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Research article

The vasculature in HFpEF vs HFrEF: differences in contractile protein expression produce distinct phenotypes

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ABSTRACT

Both heart failure with reduced (HFrEF) and preserved (HFpEF) ejection fraction are associated with abnormalities of the vasculature, including a resting vasoconstriction and a decrease in sensitivity to nitric oxide (NO) mediated vasodilation. Vascular tone is controlled by the expression and activation of both smooth muscle (SM) and nonmuscle (NM) myosin, and NO mediated vasodilation is regulated by the expression of the leucine zipper positive (LZ+) isoform of the myosin targeting subunit (MYPT1) of myosin light chain phosphatase (MLCP). This study was designed to determine the expression of these contractile proteins in humans with HFrEF and HFpEF vs normal controls. We isolated tertiary mesenteric vessels from remnant biospecimens of patients undergoing partial or total colectomy at Mayo Clinic Rochester from August 2017 to December 2018, and examined the expression of MYPT1 and the LZ + MYPT1 isoform with immunoblots, while 2D SDS-PAGE was used to resolve the phosphorylated and nonphosphorylated regulatory light chains of NM and SM myosin. Our data show that NM myosin expression, as a percentage of total myosin, was 12 ± 3% (controls, n = 6), 7 ± 5% (HFpEF, n = 4) and 37 ± 18% (HFrEF, n = 5, $p < 0.05$). Total MYPT1 expression was significantly reduced ($p < 0.05$) in both HFpEF (70 ± 11%) and HFrEF (48 ± 6%); and in HFrEF, LZ + MYPT1 was also depressed (62 ± 19%, <0.05). These results demonstrate that HFrEF and HFpEF are distinct vascular entities, and the changes in protein expression contribute to the vascular abnormalities associated with these diseases. Further in HFpEF, the decrease in MYPT1 would explain why pharmacologic therapies that are designed to activate the NO/cGMP/PKG signaling pathway do not produce a clinical benefit.

1. Introduction

Heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF) have similar clinical presentations, but are clearly two distinct entities. Therapies that improve outcomes in HFrEF [1] have shown no benefit in patients with HFpEF [2, 3]. Although a resting vasoconstriction and reduced sensitivity to nitric oxide (NO) are associated with both types of heart failure, the molecular basis for these changes in the vasculature are poorly understood. An increase in vascular tone can be produced by changes in the expression of contractile proteins within the smooth muscle cell or alterations in the vessel wall, while a decrease in NO sensitivity could be the result of endothelial dysfunction or a decrease in the vascular response to NO.

Overall vascular tone is determined by the level of phosphorylation of the smooth muscle myosin regulatory light chain (RLC), which is controlled by the activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) [4]. MLCK is regulated by Ca^{2+}-calmodulin [5], and an increase in MLCK activity results in an increase in the phosphorylation of the RLC, which produces vasoconstriction. MLCP is regulated by a number of signaling pathways, which either inhibit MLCP to increase RLC phosphorylation and vascular tone or activate MLCP which decreases RLC phosphorylation and vascular tone [6, 7].

The signaling pathway for NO mediated vasodilatation has been well described [7]. Briefly, NO diffuses into smooth muscle cells and stimulates soluble guanylate cyclase, which hydrolyzes GTP to cGMP, which activates protein kinase G (PKG). Subsequently, PKG phosphorylates a number of targets to produce a decrease in intracellular Ca^{2+}, and also phosphorylates the myosin-targeting subunit (MYPT1) of MLCP, which dephosphorylates the RLC of SM myosin to produce a Ca^{2+} independent relaxation. Alternative mRNA splicing produces 2 MYPT1 isoforms, leucine zipper (LZ+ and LZ-), which differ by the presence or absence of a COOH-terminal LZ domain [7, 8]. The amino acid sequence of the

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MYPT1 LZ domain is identical from worm to human [8], which suggests that this domain could play a prominent role in the regulation of MLCP. Others have demonstrated that PKG only phosphorylates and therefore only activates the LZ + MYPT isoform [9, 10]. Thus, the sensitivity to NO is determined by LZ + MYPT1 expression [9, 10, 11]. Further, the expression of LZ+/LZ- MYPT1 isoforms is modulated in animal models of sepsis [12], pre-eclampsia [13], pulmonary arterial hypertension [14], and HFrEF [15, 16, 17], which suggests that changes in MYPT1 expression could be important in humans with HFpEF and/or HFrEF.

Smooth muscle expresses both smooth muscle (SM) and nonmuscle (NM) myosin [18]. The kinetics of NM myosin are slow [19, 20, 21] and therefore an increase in NM myosin expression increases vascular tone and force [18]. NM myosin expression has also been demonstrated to increase in pulmonary arterial hypertension [14] as well as hypertension [22], suggesting that an increase in NM myosin expression could also occur in patients with heart failure.

Therefore, focusing on the vascular etiologies of both HFpEF and HFrEF could further define pathophysiologic characteristics, and potentially explain the differences in response to specific therapy observed in patients with HFrEF versus HFpEF. This study was designed to determine the expression of smooth muscle contractile proteins, specifically SM myosin, NM myosin, total MYPT1, and the LZ + MYPT1 isoform in humans with HFrEF and HFpEF compared with normal controls.

2. Material and methods

2.1. Patient population

Patients undergoing partial or total colectomy at Mayo Clinic in Rochester from August 2017 until December 2018 were screened for inclusion. No patient underwent an emergent procedure, and all had colon cancer. Prior to surgery, patient records were reviewed for a diagnosis of heart failure. In patients with heart failure, echocardiographic data was reviewed to determine HFrEF versus HFpEF based on an ejection fraction of <40% for HFrEF and >50% for HFpEF. Baseline patient characteristics are included in Table 1. Electronic medical records were reviewed for the normal control group as well, and the controls consisted of patients with no history of any cardiovascular disease and not treated with any cardiac medication. At the time of surgery, tertiary mesenteric arterial vessels were isolated from remnant biospecimens, placed in liquid nitrogen, and stored at -80 °C. The protocol for tissue procurement was reviewed by the Institutional Review Board of the Mayo Clinic and approved (IRB:17-006365), and the tissue requested (remnant biospecimens) is considered exempt from requiring informed consent by institutional policy (Surgical/Procedural Specimen Exemption from Pathology Examination and Gross Examination Only Policy; http://mayocontent.mayo.edu/practiceadmin/DOCMAN-0000132386?redirected=1).

2.2. Immunoblotting

As previously described [9, 23, 24, 25, 26, 27], Western blots were used to determine protein expression in human mesenteric arteries. Briefly, mesenteric artery samples were homogenized in SDS sample buffer, and total extracted protein was then resolved by SDS-PAGE using a Bis-Tris buffer and 7.5% gels. For the Western blots, we used the actin band on the Coomassie stained gels to normalize protein loading. After SDS-PAGE, proteins were transferred onto a Hybond™ (GE Healthcare) membrane. MYPT1, LZ + MYPT1, and actin were visualized using appropriate antibodies; a rabbit polyclonal anti-MYPT1 (Cell Signaling Cat#: 2634), a monoclonal mouse anti-LZ + MYPT1 [23], and a rabbit polyclonal anti-actin (A2066, Sigma). Following washing, the blots were incubated with appropriate antibodies, scanned on a Kodak imager, and analyzed using ImageQuant TL software. The density of MYPT1 was divided by the actin signal to control for relative expression, and then indexed to the level in controls, set as 1. To normalize the data between blots, one sample was chosen as a standard, which was loaded on every gel [28].

2.3. Determination of NM and SM expression

NM and SM myosin expression was determined using 2D SDS-PAGE, as previously described [18, 24, 26]. We have previously used mass spectroscopy to demonstrate that this technique resolves the non-phosphorylated and phosphorylated SM RLCs and NM myosin RLCs as four distinct spots [18]. Briefly, after the mesenteric artery was separated from the surrounding connective tissue and adipose, and the artery was homogenized in 2D gel extraction buffer (7M urea, 2M thiourea, 4% CHAPS, 1% 3–5.6 pH) immobilized pH gradient (IPG) buffer and EDTA-free Protease Inhibitor and PhosStop Phosphatase Inhibitor (Roche, Indianapolis, Ind., USA)). The homogenates were cleared of lipids and extraneous salts using the 2D Gel Clean up Kit (GE Healthcare) prior to isoelectric focusing. The acidic halves of 13-cm IPG DryStrip gels were rehydrated in rehydration buffer solution (7M urea, 2M thiourea, 2% CHAPS, 0.5% pH 3.5–5 IPG buffer, 0.002% bromophenol blue and 12 μM/ml Destreak Reagent) for 10–12 h with the gel side in the 'face-down' mode on the Ettan IPG rehydration tray and then resolved by completing the isoelectric focusing in the 'face-up' mode on an Ettan IPGphor III (GE Healthcare). After isoelectric focusing was completed, the gel strips were equilibrated in 6M urea, 50 mM Tris-HCl, pH 6.4, 30% glycerol, 2% (w/v) SDS and 0.002% bromophenol blue, first containing 100 mg DTT for 15 min and then 125 mg iodoacetamide for 15 min before undergoing SDS-PAGE for protein separation by molecular weight using the Bis-Tris buffering system with 12% gels. Subsequently, resolved 2D SDS-PAGE gels were silver stained. Gels were scanned using a Personal Densitometer SI, and the spots representing the NM and SM RLCs were quantified using ImageQuant TL software. The two spots nearest the negative (-) sign on the gel have the highest negative charge (spots 1 & 2) and we have demonstrated that these two spots represent the phosphorylated and nonphosphorylated NM RLC [18], while the two spots nearest the positive (+) sign on the gel have the highest positive charge (spots 3 & 4) and we have shown that these spots represent the phosphorylated and nonphosphorylated SM RLC [18]. The expression and phosphorylation of NM and SM myosin can then be calculated [18]; NM myosin expression is the density of spots [(1 + 2)/(1 + 2 + 3 + 4)] x 100%].

2.4. Statistics

Data is presented as mean ± SEM, where n = number of patients in each group. Results are compared using a student's t-test with a $p < 0.05$ defining a significant difference.

3. Results

3.1. Patient population

Patient samples included six normal controls, four patients with HFpEF, and five patients with HFrEF (Table 1). Average age of the six controls was 63 ± 5 years, and four (67%) were female. Mean age for the

Table 1. Patient characteristics.

	Control	HFpEF	HFrEF
n	6	4	5
Age (years)	63 ± 5	80 ± 2	83 ± 2
Male (%)	4 (67)	3 (75)	1 (20)
Ejection Fraction (%)		64 ± 4	30 ± 5
Coronary Artery Disease		1	4
Hypertension		4	5
Diabetes Mellitus		0	2
Obseity		1	3
Body Mass Index (kg/m^2)		28 ± 3	34 ± 2
Obstructive Sleep Apnea		0	2

Characteristics of control and patients with HFrEF and HFpEF.

HFrEF group was 80 ± 2 years, and only one was female. Mean ejection fraction was 30 ± 5%, and medial E/e' was 20 ± 1, and four (80%) had an ischemic cardiomyopathy. All patients with HFrEF were treated with a β-blocker and ACE inhibitor or ARB ± a diuretic, spironolactone and amiodarone. The mean age of the HFpEF group was 83 ± 2 years, and mean ejection fraction 64 ± 4%. Mean right ventricular systolic pressure for this group was 40 ± 7 mmHg, and medial E/e' was 22 ± 5. Cardiac medications in the HFpEF group were variable and included β-blockers, ARBs, diuretics, digoxin and amiodarone.

3.2. NM myosin expression

Smooth muscle contains both SM myosin and nonmuscle (NM) myosin, both of which are regulated by phosphorylation of their respective RLCs [29]. As described above and previously published [18], the non and phosphorylated RLCs of NM and SM myosin are resolved by 2D SDS-PAGE as four distinct spots (Figure 1); our data show that NM expression, as a percentage of total myosin, is 12 ± 3% (controls, n = 6), 7 ± 5% (HFpEF, n = 4) and 37 ± 18% (HFrEF, n = 5, $p < 0.05$). Figure 2 shows NM myosin expression as a function of age, and because of the small number of samples, these data are only hypothesis generating. However, NM myosin expression appears to decrease with age in the controls as well as HFpEF, but NM expression is higher in HFrEF.

3.3. MYPT1 expression

MYPT1 expression (Figure 2) was significantly lower ($p < 0.05$) in both HFpEF (70 ± 11%) and HFrEF (47 ± 6%). However, LZ + MYPT1 expression was similar in the controls and patients with HFpEF (90 ± 14% vs 99 ± 7%, control vs HFpEF), while LZ + MYPT1 expression was lower in patients with HFrEF (62 ± 19%, $p < 0.05$). In the normal controls, both MYPT1 expression and LZ + MYPT1 expression appear to increase with age (Figure 4), and the expression of both MYPT1 and LZ + MYPT1 is lower in both patients with HFpEF and HFrEF.

4. Discussion

Heart failure with preserved ejection fraction and heart failure with reduced ejection fraction represent opposite ends of the spectrum of heart failure. However, HFpEF and HFrEF are both associated with a resting vasoconstriction and a decrease in the sensitivity to NO mediated vasodilation, which can be produced by alterations in the contractile protein expression and signaling pathways in smooth muscle, or outside the smooth muscle cell, by vessel fibrosis and endothelial dysfunction. The purpose of our study was to define the expression of smooth muscle contractile proteins in HFpEF and HFpEF versus normal controls, and to determine if changes in contractile protein expression explain the vascular abnormalities in patients with HF.

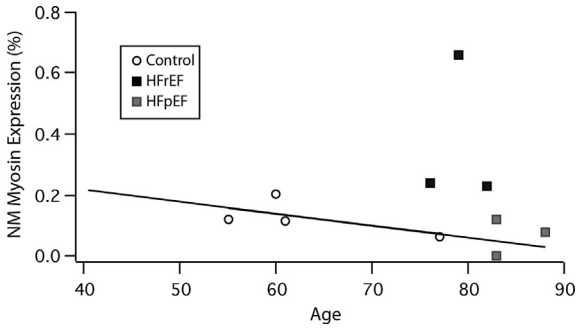

Figure 2. *NM myosin expression declines with age.* Although only hypothesis generating, the expression of NM myosin appears to slowly decline with age, but is higher in patients with HFrEF.

The vasodilatory response to nitric oxide (NO), or flow mediated vasodilatation, is a fundamental response of the vasculature [30] and the sensitivity to NO is defined by LZ+/LZ- MYPT1 expression [9, 10, 11]. Further, the fundamental role of the LZ + MYPT1 isoform in regulating peripheral vascular resistance (PVR) and blood pressure has been demonstrated in transgenic mice; a decrease in LZ + MYPT1 expression results in hypertension [31], while an increase in LZ + MYPT1 expression decreases blood pressure [32].

In animal models of HFrEF, LZ + MYPT1 expression is lower than in normal controls [15, 16, 17], and the decrease in LZ + MYPT1 expression has been demonstrated to produce a decrease in sensitivity to NO [15, 16]. Our data show that in patients with both HFpEF and HFrEF, MYPT1 expression is significantly lower than patients without cardiac disease (70 ± 11% and 47 ± 6% of control, respectively, Figures 3 and 4). The phosphorylation of the RLC of smooth muscle myosin is regulated by the expression and activities of MLCK and MLCP [4], and thus a decrease in MYPT1 would increase RLC phosphorylation and contribute to the increase in vascular tone associated with both HFpEF and HFrEF.

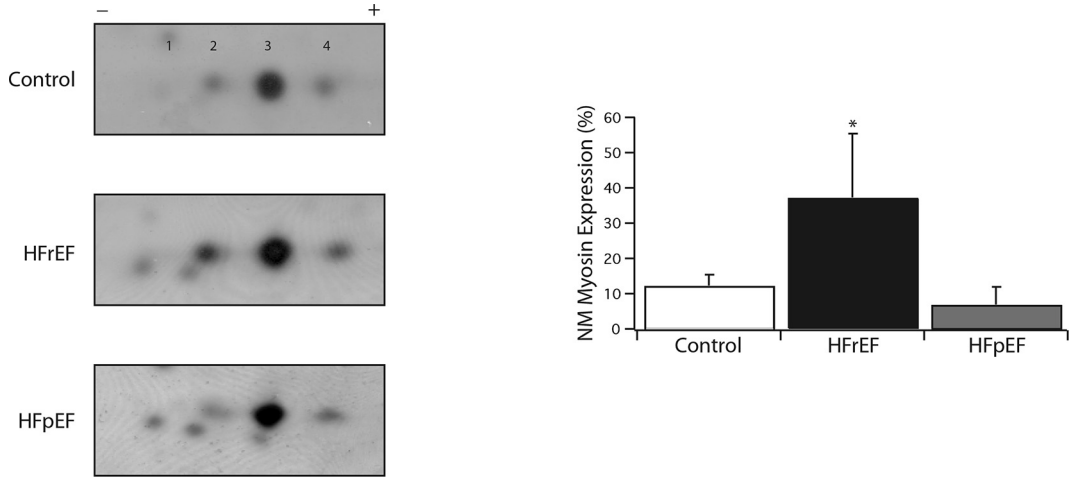

Figure 1. *NM myosin expression is increased in HFrEF.* Separation of NM and SM regulatory light chains by two-dimensional gel electrophoresis. Spots 1 and 2 represent the phosphorylated and nonphosphorylated RLCs of NM myosin, and Spots 3 and 4 represent the phosphorylated and nonphosphorylated RLCs of SM myosin [18]. Histogram demonstrates the expression of NM myosin in normal controls (n = 6), HFrEF (n = 5) and HFpEF (n = 4), and the expression of NM myosin is significantly higher in patients with HFrEF (*, $p < 0.05$). Uncropped images are provided in Supplemental Figure 1.

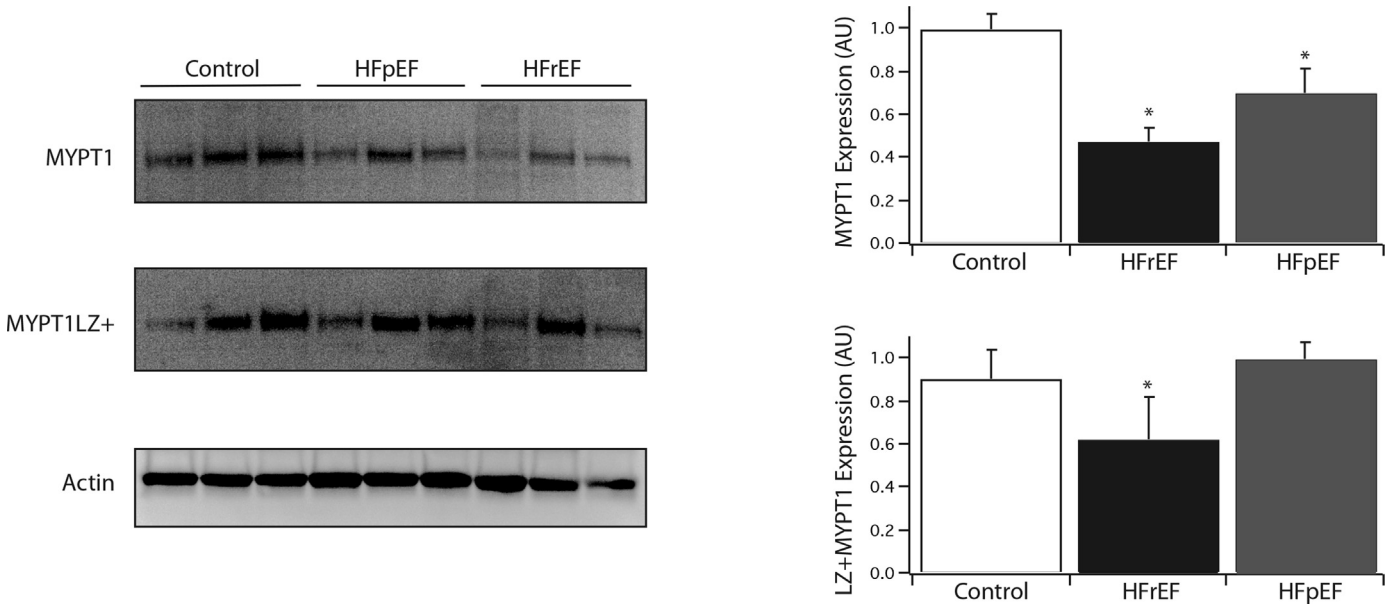

Figure 3. *MYPT1 and LZ + MYPT1 expression is lower in HF.* Immunoblots demonstrate the expression of MYPT1 and the LZ + MYPT1 isoform. Histograms demonstrates MYPT1 and LZ + MYPT1 expression in normal controls (n = 6), HFrEF (n = 5) and HFpEF (n = 4), and MYPT1 and LZ + MYPT1 expression is lower in HF (*, $p < 0.05$). Uncropped images are provided in Supplemental Figure 2.

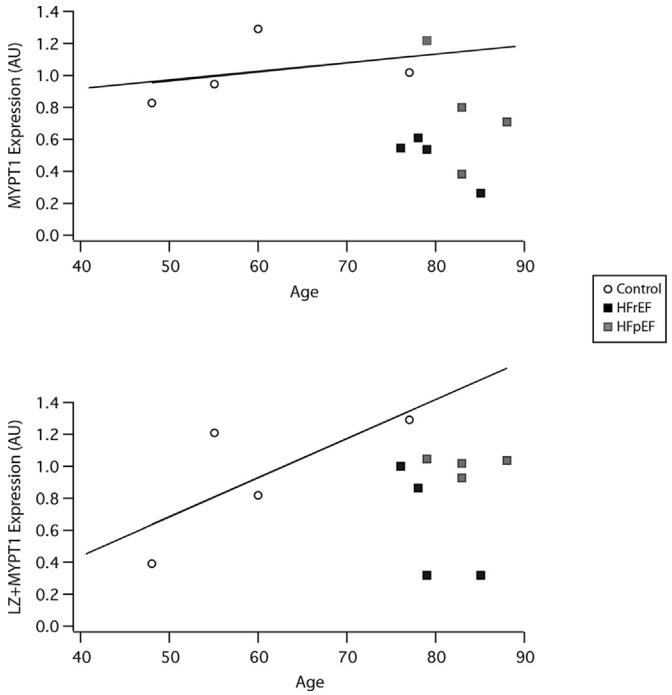

Figure 4. *MYPT1 and LZ + MYPT1 expression increase with age.* In normal controls, MYPT1 and LZ + MYPT1 expression appears to increase with age (hypothesis generating), but is significantly lower in patients with HF.

Compared to controls, our data demonstrate that LZ + MYPT1 expression was significantly lower in HFrEF patients (62 ± 19%), which indicates that the decrease in sensitivity to NO-mediated relaxation in HFrEF is beyond the level of the endothelium and cGMP signaling; it is at the level of the smooth muscle cells and is due to the decrease in LZ + MYPT1 expression. In HFpEF, total MYPT1 expression was 30% lower than the control group without cardiac disease (Figure 3) and both total MYPT1 and relative LZ + MYPT1 expression were lower than controls (Figure 4). Therefore, the decrease in sensitivity to NO is produced by the decrease in MYPT1 and LZ + MYPT1 expression in both HFrEF and HFpEF patients. However, our data demonstrate that the decrease in MYPT1 and LZ + MYPT1 expression is more profound in HFrEF compared to HFpEF, which suggests the fall in MYPT1 plays a more prominent role in producing the decrease in NO sensitivity in HFrEF compared to HFpEF.

It is speculated that higher overall arterial stiffness produces the resting vasoconstriction associated with HFpEF [33]. Arterial stiffness is regulated by both changes within the smooth muscle cells and vessel wall. Smooth muscle contains both NM and SM myosin, both of which are regulated by phosphorylation [29]. The kinetics for the NM myosin ATPase are slower than cardiac, skeletal, and SM myosin [19, 20], and in the presence of actin, the ADP affinity of NM myosin increases [21]. Therefore, an increase in NM myosin expression increases vascular tone and resistance [18].

In our samples, cancer and other co-morbidities may have alter the expression of MYPT1 or NM myosin, and in animal models of HFrEF, treatment with ACE-inhibitors and ARBs has been demonstrated to increase LZ + MYPT1 expression [16, 17]. However, the changes in MYPT1 in the patients with cancer and HFrEF are similar to those documented in animal models of HFrEF [15, 16, 17, 34]. Further in the present study of humans with colon cancer and either HFpEF or HFrEF, the changes in MYPT1 and NM myosin expression were distinct. Thus, although possible, it is unlikely that cancer, co-morbidities or medications were responsible for the unique changes in the expression of these contractile proteins in controls vs HFpEF vs HFrEF. In animal models of HFrEF and PAH, we have previously demonstrated that changes in MYPT1 expression are similar in the aorta, iliac artery, pulmonary artery and tertiary mesenteric vessels [14, 15, 25], and thus, the changes in MYPT1 and NM myosin expression observed in the mesenteric vessels of patients with HF are likely to be representative of other vascular beds.

We were unable to find age matched controls without evidence of cardiovascular disease and on no cardiac medications, and thus, our controls were significantly younger than the patients with HFrEF and HFpEF. The data relating age and protein expression data are only hypothesis generating; in controls, NM myosin expression decreases with age (Figure 2) and both MYPT1 and LZ + MYPT expression increase with age (Figure 4). Arterial stiffness has been demonstrated to increase with age [35], possibly due to alterations within the vessel wall. The decrease in NM myosin and the increase in MYPT1 in the controls would then

represent a compensatory mechanism to decrease vascular resistance to maintain normotension. The increase in NM myosin expression in HFrEF and decrease in LZ + MYPT1 and MYPT1 in both HFpEF and HFrEF would be maladaptive, and would produce vasoconstriction and an increase in vascular resistance.

The increase in NM myosin in patients with HFrEF compared to HFpEF demonstrates a significant difference, at the level of the smooth muscle contractile proteins, between the two heart failure phenotypes. These data could suggest that the increase in arterial stiffness is primarily at the level of the smooth muscle cell contractile proteins (NM myosin and MYPT1) in HFrEF, but in HFpEF there are contributions from both the vascular smooth muscle cells (the decrease in MYPT1) and within the vessel wall itself, possibly fibrosis produced by the inflammatory state associated with HFpEF [36]. Further, not all patients with hypertension and the other comorbidities associated with HFpEF develop heart failure. This variable penetrance of heart failure is similar to hypertrophic cardiomyopathy and could suggest that a gene(s) mutation is necessary to develop HFpEF.

Our study highlights that HFpEF and HFrEF are two distinct vascular phenotypes, and thus, it is not unexpected that these distinct clinical phenotypes do not have the same clinical response to therapies. In HFpEF, there is a decrease in MYPT1 and LZ + MYPT1, whereas HFrEF is associated with more pronounced decreases in MYPT1 and LZ + MYPT1, as well as an increase in NM myosin expression. HFrEF is characterized by abnormal vasoconstriction and reduced sensitivity to NO mediated vasodilation, and our data suggest that these vascular abnormalities are produced by an increase in NM myosin expression and the decreases in MYPT1 and LZ + MYPT1 isoform expression. On the other hand, HFpEF is also associated with increased arterial stiffness, resting vasoconstriction, and decreased sensitivity to NO, and our data suggest that these vascular abnormalities are due to the decrease in MYPT1 expression in addition to changes outside the contractile proteins of the vascular smooth muscle cells.

No therapy has demonstrated a clinical benefit in patients with HFpEF. Our data demonstrate that the decrease in sensitivity to NO and resting vasoconstriction, in part, are due to changes within the smooth muscle cell, particularly the decrease in MYPT1, in addition to changes outside the vascular smooth muscle cells, possibly fibrosis and inflammation. These data provide a mechanism to explain why therapies designed to increase NO/cGMP signaling, whether produced by the inhibition of PDE5 [37], nitrates [38], nitrites [39], or inhibition of neprilysin [40, 41] have not shown a significant clinical benefit. Further, serum neprilysin levels are lower in patients with HFpEF compared with normal controls without diastolic dysfunction [42], which represents another reason that the trial of inhibition of neprilysin in HFpEF [40, 41] did not improve relevant clinical endpoints.

5. Conclusions

Our data highlights that HFrEF and HFpEF represent two very different vascular entities, and it is not surprising that therapies with proven benefit in HFrEF [1] are not beneficial in HFpEF [2, 3]. In animal models of HFrEF, treatment with both ACE-inhibition [16] and ARBs [17], but not other vasodilators [16], has been demonstrated to preserve the normal expression of the LZ + MYPT1 isoform and vascular reactivity, which could represent a mechanism that contributes to the benefit of these therapies in patients with HFrEF.

Until recently [43], there were no small animal models of HFpEF, which has limited our understanding of the pathophysiology and the identification of novel targets for rational drug design. Hypertension in addition to the other comorbidities associated with HFpEF produce a chronic inflammatory state [36]. This persistent inflammatory state is thought to result in myocardial fibrosis [44], as well as the accumulation of collagen and elastin [45] and advanced glycation end products that cross-link proteins [46] in the vasculature, which contribute to the increased myocardial and vascular stiffness seen in HFpEF. Thus, it is not unexpected that in patients with HFpEF, these changes within the myocardium and vasculature produce a disease that is resistant to therapy. If this is the case, early and more judicious treatment of hypertension, possibly with an ACE inhibition or ARBs could preserve normal MYPT1 expression and vascular reactivity, and thus, could prevent the development of HFpEF.

Declarations

Author contribution statement

F. Brozovich: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

M. Lyle: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Alabdaljabar: Performed the experiments.

Y. Han: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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