Intravenous AAV8 Encoding Urocortin-2 Increases Function of the Failing Heart in Mice

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Abstract

Urocortin-2 (UCn2) peptide infusion increases cardiac function in patients with heart failure, but chronic peptide infusion is cumbersome, is costly, and provides only short-term benefits. Gene transfer would circumvent these shortcomings. We previously showed that a single intravenous (IV) injection of AAV8.UCn2 increases plasma UCn2 and left ventricular (LV) systolic and diastolic function for at least 7 months in normal mice. Here we test the hypothesis that IV delivery of AAV8.UCn2 increases function of the failing heart. Myocardial infarction (MI, by coronary ligation) was used to induce heart failure, which was assessed by echocardiography 3 weeks after MI. Mice with LV ejection fraction (EF) <25% received IV delivery of AAV8.UCn2 (5×10^{11} gc) or saline, and 5 weeks later echocardiography showed increased LV EF in mice that received UCn2 gene transfer ($p=0.01$). *In vivo* physiological studies showed a 2-fold increase in peak rate of LV pressure development (LV +dP/dt; $p<0.0001$) and a 1.6-fold increase in peak rate of LV pressure decay (LV –dP/dt; $p=0.0007$), indicating increased LV systolic and diastolic function in treated mice. UCn2 gene transfer was associated with increased peak systolic Ca^{2+} transient amplitude and rate of Ca^{2+} decline and increased SERCA2a expression. In addition, UCn2 gene transfer reduced Thr286 phosphorylation of Cam kinase II, and increased expression of cardiac myosin light chain kinase, findings that would be anticipated to increase function of the failing heart. We conclude that a single IV injection of AAV8.UCn2 increases function of the failing heart. The simplicity of IV injection of a vector encoding a gene with beneficial paracrine effects to increase cardiac function is an attractive potential clinical strategy.

Introduction

CONGESTIVE HEART FAILURE (CHF) is associated with unacceptably high morbidity and mortality. Even with optimal medical and device management, 50% of class III and class IV CHF patients die within 4 years. In the United States, the prevalence for CHF is 6 million patients; 670,000 new cases are diagnosed annually.¹ Because the prevalence of CHF is increasing and the outlook remains dismal, gene transfer is rational and may fulfill an unmet medical need.

Current methods of gene transfer for heart diseases include intramuscular (IM) injection into heart muscle and intracoronary delivery, which are cumbersome to apply. Gene transfer trials for heart disease have thus far been

disappointing perhaps because of inadequate levels of gene expression in the heart. To circumvent this impediment, we have proposed a different approach: intravenous (IV) delivery of a vector encoding a transgene with paracrine activity affecting the heart. In this strategy, a peptide with beneficial cardiovascular effects is released to the circulation from distant sites after systemic delivery of a long-term expression vector encoding the peptide. This approach enables gene transfer via a simple IV injection during an office visit, circumvents the need for more invasive delivery methods, and potentially may give gene transfer for heart failure a much needed boost.

Clinical trials of systemic delivery of genes are underway for hemophilia B^{2,3} and $\alpha 1$ -antitrypsin deficiency⁴ and others are in

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development. However, systemic delivery of vectors encoding peptides with beneficial paracrine activity has not been performed in clinical CHF trials, and is uncommon in preclinical studies.^{5,6} Intravenous infusion of potentially beneficial peptides such as urodilatin,⁷ relaxin-2,⁸ urocortin-2 (UCn2),^{9,10} and stresscopin¹¹ shows promise in clinical CHF trials, but gene transfer of these peptides has not been attempted.

Urocortins 1, 2, and 3 (38–41 amino acids) belong to the corticotropin-releasing factor (CRF) family. These peptides stimulate CRF receptors 1 and 2 (CRFR1 and CRFR2). UCn1 binds to CRFR1 and CRFR2, but UCn2 and UCn3 exclusively bind to CRFR2, which is expressed in cardiac myocytes, vasculature, gut, brain, and skeletal muscle.^{12–14} Through selective CRFR2 activation, UCn2 mediates protean beneficial effects, including reduced renin-aldosterone activation, and is a potent inotrope with minimal effects on cardiac cAMP.¹⁵ In preclinical and clinical HF, peptide infusions of UCn2 or stresscopin, a related peptide, have shown favorable effects on left ventricular (LV) function.^{9–11,16}

Since plasma half-life of UCn2 is 15 min,¹⁰ chronic infusion is required for sustained effects. The inconvenience, need for hospitalization and associated hazards, and the expense of therapeutic peptide infusions for CHF are considerable, and impede the broad use of these otherwise attractive peptides. The present study was conducted to address and resolve these shortcomings using UCn2 gene transfer in a murine model of CHF.

Adeno-associated virus (AAV) vectors enable prolonged transgene expression. For example, persistent transgene expression has been shown in nonhuman primates for sustained periods after IM¹⁷ or IV delivery of AAV vectors.^{18–20} We have confirmed this in rats.⁶ Although recent clinical trials have found that some AAV serotypes incite immune responses after IM injection,^{21,22} newer generation AAV vectors (AAV5, 6, 8, 9, rh-10) do not appear to have similar problems in nonhuman primates.^{19,20,23,24} IV AAV delivery is superior to IM *vis-à-vis* serum transgene levels, and AAV9 and AAV8 are superior to AAV5.²⁵ Moreover, preexisting anti-AAV8 antibodies are not as prevalent in humans (19%) as are other AAV serotypes, including AAV1 and AAV2 (50–59%).²⁶ These data suggest that IV AAV8 may be an attractive delivery route and vector to attain sustained increased levels of plasma UCn2. IV delivery of an AAV vector encoding a paracrine gene, as compared with IV peptide infusion, has the potential to circumvent infection and reduce repeated and prolonged hospital stays, thereby reducing costs. Systemic vector delivery may be an advantage in this approach—it provides the highest level of expression for any given AAV dose—by exploiting widespread distribution of the vector.

In a previous study we showed that a single IV injection of AAV8.UCn2 increases plasma UCn2 and increases LV systolic and diastolic function for at least 7 months in normal mice.²⁷ The present study was conducted to test the hypothesis that IV delivery of AAV8.UCn2 will increase function of the failing heart.

Methods

AAV8.UCn2 vector production

A helper virus-free AAV8 vector encoding murine UCn2 driven by a chicken β -actin (CBA) promoter (AAV8.CBA.UCn2; Fig. 1) was produced by transient transfection of

FIG. 1. AAV8.CBA.UCn2 map and experimental protocol. (A) AAV8.CBA.UCn2 vector map. CBA, chicken β -actin promoter; CMV.en, human cytomegalovirus enhancer; ITR, inverted terminal repeat; SVpA, polyA from SV40 viral genome; UCn2, urocortin-2. (B) Experimental protocol. Normal mice underwent myocardial infarction (MI, by proximal left coronary ligation) to induce HF, which was assessed by echocardiography 3 weeks after MI. Mice with EF < 25% were then randomized to receive AAV8.UCn2 (5×10^{11} gc, IV) or IV saline. Five weeks later, echocardiography was used to assess LV size and function. *In vivo* physiological studies were conducted to evaluate rates of LV pressure development (LV + dP/dt) and decay (LV - dP/dt), to assess LV systolic and diastolic function. Cross sections of LV (midpapillary level) show that the infarction is extensive (lower section), comprising the majority of the LV free wall, with only the interventricular septum spared. Data acquisition and analysis were blinded to group treatment. Color images available online at www.liebertpub.com/hum

HEK293T cells with the vector plasmid pRep2/Cap8 and pAd-Helper plasmid.²⁸ Plasmid pRep2/Cap8 was obtained from the University of Pennsylvania Vector Core. Cell lysates prepared after 72 hr of transfection were treated with benzonase and viruses were consolidated through 25% sucrose-cushion ultracentrifugation. The pellets were resuspended for further purification of the virus through anion-exchange column chromatography (Q-Sepharose; GE Health Science) and concentrated by 25% sucrose-cushion ultracentrifugation.^{29,30} Subsequently, the pellets were resuspended in 10 mM Tris-HCl (pH 7.9, 1 mM MgCl₂, 3% sucrose). Virus titers were determined by real-time qPCR with virus genome DNA prepared from purified virus.

Heart failure model

The Animal Use and Care Committee of the VA San Diego Healthcare System approved the studies. Two hundred thirty-one male C57BL/6J mice (Jackson Laboratories) aged 10–12 weeks, weighing 26.1 ± 0.2 g, were used. We used coronary occlusion to induce large anterior wall myocardial infarction (MI) and CHF as described in detail previously.^{31,32} MI size deliberately was large—50% of LV, comprising most of the LV free wall (Fig. 1). Consequently, this model is associated with a high initial mortality. Of 231 mice that underwent coronary occlusion, 125 (54%) died before randomization (AAV8.UCn2 or saline) primarily in the first few days after MI. An additional 45 mice (19%) did not show sufficient LV dysfunction 3 weeks after MI to be randomized. Sixty-one mice (26%) had sufficiently low LV ejection fractions (EF < 25%) and were randomized, and 11 of these mice died before the final study 5 weeks after

randomization: 4 UCn2 (mortality 13%) and 7 saline (mortality 23%). The primary end point was LV function 5 weeks after IV delivery of AAV8.UCn2 versus saline in mice with severe heart failure (Fig. 1). Data were acquired and analyzed without knowledge of group identity.

AAV8.UCn2 delivery

Under anesthesia (1.5% isoflurane via nose cone), a small incision was made to expose the jugular vein for IV delivery of AAV8.UCn2 (5×10^{11} genome copies [gc] in 100 μ l) or a similar volume of saline (control).

Effects of UCn2 gene transfer on heart rate and blood pressure

These studies were conducted to assess the effects of UCn2 gene transfer on heart rate and blood in unsedated mice with heart failure. Impaired LV EF was confirmed 3 weeks after MI, and mice received IV AAV8.UCn2 (5×10^{11} gc) or saline. Systolic and diastolic blood pressure and heart rate were measured by tail cuff (Visitech Systems) in unsedated mice.

Echocardiography

Echocardiography was performed as previously described.³³ Echocardiography was performed 3 weeks after MI to document reduced LV function (EF <25%) and to record LV chamber dimensions. Echocardiographic assessment was then repeated 5 weeks after randomization of mice to receive IV delivery of AAV8.UCn2 or saline.

LV systolic and diastolic function

Mice were anesthetized with sodium pentobarbital (80 mg/kg, ip) and a 1.4F conductance-micromanometer catheter (SPR 839; Millar Instruments) was advanced via the right carotid artery across the aortic valve and into the LV cavity. LV pressure was recorded and stored digitally for processing (IOX1.8; Emka Technologies) as previously reported.⁶ Subsequently, blood and tissue samples were obtained. After acquisition, the first derivatives of LV pressure development (LV +dP/dt) and decline (LV -dP/dt) were used to assess LV systolic and diastolic function. Data were acquired and analyzed without knowledge of group identity.

Cardiac myocyte isolation

Cardiac myocytes were isolated as previously described.³³

Ca²⁺ transients

Cytosolic Ca²⁺ transients were measured using Indo-1 as described previously^{27,34} with modifications. Cardiac myocytes were plated onto laminin-coated glass cover slips and loaded with indo-1/AM (3 μ M; Calbiochem) and dispersing agent, pluronic F-127 (0.02 mg/ml; Calbiochem), for 30 min. Following dye loading, cover slips were mounted in a superfusion chamber, rinsed to remove excess indo-1-AM, and mounted on a Nikon Diaphot epifluorescence microscope equipped with a 40 $\times$ objective interfaced to a Photon Technologies photometry system with the excitation wavelength set to 365 nm via a monochromator. Fluorescence emission was split and directed to two photomultiplier tubes

through 20 nm band-pass filters centered at 405 and 485 nm, respectively. The ratio F405/F485 represents a measure for [Ca²⁺]_i. During these measurements, cardiac myocytes were superfused with 25 mM HEPES (pH 7.3) containing 2 mM CaCl₂. Myocytes were field-stimulated at 0.3 Hz. Ca²⁺ transients were recorded from 144 cardiac myocytes obtained from 6 hearts (3 per group). Diastolic and systolic intracellular Ca²⁺ levels were inferred from the basal and maximal indo-1 ratio per cycle, respectively. Diastolic decay time (τ) was calculated from the normalized Ca²⁺ transient.

Quantitative RT-PCR and immunoblotting

LV and liver samples were collected and stored at -80°C for quantitative RT-PCR and Western blotting.

Quantitative RT-PCR. LV and liver RNA was isolated using RNeasy mini kit (Qiagen) and qRT-PCR conducted as previously described²⁷ under the following conditions: 5 min at 98°C, 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. RNA equivalents were normalized to simultaneously determined glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in each sample. Primers are listed in Table 1.

Immunoblotting. Immunoblotting was performed as described previously.³⁵ The following antibodies were used: cMLCK (Abgen/Thermo Scientific); p286 CamKII (Santa Cruz); phospho-PKA catalytic subunit, PKA catalytic subunit, troponin I (TnI), and 22/23-phospho-TnI (Cell Signaling Technology); phospholamban (PLB) (Thermo Fisher Scientific); Ser 16 and Thr 17-phospho-PLB (Badrilla, Ltd.); and SERCA2a (Enzo Life Sciences).

Cyclic AMP and protein kinase A activity

Transmural LV samples underwent cAMP measurement before and after stimulation with isoproterenol (10 mM, 10 min) and NKH477 (10 mM, 10 min), and cAMP was measured using the Biotrak Enzymeimmunoassay System (GE Healthcare) as previously described.³⁶ PKA activity was determined as previously described.²⁷ Cardiac myocytes underwent cAMP measurement before and after stimulation with isoproterenol (10 μ M, 10 min) and NKH477 (10 μ M, 10 min) and subsequently homogenized in buffer A, 20 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 0.5 mM EDTA, and protease inhibitor cocktail (Invitrogen), and centrifuged (14,000 $\times$ g, 5 min, 4°C). The supernatant was incubated with PKA biotinylated peptide substrate (SignaTECT cAMP-Dependent Protein Kinase Assay System; Promega) in the presence of [γ -³²P]ATP. The ³²P-labeled biotinylated substrate was recovered with a streptavidin matrix and the specific activity of PKA determined.

Histology

Samples of liver and transmural sections of the uninfarcted LV septum were formalin fixed and paraffin embedded. Five-micrometer sections were mounted and counterstained with hematoxylin and eosin and with Masson's trichrome. For quantitative assessment of LV fibrosis, images of a short-axis midwall LV ring were obtained with a

TABLE 1. PRIMERS

Gene	Forward	Reverse
ANF	5'-CCTCGTCTTGGCCTTTTGG	5'-CATCTTCTACCGGCATCTTC
α -MHC	5'-AAAGGCTGAGAGGAACCTACC	5'-ACCAGCCTTCTCCTCTGC
α -Cd-actin	5'-GTGTTACGTCGCCCTTGATT	5'-TGAAAGAGGGGCTGGAAGAGA
α -SK-actin	5'-GTGTCACCCACAACGTGC	5'-AGGGCCACATAGCACAGC
β -MHC	5'-GCTGAAAGCAGAAAGAGATTATC	5'-TGGAGTTCTTCTCTTCTGGAG
BNP	5'-GAAGTCCTAGCCAGTCTCC	5'-CAGCTTGAGATATGTGTACC
Coll1 α 1	5'-GCCAAGAAGACATCCCTGAAG	5'-GGGTCCCTCGACTCCTAC
Coll3 α 1	5'-GCACAGCAGTCCAACGTAGA	5'-TCTCCAAATGGGATCTCTGG
GAPDH	5'-CATGTTCCAGTATGACTCCACTC	5'-GGCCTCACCCCATTTGATGT
MEF2	5'-GAGCCTCATGAAAGCAGGAC	5'-GAAGTTCTGAGGTGGCAAGC
MMP2	5'-GAGTTGCAACCTCTTTGTGC	5'-CAGGTGTGTAACCAATGATCC
MMP8	5'-GACTCTGGTGATTTCTTGCTAAC	5'-CACCATGGTCTCTTGAGACG
MMP9	5'-CGTCGTGATCCCCACTTACT	5'-GAACACACAGGGTTTGCCTTC
TIMP1	5'-GACAGCTTTCTGCAACTCGG	5'-CTTGTGGACATATCCACAGAGG
TIMP2	5'-GCAATGCAGACGTAGTGATCAG	5'-CCTTCTTTCTCCCAACGTCC
TIMP3	5'-CTTCTGCAACTCCGACATCG	5'-CCTGTGCAGCAGGTACTGG
TIMP4	5'-CAAGGATATTTCAGTATGTCTACACG	5'-CTGGTGGTAGTGATGATTTCAGG
UCn2	5'-ACTCCTATCCCCACCTTCCA	5'-AAGATCCGTAGGAGGCCAAT

ANF, atrial natriuretic peptide; BNP, brain natriuretic peptide; Coll, collagen; MEF2, myocyte enhancer factor-2; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; UCn2, urocortin 2; α -Cd-actin, alpha-cardiac actin; α -MHC, alpha-myosin heavy chain; α -SK-actin, alpha-skeletal actin; β -MHC, beta-myosin heavy chain.

Nikon Eclipse Ti-U microscope. Blinded analysis of the degree of fibrosis in the viable LV region (excluding the infarcted region) was conducted using NIS-Elements AR 3.10 software (Nikon Inc.). A similar analytical process was performed on fixed and counterstained liver samples.

Statistical analysis

Data represent mean $\pm$ SE; group differences were tested for statistical significance with ANOVA followed by Bonferroni *t*-test. Between-group comparisons were made using Student's *t*-test (unpaired, two-tailed). The null hypothesis was rejected when $p < 0.05$.

Results

Heart rate and blood pressure in unsedated mice

No group differences were seen in heart rate or systolic, diastolic, or mean arterial blood pressure 5 weeks after UCn2 gene transfer (Table 2), although heart rates tended to be quite high in the untreated group and closer to normal in mice that had received UCn2 gene transfer.

Urocortin 2 expression

Five weeks after IV delivery of AAV8.UCn2 (5×10^{11} gc; $n=6$), UCn2 mRNA was increased 15,263-fold in liver ($p < 0.0001$) and 70-fold in LV ($p=0.03$) versus endogenous UCn2 mRNA.

Echocardiography

Intravenous delivery of AAV8.UCn2 to mice with HF was associated with increased EF ($p=0.01$), and velocity of circumferential fiber shortening was increased but did not reach statistical significance ($p=0.09$). Mice that received AAV8.UCn2 also exhibited reductions in LV end-diastolic diameter (EDD; $p < 0.001$) and LV end-systolic diameter (ESD; $p=0.002$). The saline-treated mice showed

an 11% increase in LV EDD, while the UCn2-treated group showed a 2% decrease in LV EDD. Similarly, the saline group showed a 16% increase in LV ESD, while the UCn2 group experienced a 6% reduction. Although these changes in LV dimension are small, volume is a cubic function of dimension, so the changes are considerable—a calculated 64% increase in ESD (saline vs. UCn2) and a 46% increase in EDD (saline vs. UCn2). Posterior and septal wall thickness showed no group differences (Table 3).

LV systolic and diastolic function

In vivo assessment of LV pressure development showed substantial increases in rates of LV pressure development (LV +dP/dt; $p < 0.0001$) and in LV relaxation (LV -dP/dt; $p < 0.0007$) (Fig. 2 and Table 4). There were no group differences in mean arterial pressure (Table 4). Heart rate during these studies, conducted under anesthesia, was somewhat

TABLE 2. EFFECTS OF UCn2 GENE TRANSFER ON HEART RATE AND BLOOD PRESSURE IN MICE WITH HEART FAILURE

	HF (n)	HF + UCn2 (n)	p
Heart rate, beats/min	693 $\pm$ 54 (4)	601 $\pm$ 96 (5)	0.13
Systolic pressure, mmHg	123 $\pm$ 23 (5)	105 $\pm$ 17 (5)	0.20
Diastolic pressure, mmHg	89 $\pm$ 18 (5)	73 $\pm$ 14 (5)	0.16
Mean arterial pressure, mmHg	100 $\pm$ 19 (5)	83 $\pm$ 16 (5)	0.28

The effects of UCn2 gene transfer on blood pressure and heart rate (HR) were assessed in unsedated mice with heart failure (HF) 5 weeks after UCn2 gene transfer (HF + UCn2, 5×10^{11} gc, IV) or IV saline (HF). Systolic and diastolic blood pressure was measured by tail cuff and mean blood pressure calculated. No group differences were seen in heart rate or blood pressure. Values denote mean $\pm$ SE; *p*-values are from Student's *t*-test (unpaired, 2-tailed).

TABLE 3. ECHOCARDIOGRAPHY BEFORE AND AFTER UCn2 GENE TRANSFER VS. SALINE FOR HF

	HF (12)			HF+UCn2 (13)			
	3 weeks after MI	5 weeks after Saline	Post-Pre	3 weeks after MI	5 weeks after UCn2	Post-Pre	p
HR (bpm)	542±18	513±13	-29±21	503±12	525±12	22±13	0.045
EDD (mm)	5.3±0.3	5.9±0.3	0.6±0.1	5.3±0.3	5.2±0.3	-0.1±0.1	<0.001
ESD (mm)	4.5±0.4	5.2±0.4	0.7±0.2	4.7±0.4	4.4±0.4	-0.3±0.2	0.002
LV EF (%)	19±2	12±1	-7±2	17±2	20±4	3±3	0.01
VCFc (circ/sec)	3.3±0.9	3.0±0.8	-0.3±0.6	3.5±0.7	4.7±0.8	1.2±0.6	0.09
PW Th (mm)	0.5±0.03	0.5±0.03	-0.05±0.03	0.5±0.03	0.5±0.03	-0.01±0.02	0.20
IVS Th (mm)	0.5±0.04	0.5±0.04	0.01±0.02	0.5±0.04	0.5±0.05	-0.02±0.04	0.43

bpm, beats per minute; EDD, LV end-diastolic diameter; ESD, LV end-systolic diameter; HF, heart failure; HR, heart rate; IVS Th, interventricular wall thickness at end-diastole; LV EF, left ventricular ejection fraction; Post-Pre, the value 5 weeks after saline or UCn2 gene transfer minus the value before; PW Th, posterior wall thickness at end-diastole; UCn2, urocortin-2; VCFc, velocity of circumferential fiber shortening (corrected for heart rate).

p-Values from Student's *t*-test (paired data, 2-tailed) for group difference in change, Post-Pre.

higher in mice that had received UCn2 gene transfer, but the difference did not reach statistical significance.

Cytosolic Ca²⁺ transients and related genes

Basal Ca²⁺ released (systolic-diastolic Ca²⁺) was increased in cardiac myocytes from heart failure mice that had received UCn2 gene transfer (*p*=0.0001; Fig. 3A and B). UCn2 gene transfer was also associated with a reduced Ca²⁺ decline time (*t*_{1/2}, Tau) in cardiac myocytes from mice with heart failure 5 weeks after UCn2 gene transfer *p*=0.001; Fig. 3C and D). Increased UCn2 was associated with increased expression of SERCA2a mRNA and protein in normal and failing LV (Fig. 3E). However, no group differences were seen in LV protein expression and phosphorylation of PLB or TnI (data not shown).

FIG. 2. LV function *in vivo*. (A and B) Five weeks after AAV8.UCn2 (5×10^{11} gc, IV) or saline (HF) infusion, *in vivo* studies were performed to measure the rate of LV pressure development: LV +dP/dt (A) and decay LV -dP/dt (B). AAV8.UCn2 increased LV +dP/dt and LV -dP/dt 5 weeks after gene transfer, indicating that UCn2 gene transfer increases LV systolic function. (C and D) Heart rate tended to be higher (D). LV-developed pressure was increased by UCn2 gene transfer (C). Studies were performed without knowledge of group identity. *p*-Values are from Student's *t*-test (unpaired, 2-tailed). Data represent mean $\pm$ SE, and numbers in bars denote group size.

Cyclic AMP and PKA activity

LV samples and cardiac myocytes isolated from hearts of both groups showed no differences in cAMP or PKA activity (Fig. 4). Cyclic AMP production and PKA activity were assessed before and after stimulation with isoproterenol or NKH477, a water-soluble forskolin analog that stimulates adenylate cyclase independently of β -adrenergic receptors. No group differences were seen in basal, Iso, or NKH477-stimulated cAMP production (Fig. 4A) or in PKA activity (Fig. 4B). Expression of PKA family proteins (catalytic α unit and regulatory α and β subunits and their phosphorylation) was not altered (data not shown).

CamKII and cMLCK

To seek mechanisms to explain increased function of the failing heart evoked by UCn2 gene transfer, we measured LV expression and phosphorylation of calcium/calmodulin-dependent protein kinase II (CamKII) and expression of cardiac myosin light chain kinase 3 (cMLCK). CamKII phosphorylation at Ser286 was reduced in LV samples from HF mice after UCn2 gene transfer (47% reduction, *p*=0.04; Fig. 4C), although total CamKII protein expression showed no group difference. Seeking alterations in myofilament sensitivity to Ca²⁺, we assessed LV cardiac myosin light chain kinase 3 (cMLCK) expression after UCn2 gene transfer, finding a 1.6-fold increase (*p*<0.04) (Fig. 4D).

TABLE 4. PHYSIOLOGICAL DATA

	Saline (11)	UCn2 (9)	p
LVP (mmHg)	68±3	83±2	<0.004
LV +dP/dt (mmHg/s)	3225±287	6354±451	<0.0001
LV -dP/dt (mmHg/s)	-3127±370	-4974±215	<0.0007
MAP (mmHg)	56±3	63±3	0.10
HR (bpm)	404±23	461±18	0.08

HR, heart rate; LV, left ventricle; LVP, left ventricular developed pressure; MAP, mean arterial pressure; UCn2, urocortin-2 gene transfer.

Three weeks after myocardial infarction, mice received intravenous saline or AAV8.UCn2 (5×10^{11} gc). Mice underwent physiological studies 5 weeks later. Values represent mean $\pm$ SE. *p*-Values are from Student's *t*-test (unpaired, 2-tailed).

FIG. 3. Cytosolic Ca^{2+} transients in cardiac myocytes from mice with heart failure (HF) 5 weeks after IV AAV8.*UCn2* (HF+UCn2) or IV saline. (**A** and **B**) Basal Ca^{2+} released (systolic–diastolic Ca^{2+}) was increased in cardiac myocytes from HF+UCn2 mice ($p=0.0001$). (**A**) Representative Indo-1 Ca^{2+} transient recordings from one heart in each group showed increased peak Ca^{2+} in cardiac myocytes isolated from mice with heart failure 5 weeks after *UCn2* gene transfer. (**B**) Summary data from three mice per group are shown. (**C** and **D**) Time to Ca^{2+} decline ($t_{1/2}$, Tau) was shortened in cardiac myocytes from mice with heart failure 5 weeks after *UCn2* gene transfer. (**C**) Representative normalized Ca^{2+} transients from cardiac myocytes from one heart in each group. (**D**) Summary data from three mice per group are shown. For (**A**) and (**C**), each curve is the average of 30 cardiac myocytes from one heart from each group. For (**B**) and (**D**), summary data from 3 animals per group include analysis of 146 individual cardiac myocytes (86, saline; 60, AAV8.*UCn2*). For (**B**) and (**D**), bars denote mean + SE; numbers in bars denote number of cardiac myocytes; numbers above bars indicate p -values from Student's t -test (unpaired, 2-tailed). (**E**) Summary (top panel) of immunoblotting data (bottom panel) indicates that *UCn2* gene transfer increased SERCA2a protein in LV from normal mice and from mice with heart failure. Expression and phosphorylation of phospholamban (PLB) and troponin I (TnI) were not affected. Bars denote mean + SE; numbers in bars denote group size; numbers above bars from Student's t -test (unpaired, 2-tailed vs. control).

FIG. 4. Cardiac myocyte cAMP-PKA signaling. LV samples (**A**, **C**, **D**) or cardiac myocytes (**B**) were obtained from mice with heart failure (HF) and from mice with HF that had received AAV8.*UCn2* (UCn2). Cyclic AMP and PKA activity were assessed in the unstimulated (basal) state and after stimulation with isoproterenol (Iso, 10 μM , 10 min) and, in separate experiments, NKH477 (NKH, 10 μM , 10 min), a water-soluble forskolin analog that stimulates adenylate cyclase independent of β -adrenergic receptors. Numbers in bars denote group size. (**A**) cAMP production: no group differences were seen in basal, Iso, or NKH477-stimulated cAMP production. (**B**) PKA activity: no group differences were seen in basal, Iso, or NKH477-stimulated conditions. (**C**) CamK II expression and phosphorylation: *UCn2* gene transfer was associated with reduced Thr286 phosphorylation of CamK II (left panel, normalized to GAPDH). Total CamK II was unchanged. (**D**) Cardiac myosin light chain kinase: *UCn2* gene transfer was associated with increased cardiac myosin light chain kinase (cMLCK) protein (left panel, normalized to GAPDH). In all graphs, bars denote mean + SE; numbers in bars denote group size, numbers above bars from Student's t -test (unpaired, 2-tailed vs. control groups).

TABLE 5. NECROPSY

	Saline (17)	UCn2 (17)	p
BW, g	30±1	31±1	<0.17
LV, mg	154±7	139±5	0.095
LV/BW, mg/g	5.1±0.2	4.5±0.1	0.01
Liver, mg	1489±53	1405±43	<0.2
Lung, mg	212±19	213±13	0.97

BW, body weight; LV, left ventricle; UCn2, urocortin-2 gene transfer.

Three weeks after myocardial infarction, mice received intravenous saline or AAV8.UCn2 (5×10^{11} gc). Mice were killed 6 weeks later and necropsy conducted. Values represent mean±SE. *p*-Values are from Student's *t*-test (unpaired, 2-tailed).

Necropsy

Liver, lung, and body weights showed no group differences. UCn2 gene transfer tended to reduce LV weight, and LV-to-body weight ratio was reduced (12% reduction; $p=0.01$) (Table 5).

Markers of stress, inflammation, and tissue injury

The expression of several markers of LV stress, inflammation, and tissue injury was examined using RT-PCR. HF altered the expression of most of these genes (Table 6). Increased UCn2 expression did not influence alterations associated with HF. However, in normal mice, increased UCn2 expression was associated with reduced expression of ANF ($p=0.007$), BNP ($p=0.01$), β -MyHC, and α -SK-actin ($p=0.03$).

LV and liver histology

Hematoxylin and eosin staining of samples of liver and LV showed no evidence of group differences (data not

shown). Masson's trichrome staining revealed no group differences in fibrosis in liver ($p=0.79$).

Discussion

The most important finding in this study is that a single IV injection of AAV8.UCn2 increased function of the failing heart, demonstrating the feasibility and effectiveness of IV delivery of a long-term expression vector encoding a peptide with beneficial paracrine effects to treat heart failure.

Two measures of cardiac function confirmed increased LV function 5 weeks after IV AAV8.UCn2 delivery to animals with severely dysfunctional left ventricles. Echocardiography showed increases in LV EF, and reductions in LV volumes (Table 3). Second, UCn2 gene transfer increased peak LV +dP/dt, indicating enhanced LV contractile function, and reduced LV−dP/dt, indicating enhanced LV diastolic function (Table 4 and Fig. 2).

Although the absolute degree of LV EF change was only 8% units (HF: $12\% \pm 1\%$; HF+UCn2: $20\% \pm 4\%$), the relative increase was 67%. The small absolute change reflects the large size of the infarction—the mean prerandomization LV EFs were $\leq 20\%$ in both groups. Despite such large infarctions, UCn2 gene transfer attenuated LV chamber dilation and increased EF, while saline-treated mice showed progressive LV chamber dilation and further deterioration of LV EF. One would not expect UCn2 gene transfer to remedy the problems associated with such a large area of scar, representing virtually the entirety of the LV free wall. The cardiac benefits of UCn2 gene transfer would be anticipated to be limited to the viable portion of the LV, which, in the current model, represents the interventricular septum. EF in this setting may underestimate the benefits on LV function, especially since we observed dyskinesia of the infarcted

TABLE 6. MRNA EXPRESSION IN LEFT VENTRICLE

Gene	Normal		HF		Interaction	UCn2 effect	HF effect
	Control	UCn2	Control	UCn2			
ANF	100±17	38±7	2393±591	2458±728	ns	ns	0.0001
α -MHC	100±10	83±26	837±90	714±76	ns	ns	0.0001
α -Cd-actin	100±7	164±70	1160±94	1368±134	ns	ns	0.0001
α -sk-actin	100±32	18±4	56±12	51±14	0.05	0.03	ns
β -MHC	100±33	11±3	104±23	74±20	ns	0.016	ns
BNP	100±16	44±9	484±098	525±152	ns	ns	0.0001
MMP2	100±9.5	102±14	707±304	601±41	ns	ns	0.002
MMP8	100±38	68±9.6	96±36	90±50	ns	ns	ns
MMP9	100±44	68±2.3	57±20	44±21	ns	ns	ns
TIMP1	100±47	69±6	250±62	341±49	ns	ns	0.0002
TIMP2	100±7	122±16	500±65	719±106	ns	ns	0.0001
TIMP3	100±13	52±4	207±42	269±43	ns	ns	0.0001
TIMP4	100±22	86±16	239±50	164±21	ns	ns	0.002
Coll1 α 1	100±10	152±7	183±45	257±38	ns	ns	0.005
Coll3 α 1	100±11	140±17	281±80	376±62	ns	ns	0.0006
MEF2	100±9	132±78	1486±174	1682±155	ns	ns	0.0001

ANF, atrial natriuretic peptide; BNP, brain natriuretic peptide; Coll, collagen; MEF2, myocyte enhancer factor-2; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; α -Cd-actin, α -cardiac actin; α -MHC, α -myosin heavy chain; α -SK-actin, α -skeletal actin; β -MHC, β -myosin heavy chain.

RT-PCR detection of gene expression in LV tissues of normal and HF mice. RNA equivalents were normalized to simultaneously determined glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in each sample and data are normalized to control in normal LV.

wall during ejection. Assessment of LV contractile function using peak LV $+dP/dt$ reveals a larger absolute increase in LV function—an increase of 3129 mmHg/sec in peak LV $+dP/dt$, and a 1847 mmHg/sec increase in peak $-dP/dt$ conferred by *UCn2* gene transfer. These represent a 2-fold increase in peak LV $+dP/dt$, and a 1.6-fold increase in peak $-dP/dt$. A doubling of peak LV $+dP/dt$ in clinical heart failure would normalize LV contractile function.^{37,38}

Heart rate and blood pressure in the unsedated state are not affected by IV delivery of AAV8.*UCn2* despite sustained high levels of transgene *UCn2* in normal mice²⁷ or in mice with CHF, as shown in the current study. Similarly, in clinical trials of peptide infusions of *UCn2* and stresscopin (similar to *UCn3*), the rate-pressure product is unchanged.^{9–11} One would, therefore, not anticipate an increase in cardiac metabolic demands associated with *UCn2* gene transfer, but more direct metabolic studies must be performed to know this with certainty.

The present study focused on the feasibility and physiological consequences of IV delivery of AAV8.*UCn2* in the setting of a severely compromised and failing heart, and we found a pronounced positive effect. The mechanisms by which *UCn2* gene transfer evoked beneficial physiological changes, although not the primary focus of the present study, were also examined.

For example, we found that *UCn2* gene transfer was associated with (1) increased peak systolic Ca^{2+} transient amplitude and increased rate of Ca^{2+} decline in cardiac myocytes isolated from HF mice (Fig. 3A–D), and (2) increased SERCA2a expression (Fig. 3E) as we previously reported in mice with normal hearts.²⁷ Increased LV SERCA2a expression provides a mechanism by which LV contractile function and relaxation would be increased, as was observed (Fig. 2). SERCA2a returns cytosolic Ca^{2+} to the sarcoplasmic reticulum. An increased amount of SERCA2a would be anticipated to yield a more rapid cytosolic Ca^{2+} decline, which is what we found (Fig. 3C and D), and consequently to increase the rate of LV pressure decline (LV $-dP/dt$), as we also found (Fig. 2B).

In addition, we found alterations in LV expression of two additional proteins that are likely to have been of mechanistic importance in the observed beneficial effects of *UCn2* gene transfer on function of the failing LV: reduced Thr286 phosphorylation of Ca^{2+} /calmodulin-dependent kinase II (CaMKII), and increased LV expression of cardiac myosin light chain kinase (cMLCK) (Fig. 4).

CaMKII Thr286 phosphorylation

Our data show that *UCn2* gene transfer was associated with reduced Thr286 phosphorylation of CaMKII (Fig. 4C). CaMKII expression and activation are important determinants of cardiac function.³⁹ For example, cardiac-directed expression of CaMKII results in heart failure in mice.⁴⁰ Others have shown increased CaMKII activity and expression in MI-induced heart failure in mice.⁴¹ The clinical relevance of these findings was demonstrated recently by the demonstration that inhibition of LV CaMKII increases function of the failing human heart.⁴² Although we speculate that reduced Thr286 phosphorylation of CaMKII may have been important mechanistically in the observed increase in LV function, we were unable to determine the

pathway by which increased *UCn2* reduces Thr286 CaMKII phosphorylation, which will require focused studies in cultured cardiac myocytes that are underway.

Cardiac myosin light chain kinase

We found increased cardiac myosin light chain kinase (cMLCK) expression associated with *UCn2* gene transfer (Fig. 4D). Phosphorylation of cardiac myosin light chain 2v by cMLCK increases the rate of cross-bridge recruitment in cardiac myocytes and influences contractile function.^{43,44} Increased levels cMLCK are associated with increased LV function in the setting of MI-induced heart failure.⁴⁵ In contrast, the deletion of cMLCK reduces cardiac performance.⁴⁶ Sadly, there is no antibody available to assess myosin light chain 2v phosphorylation, and so the biological importance of the increase in cMLCK associated with *UCn2* gene transfer in the present study must remain speculative.

UCn2 gene transfer was associated with a doubling in the peak rate of LV pressure development (LV $+dP/dt$; Table 4 and Fig. 2). This finding was supported by evaluation of LV dimension and function by echocardiography (Table 3), enhanced Ca^{2+} handling (Fig. 3), and signaling changes in LV predicted to increase contractile function, including increased SERCA2a protein expression (Figs. 3 and 4). Because of the consistency of these findings, which reverberated from isolated cardiac myocytes to *in vivo* physiology, we were less concerned by the absence of group differences in BNP and ANF mRNA in LV (Table 6). Perhaps plasma levels or BNP/ANF expression in LA would have revealed group differences that LV mRNA levels missed. It is also possible that despite increased LV contractile function there was sufficient persistent chamber dilation—owing to infarction of the entire LV free wall—to provide ongoing stimulation of ANF and BNP expression.

We saw no group difference in lung or liver weight (Table 5). Liver weights were not increased in mice with heart failure compared with normal mice,²⁷ and so, despite severe LV failure, there is no liver congestion. Whether this is unique to MI-induced CHF in mice is unknown. Lung weights increased by 23% versus normal age-matched mice,²⁷ but did not show a group difference. We speculate that despite a doubling of LV contractile function (peak $+dP/dt$) conferred by *UCn2* gene transfer (Table 4 and Fig. 2), there may have been persistent left-sided congestion 5 weeks after treatment.

Clinical application

Intravenous delivery of AAV8 enables transfection of many organs and is especially effective in liver, skeletal muscle, and heart.⁴⁷ These organs, because they comprise an enormous mass of tissue and therefore can release abundant transgene *UCn2*, will enable us to reduce the vector dose. Indeed, a vector dose 10-fold lower (5×10^{10} gc per mouse or 2×10^{12} gc/kg) is still effective in increasing LV $+dP/dt$.²⁷ A dose of 2×10^{12} gc/kg of AAV8 encoding human factor IX was delivered intravenously safely and effectively in a clinical trial in subjects with hemophilia B.²

An additional feature to consider in translating our findings to clinical applications is the use of a regulated expression system,^{5,6} which would enable turning *UCn2* expression on or off at will. We have designed such AAV8

vectors using tetracycline and rapamycin regulation systems and are conducting preclinical studies with these regulated expression vectors.

LV Ca^{2+} handling is different in humans than in mice,⁴⁸ but peptide infusions of UCn2 or stresscopin (similar to UCn3) in patients with HF increases LV function.^{9–11} Whether this is through Ca^{2+} handling is unknown because Ca^{2+} transients and Ca^{2+} handling proteins have not been assessed in cardiac myocytes or myocardium before and after UCn2 peptide infusions in humans.

Finally, now that we have demonstrated that UCn2 gene transfer increases function of the severely failing heart, it will be important to determine how long the effect persists and whether it reduces mortality. Such studies using a less severe model of CHF with better long-term survival are planned.

Conclusions

A single IV injection of AAV8.UCn2 increases both systolic and diastolic function of the severely failing heart. Systemic delivery of the vector ensures that the transgene is expressed in the heart, but also is continuously released into the circulation, thereby providing sustained benefits that would otherwise not be possible. Other theoretical advantages of gene transfer as compared with IV infusion of paracrine-acting peptides include reduction in catheter-based infections, no need for hospitalization, and reduced costs. Whether this strategy will be effective in increasing function of the failing heart in clinical settings will require additional studies.

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