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Authors

Lai, N
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Giamouridis, Dimosthenis
et al.

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Urocortin 2 Gene Transfer for Systolic and Diastolic Dysfunction Due to Chronically Increased Left Ventricular Pressure

N. Chin Lai,[1,2,†] Zhen Tan,[1,2,†] Dimosthenis Giamouridis,[1,2] Mei Hua Gao,[1,2] and H. Kirk Hammond[1,2,*]

[1]*Veterans Affairs San Diego Healthcare System, San Diego, California, USA;* [2]*Department of Medicine, University of California San Diego, San Diego, California, USA.*
[†]*These authors contributed equally to this work.*

We used transverse aortic constriction (TAC) in mice to test the hypothesis that *urocortin 2* (*Ucn2*) gene transfer would increase left ventricular (LV) systolic and diastolic function in the pressure-stressed LV. Three groups were studied: (1) control mice (no TAC); (2) mice that received saline 6 weeks after TAC; and (3) mice that received *Ucn2* gene transfer 6 weeks after TAC, using adeno-associated virus 8 encoding murine *Ucn2* (AAV8.m*Ucn2*; 2×10^{13} genome copies (gc)/kg, i.v. per mouse). Echocardiography was performed 6 and 12 weeks after TAC. In terminal studies 12 weeks after TAC, rates of LV pressure development and decay and Tau were measured, and LV cardiac myocytes (CMs) were isolated and cytosolic Ca^{2+} transients and sarcomere shortening rates recorded. Reverse transcription polymerase chain reaction and immunoblotting were used to measure key proteins in LV samples. A CM cell line (HL-1) was used to explore mechanisms. Concentric LV hypertrophy was evident on echocardiography 6 weeks after TAC. Twelve weeks after TAC, LV ejection fraction (EF) was higher in mice that received *Ucn2* gene transfer (TAC-saline: 65% ± 3%; TAC-Ucn2: 75% ± 2%; $p = 0.01$), as was LV peak +dP/dt (1.9-fold increase; $p = 0.001$) and LV peak −dP/dt (1.7-fold increase; $p = 0.017$). Tau was more rapid (23% reduction, $p = 0.02$), indicating improved diastolic function. The peak rates of sarcomere shortening ($p = 0.002$) and lengthening ($p = 0.002$) were higher in CMs from TAC-Ucn2 mice, and Tau was reduced ($p = 0.001$). LV (Ser-16) phosphorylation of phospholamban (PLB) was increased in TAC-Ucn2 mice ($p = 0.025$), and also was increased in HL-1 cells treated with angiotensin II to induce hypertrophy and incubated with Ucn2 peptide ($p = 0.001$). *Ucn2* gene transfer in TAC-induced heart failure with preserved ejection fraction increased cardiac function in the intact LV and provided corresponding benefits in CMs isolated from study animals, including increased myofilament Ca^{2+} sensitivity during contraction. The mechanism includes enhanced CM Ca^{2+} handling associated with increased (Ser-16)-PLB.

Keywords: TAC, Tau, HL-1 cell line, gene transfer

INTRODUCTION

IN THE PRESENT article, we ask whether *urocortin 2* (*Ucn2*) gene transfer, which has beneficial effects on the dilated failing heart[1] and on the aged heart,[2] can safely and effectively treat heart failure with preserved ejection fraction (HFpEF). Transverse aortic constriction (TAC) provides a pressure load on the heart, resulting in concentric left ventricular hypertrophy (LVH), preserved EF, reduced left ventricular (LV) peak −dP/dt, and prolonged Tau. Tau is the time constant for LV isovolumic relaxation and is a physiological correlate of LV diastolic function.[3]

In the current study we used 129S1/SvImJ mice, which maintain normal EF 8 weeks after TAC with low mortality,[4] to test the hypothesis that *Ucn2* gene transfer would safely and effectively increase LV systolic and diastolic function in mice with HFpEF induced by pressure overload, and to determine mechanisms for possible benefits. Although aging-related LV dysfunction mimics aspects of

*Correspondence: Dr. H. Kirk Hammond, Department of Medicine, University of California San Diego, 3350 La Jolla Village Drive, San Diego, CA 92161, USA. E-mail: khammond@ucsd.edu

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clinical HFpEF,[2] to obtain a more complete picture of the potential benefits of *Ucn2* gene transfer, studies in pressure overload—a cause of clinical HFpEF—are potentially useful and may predict outcomes in clinical settings.

The definition of clinical HFpEF includes normal EF (≥50%, generally) and symptoms of HF. Some clinical trials require that patients also have abnormalities in diastolic function on echocardiography or elevation in B-type natriuretic peptide (BNP) or N-terminal-proBNP. Since symptoms of HF are not accessible in mice, echocardiography is often used to select rodents with abnormal LV filling and EF ≥50%. The efficacy endpoints focus on LV filling using echocardiography, LV peak −dP/dt, and Tau. A recent article highlighted the importance of selecting for nonclinical study suitable animal models of clinical HFpEF.[5] Mice subjected to TAC possess features consistent with HFpEF.

Although infused urocortin 2 (Ucn2) peptide has beneficial cardiac effects in patients with HF,[6,7] it is not suited for a chronic therapy due to its short half-life (15 min).[8] Gene transfer circumvents this impediment by providing sustained plasma Ucn2 concentrations for >20 months after a single intravenous (i.v.) injection of AAV8.m*Ucn2* in mice.[2] The present study tested the hypothesis that *Ucn2* gene transfer, through adaptations in CM Ca^{2+} handling, would preserve LV function in the setting of severe LV pressure stress.

METHODS

Mice and study design

The Animal Use and Care Committee of the Veterans Affairs San Diego Healthcare System approved the studies. Three groups of 129S1/SvImJ mice[4] were studied: (1) control mice that did not receive TAC ($n=18$); (2) mice that received saline 6 weeks after TAC ($n=29$; 14 used for isolation of cardiac myocytes (CMs) for Ca^{2+} transient and sarcomere shortening studies alone); and (3) mice that received *Ucn2* gene transfer 6 weeks after TAC ($n=21$; 14 used for isolation of CMs for Ca^{2+} transient and sarcomere shortening studies alone). Final physiological data were collected 12 weeks after TAC, 6 weeks after randomization to *Ucn2* gene transfer or saline, and tissues were collected for additional studies. We used a total of 72 129S1/SvlmJ mice (41M, 31F; Jackson Laboratories, Bar Harbor, ME, USA). Mice were 5.3 ± 1.5 weeks old at entry, weighing 21 ± 3 g.

Of the 72 mice, 54 underwent TAC surgery. Four died perioperatively, and surviving mice were randomized to receive *Ucn2* gene transfer ($n=21$) or saline ($n=29$) 6 weeks after TAC (Fig. 1A). Mice were provided a cereal-based diet (Harlan Teklad Laboratory, Madison WI) and tap water *ad libitum*. Environmental temperature was 20–21°C and lights were off from 6 pm to 6 am daily.

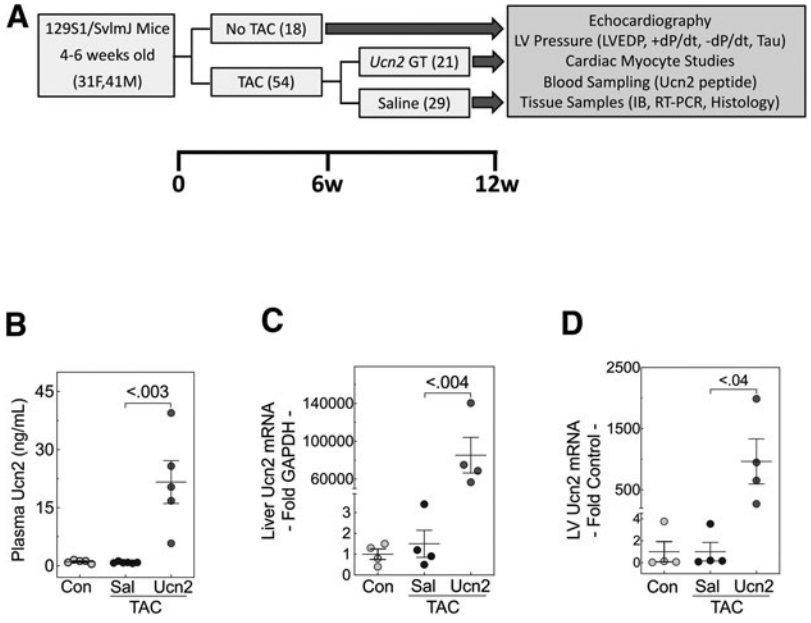

Figure 1. Study design and *Ucn2* expression. **(A)** Mice ($n=54$) underwent TAC; a control group ($n=18$) did not receive TAC. Six weeks later, 21 mice received *Ucn2* gene transfer (AAV8.*mUcn2*, 2×10^{13} gc/kg, i.v.) and 29 mice received saline, i.v. The control group received no intervention. Six weeks later, mice underwent the final studies depicted. **(B)** Mice that received *Ucn2* gene transfer (TAC-Ucn2) showed a mean 20-fold increase in plasma Ucn2 versus TAC-saline animals ($p<0.003$). **(C, D)** *Ucn2* mRNA expression was increased in the liver (>85,000-fold control; $p<0.004$) and LV (>900-fold control, $p<0.04$). *p* Values from Student's *t*-test (unpaired and two tailed). Data denote mean value ± SE. IB, immunoblotting; LV, left ventricular; SE, standard error; TAC, transverse aortic constriction; *Ucn2* GT, *urocortin 2* gene transfer.

Transverse aortic constriction

We followed our previously published methods for TAC.[9] Mice were anesthetized with 5% isoflurane in oxygen (1 L/min), intubated, and ventilated. Anesthesia was maintained with 1% isoflurane in oxygen. A segment of the aortic arch between the innominate and left carotid arteries was identified and a 7-0 silk suture was tied against a 26-gauge needle, which yielded aortic constriction. These methods yield >45 mmHg peak systolic pressure gradient across the constriction[10] similar to what was previously reported in 129S1/SvImJ mice undergoing TAC.[4]

Ucn2 gene transfer

AAV8.m*Ucn2* vector. HEK293T cells were transfected with the pRep2/Cap8 and pAd-Helper plasmids,[11] for the production of an AAV8 vector encoding murine *Ucn2* driven by a chicken β-actin promoter. The University of Pennsylvania Vector Core provided plasmid pRep2/Cap8. Virus vectors were purified and concentrated as previously described.[2,11] To quantify virus titers, real-time PCR with virus genome DNA prepared from purified virus was used.[2] *Vector Injection.* Under anesthesia (1.5% isoflurane via nose cone), the jugular vein was exposed. AAV8.m*Ucn2* (2×10^{13} gc/kg in 100 μL) or a similar volume of saline (in controls) was delivered using a syringe with a 31G needle.[2] *Detection of plasma Ucn2.* In the terminal study, 12 weeks after TAC, blood was collected (with ethylenediaminetetraacetic acid), centrifuged, and plasma stored at −80°C. Plasma Ucn2 levels were measured using a mouse Ucn2 enzyme immunoassay (Kamiya Biomedical, Seattle WA, USA).[2]

Echocardiography

Echocardiography was performed at 6 weeks (before randomization to *Ucn2* gene transfer vs. saline) and at 12 weeks. We used a Vevo 3100 system with an MX550s (32–55 MHz) transducer (FUJIFILM VisualSonics, Inc., Toronto, Canada), as previously described.[11] Inter-observer agreement for murine echocardiography measurements in our laboratory is excellent ($R^2=0.89$).[9] Mice not subjected to TAC ($n=12$) served as controls. Light sedation not requiring intubation was achieved using isoflurane (1%) at a flow rate of 1 L/min oxygen through nose cone. The velocity of LV circumferential fiber shortening (VCF) was taken at the midwall using the following formula: VCF (circ/s) = LVEDD − LVESD/LVEDD × LVET, where EDD is end-diastolic diameter; ESD, end-systolic diameter; and LVET, LV ejection time. We then normalized VCF to heart rate (HR):VCFc = VCF divided by the square root or R − R interval.[12]

LV systolic and diastolic function

We followed our previously published methods.[1,11,13] Mice received sodium pentobarbital (80 mg/kg, IP), and a 1.4 F catheter (SPR 839; Millar Instruments, Houston, TX, USA) was placed in the LV cavity through the unimpeded right carotid artery to measure LV pressure. Data were analyzed using IOX v2.9 (Emka Technologies, Christchurch, VA), as previously reported.[1,11,13] We analyzed peak LV −dP/dt, peak LV +dP/dt, and, in addition, we assessed LV −dP/dt and LV +dP/dt at LV developed pressure (LVP) of 40 mmHg.

CM isolation, Ca^{2+} transients, and sarcomere shortening

CMs were isolated using previously described methods.[14,15] Cytosolic Ca^{2+} transients in isolated CMs were measured using indo-1 AM, following the techniques that we have recently published.[15]

Reverse transcription PCR

Quantitative reverse transcription-PCR was conducted, as previously described,[15] to measure mRNA levels of stress-related peptides/proteins. In addition, we assessed *Ucn2* mRNA expression in the liver and LV. The primers used were as follows: atrial natriuretic peptide (ANP)—Forward: 5′-CCTCGTCTTGGCCTTTTGG, Reverse: 5′-CATCTTCTACCGGCATCTTC; BNP—Forward: 5′-GAAGTCCTAGCCAGTCTCC, Reverse: 5′-CAGCTTGAGATATGTGTCACC; β-myosin heavy chain (β-MHC)—Forward: 5′-GCTGAAAGCAGAAAGAGA TTATC, Reverse: 5′-TGGAGTTCTTCTCTTCTGGAG; and Ucn2—Forward, 5′-ACTCCTATCCCCACCTTCCA, Reverse: 5′-AAGATCCGTAGGAGGCCAAT.

Immunoblotting

Western blots were performed to assess the expression of total or phosphorylated proteins from frozen LV samples. The following antibodies were used: SERCA2a (1:500 dilution; ENZO, Exeter, United Kingdom); p16-phosphorylation of phospholamban (PLB) (1:5,000 dilution; Badrilla, Leeds, United Kingdom); total PLB (1:5,000 dilution; Thermo Scientific, Carlsbad, CA, USA); phosphorylation of eukaryotic elongation factor (eEF2) at threonine 56 (1:1,000 dilution; Cell Signaling Technologies, Danvers, MA, USA), GAPDH (1:20,000 dilution; Fitzgerald, Acton, MA, USA); and Vinculin (1:1,000,000 dilution; Sigma, St Louis, MO, USA). Protein quantification was conducted using Image Lab Software (Bio-Rad, Hercules, CA, USA).

Studies in HL-1 CM line

HL-1 cells (Sigma) were cultured in Claycomb medium (Sigma) and exposed to angiotensin II (AngII 100 mM, 24 h) to induce hypertrophy, followed by Ucn2 peptide incubation (100 nM, 15 min) in media containing 1% serum. Cells were lysed in lysis buffer and p16-PLB and pEF2 were detected by immunoblotting.

Necropsy and histology

Body, LV, liver, and lung weights were recorded. LV samples were fixed in 10% formalin (Sigma-Aldrich, St. Louis, MO, USA), paraffin embedded, sliced into 5 μm transmural sections, mounted, and counterstained with hematoxylin and eosin and Masson's trichrome. Five CMs in short-axis orientation were measured in each of 12 mice (control: $n=3$; TAC-saline: $n=5$; TAC-Ucn2: $n=4$). An Axio Scan Z1 slide scanner (Carl Zeiss, Oberkochen, Germany) enabled quantitative assessment of fibrosis, and CM cross-sectional area using ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

Data acquisition and analysis were done blinded to treatment group. Power calculations were used to estimate required group sizes. Similar numbers of female and male mice were used (reported in Tables 1 and 3). An age- and sex-matched control group of the same strain of mice did not undergo TAC to establish that TAC induced the expected alterations in LV structure and function. The hypothesis was that *Ucn2* gene transfer would preserve LV function in the pressure-stressed and hypertrophied LV. The key comparison was between TAC groups that received saline versus *Ucn2* gene transfer. Comparisons were kept to a minimum to reduce type 1 statistical errors. The effective concentration for 50% effect (EC50) was calculated from multiple CMs from each animal of each group and tested for group differences for both shortening and relaxation phases.

The D'Agostino and Pearson omnibus normality test confirmed normal distribution and group differences, and therefore, group differences were tested via Student's *t*-test (unpaired and two tailed). The null hypothesis was rejected when $p<0.05$. Bonferroni corrections were used when multiple comparisons were made. Data in graphs and tables denote mean ± standard error (SE). Analyses were performed using GraphPad Prism, version 9.0 (GraphPad Software, Inc., San Diego, CA, USA).

Table 1. *Echocardiography*

	Control (No-TAC), n = 12 (6F, 6M)	*6 Weeks After Randomization (12 Weeks After TAC)*		
		TAC-Saline, n = 21 (10F, 11M)	*TAC-Ucn2, n = 18 (9F, 9M)*	p
HR (bpm)	525 ± 9	528 ± 8	538 ± 5	0.43
EDD (mm)	3.7 ± 0.1	3.9 ± 0.1	3.3 ± 0.1	0.0002
ESD (mm)	2.3 ± 0.1	2.5 ± 0.1	1.9 ± 0.1	0.0002
EF (%)	69 ± 2	65 ± 3	75 ± 2	0.01
PWd (mm)	0.78 ± 0.03	1.01 ± 0.03[a]	1.14 ± 0.04	0.024
IVSd (mm)	0.80 ± 0.03	0.93 ± 0.02[b]	1.05 ± 0.04	<0.02
VCFc (circ)	24 ± 1	21 ± 1	27 ± 2	0.008

Echocardiography was performed 12 weeks after TAC placement, 6 weeks after randomization to saline versus *Ucn2* gene transfer (AAV8.-m*Ucn2*; 2×10^{13} gc/kg, i.v.) to determine the effects of *Ucn2* gene transfer in the setting of HFpEF induced by chronic LV pressure stress. Values are means ± SE. Between-group differences (TAC-saline vs. TAC-Ucn2) were tested using Student's *t*-test (two tailed and unpaired) and are shown in the column at right.

[a] $p=0002$ versus control.

[b] $p<0.002$ versus control.

circ, circumference; EDD, LV end-diastolic diameter; EF, ejection fraction; ESD, LV end-systolic diameter; HR, heart rate; IVSd, intraventricular septum thickness at end diastole; PWd, posterior wall thickness at end diastole; SE, standard error; TAC, transverse aortic constriction; Ucn2, urocortin 2; VCFc, velocity of circumferential fiber shortening (corrected for HR).

Table 2. *Left ventricular expression (mRNA) of stress-related proteins*

LV mRNA	*Control (No-TAC) (4)*	*6 Weeks After Randomization (12 Weeks After TAC), Fold Control*		
		TAC-Saline (4)	*TAC-Ucn2 (4)*	p
ANP	1 ± 0.4	9.9 ± 3.5[a]	6.2 ± 3.1	0.45
BNP	1 ± 0.3	11.3 ± 4.2[b]	4.1 ± 2.3	0.18
β-MHC	1 ± 0.2	15.9 ± 4.8[c]	6.9 ± 2.6	0.15

The effects of *Ucn2* gene transfer in the setting of HFpEF induced by TAC. Values are means ± SE. Data from RT-PCR, normalized to GAPDH, and expressed as fold control (normal mice, no TAC). Between-group differences (TAC-saline vs. TAC-Ucn2) were tested using Student's *t*-test (two tailed and unpaired) and are shown in the column at right.

[a] $p=0.04$ versus control.

[b] $p=0.05$ versus control.

[c] $p=0.02$ versus control.

β-MHC, β-myosin heavy chain; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; LV, left ventricular.

RESULTS

Ucn2 gene transfer

Mice that received *Ucn2* gene transfer (TAC-Ucn2) showed a mean 20-fold increase in plasma Ucn2 versus TAC-saline animals 6 weeks after randomization

Table 3. *Necropsy*

	Control (No-TAC), n = 9 (6F, 3M)	*6 Weeks After Randomization (12 Weeks After TAC)*		
		TAC-Saline, n = 10 (3F, 7M)	*TAC-Ucn2, n = 7 (3F, 4M)*	p, *TAC-Saline Versus TAC-Ucn2*
BW (g)	25.1 ± 0.9	25.9 ± 1	26.3 ± 1	0.80
LV + IVS (mg)	88 ± 6	149 ± 10[a]	171 ± 21	0.32
LV/BW (mg/g)	3.5 ± 0.2	5.8 ± 0.2[a]	6.4 ± .6	0.27
LA weight (mg)	3 ± 0.1	8 ± 2[b]	12 ± 5	0.42
Liver (mg)	948 ± 59	917 ± 59	921 ± 87	0.95
Lung (mg)	141 ± 7	160 ± 23	200 ± 41	0.37
LV fibrosis (%)	1.3% ± 0.3% ($n=7$)	8.7% ± 0.8%[c]	7.9 ± 1.1 ($n=8$)	0.55
CM CSA (μm^2)	177 ± 6 ($n=3$)	318 ± 8 ($n=5$)[c]	307 ± 12 ($n=4$)	0.44

The effects of *Ucn2* gene transfer in the setting of HFpEF induced by TAC. Values are means ± SE. Between-group differences (TAC-saline vs. TAC-Ucn2) were tested using Student's *t*-test (two tailed and unpaired) and are shown in the column at right.

[a] $p=0.0002$ versus control.

[b] $p=0.06$ versus control.

[c] $p<0.0001$ versus control.

BW, body weight; CM CSA, cardiac myocyte cross-sectional area (histological analysis); IVS, interventricular septum; LA, left atrium; LV, left ventricle.

($p < 0.003$; Fig. 1A). *Ucn2* mRNA expression was increased in the liver (>85,000-fold saline, $p < 0.004$; Fig. 1B) and LV (>900-fold saline, $p < 0.04$; Fig. 1C).

TAC model of HFpEF

Comparison of the control (no TAC) group versus TAC-saline showed that LV pressure overload produced concentric LVH, left atrial enlargement, increased LV BNP expression, reduced LV peak +dP/dt and LV peak –dP/dt, and prolongation of Tau (Tables 1–3 and Fig. 2A–C). These findings, in combination with EF >50%, established that animals met the criteria for HFpEF.

Echocardiography

Concentric LVH was evident on echocardiography 6 weeks after TAC, but LV EF and all other echocardiographic measures were unaltered by TAC compared with the control group at 6 weeks. However, at 12 weeks (6 weeks after randomization), mice that received *Ucn2* gene transfer showed higher LV EF (TAC-saline: 65% ± 3%, $n = 21$; TAC-Ucn2: 75% ± 2%, $n = 18$; $p = 0.01$), lower LV end-diastolic ($p = 0.0002$) and end-systolic ($p = 0.0002$) diameters, and higher velocity of circumferential fiber shortening (corrected for HR) (VCFc), an echocardiographic estimate of LV contractility ($p = 0.008$) (Table 1). HRs were unchanged.

LV dP/dt

At 12 weeks mice that had received *Ucn2* gene transfer (vs. TAC-saline) showed higher values in LV peak –dP/dt (Fig. 2A; $p = 0.017$) and in LV peak +dP/dt (Fig. 2B; $p = 0.001$). The values at an LVP of 40 mmHg also showed group differences: LV $-dP/dt_{40}$: TAC-saline: 3128 ± 162 mmHg/s, $n = 9$; TAC-Ucn2: 4491 ± 529 mmHg/s, $n = 7$; $p = 0.016$; LV $+dP/dt_{40}$: TAC-saline: 3521 ± 310 mmHg/s, $n = 9$; TAC-Ucn2: 7058 ± 753 mmHg/s, $n = 7$; $p = 0.0003$. The TAC-Ucn2 group also had lower Tau (Fig. 2C; $p = 0.02$), indicating a more rapid time constant of LV pressure decay, a hallmark for improved LV diastolic function.

TAC was associated with increased LVEDP (Fig. 2D; control: 2.4 ± 0.9 mmHg; TAC-saline 8.0 ± 1.3 mmHg; $p = 0.002$). TAC-Ucn2 showed a nonsignificant reduction in LVEDP versus TAC-saline (6.6 ± 1.8 mmHg, $p = 0.52$). LVP (Fig. 2D; $p = 0.015$) and HRs (Fig. 2F; $p = 0.0003$) were higher in TAC-Ucn2 mice.

Ca^{2+} transients and sarcomere shortening

Ca^{2+} transients were measured in CMs isolated from age- and strain-matched control mice (no TAC), TAC-saline mice, and TAC-Ucn2 mice. First, we compared TAC-saline mice with control mice to see what abnormalities TAC alone caused. TAC-saline mice showed a

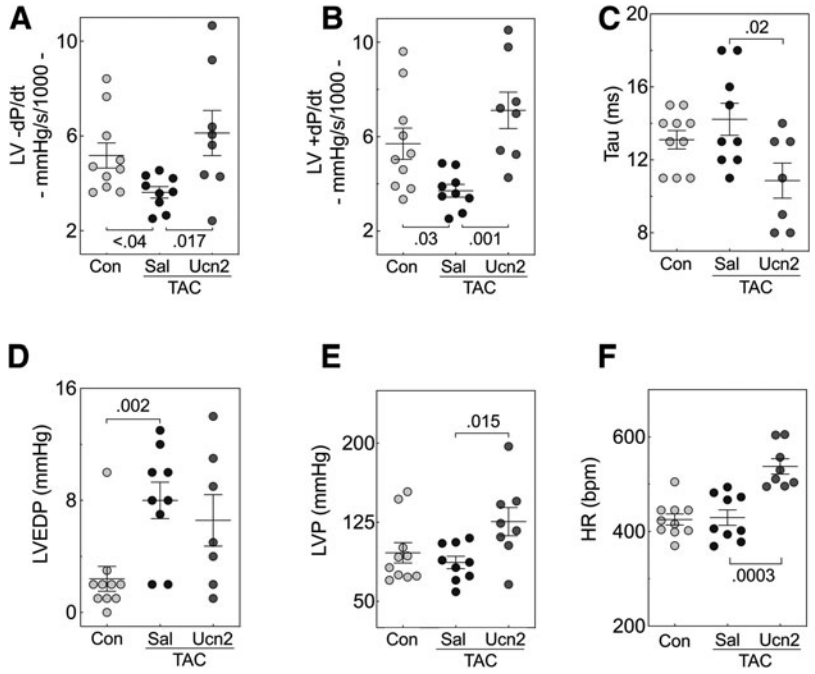

Figure 2. Effects of *Ucn2* gene transfer on LV function. Mice underwent TAC, were randomized 6 weeks later to receive i.v. saline or *Ucn2* gene transfer (AAV8.m*Ucn2*, 2×10^{13} gc/kg, i.v.), and were studied 6 weeks after randomization. A control group (age- and strain-matched) did not undergo TAC (see Fig. 1A). Data were obtained from anesthetized mice using Millar pressure catheters in terminal studies. **(A)** TAC was associated with reduced LV peak –dP/dt ($p < 0.04$ vs. control). Mice that received *Ucn2* gene transfer showed a higher peak rate of LV pressure decline (–dP/dt; $p = 0.017$), which was similar to normal mice. **(B)** TAC was associated with reduced LV peak +dP/dt ($p = 0.03$ vs. control). Mice that received *Ucn2* gene transfer showed a higher rate of LV pressure rise, which was similar to normal mice (+dP/dt; $p = 0.001$). **(C)** TAC-Ucn2 mice showed more rapid Tau ($p = 0.02$; time constant of LV pressure decay), indicating increased LV diastolic function. **(D)** TAC was associated with increased LVEDP ($p = 0.002$). **(E)** TAC-Ucn2 mice showed increased LVP ($p = 0.015$). **(F)** TAC-Ucn2 mice showed a higher HR during anesthesia ($p = 0.0003$). *p* Values from Student's *t*-test (unpaired and two tailed). Data denote mean values ± SE. HR, heart rate; LVEDP, LV end-diastolic pressure; LVP, LV developed pressure.

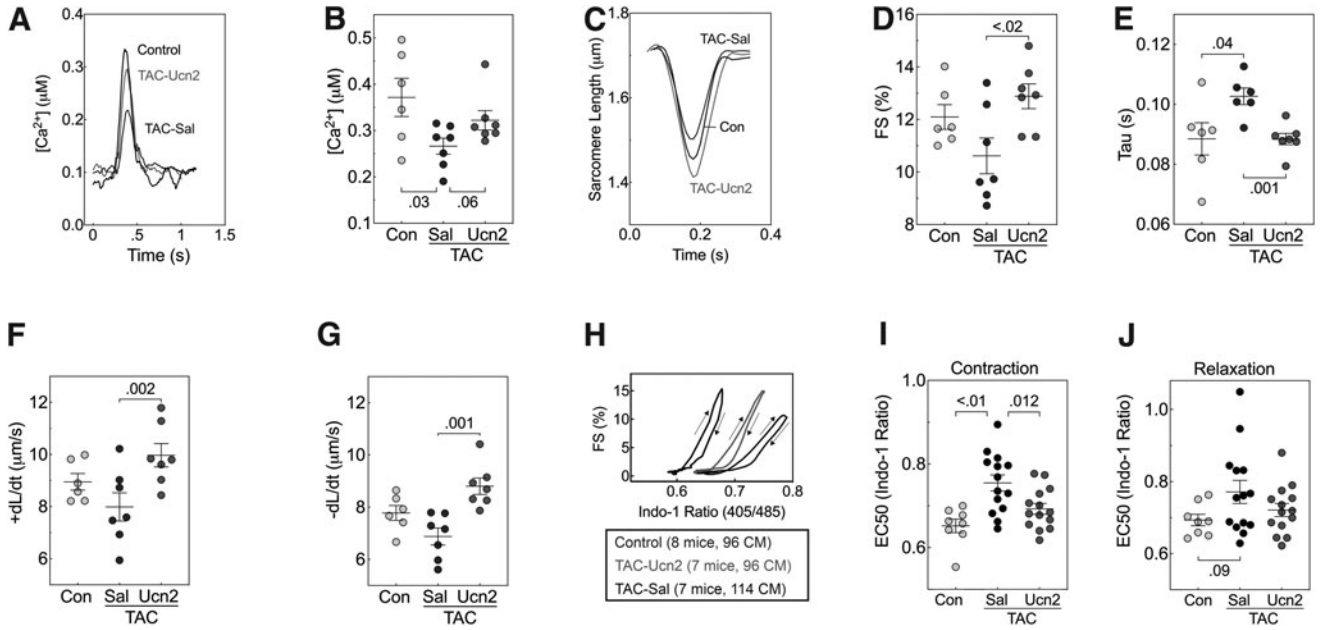

Figure 3. Ca^{2+} transients and sarcomere shortening. **(A)** Representative Ca^{2+} transients from each group. **(B)** Peak rates of Ca^{2+} transients were lower in CMs from TAC-saline mice versus control (**B**, $p=0.03$) and tended to be higher in CMs from TAC-Ucn2 versus TAC-saline mice (**B**, $p=0.06$). **(C–G)** Sarcomere shortening. **(C)** Representative sarcomere shortening from each group. **(D)** Sarcomere fractional shortening was higher in CMs from TAC-Ucn2 versus TAC-saline mice ($p<0.02$). **(E)** TAC was associated with prolonged Tau, the time constant of sarcomere relaxation ($p=0.04$ vs. control), indicating impaired relaxation. In contrast, Tau was more rapid in CM from TAC-Ucn2 versus TAC-saline mice ($p=0.001$), indicating enhanced diastolic function. **(F)** The peak rate of sarcomere shortening (+dL/dt) was more rapid in CM from TAC-Ucn2 versus TAC-saline mice (**F**, $p=0.002$). **(G)** Likewise, the peak rate of sarcomere lengthening (–dL/dt) was more rapid in CM from TAC-Ucn2 versus TAC-saline mice (**G**, $p=0.001$). **(H)** Myofilament sensitivity. Myofilament sensitivity to Ca^{2+} was analyzed from Ca^{2+} transients and the coinciding sarcomere shortening and displayed as mean phase loops from CMs from each group. Arrows indicate loop direction. The loop from CMs from TAC-Ucn2 12 weeks after TAC shows a leftward shift compared with the TAC-saline group, indicating increased myofilament sensitivity to cytosolic Ca^{2+}. **(I)** During contraction, EC50 for cytosolic Ca^{2+} (Indo-1 ratio) was reduced in CM from TAC-Ucn2 mice during the contraction phase ($p=0.012$), indicating increased myofilament Ca^{2+} sensitivity. **(J)** There were no group differences during the relaxation phase. *p* Values from Student's *t*-test (unpaired and two tailed). Data denote mean values ± SE. CM, cardiac myocyte.

lower peak rate of Ca^{2+} rise (Fig. 3A, B; $p=0.03$); and prolonged Tau (Fig. 3E; $p=0.04$), establishing significant impairment in Ca^{2+} handling in CMs associated with TAC. Then we compared CMs from TAC-saline and TAC-Ucn2 mice to uncover the possible benefits of *Ucn2* gene transfer. The peak rate of Ca^{2+} cytosolic rise tended to be more rapid in CMs from TAC-Ucn2 mice (Fig. 3B; $p=0.06$). Measurement of sarcomere length changes (Fig. 3C) showed that CMs from TAC-Ucn2 mice exhibited higher fractional shortening (FS%) versus TAC-saline mice (Fig. 3D; $p<0.02$).

Tau, the rate constant for sarcomere lengthening, was more rapid in CMs from TAC-Ucn2 versus TAC-saline mice (Fig. 3E; $p=0.001$) and was less rapid in CMs from TAC-saline versus control mice (Fig. 3E; $p=0.04$). The peak rates of sarcomere shortening (+dL/dt) and lengthening (–dL/dt) were more rapid in CMs from TAC-Ucn2 versus TAC-saline mice (Fig. 3F; $p=0.002$; Fig. 3G; $p=0.001$). Measurement of CM sarcomere sensitivity to Ca^{2+} showed that the EC50 for contraction increased with TAC and returned toward normal with *Ucn2* gene transfer (Fig. 3H, I), but there were no group differences in the relaxation phase (Fig. 3J).

Ca^{2+} handling proteins

There were no group differences in LV SERCA2a protein expression (Fig. 4A). LV PLB phosphorylation at Ser16 in TAC-saline mice was not different from control, but was higher in TAC-Ucn2 mice compared with TAC-saline mice (Fig. 4B; $p=0.025$). Total LV PLB was higher after TAC ($p=0.001$), but this was not altered by *Ucn2* gene transfer (Fig. 4C).

In vitro studies of HL-1 cells

AngII was used to mimic TAC *in vitro* to enable mechanistic studies (Fig. 4D). Hypertrophy was documented by reduced phosphorylation of eEF2 at threonine 56 in HL-1 cells.[16,17] Ucn2 peptide stimulation increased PLB phosphorylation at Serine 16 in both normal and hypertrophied HL-1 cells (Fig. 4D).

LV expression of stress-related proteins

In mice, pressure overload is associated with increased expression of β-MHC and LV expression of ANP and BNP, commonly used as indicators of LV stress.[18] At 12 weeks, all three of these indicators of LV stress were elevated comparing TAC-saline versus control: β-MHC,

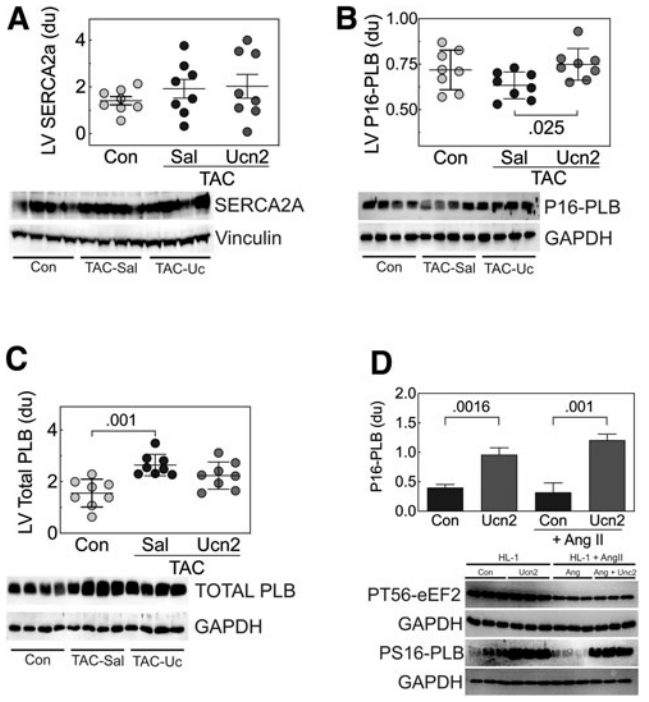

Figure 4. LV immunoblotting. **(A)** Neither TAC nor *Ucn2* gene transfer altered LV SERCA2a content. **(B)** LV PLB phosphorylation at Ser16 was restored to normal levels by *Ucn2* gene transfer ($p=0.025$). **(C)** LV total PLB was higher after TAC ($p=0.001$), but there was no difference between TAC-saline versus TAC-Ucn2. **(D)** Cultured HL-1 cells were exposed to AngII (100 nM, 24 h) to induce hypertrophy, documented by reduced phosphorylation at Thr56 of eEF2.[16] The addition of Ucn2 peptide (100 nM, 15 min) increased PLB phosphorylation at Ser16, both in the absence ($p=0.0016$) and presence of AngII ($p=0.001$). Data displayed are normalized to vinculin **(A)** or GAPDH **(B–D)**. *p* Values from Student's *t*-test. Data denote mean value ± SE. AngII, angiotensin II; Con, control; eEF2, eukaryotic elongation factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PLB, phospholamban; Sal, saline; Uc, Ucn2.

15.9-fold increase ($p=0.02$); BNP, 11.3-fold increase ($p=0.05$); and ANP, 9.9-fold increase ($p=0.04$). TAC-Ucn2 mice showed lower levels of BNP mRNA than TAC-saline mice (64% reduction) and β-MHC mRNA (57% reduction). However, these data had high variability and the reductions were not statistically significant (Table 2).

Necropsy and cardiac histology

At necropsy, TAC-saline mice showed substantial LVH with a 1.7-fold increase in LV/BW versus control mice (Table 3; $p=0.0002$). TAC-Ucn2 mice showed similar increases in LV/BW (1.8-fold vs. control), with no between-group difference (Table 3; $p=0.27$). TAC was associated with increased LV fibrosis of similar degrees in both the TAC-saline and TAC-Ucn2 groups (control: $1.3\pm0.8\%$, $n=7$; TAC-saline: $8.7\pm0.8\%$, $n=10$ [$p<0.0001$ vs. control]; and TAC-Ucn2: $7.9\pm1.1\%$, $n=8$ [$p=0.55$ vs. TAC-saline]; Table 3). TAC was associated with increased CM cross-sectional area of similar degrees in both TAC-saline and TAC-Ucn2 groups (control: $177\pm6\,\mu m^2$, $n=3$; TAC-saline: $318\pm8\,\mu m^2$, $n=5$ ($p<0.0001$ vs. control); and TAC-Ucn2: $307\pm12\,\mu m^2$, $n=4$ ($p=0.44$ vs. TAC-saline). Inflammatory infiltrates were absent in all the three groups.

DISCUSSION

Despite LVH and LV systolic and diastolic dysfunction being associated with TAC, *Ucn2* gene transfer improved systolic and diastolic LV function. These data indicate that *Ucn2* gene transfer has therapeutic benefits in pressure-induced LVH.

Use of the 129S1/SvImJ mouse strain served three functions. First, we wanted to perform gene transfer only after pressure-induced LVH was present, but not during its development—our goal was to treat LV dysfunction, not to prevent it from occurring. Previous studies had shown that 5 weeks after TAC placement, 129S1/SvImJ mice exhibit LVH, and EF remains normal.[4] Second, we needed adequate time (4–6 weeks) after gene transfer to allow exogenous *Ucn2* transfer to have an effect. Third, we wanted a low mortality model. Mortality rates in more conventional strains after TAC are excessively high: 3 weeks mortality is 61% in C57Bl/6-Harlan[9] versus no mortality 8 weeks after TAC in 129S1/SvImJ mice.[4] After an initial 7% perioperative mortality during TAC placement in the present study, there were no deaths in the subsequent 12 weeks.

Our goal was to determine whether *Ucn2* gene transfer would improve LV function, with a special focus on LV diastolic function. TAC was performed in 4–5-week-old mice. Six weeks later, mice were randomized to receive *Ucn2* gene transfer (AAV8.m*Ucn2*, 2×10^{13} gc/kg, i.v.) or saline, i.v., and 6 weeks after that, TAC-Ucn2 mice showed higher rates of LV peak −dP/dt (Fig. 2A), reduced Tau, indicating superior LV filling (Fig. 2C), higher rates of LV peak +dP/dt (Fig. 2B), and corresponding higher rates of sarcomere shortening and lengthening in studies conducted on isolated CMs (Fig. 3F, G). CMs from TAC-Ucn2 mice showed reduced time constant of cytosolic Ca^{2+} decline (Tau), providing a physiological and mechanistic basis for improved LV diastolic function (Fig. 3E).

LV mass, as assessed by LV/BW ratio, increased 1.7–1.8-fold after TAC ($p=0.0002$ vs. no TAC), which was unaffected by *Ucn2* gene transfer (Table 3). EF, which was not significantly decreased in TAC-saline mice compared with control, was higher in TAC-*Ucn2* mice (TAC-saline: $65\%\pm3\%$, $n=21$; TAC-Ucn2: $75\%\pm2\%$, $n=18$; $p=0.01$; Table 1).

LV EF, an ejection phase index of LV function that is affected by loading conditions, is a crude estimate of LV function. LV peak +dP/dt is a better indicator of contractile function,[19] which was decreased 35% by TAC ($p=0.03$), and subsequently increased 1.9-fold by *Ucn2* gene transfer ($p=0.001$; Fig. 2B). LV dP/dt at a developed pressure of 40 mmHg also showed superior LV +dP/dt and LV −dP/dt

in TAC mice that received *Ucn2* gene transfer versus TAC mice that received saline. HRs were higher in TAC-Ucn2 mice under deep anesthesia (Fig. 2F) but showed no group difference under light anesthesia during echocardiographic studies (Table 1), where increases in VCFc was seen in mice that had received *Ucn2* gene transfer, indicating increased contractile function at a similar HR.

Group difference in HR (Fig. 2F) may have affected LV peak dP/dt through the force–frequency relationship. However, LV peak −dP/dt was increased 69% and LV peak +dP/dt was increased 90% in TAC mice that received *Ucn2* gene transfer versus saline (Fig. 2A, B). The force–frequency effect in closed-chest anesthetized mice over the range reported in our study (429–538 bpm; Fig. 2F) accounts for a smaller change: ~8% increase in both LV peak +dP/dt and LV peak −dP/dt in closed-chest anesthetized mice.[20] Previously reported telemetry studies in ambulatory unanesthetized mice indicated that *Ucn2* gene transfer is not associated with increased mean daily HRs.[21]

Studies conducted on isolated CMs (Fig. 3) confirmed studies conducted in the intact heart (Table 1 and Fig. 2). The increase in LV EF in TAC-Ucn2 versus TAC-saline mice (Table 1; $p = 0.01$) aligned with a corresponding increase in sarcomere fractional shortening (Fig. 3D; $p < 0.02$). Tau was reduced by *Ucn2* gene transfer in both the intact LV (Fig. 2C) and in isolated CM (Fig. 3E). The increased rates of sarcomere shortening and lengthening (Figs. 3F, G) mirrored the increases seen in peak −dP/dt and peak +dP/dt in the intact LV (Fig. 2A, B). Finally, there was increased myofilament Ca^{2+} sensitivity during contraction in CM isolated from TAC mice that had received *Ucn2* gene transfer versus TAC-saline mice (Fig. 3H, I; $p = 0.012$).

The tight correlation between these two domains (intact LV and isolated CM) provides confidence in the validity of the findings *vis-à-vis* the beneficial effects of *Ucn2* gene transfer on heart function. The consistency between the physiological responsiveness of the intact heart and isolated CMs, while expected, merits comment. Unlike the intact heart, which is perfused constantly with supranormal levels of Ucn2 peptide after *Ucn2* gene transfer, isolated CMs do not have access to plasma Ucn2. We speculate that CMs isolated from mice that received *Ucn2* gene transfer are continuing to synthesize transgene *Ucn2 in vitro*, which, when released to the media, provides autocrine- or paracrine-based stimulation of cell surface corticotropin releasing hormone receptor 2s (CRHR2s). LV samples from TAC mice that received *Ucn2* gene transfer showed a 900-fold increase in *Ucn2* mRNA versus LV samples from TAC mice that received saline (Fig. 1D).

While *Ucn2* gene transfer improved cardiac function, it did not reduce LVH caused by TAC. A reduction in LV mass was not expected. Although Ucn2 has vasodilating effects and reduces ambulatory blood pressure in conscious mice,[21] this was not expected to reduce the degree of LVH because of the nature of TAC, which is a mechanical constriction of LV outflow that is independent of systemic vascular resistance. Indeed, systemic arterial blood pressure is normal in TAC.[22] Our purpose, rather, was to determine whether, in a suitable model of HFpEF, *Ucn2* gene transfer would have beneficial effects on LV function *per se*, especially LV diastolic function. *Ucn2* gene transfer improved cardiac function even in a model where benefits that could come from afterload reduction were blocked, underscoring the importance of the beneficial effects of *Ucn2* gene transfer on the heart *per se*.

However, although TAC has many advantages, its clinical correlates are aortic stenosis or coarctation of the aorta, conditions best remedied by transcatheter aortic valve replacement or surgery. The benefits of *Ucn2* gene transfer in nonclinical models of systemic hypertension are currently undergoing study.

In previous pathophysiological models of cardiovascular diseases including HFrEF,[1] aging,[2] diabetes,[23,24] and in normal mice[11,21] that underwent *Ucn2* gene transfer, a consistent finding has been the increased function of the intact LV and enhanced Ca^{2+} handling in isolated CMs. These findings were present in the current study (Figs. 2 and 3). However, many past studies showed increases in LV SERCA2a protein content[1,2,11,21] and reductions in LV CaMKII,[1,2,21] but no changes in PLB expression or phosphorylation.

In the present study, there was no change in LV SERCA2a expression or CaMKII, while LV PLB phosphorylation at Ser16 was increased in TAC-Ucn2 mice versus TAC-saline mice ($p = 0.025$; Fig. 4B). This increase likely contributes to improved LV function (Fig. 2) and enhanced sarcomere shortening (Fig. 3). The absence of increased LV SERCA2a protein after *Ucn2* gene transfer may reflect the model (TAC) or difference in mouse strain used in these studies (129S1/SvImJ)—all prior work was performed using C57Bl/6 mice.

To determine the underlying mechanisms by which *Ucn2* gene transfer had beneficial effects, we performed studies in HL-1 cells, a cardiac-muscle cell line (Fig. 4D). Cultured HL-1 cells were exposed to AngII to induce hypertrophy and then incubated with Ucn2 peptide. AngII slightly reduced PLB phosphorylation at Serine 16. However, Ucn2 peptide increased PLB phosphorylation in the presence of hypertrophy, which mimicked what was seen in the intact LV. PLB phosphorylation likely is of mechanistic importance in the effects of *Ucn2* gene transfer on enhanced Ca^{2+} handling in CMs, improved sarcomere shortening and relaxation rates (Fig. 3), and function of the intact hypertrophied LV (Fig. 2).

Although we found benefits in multiple domains after *Ucn2* gene transfer in TAC, CM-targeted CRHR2 *deletion* was reported to increase LV response to TAC.[25] This seems counterintuitive—Ucn2's cognate receptor is CRHR2. The authors suggested that CRHR2 stimulation may be deleterious in HF. However, the effects of infused Ucn2, Ucn3, or stresscopin peptides (which stimulate

CRHR2) have been beneficial and safe in animal models of HF,[26,27] and in randomized double-blinded clinical trials enrolling patients with HF.[6,7,8,28] Finally, we have found that *Ucn2* gene transfer and sustained plasma elevation of Ucn2 for 5 weeks to 21 months has benefits in HF with reduced EF[1] and in age-related HFpEF.[2] Given the broad and consistent evidence of benefits of Ucn2 treatment of HF indicates that cardiac CRHR2 deletion may affect LV function independently of CRHR2 signaling *per se*.

We found a single published article in which a similar protocol used in the present article was followed—that is, performing gene transfer after TAC-induced hypertrophy was already present, as a treatment strategy. In this article, 4 weeks after TAC in rats, an intramyocardial injection of an adenovirus vector encoding relaxin-2 was performed, and 12 days later, echocardiography showed improvement in diastolic function. LV samples showed increased phosphorylation of Akt and PLB and increased SERCA2a activity.[29] LV peak −dP/dt and Tau were not assessed, and no studies were performed on isolated CMs.

Limitations

Whether the TAC model is suitable for clinical HFpEF can be questioned. However, diastolic abnormalities in the intact heart and in isolated CMs, elevation of LVEDP, and increased LV BNP expression are consistent with a congested state. Not all of the features of HFpEF were improved by *Ucn2* gene transfer. For example, we did not see reductions in LVEDP or LA mass, both of which were increased by TAC but refractory to *Ucn2* gene transfer, probably reflecting persistent mechanically produced pressure overload. Although *Ucn2* gene transfer decreased LV BNP expression by 64%, the reduction was not statistically significant ($p = 0.18$ vs. TAC-Saline). This was due to high individual animal variability, an inherent problem of mRNA assessment. Plasma levels of BNP peptide or NT-Pro-BNP would have been preferred, but murine anti-BNP antibodies are not readily available.

Not uncommonly, patients with severe aortic stenosis present with pulmonary congestion, concentric LVH, and preserved LV EF—so prolonged TAC, as we presented here, is a suitable yet imperfect model for aortic stenosis, although transcatheter aortic valve replacement rather than gene transfer would be the best treatment for aortic stenosis. Nonclinical studies conducted to test new therapies for clinical HFpEF are fraught because HFpEF has variable etiology and manifestations, but are nevertheless useful for future clinical translation.

CONCLUSIONS

Ucn2 gene transfer was associated with beneficial effects on LV function in mice with pressure-induced HFpEF. These benefits on global LV function were mirrored by increased rates of lengthening and relaxation in CMs isolated from study animals. A contributing mechanism for improved LV function derived from enhanced Ca^{2+} handling was associated with increased LV PLB phosphorylation. These findings, combined with the other benefits of *Ucn2* gene transfer: vasodilation and enhanced LV relaxation (Tau), make *Ucn2* gene transfer a promising potential therapeutic in HFpEF.

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AUTHORS' CONTRIBUTIONS

N.C.L. Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing (review and editing), visualization, project administration, and funding acquisition; Z.T. Methodology, formal analysis, investigation, writing (review and editing), and visualization; D.G. Methodology, investigation, and writing (review); M.H.G. Methodology, investigation, writing (review and editing), project administration, and funding acquisition; H.K.H. Conceptualization, validation, formal analysis, investigation, writing (initial draft, review, and editing), visualization, supervision, project administration, and funding acquisition. All authors have read and agree with the data presented in the article, which are not being considered elsewhere for publication.

AUTHOR DISCLOSURE

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