soleus muscle phenotypic switching with reduced type-1 oxidative fibers and mitochondrial dysfunction similar to that of HFpEF SAUNA mice.[9] Thus, regardless of fat mass, the above findings are not model nor comorbidity-specific.

Musclin and PGC-1α in Patients With HFpEF

Musclin and PGC-1α expressions were measured in the vastus lateralis muscle (oxidative-predominant) of patients with HFpEF from the University of Pennsylvania cohort. Similar to the above findings from the HFpEF SAUNA mice and obese ZSF1 HFpEF rats, musclin gene (*OSTN*) and protein expressions were also increased in the muscle of these patients with HFpEF by 180% ($P=0.009$) and 38% ($P=0.048$), respectively, versus control subjects (Figure 2A and 2B). Although PGC-1α gene (*PPARGC1A*) expression was no different between patients with HFpEF and controls (Figure 2C), protein expression was significantly decreased by 16% ($P=0.003$) in patients with HFpEF versus control subjects (Figure 2D). As previously described,[10] the University of Pennsylvania cohort consisted of chronic, stable patients with HFpEF with 50% being women, with a mean age of 64.7±4.3 years, and had comorbidities commonly seen in HFpEF (hypertension being the most common [>90%]); obesity, type 2 diabetes, atrial fibrillation/flutter, and coronary artery disease were others. NT-proBNP (N-terminal pro-B-type natriuretic peptide) levels in patients with HFpEF were elevated versus healthy control subjects (137.5±33.7 versus 55.4±10.1 pg/mL; $P=0.030$). Control subjects were 31% women with a mean age of 54.6±4.6 years and no evidence of adverse cardiac remodeling or diastolic dysfunction by echocardiogram.

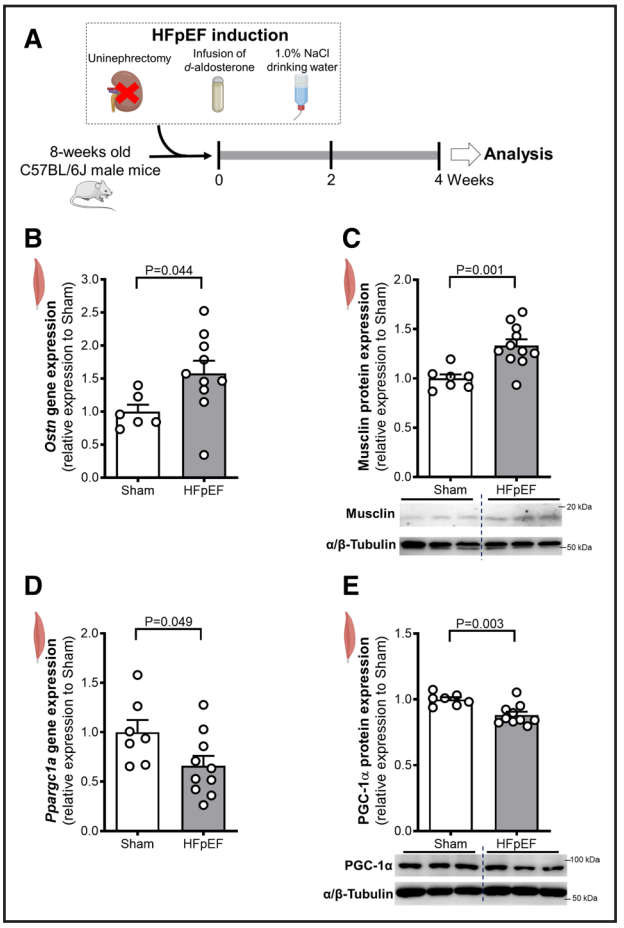

Figure 1. Increased musclin and decreased PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1 alpha) expression in heart failure with preserved ejection fraction (HFpEF) mice.

A, HFpEF in C57BL/6J male mice was induced by uninephrectomy, chronic infusion of d-aldosterone, and 1.0% sodium chloride (NaCl) drinking water for 4 weeks. In the soleus muscle of Sham (n=6–7) and HFpEF mice (n=10–11), quantitative analysis showing musclin (*Ostn*) gene expression (**B**), musclin protein expression and representative blots (**C**), PGC-1α (*Ppargc1a*) gene expression (**D**), and PGC-1α protein expression and representative blots (**E**). Data are presented as mean±SEM. Statistical analysis by the Student *t* test.

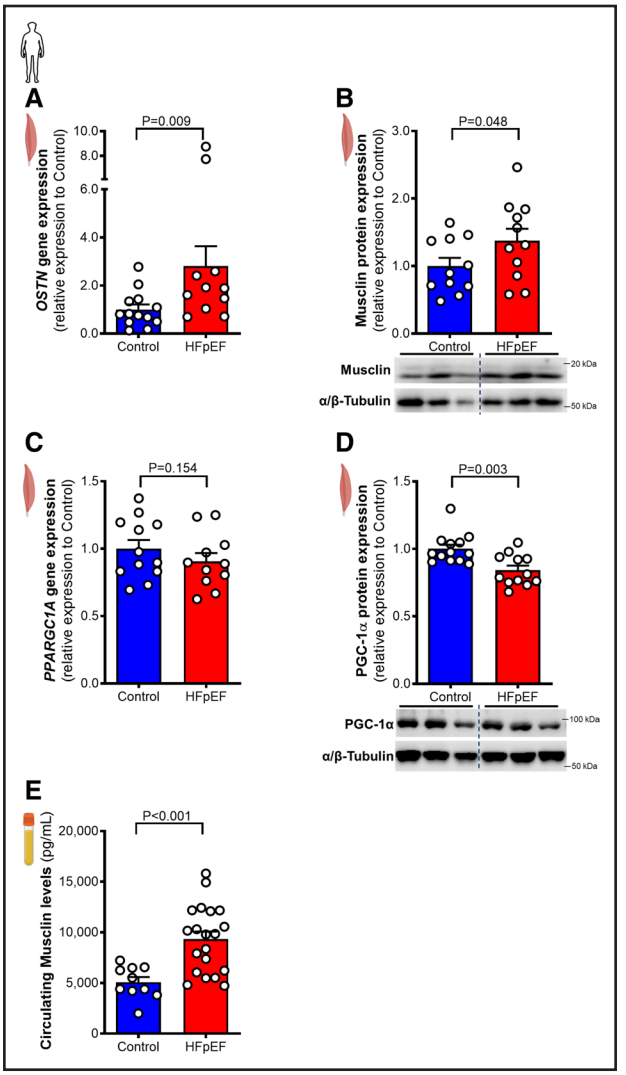

Figure 2. Increased musclin and decreased PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1 alpha) expression in patients with heart failure with preserved ejection fraction (HFpEF).

In the vastus lateralis muscle of control subjects (n=12–13) and patients with HFpEF (n=11–12) from the University of Pennsylvania cohort and quantitative analysis demonstrating musclin (*OSTN*) gene expression (**A**), musclin protein expression and representative blots (**B**), PGC-1α (*PPARGC1A*) gene expression (**C**), and PGC-1α protein expression and representative blots (**D**). Data are presented as mean±SEM. Statistical analysis by the Student *t* test for normally distributed data (musclin protein expression and *PPARGC1A* gene expression) or the Mann-Whitney *U* test for nondistributed data (*OSTN* gene expression and PGC-1α protein expression).
E, Quantitative analysis of circulating musclin levels in control subjects (n=10) and patients with HFpEF (n=20) from the Boston Medical Center cohort. Data are presented as mean±SEM. Statistical analysis by the Student *t* test.

Additional analysis was performed in a second cohort of patients with HFpEF (the Boston Medical Center cohort) to measure circulating musclin levels, which were significantly increased by 83% in patients with HFpEF versus control subjects (9341±741 versus 5087±509 pg/mL; $P<0.001$; Figure 2E). The BMC cohort included patients with HFpEF with a mean age of 74±3.1 years, predominantly women (65%) and Black (65%), mean body mass index (BMI) of 29.7±0.8 kg/m^2, and elevated circulating BNP levels (Table S1). They were predominantly New York Heart Association functional class II (55%) and class III (25%). Hypertension was the most common comorbidity (90%). Obesity (50%), type 2 diabetes (35%), and coronary artery disease (45%) were also prevalent. Echocardiography showed preserved left ventricular ejection fraction (61.9±1.3%) with increased left ventricle (LV) mass (175.3±13.1 g) and relative wall thickness (0.51±0.04). Diastolic dysfunction was present in 77% of the patients. Control subjects were healthy with no HFpEF

history, a mean age of 57.7±4.3 years, and a mean BMI of 26.8±1.1 kg/m^2. Hypertension (40%) and obesity (20%) were present in the controls. Blood samples were obtained from 2015 to 2016, and guideline-directed medical therapy did not include sodium-glucose cotransporter-2 inhibitors at that time for HFpEF.

Exogenous Musclin Administration to HFpEF Mice

Despite the increase in endogenous circulating musclin levels, which should, and may initially, potentiate NP signaling and, in turn, enhance SkM oxidative capacity,[12,13] this was not seen in HFpEF mice and patients with HFpEF.[7,10] Exogenous musclin (0.2 µg/g) or saline was administered intraperitoneally once, every other day, for 14 days to a second cohort of HFpEF male mice (Figure 3A and 3B) to determine whether further increasing musclin levels could augment NP signaling and counteract the soleus muscle phenotypic switching.[10] A pilot dose-response experiment demonstrated that 0.2-µg/g dose of musclin was effective in significantly reducing systolic blood pressure (SBP) in healthy mice, and as such, this was selected as the relevant dose (Supplemental Material; Figure S2B).

Enzyme-linked immunosorbent assay analysis demonstrated successful delivery of exogenous musclin, as musclin levels increased by 21% in musclin-treated HFpEF mice versus untreated HFpEF mice (416±17 versus 343±24 pg/mL; *P*=0.036; Figure 3C). As expected, circulating BNP levels were increased in untreated HFpEF by 61% versus Sham mice (7.1±0.7 versus 4.8±0.5 pg/mL; *P*=0.007; Figure 3D); however, BNP levels were not different between musclin-treated (8.5±0.6 pg/mL) and untreated HFpEF mice (*P*=0.963).

Characteristics of Musclin-Treated HFpEF Mice

As previously shown,[10,29–31] untreated HFpEF mice demonstrated moderately elevated SBP (138.6±1.7 versus 124.6±3.5 mm Hg in Sham mice; *P*=0.002). However, SBP remained persistently elevated in musclin-treated HFpEF mice (140.9±2.1 mm Hg; *P*=0.734) versus untreated HFpEF mice despite this dose lowering SBP in healthy mice (Figure S2B).

As noted previously, HFpEF mice have calf muscle atrophy, with a weight reduction in both gastrocnemius and soleus muscles.[10] Treatment with musclin had no effect on the weight (normalized to tibial length) of the gastrocnemius muscle (glycolytic-predominant) in musclin-treated versus untreated HFpEF mice (61.0±2.0 versus 58.5±2.1 mg/cm; *P*=0.626). However, the weight of the soleus muscle (oxidative-predominant) was significantly increased in musclin-treated versus untreated HFpEF mice (5.5±0.2 versus 4.4±0.3 mg/cm; *P*=0.012).

Furthermore, 4 weeks after HFpEF induction, survival in untreated HFpEF mice was 92% versus 100% survival in musclin-treated HFpEF mice (*P*=0.368), which was comparable to Sham mice.

Exogenous Musclin Enhances NP Signaling in the SkM of HFpEF Mice

Because we hypothesized that NP resistance may contribute to impaired SkM oxidative capacity in HFpEF, and exogenously administered musclin may augment NP signaling, the relative ratio of the gene expression of the NPRA (*Npr1*) and NPRC (*Npr3*) as an indicator of NP signaling activity was determined. There were no differences in the *Npr1*/*Npr3* ratio between untreated HFpEF and Sham mice; however, this ratio was significantly increased by 43% in the soleus muscle of musclin-treated versus untreated HFpEF mice (*P*=0.027; Figure 3E), driven mainly by a decreasing trend in *Npr3* gene expression in musclin-treated HFpEF mice (*P*=0.146; Figure S3), suggesting that exogenous musclin increased NP signaling activity.

To investigate this further, second messenger cGMP levels were measured in the soleus muscle. The cGMP levels were significantly decreased in the soleus muscle of HFpEF mice versus Sham mice (1.95±0.2 versus 2.86±0.4 pmol/mL; *P*=0.028) but were no different between musclin-treated (2.31±0.2 pmol/mL; *P*=0.391) and untreated HFpEF mice (Figure 3F).

Because cGMP can be rapidly hydrolyzed by PDEs (phosphodiesterases) and, thus, deplete the intracellular cGMP levels (Figure 3B) and diminish NP signaling activity, gene expression of the cGMP-specific PDEs, *Pde5a* and *Pde9a*, was measured. While *Pde5a* gene expression was increased in the soleus muscle of untreated HFpEF (152%; *P*=0.001) versus Sham mice, exogenous musclin did not affect *Pde5a* gene expression in musclin-treated HFpEF mice (Figure 3G). *Pde9a* gene expression was unchanged in Sham, untreated HFpEF, and musclin-treated HFpEF mice.

As shown in Figure 3B, the activity of PKG1 (a primary effector of the NP signaling cascade) was then determined by measuring the phosphorylation level of its downstream substrate, VASP (vasodilator-stimulated phosphoprotein). Western blot analysis showed that phosphorylated VASP at serine 239 was similar between untreated HFpEF and Sham mice. This phosphorylation site is specific for PKG1 and is used to monitor cGMP/PKG1 signaling activity.[32] However, phosphorylated VASP was significantly increased by 45% in musclin-treated HFpEF mice versus untreated HFpEF mice (*P*=0.035; Figure 3H). Moreover, PGC-1α protein expression was significantly increased in musclin-treated versus untreated HFpEF mice (*P*=0.043; Figure 3I) and reverted to similar levels as the Sham mice, indicating that exogenous musclin was able to increase PKG1 activity and PGC-1α protein expression.

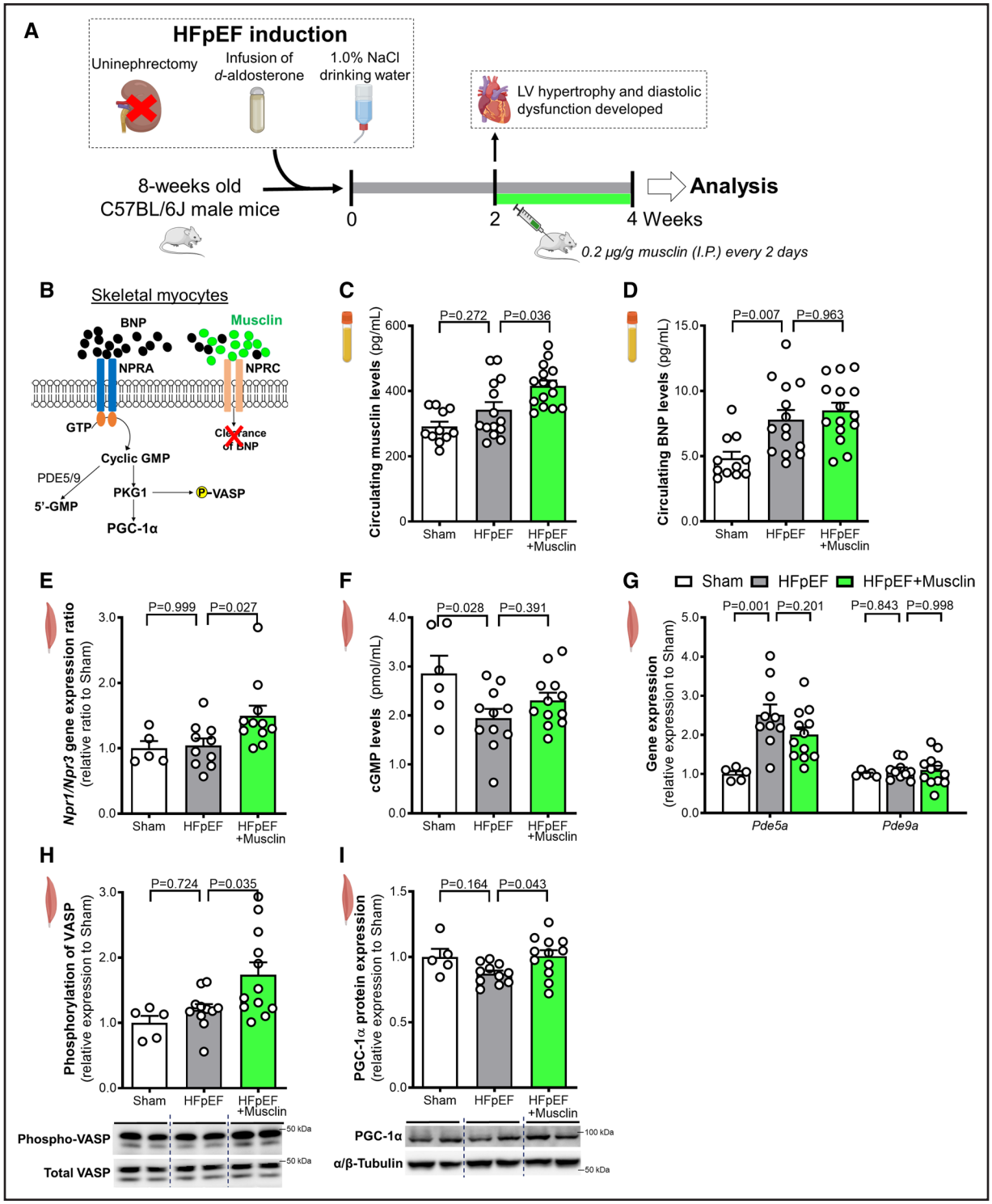

Figure 3. Exogenous musclin activates NPRA (natriuretic peptide receptor-A)/cyclic guanosine monophosphate (cGMP)/PKG1 (protein kinase G1)/PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1 alpha) signaling in heart failure with preserved ejection fraction (HFpEF) mice.

A, Exogenous musclin (0.2 μg/g, every 2 days) was injected intraperitoneally into HFpEF mice in the last 2 weeks of HFpEF induction. **B**, Illustrative scheme describes musclin's role in preventing NP (natriuretic peptide) degradation by binding to NPRC (natriuretic peptide clearance receptor) and so further activating NP signaling cascade. In serum samples of Sham (n=11), untreated HFpEF (n=14), and musclin-treated HFpEF mice (n=15), quantitative analysis showing the circulating levels of musclin (**C**) and BNP (brain natriuretic peptide; **D**). Data are presented as mean±SEM. Statistical analysis by the Kruskal-Wallis test with the Dunn multiple comparison test. In the soleus muscle of Sham (n=5–6), untreated HFpEF (n=10–11), and musclin-treated HFpEF mice (n=11–12), quantitative analysis showing NPRA (*Npr1*)-to-NPRC (*Npr3*) gene expression ratio (**E**), cGMP levels (**F**), *Pde5a* and *Pde9a* (phosphodiesterase 9A [PDE9]) gene expression (**G**), VASP (vasodilator-stimulated phosphoprotein) phosphorylation (**H**), and PGC-1α protein expression with representative (*Continued*)

Further analyses were performed to investigate the role of musclin in vitro. Cultured primary mouse myotubes stimulated with the combination of aldosterone (10 µmol/L) plus BNP (5 µmol/L), to recapitulate the in vivo HFpEF milieu, showed increased musclin protein expression by 16% (P=0.035) versus vehicle-treated myotubes (Figure S4A), supporting that elevated endogenous musclin is likely a compensatory response. Under the same experimental condition, PGC-1α protein expression was significantly reduced by 42% versus vehicle-treated myotubes (P=0.004; Figure S4B). These findings are consistent with the findings seen in HFpEF mice and patients with HFpEF (Figures 1 and 2). The addition of 0.1-µmol/L musclin to myotubes treated with the combination of aldosterone plus BNP increased PGC-1α protein expression by 63% versus myotubes stimulated with only aldosterone plus BNP without musclin (P=0.012; Figure S4B). These findings are consistent with the in vivo findings in musclin-treated HFpEF mice (Figure 3I) and demonstrate that exogenous musclin can activate NP signaling in skeletal myotubes.

NP Signaling in the SkM of Patients With HFpEF

To determine the clinical relevance of our findings in mice, the *NPR1/NPR3* ratio, cGMP levels, and *PDE5A* and *PDE9A* gene expression were measured in the vastus lateralis muscle from the University of Pennsylvania patients with HFpEF. The *NPR1/NPR3* ratio was significantly reduced by 40% in patients with HFpEF versus control subjects (P=0.008; Figure 4A), mainly driven by a significant increase in *NPR3* gene expression (P=0.005; Figure S5), suggesting diminished NP signaling activity. There was a trend to decreased cGMP levels in patients with HFpEF versus control subjects (0.25±0.02 versus 0.36±0.07 pmol/mL; P=0.268), but this was not statistically different likely due to the limited sample size (n=7–8/group; Figure 4B). Similarly, there was a trend of increasing *PDE5A* gene expression in patients with HFpEF, which was not different from control subjects. However, *PDE9A* gene expression was increased by 53% in patients with HFpEF versus control subjects (P=0.047; Figure 4C). Altogether, these clinical findings suggest a comparable degree of dysregulated NP signaling possibly due to NP resistance as seen in the HFpEF mice (Figure 3F and 3G).

Exogenous Musclin Induces Fiber Transition in the SkM of HFpEF Mice

To investigate the changes seen with exogenous musclin administration in HFpEF mice, immunofluorescent staining was performed to examine the fiber composition of the soleus muscle. As expected, the soleus muscle of untreated HFpEF mice demonstrated a reduced abundance of oxidative-type fibers versus Sham mice (86.8±0.5 versus 93.0±1.6%; P=0.014; Figure S6A). Glycolytic-type fibers were increased in abundance in HFpEF (13.3±0.5 versus 7.0±1.6%; P=0.866) but were no different between musclin-treated and untreated HFpEF mice.

However, by comparing the abundance of specific fiber type in the soleus muscle of musclin-treated versus untreated HFpEF mice, there was increased abundance of type-1/2A hybrid fibers (1.8±0.4 versus 0.6±0.1%; P=0.015; Figure 5A and 5B) but unchanged in the abundance of type-1, type-2A, type-2X, and type-2B fibers (Figure S6B), indicating that with this increase in hybrid fibers, the soleus muscle is in the process of fiber type switching.[33] To confirm these findings, protein expression of specific MHC (myosin heavy chain) isoforms was quantitatively measured. MHCI, the main MHC isoform in type-1 fiber, was increased by 25% in the soleus muscle of musclin-treated HFpEF mice versus untreated HFpEF mice (P=0.003), and conversely, MHCIIA, the main MHC isoform in type-2A fiber, protein expression was decreased by 15% in musclin-treated HFpEF mice versus untreated HFpEF mice (P=0.005; Figure 5C), indicating a fiber transition from type-2A to type-1 fibers. Although both type-1 and type-2A are oxidative-type fibers, MHCI in type-1 fiber has the slowest contraction speed (slow-twitch), and hence, it is resistant to fatigue. Conversely, MHCIIA in type-2A fiber contracts and fatigues faster than type-1 (fast-twitch).[15,34]

Exogenous Musclin Enhances Mitochondrial Succinate Dehydrogenase Activity in the SkM of HFpEF Mice

Because PGC-1α enhances mitochondrial activity and biogenesis in the SkM,[14,15,17] the activity of mitochondrial complex II SDH (succinate dehydrogenase; a marker for muscle oxidative capacity[35]) was measured. SDH activity was increased in the soleus muscle of musclin-treated HFpEF mice versus untreated HFpEF mice (0.76±0.004 versus 0.74±0.004 blue pixel/µm^2, P=0.009; Figure 5D and 5E).

Exogenous Musclin Increases Hindlimb Blood Flow and SkM Capillary Density in HFpEF Mice

Laser Doppler imaging of the hindlimb of mice showed a 26% reduction in blood flow in untreated HFpEF mice versus Sham mice (P<0.001). This reduction was

Figure 3 Continued. blots (**I**). Data are presented as mean±SEM. Statistical analysis by the 1-way ANOVA with the Tukey multiple comparison test for normally distributed data (cGMP levels, *Pde5a* and *Pde9a* gene expression, VASP phosphorylation, and PGC-1α protein expression) or the Kruskal-Wallis test with the Dunn multiple comparison test for nondistributed data (*Npr1*-to-*Npr3* gene expression ratio). 5'-GMP, Pde5a indicates phosphodiesterase 5A (PDE5); and GTP, guanosine triphosphate.

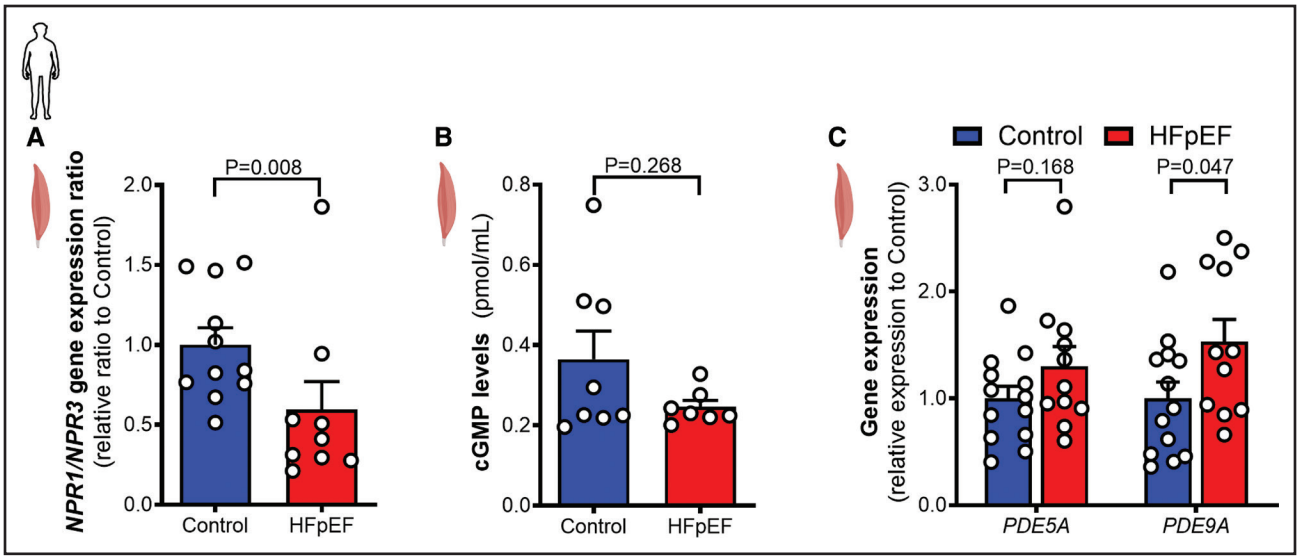

Figure 4. NPRA (natriuretic peptide receptor-A)/cyclic guanosine monophosphate (cGMP) signaling in patients with heart failure with preserved ejection fraction (HFpEF).
In the vastus lateralis muscle of control subjects and patients with HFpEF from the University of Pennsylvania cohort, quantitative analysis showing NPRA (*NPR1*)-to-*NPR3* (natriuretic peptide receptor 3 [NPRC]) gene expression ratio (n=9–11/group; **A**), cGMP levels (n=7–8/group; **B**), and *PDE5A* and *PDE9A* gene expression (n=11–13/group; **C**). Data are presented as mean±SEM. Statistical analysis by the Student *t* test for normally distributed data (*PDE5A* and *PDE9A* gene expression) or the Mann-Whitney *U* test for nondistributed data (*NPR1*-to-*NPR3* gene expression ratio and cGMP levels).

ameliorated by exogenous musclin administration with an increase in blood flow of 20% in HFpEF versus untreated HFpEF mice (P=0.006; Figure 6A and 6B). Additional immunofluorescent analysis of the soleus muscle showed that the number of isolectin-stained endothelial cells per fiber was reduced by 46% in untreated HFpEF mice versus Sham mice (2.5±0.1 versus 4.6±0.2; P<0.001). Exogenous musclin increased the isolectin-stained endothelial cells per fiber by 24% (3.1±0.1; P=0.044) versus untreated HFpEF mice (Figure 6C and 6D). Although proangiogenic *Pdgfb* gene expression was no different in the soleus muscle between untreated HFpEF and Sham mice (Figure 6E), *Pdgfb* expression was increased by 33% in musclin-treated HFpEF mice versus untreated HFpEF mice (P=0.044). *Angpt2* gene expression was no different between Sham, untreated HFpEF, and musclin-treated HFpEF mice.

Exogenous Musclin Mitigates Cardiac Hypertrophy in HFpEF Mice Independent of an Effect on SBP

HFpEF mice demonstrated cardiac hypertrophy, impaired diastolic function, and preserved left ventricular ejection fraction, as previously described (Table S2).[30,31] Echocardiography demonstrated that exogenous musclin reduced LV mass in HFpEF mice (120.7±4.1 versus 140.8±6.6 mg; P=0.045) and posterior wall thickness (0.92±0.03 versus 1.02±0.02 mm; P=0.039) compared with untreated HFpEF mice (Table S2). There was a trend to decreased LV end diastolic diameter and relative wall thickness in musclin-treated HFpEF mice but did not differ significantly from untreated HFpEF mice. Although musclin-treated HFpEF mice still developed diastolic dysfunction, only 40% of these mice exhibited a grade III (restrictive) pattern, the most severe form of diastolic dysfunction, versus 60% of the untreated HFpEF mice (Figure S7). The left atrium area was increased in untreated HFpEF mice versus Sham mice (10.9±0.9 versus 9.6±0.4 mm^2; P=0.045) and was no different after musclin treatment (9.8±0.4 mm^2; P=0.082; Table S2).

Given the above echocardiographic findings, the cross-sectional area of the cardiomyocytes was determined after hematoxylin and eosin staining. Cardiomyocyte size was increased by 21% in untreated HFpEF mice versus Sham mice (417±19 versus 344±9 μm^2; P=0.015), but treatment with exogenous musclin decreased it by 18% versus untreated HFpEF mice (341±13 μm^2; P=0.006; Figure 7A and 7B). Consistent with these findings, BNP gene (*Nppb*) expression was increased by 220% in untreated HFpEF mice versus Sham mice (P=0.002), and musclin treatment decreased *Nppb* expression by 51% in musclin-treated HFpEF mice versus untreated HFpEF mice (P=0.018; Figure 7C). Therefore, exogenous musclin, independent of SBP, exerted a direct effect on the heart by mitigating cardiomyocyte hypertrophy.

Cardiac fibrosis, measured by picrosirius red staining, was increased in the LV of untreated HFpEF mice versus Sham mice (7.4±0.3 versus 5.4±0.9%; P=0.043) but unaffected by exogenous musclin (7.5±0.6%; P=0.993; Figure 7D and 7E).

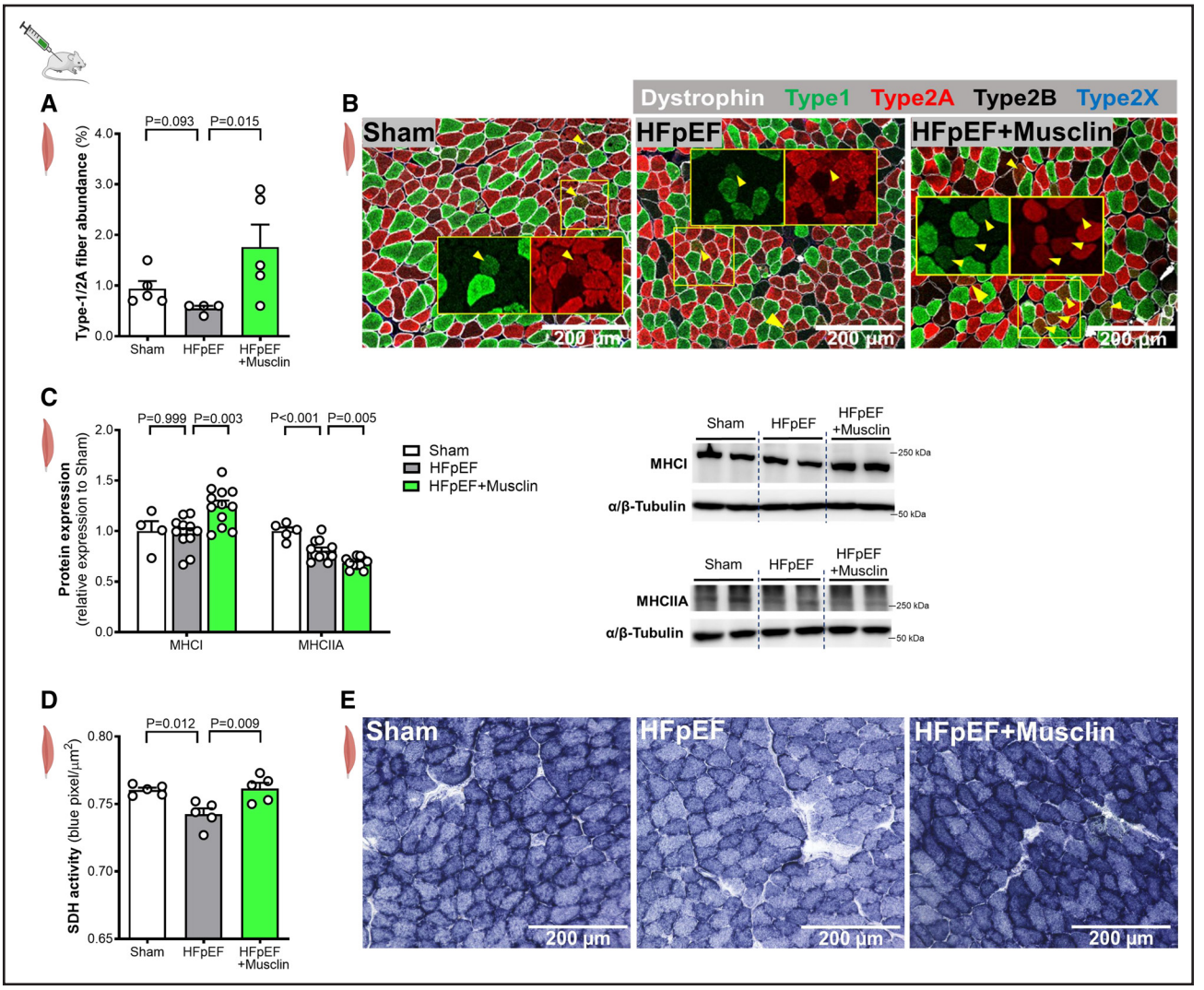

Figure 5. Exogenous musclin enhances fiber transition and SDH (succinate dehydrogenase) activity in heart failure with preserved ejection fraction (HFpEF) mice.

A, Quantitative analysis showing the abundance of type-1/2A hybrid fibers in the soleus muscle of Sham (n=5), untreated HFpEF (n=4), and musclin-treated HFpEF mice (n=5). Data are presented as mean±SEM. Statistical analysis by the Kruskal-Wallis test with the Dunn multiple comparison test. **B**, Representative confocal images showing staining of dystrophin (white), MHCI (myosin heavy chain I) in type-1 fiber (green), MHCIIA (myosin heavy chain IIA) in type-2A fiber (red), and MHCIIX (myosin heavy chain IIX) in type-2X fiber (blue) in the soleus muscle. Type-1/2A hybrid fibers (denoted by the yellow arrows) are coexpressing MHCI (green) and MHCIIA (red). **C**, Qualitative immunoblot and quantitative analysis showing MHCI and MHCIIA protein expression and representative blots in the soleus muscle of Sham (n=4–5), untreated HFpEF (n=11), and musclin-treated HFpEF mice (n=11–12). Data are presented as mean±SEM. Statistical analysis by the 1-way ANOVA with the Tukey multiple comparison test. **D**, Quantitative analysis showing the SDH activity in the soleus muscle of Sham (n=5), untreated HFpEF (n=5), and musclin-treated HFpEF mice (n=5). Data are presented as mean±SEM. Statistical analysis by the 1-way ANOVA with the Tukey multiple comparison test. **E**, Representative brightfield images showing the SDH activity in the soleus muscle.

Exogenous Musclin Improves Exercise Capacity in HFpEF Mice

HFpEF mice have impaired exercise capacity as demonstrated by reduced running distance and time on a treadmill.[30,36] Treadmill exercise exhaustion test was performed in a blinded manner to examine the effect of exogenous musclin in HFpEF. Exogenous musclin in HFpEF mice improved running distance by 33% versus untreated HFpEF mice (541±43 versus 405±35 meters; *P*=0.020; Figure 8A). Running time was increased by 17% in musclin-treated HFpEF mice versus untreated HFpEF mice (28.2±1.3 versus 24.1±1.3 minutes; *P*=0.016; Figure 8B).

DISCUSSION

In our study, musclin expression was increased with a concurrent decrease in PGC-1α expression in the oxidative-predominant SkM of rodents and patients with HFpEF. Decreased PGC-1α expression can be explained, at

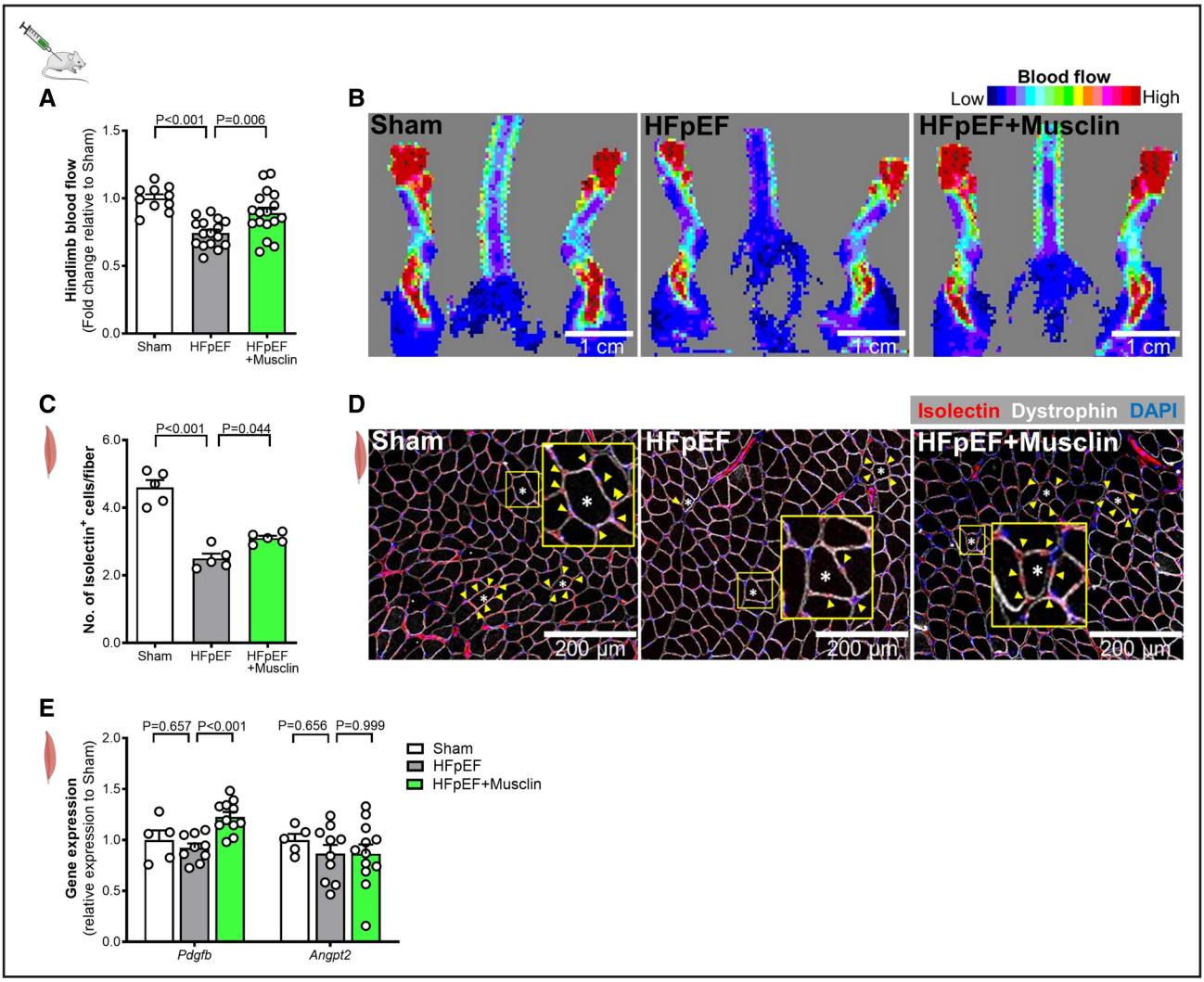

Figure 6. Exogenous musclin increases hindlimb blood flow and capillary density in heart failure with preserved ejection fraction (HFpEF) mice.

A, Quantitative analysis and showing the blood flow in the hindlimbs of Sham (n=10), untreated HFpEF (n=16), and musclin-treated HFpEF mice (n=17). Data are presented as mean±SEM. Statistical analysis by the 1-way ANOVA with the Tukey multiple comparison test. **B**, Representative laser Doppler images showing blood flow in the hindlimbs of mice. **C**, Quantitative analysis showing the density of capillaries in the soleus muscle of Sham (n=5), untreated HFpEF (n=5), and musclin-treated HFpEF mice (n=5). Data are presented as mean±SEM. Statistical analysis by the 1-way ANOVA with the Tukey multiple comparison test. **D**, Representative confocal images showing staining of dystrophin (white; white asterisk indicates a single fiber), isolectin-stained endothelial cells (red; denoted by the yellow arrows), and nuclei (blue) in the soleus muscle. **E**, Quantitative analysis showing the relative gene expression of *Pdgfb* and *Angpt2* in the soleus muscle of Sham (n=5), untreated HFpEF (n=9–10), and musclin-treated HFpEF mice (n=11–12). Data are presented as mean±SEM. Statistical analysis by the 1-way ANOVA with the Tukey multiple comparison test.

least in part, by NP resistance, thus contributing to defective NP signaling in SkM in HFpEF. The evidence that NP resistance occurred in HFpEF is demonstrated by increased circulating BNP levels but unchanged (mice)/decreased (humans) *Npr1*/*Npr3* ratio, decreased cGMP levels, and unchanged PKG1 activity with a concomitant increase in cGMP-specific PDEs. Importantly, exogenous musclin administration in HFpEF mice overcame this NP resistance as shown by an increase in the *Npr1*/*Npr3* ratio, PKG1 activity, and PGC-1α expression. This led to SkM function improvement that included, in addition to improved exercise capacity, a transition of fast-twitch type-2A to slow-twitch type-1 fibers, increased mitochondrial activity, and enhanced hindlimb blood flow accompanied by increased capillary density in the soleus muscle. Exogenous musclin also ameliorated cardiomyocyte hypertrophy as demonstrated by a reduction in LV mass and BNP gene expression. Altogether, these findings demonstrate the potential therapeutic benefit of musclin as an SkM-directed approach with added benefits on cardiac structure in HFpEF independent of an effect on blood pressure (Figure S8).

Impaired oxidative capacity is a major feature of SkM dysfunction in HFpEF (Figure S8)[6–11] and, as previously

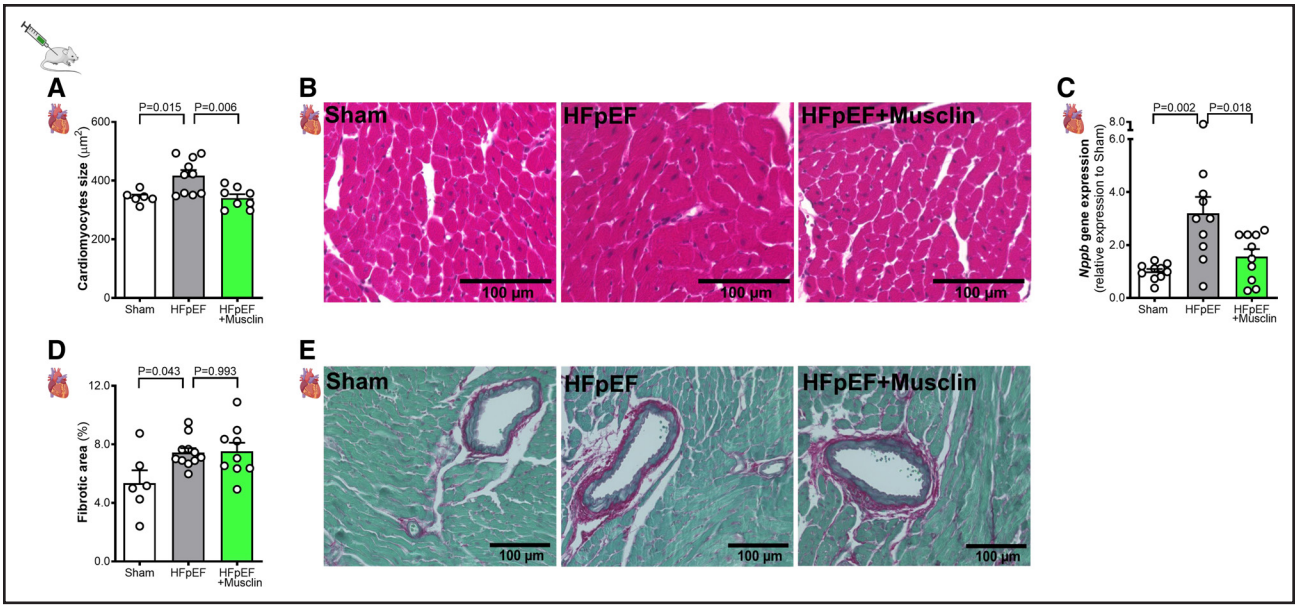

Figure 7. Exogenous musclin reduces cardiomyocyte hypertrophy and *Nppb* gene expression in the left ventricle (LV) of heart failure with preserved ejection fraction (HFpEF) mice.

A, Quantitative analysis showing the cardiomyocyte size in the LV of Sham (n=6), untreated HFpEF (n=10), and musclin-treated mice (n=8). Data are presented as mean±SEM. Statistical analysis by the 1-way ANOVA with the Tukey multiple comparison test. **B**, Representative brightfield images of hematoxylin and eosin-stained LV sections. **C**, Quantitative analysis showing the relative gene expression of *Nppb* in the LV of Sham (n=10), untreated HFpEF (n=10), and musclin-treated HFpEF mice (n=10). Data are presented as mean±SEM. Statistical analysis by the 1-way ANOVA with the Tukey multiple comparison test. **D**, Quantitative analysis showing the fibrotic area in the LV of Sham (n=6), untreated HFpEF (n=11), and musclin-treated mice (n=9). Data are presented as mean±SEM. Statistical analysis by the 1-way ANOVA with the Tukey multiple comparison test. **E**, Representative brightfield images of picrosirius red–stained LV sections.

reported, seen in muscle samples from patients with HFpEF and SAUNA HFpEF mice.[10] While the exact causative mechanisms are unknown, NP resistance has a high likelihood of contributing to this phenotype of impaired SkM oxidative capacity in HFpEF.

Prior therapies developed for HF with reduced ejection fraction to overcome NP resistance and the presumed relative deficiency of the active forms of NPs include the ANP analog, carperitide; the BNP analog, nesiritide; and sacubitril (neprilysin inhibitor; in combination with angiotensin receptor inhibitor valsartan).[25,37,38] In our study, similar to human HF, the initial increase in endogenous musclin in HFpEF may be compensatory to overcome SkM NP resistance. That being said, such an elevation in endogenous musclin is apparently insufficient to overcome NP resistance, and impaired oxidative capacity is observed in both HFpEF mice and patients with HFpEF. Other possible contributing factors that prevent endogenous musclin from augmenting NP signaling may include the relative deficiency of circulating NPs (although increased, is apparently insufficient),[39] increased inactive form of NPs,[40] and increased NP degradation via other mechanisms such as neprilysin.[41] Thus, it is reasonable to administer exogenous musclin to HFpEF mice with the same rationale as carperitide, nesiritide, and sacubitril, so as to augment NP signaling. However, what makes musclin (and sacubitril) different from carperitide and nesiritide is that musclin prolongs the half-life of existing circulating NPs rather than directly increasing it.

Our study is dissimilar to Szaroszyk et al[13] who reported downregulation of musclin in the SkM of patients with sarcopenia/cachexia with chronic HF with reduced ejection fraction and HF with reduced ejection fraction mice. However, it is important to note that neither HFpEF mice nor patients with HFpEF were cachectic in our study and that HFpEF and HF with reduced ejection fraction are distinct diseases with different organ pathophysiologies and molecular signatures although the clinical presentation of effort intolerance and SkM phenotypic switching appears similar.[42]

Prior studies demonstrate that musclin potentiates NP signaling.[12,13] In SkM, NPs bind their receptors and activate NP signaling, generating cGMP, activating PKG1, and leading PGC-1α to transcriptionally enhance downstream signaling pathways involved in oxidative capacity.[14–17] In our study, while we initially postulated that exogenous musclin would further increase the circulating BNP levels in HFpEF, these levels, however, remained comparable between musclin-treated and untreated HFpEF mice. This finding can be explained by inadequate BNP production from the hearts of musclin-treated HFpEF mice, but this also indicates that exogenous musclin, by binding to NRPC, was effective at preventing BNP degradation and, thus, prolonging the

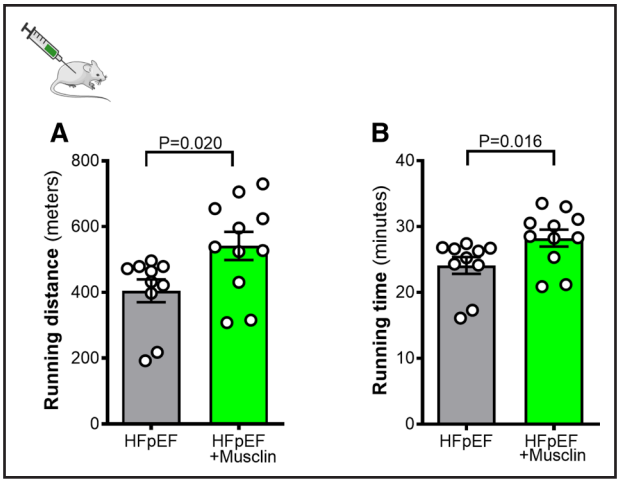

Figure 8. Exogenous musclin improves treadmill exercise exhaustion capacity in heart failure with preserved ejection fraction (HFpEF) mice.
A quantitative analysis demonstrating treadmill running distance (**A**) and running time (**B**) of untreated HFpEF and musclin-treated HFpEF mice (n=10–11/group). Data are presented as mean±SEM. Statistical analysis by the Mann-Whitney *U* test.

half-life of BNP in the circulation in HFpEF mice. Furthermore, cGMP levels were decreased in the SkM of untreated HFpEF mice with a trend to decrease cGMP levels in the SkM of patients with HFpEF. This is, at least partly, due to the enhanced hydrolytic action of PDEs, leading to impaired signaling transduction. Administration of exogenous musclin did not markedly increase cGMP levels but resulted in a small elevation, as it is possible that the newly synthesized cGMP is being hydrolyzed by PDEs simultaneously. Nevertheless, this small elevation was sufficient to activate cGMP-dependent PKG1 as demonstrated by increased phosphorylation at serine 239 of VASP (PKG1-specific substrate)[32] and increased PGC-1α expression in the soleus muscle of musclin-treated HFpEF mice, thus overcoming the hydrolytic action of PDEs.

PGC-1α transcriptionally coactivates signaling pathways that lead to glycolytic-to-oxidative fiber transition (this occurs in the direction of 2B→2X→2A→1[33]) and regulation of mitochondrial function/biogenesis to meet energy demand and maintain oxidative capacity.[14,15,17] Type-1 (slow-twitch) oxidative fibers are enriched with mitochondria primarily utilizing oxidative metabolism for fuel; type-2X and type-2B have less mitochondria and predominantly use glycolytic metabolism. Type-2A oxidative (fast-twitch) has both high oxidative and glycolytic capacity and intermediate contractile properties.[10] In humans with HFpEF, decreased type-1 oxidative fiber number and mitochondrial dysfunction are closely associated with exercise intolerance.[6,7] Our findings showed that exogenous musclin supplementation in HFpEF mice significantly increased the abundance of hybrid fibers in the soleus muscle. Additional quantitative analysis showed increased MHCI and decreased MHCIIA expression in the soleus muscle of musclin-treated HFpEF mice, indicating ongoing fiber transition from type-2A to type-1.[33] These findings, in addition to the lack of hypertrophy in the glycolytic-predominant gastrocnemius muscle, indicate that musclin has no effect on glycolytic-type fibers in HFpEF. Moreover, similar to others,[12] exogenous musclin also increased mitochondrial complex II SDH activity in the soleus muscle of HFpEF mice. Although mitochondrial biogenesis and number were not measured in our study, findings here are consistent with those reported by Engeli et al,[14] where NP/cGMP/PGC-1α signaling increased mitochondrial function without significant changes in mitochondrial DNA content and mass in human primary myotubes.

PGC-1α plays an essential role in mediating exercise-induced angiogenesis in SkM.[43] It coactivates with the ERR (estrogen-related receptor)-α to increase gene expression of proangiogenic factors such as *Angpt2* and *Pdgfb* during normoxia.[16,43] We previously showed decreased capillary density in the soleus muscle of HFpEF mice,[10] which is seen in humans with HFpEF.[6] In the current study, exogenous musclin enhanced hindlimb blood flow and capillary density in HFpEF soleus muscle. This was accompanied by increased *Pdgfb* gene expression. PDGF-β induces splitting angiogenesis and maintains vascular integrity by recruiting pericytes to the basement membrane of vessels.[44,45] Splitting angiogenesis is a common form of intravascular remodeling that contributes to capillary expansion with new microvessels formed from preexisting microvessels that splits into 2 without sprouting[46] It is a faster, more energy-efficient process than sprouting angiogenesis, which requires intense endothelial cell proliferation or migration. Splitting involves only the reorganization of existing endothelial cells. Our findings suggest that exogenous musclin may induce splitting angiogenesis by activating PDGF-β via PGC-1α to rapidly increase the supply of oxygen and nutrients to SkM.

Although the primary aim of our study was to explore the role of musclin in SkM in HFpEF, there were added benefits of musclin in the heart. Cardiomyocyte hypertrophy was attenuated by exogenous musclin in HFpEF mice as shown by the reduction in LV mass, reduced LV cardiomyocyte size, and decreased *Nppb* gene expression with minimal effects on systolic and diastolic function. Importantly, SBP remained elevated in the musclin-treated HFpEF mice, and such an improvement in the cardiac phenotype was independent of BP. These findings are consistent with prior studies showing that musclin alleviates cardiac hypertrophy,[13,47] and it is plausible that this effect is directly mediated through NP signaling via NPRA that is known to counteract hypertrophy.[48] Moreover, although musclin reduced SBP in healthy mice (Figure S2B) and as previously described,[47] this was not the case in HFpEF mice, which may be explained by the elevated circulating

levels of vasoconstrictors such as endothelin, angiotensin II, and norepinephrine to overcome the effects of musclin and NP signaling.[23,49] Although other studies also showed that musclin and BNP exert antifibrotic effects in the heart,[13,50] this was not seen in our study. It is possible that because musclin was only administered for 2 weeks after HFpEF induction, it was of insufficient duration, or conversely, the fibrotic process was already irreversible at this time point in this model. This contrasts with genetic models that constitutively express musclin at much greater levels before the development of cardiovascular disease, in which musclin exhibits a beneficial effect,[13] but the clinical relevance of this to a chronic disease state, such as HFpEF, is unclear.

Finally, exogenous musclin improved exercise capacity in HFpEF mice, as shown by the improved running distance and time to exhaustion on the treadmill. The totality of these findings suggests that exogenous musclin may have a therapeutic potential in humans with HFpEF and warrants future investigations.

Study Limitations

In this study, decreased cGMP levels were seen in the SkM of untreated HFpEF mice with a trend to decrease cGMP levels in the SkM of patients with HFpEF. The lack of statistical difference between patients with HFpEF and controls may be due to the relatively small sample size and the heterogeneous nature of human muscle, and the majority of patients with HFpEF were stable and well-compensated with only New York Heart Association class II symptoms (less symptomatic patients). In contrast, the HFpEF SAUNA mice, in addition to developing severe symptoms such as lung congestion and decreased exercise capacity,[10,29–31] had decreased survival, and as such, this phenotype is more comparable with patients with acutely decompensated HF. Additional studies are also warranted to evaluate whether exogenous musclin can be an effective treatment during an acute, severe HFpEF event. Of note, healthy control subjects were not age-matched to the patients with HFpEF as it remains challenging to recruit older healthy subjects without comorbidities or cardiovascular disease for invasive muscle biopsy procedures.

While significant attention has been given to obesity in HFpEF, hypertension persists as the major risk factor in both the pathogenesis and prognosis of HFpEF. Despite overwhelming evidence linking hypertension to HFpEF pathogenesis, preclinical studies (and clinical practice) often relegate hypertension to a secondary consideration. Comorbidities such as obesity and type 2 diabetes are deemed equivalent to hypertension in HFpEF development, yet HFpEF occurrences in obesity or type 2 diabetes are uncommon without concurrent hypertension (or underlying coronary artery disease). The current study utilizes a murine HFpEF model where hypertension (with renal dysfunction) is the major comorbidity. While obesity-HFpEF is increasingly common, and recent studies show a therapeutic benefit in this subtype, hypertension remains the single most common risk factor in HFpEF worldwide as seen in the 2 patient cohorts investigated in this study. Therefore, the HFpEF SAUNA preclinical model was used in this study. We also utilized the obese, hypertensive diabetic HFpEF ZSF1 rat to confirm that our pivotal finding was not model-specific. Future studies warrant combining comorbidities such as hypertension plus obesity and others to further investigate NP resistance/depletion in animal models and patients with HFpEF. Finally, the current study included only male mice. Although the observations of musclin expression changes and NP resistance/depletion in HFpEF mice were consistent with those in patients with HFpEF (of which 65% were women in this study), future studies should be performed in female mice to corroborate the current findings.

ARTICLE INFORMATION

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Disclosures

Dr Zamani consulted for Pfizer and Vyaire. Dr Chirinos is an editorial board member of the American Heart Association, the American College of Cardiology, Elsevier, and Wiley. He consulted for Bayer, Fukuda Denshi, Bristol Myers Squibb, JNJ, Edwards Life Sciences, Merck, and NGM Biopharmaceuticals. Dr Sam is a full-time employee of Eli Lilly and Co, Indianapolis, IN. None of the work was funded nor supported by Eli Lilly and Co. The other authors report no conflicts.

Supplemental Material

Supplemental Methods and Materials
Tables S1–S7
Figures S1–S10
References 51–56

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