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Urocortin 2 Gene Transfer Reduces the Adverse Effects of a Western Diet on Cardiac Function in Mice

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Diabetes mellitus is associated with increased risk of heart failure. It has been previously demonstrated in mice that a single injection of adeno-associated virus 8 encoding urocortin 2 (AAV8.*UCn2*) increases glucose disposal in models of insulin resistance and improves the function of the failing heart. The present study tested the hypothesis that *UCn2* gene transfer would reduce diabetes-related left ventricular (LV) dysfunction. Eight-week-old C57BL6 male mice were fed a Western diet (WD; 45% fat, 35% carbohydrate) for 40 weeks. At week 30, they received saline or AAV8.*UCn2* (2×10^{13} genome copies/kg) via intravenous injection. Ten weeks after gene transfer, fasting blood glucose, glucose tolerance, and cardiac function were measured via echocardiography and *in vivo* measurement of LV contractile function, and the results were compared to those of mice fed normal chow (NC; 10% fat; 70% carbohydrate). The contents of key LV signaling proteins were also measured to probe mechanisms. WD increased 12 h fasting glucose (WD: 190 ± 11 mg/dL, $n = 8$; NC: 105 ± 12 mg/dL, $n = 7$; $p = 0.0004$). WD tended to reduce LV peak +dP/dt ($p = 0.08$) and LV peak -dP/dt ($p = 0.05$). LV ejection fraction was unchanged. Among WD-fed mice, *UCn2* gene transfer reduced 12 h fasting glucose (WD-*UCn2*: 149 ± 6 mg/dL, $n = 8$; WD-Saline: 190 ± 11 mg/dL, $n = 8$; $p = 0.012$), increased LV peak +dP/dt ($p < 0.001$) and LV peak -dP/dt ($p = 0.013$), and reduced Tau ($p < 0.02$), indicating beneficial effects on systolic and diastolic LV function. In addition, among WD-fed mice, *UCn2* gene transfer increased LV ejection fraction ($p < 0.005$) and the velocity of circumferential fiber shortening ($p = 0.0005$). Finally, a reduction was seen in fatty infiltration of the liver in WD-fed mice that had received *UCn2* gene transfer. LV samples from WD-*UCn2* mice showed increased phosphorylation of the protein kinase A catalytic domain ($p = 0.03$). In conclusion, *UCn2* gene transfer increased LV systolic and diastolic function and reduced blood glucose in mice with diabetes-related LV dysfunction, indicating that *UCn2* gene transfer may be of potential therapeutic benefit.

Keywords: type 2 diabetes, insulin resistance, gene therapy, adeno-associated virus

INTRODUCTION

DIABETES MELLITUS AFFECTS 9% of adults worldwide¹ and increases the risk of developing cardiovascular diseases. In addition, among adults with diabetes, heart disease death rates are two to four times higher.^{2,3} Left ventricular (LV) dysfunction often accompanies diabetes, even in the absence of obstructive coronary atherosclerosis or hypertension.⁴ Structural alterations of the LV associated with diabetes-related LV dysfunction often include increased wall thickness and fibrosis accompanied by diastolic or systolic dysfunction.^{4,5} Uncontrolled

high blood glucose, insulin resistance, and impaired cardiac insulin signaling contribute to the development of diabetes-related LV dysfunction.⁶ Despite advances in understanding its pathophysiological mechanisms, diabetic-related LV dysfunction represents an unmet medical need, warranting development of new therapeutic approaches.

The mammalian corticotropin-releasing factor (CRF) family peptides consist of CRF and urocortins 1, 2, and 3. These hormonal peptides regulate stress responses and energy balance by activating their cognate receptors, corticotropin-releasing hormone

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receptor type 1 (CRHR1) and 2 (CRHR2), which are expressed in the central nervous system and periphery.^{7,8} Among the CRF family peptides, UCn2 and UCn3 share 42% homology in amino acid sequence, and *in vitro* pharmacokinetic studies have demonstrated that both peptides preferentially bind to CRHR2 with small differences in receptor affinity.^{9–11} In preclinical and clinical studies, peptide infusion of UCn2, UCn3, and the related stresscopin showed beneficial cardiovascular effects in animal models and in clinical heart failure.^{12–16} Recent studies showed that sustained elevation of plasma UCn2 or UCn3 via intravenous (i.v.) delivery of an adeno-associated virus type 8 (AAV8) vector encoding UCn2 or UCn3 increases LV contractile function in mice.^{17–19} Interestingly, UCn2 and UCn3 have dissimilar effects on glucose metabolism. For example, UCn2 gene transfer increases insulin sensitivity and decreases fasting blood glucose in both normal and insulin-resistant mice, but UCn3 gene transfer has no effect on glucose disposal.^{19,20} These results indicate that UCn2 and UCn3 elicit distinct physiological responses, despite similar selectivity and binding affinity to CRHR2. Furthermore, UCn2 is a unique CRF family peptide that can improve LV function as well as glucose homeostasis.

The present study investigated the effects of UCn2 gene transfer on mice with diet-induced abnormalities of LV function. The hypothesis that UCn2 gene transfer would attenuate the deleterious effects of a Western diet (WD) on LV function was tested.

METHODS

Animals and protocol

Twenty-three C57BL6 male mice (8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were allocated to two groups and provided with cereal-based normal chow (NC; control group, $n = 7$; Teklad laboratory diet; Envigo, Huntingdon, United Kingdom) or a higher-fat diet ($n = 16$; cat. #D12451, Research Diets, Inc., New Brunswick, NJ). The higher-fat diet has been referred to as a WD²¹ because it is similar in composition to the diets of people in Western countries (kcal: fat 45%; carbohydrate 35%; protein 20%) and is higher in fat and lower in carbohydrate than NC (kcal: fat 10%; carbohydrate 70%; protein 20%) but lower in fat than the standard high-fat diet often used to induce insulin resistance in mice.²⁰

Mice were fed the WD for 40 weeks. At 30 weeks, WD-fed mice received i.v. saline ($n = 8$) or i.v. AAV8.UCn2 (2×10^{13} genome copies [gc]/kg; $n = 8$). Glucose homeostasis and cardiac function were

measured 10 weeks after randomization (Fig. 1A) at the end of the 40-week period. A control group of mice fed NC was included for comparison. The primary test for efficacy was a group difference in WD-fed mice that received UCn2 gene transfer compared with the group that received saline. Data acquisition and analysis were blinded to the treatment group. The use of animals was in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines and was approved by the Institutional Animal Care and Use Committee of VA San Diego Healthcare System.

AAV8 production and gene transfer

The construction and production of AAV8 encoding murine UCn2 with a chicken β -actin promoter (Fig. 1B) have been described previously.^{18,19} Mice received 2×10^{13} gc/kg of virus diluted in saline (100 μ L) through the jugular vein under anesthesia.

Plasma UCn2

Plasma was collected after a terminal study (10 weeks after gene transfer) and stored at -80°C . Plasma UCn2 levels were measured using mouse UCn2 enzyme immunoassay kits (cat. #KT-376; Kamiya Biomedical Co., Tukwila, WA) as instructed by the manufacturer.

Blood glucose and glucose tolerance testing

Mice were fasted for 12 h but were provided water *ad libitum*. Blood was drawn from a small tail incision, and glucose was measured using a Contour Blood Glucometer and test strip (Bayer [Ascensia Diabetes Care], Parsippany, NJ). After measuring 12 h fasting glucose, mice received glucose (2 g/kg) via an intraperitoneal (i.p.) injection, and blood glucose was measured every 30 min for 120 min.

Echocardiography

Mice were maintained in a lightly anesthetized state using 1% isoflurane. A Vevo 2100 system (FUJIFILM VisualSonics, Inc., Toronto, Canada) was used to assess chamber dimensions, ejection fraction (EF), and velocity of circumferential fiber shortening corrected for heart rate (VCFc), as previously described.²⁰ Vevo LAB v2.1 (VisualSonics) was used for measurement and analysis.

LV systolic and diastolic function

Mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and mechanically ventilated with oxygen (1 L/min). The right carotid artery was exposed to insert a 1.4F pressure-conductance transducer into the LV chamber followed by va-

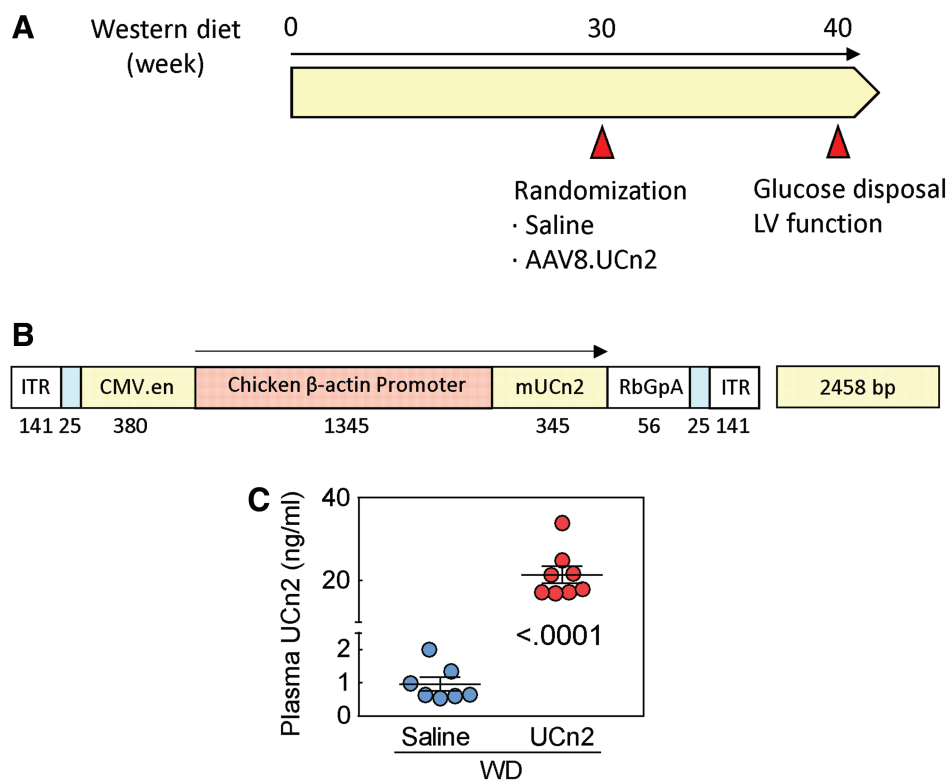


Figure 1. Experimental protocol, adeno-associated virus 8 encoding urocortin 2 (AAV8.UCn2) plasma UCn2 after gene transfer. **(A)** Experimental protocol. Eight-week-old C56BL6 mice were fed a Western diet (WD; fat: 45%, carbohydrate 35%, protein 20%) for 40 weeks. At 30 weeks, mice were randomized to receive UCn2 gene transfer or saline intravenously (i.v.). Ten weeks later (week 40 of WD), animals underwent assessment of glucose disposal (fasting glucose and glucose tolerance testing) and assessment of left ventricular (LV) function. Tissues were collected for biochemical assessment. **(B)** AAV8.mUCn2 vector map. ITR, inverted terminal repeat; CMV.en, human cytomegalovirus enhancer; mUCn2, murine urocortin 2; RbGpA, rabbit beta-globin poly A. **(C)** Plasma UCn2 concentration. Ten weeks after delivery of AAV8.UCn2 (2×10^{13} genome copies/kg, i.v.) or saline (i.v.), plasma was obtained to measure UCn2 concentration. Plasma UCn2 was increased 20-fold compared to saline-treated mice. Data are the mean \pm standard error (SE). p -values are from unpaired Student's t -tests (two-tailed). Color images are available online.

gotomy, and LV pressure was measured. Two animals were excluded from the analysis due to abnormal responses during anesthesia, one each from the WD-Sal and WD-UCn2 groups. After the procedure, mice were killed by exsanguination under deep anesthesia with sodium pentobarbital (200 mg/kg), and blood and organs were collected.

Immunoblotting

LV samples were homogenized in $1 \times$ lysis buffer (Cell Signaling Technology, Danvers, MA) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). Homogenates were sonicated on ice three times for 5 s each, and insoluble lysates were removed by centrifugation ($13,000 \times g$ for 10 min at 4°C). A total of 20–50 μg proteins were loaded per well and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated proteins were transferred to a polyvinylidene fluoride membrane. After blocking with 5% nonfat dried milk in TBS-T for 1 h, the membrane was incubated with primary antibody diluted in TBS-T

plus 5% bovine serum albumin overnight at 4°C . The primary antibodies used were: phospho-RYR S2808 (1:1,000; Abcam, Cambridge, United Kingdom), SERCA2a (1:1,000; Thermo Fisher Scientific, Waltham, MA), phospho-PLB S16 (1:5,000; Badrilla, Leeds, United Kingdom), phospho-TnI S23/24 (1:1,000; Cell Signaling Technology), phospho-PKA C T179 (1:1,000; Cell Signaling Technology), phospho-AKT S473 (1:1,000, Cell Signaling Technology), phospho-AMPKa T172 (1:1,000; Cell Signaling Technology), phospho-S6 S240/244 (1:1,000; Cell Signaling Technology), phospho-4EBP1 T37/46 (1:1,000; Cell Signaling Technology), and Vinculin (1:100,000; Sigma-Aldrich, St. Louis, MO).

Histology

Samples of LV and liver were fixed in 10% formalin overnight and then paraffin-embedded. Five-micron sections were slide mounted and stained with hematoxylin and eosin and Masson's trichrome. Microscopic images were scanned using an Axio slide scanner (Carl Zeiss, Oberkochen,

Germany), and images were analyzed using ImageJ (NIH, Bethesda, MD).

Statistical analysis

All data acquisition and analyses were performed without knowledge of group identity. GraphPad Prism v7 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis and calculation of area under the curve for data from glucose tolerance tests. Between-group comparisons were performed using Student's *t*-test (unpaired and two-tailed). Multiple tests were corrected by the Bonferroni method. The null hypothesis was rejected when $p < 0.05$.

RESULTS

Morphometrics

Forty weeks after initiation of the dietary intervention (10 weeks after randomization to the *UCn2* gene transfer group or the saline group), the mean body weight of the WD-Sal group had increased by 63% compared to the NC-Con group (Fig. 2A; $p < 0.0001$). Absolute (non-normalized) measures included: LV weight (NC-Con: 104 ± 3 g, $n = 7$; WD-

Sal: 112 ± 3 g, $n = 8$, $p = 0.07$ vs. NC-Con; WD-UCn2: 109 ± 6 g, $n = 8$); liver weight (NC-Con: $1,466 \pm 59$ g, $n = 7$; WD-Sal: $2,571 \pm 226$ g, $n = 8$, $p = 0.007$ vs. NC-Con; WD-UCn2: $2,283 \pm 197$ g, $n = 8$); tibial length (NC-Con: 18.3 ± 0.1 mm, $n = 7$; WD-Sal: 18.2 ± 0.2 mm, $n = 8$; WD-UCn2: 18.1 ± 0.04 mm, $n = 8$). LV weight–tibial length ratio was increased 9% in WD-Sal mice compared to NC-Con (Fig. 2B; $p = 0.03$). Liver weight–tibial length ratio increased by 78% in WD-Sal mice compared to NC-Con (Fig. 2C; $p = 0.0006$). No group differences were seen in WD-fed mice that received *UCn2* gene transfer compared to those receiving saline in terms of body weight, liver weight, or LV weight (Fig. 2A–C).

Plasma *UCn2*

Plasma *UCn2* concentration increased >20-fold 10 weeks after AAV8.*UCn2* delivery (Fig. 1C; saline: 1 ± 0.2 ng/mL, $n = 7$; *UCn2*: 21 ± 2 ng/mL, $n = 8$; $p < 0.0001$).

Glucose disposal

Twelve-hour fasting glucose was elevated in mice on the WD (NC-Con: 105 ± 12 mg/dL; WD-Saline: 190 ± 11 mg/dL; $p = 0.0004$) and was reduced

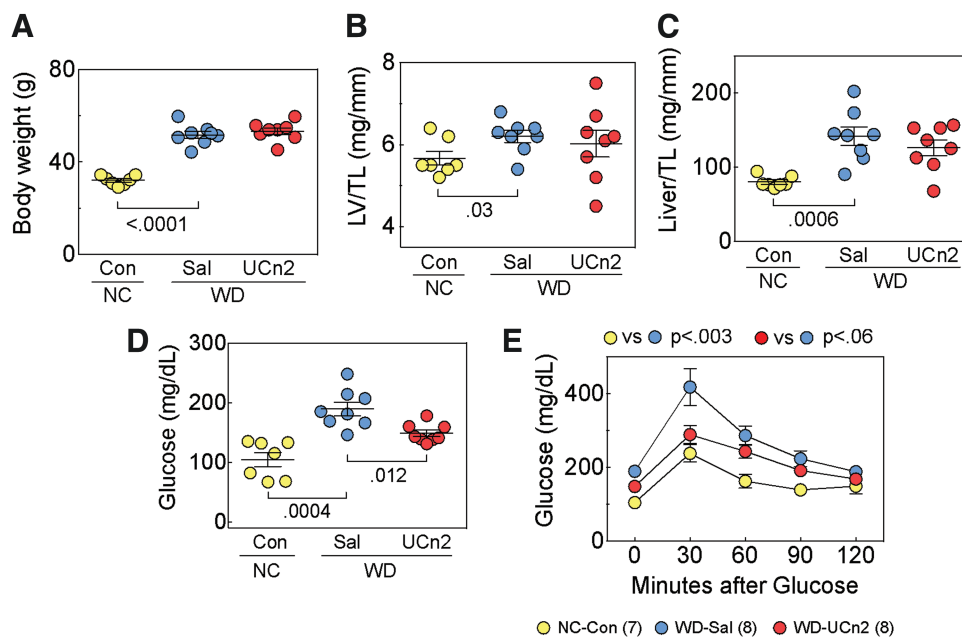


Figure 2. WD-fed mice: effects of *UCn2* gene transfer on body, LV, and liver weight and glucose disposal. **(A)** Body weight: mice gained weight after 40 weeks on the WD (NC-Con vs. WD-Sal; $p < 0.0001$). However, no group difference was seen in body weight 10 weeks after *UCn2* gene transfer. **(B)** LV weight: mice fed on the WD for 40 weeks had higher LV–tibial length ratios (NC-Con vs. WD-Sal; $p = 0.03$). However, no group difference was seen in LV–tibial length ratio 10 weeks after *UCn2* gene transfer. **(C)** Liver weight: mice fed on the WD for 40 weeks had higher liver–tibial length ratios (NC-Con vs. WD-Sal; $p = 0.0006$). However, no group difference was seen in liver–tibial length ratio 10 weeks after *UCn2* gene transfer. **(D)** Fasting blood glucose: Mice fed on the WD for 40 weeks had higher 12 h fasting blood glucose (NC-Con vs. WD-Sal; $p = 0.0004$). However, 10 weeks after *UCn2* gene transfer, fasting glucose was reduced (WD-Sal vs. WD-UCn2; $p = 0.0004$). **(E)** Glucose tolerance test: mice fed on the WD for 40 weeks showed impaired glucose disposal (NC-Con vs. WD-Sal; $p < 0.003$). However, 10 weeks after *UCn2* gene transfer, glucose disposal tended to improve (WD-Sal vs. WD-UCn2; $p < 0.06$). Symbols indicate mean values; bars indicate SE; Con, Control; Sal, Saline; UCn2, urocortin 2; NC, Normal chow; WD, Western diet. *p*-Values are from unpaired Student's *t*-tests (two-tailed), with Bonferroni correction (**D** and **E**). GTT analysis (**E**) was performed by area under the curve analysis. Color images are available online.

10 weeks after *UCn2* gene transfer (Fig. 2D; $p=0.012$). Glucose tolerance testing showed decreased glucose disposal in WD-Sal compared to NC-Con ($p<0.003$). WD-Sal mice showed impaired glucose tolerance compared to NC-Con mice (Fig. 2E; $p<0.003$), and WD-fed mice that received *UCn2* gene transfer tended to clear glucose more efficiently compared to WD-Sal mice (Fig. 2E; $p<0.06$).

Echocardiography

Echocardiography measures showed no differences between NC-Con and WD-Sal mice. However, 10 weeks after randomization to *UCn2* gene transfer or saline, group differences were evident. For example, in mice that received *UCn2* gene transfer, EF ($p<0.005$) and VCFc ($p=0.0005$) were higher, and end diastolic diameter ($p=0.01$) and end systolic diameter ($p=0.004$) were lower compared to WD-fed mice that received saline (Fig. 3A–E).

LV function

Direct measurement of the peak rates of LV pressure development (+dP/dt) and decline (–dP/dt) showed that WD-Sal mice tended to have lower values (vs. NC-Con) in peak +dP/dt (Fig. 4A; a 17% decrease; $p=0.08$) and in peak –dP/dt (Fig. 4B; a 19% decrease; $p=0.05$). However, 10 weeks after randomization to either the *UCn2* gene transfer

group or the saline group, group differences were more striking. For example, in mice that received *UCn2* gene transfer, peak +dP/dt (Fig. 4A; a 38% increase; $p<0.001$) and peak –dP/dt (Fig. 4B; a 33% increase; $p=0.013$) were higher compared to WD-fed mice that received saline. Tau was decreased (more rapid relaxation) in WD-fed mice that received *UCn2* gene transfer (Fig. 4C; $p<0.02$). No group differences were found in LV developed pressure or heart rate (Fig. 4D and E). LV end diastolic pressure tended to be lower in WD-fed mice that received *UCn2* gene transfer (Fig. 4F; $p<0.06$).

Histological analysis

Liver fatty infiltration was compared by measuring the area of lipid droplets in the liver (Fig. 5A). WD-Sal mice showed increased fatty infiltration compared to NC-Con mice ($p<0.0002$). WD-fed mice that received *UCn2* gene transfer showed lower levels of fatty infiltration compared to WD-Sal mice (Fig. 5A; a 44% reduction; $p<0.008$). Liver fibrosis was increased in the WD-Sal group compared to NC-Con mice (Fig. 5B; $p<0.02$), and *UCn2* gene transfer had no effect compared to WD-Sal mice. There were no group differences in LV fibrosis (Fig. 5B).

Relevant LV signaling proteins

Immunoblotting was used to measure LV content of signaling proteins that govern LV contrac-

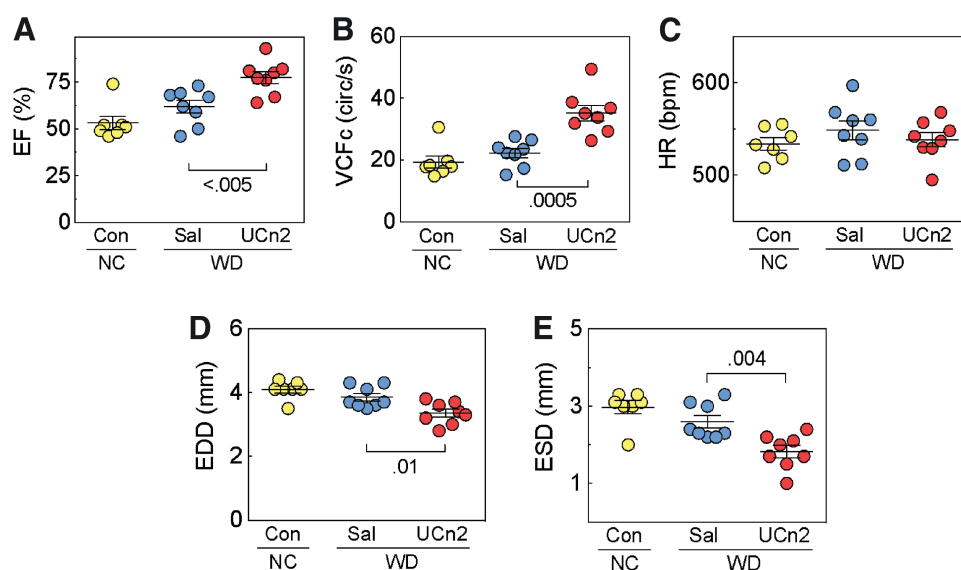


Figure 3. Echocardiography. **(A)** Ejection Fraction (EF). Mice fed on the WD for 40 weeks showed no change in EF (NC-Con vs. WD-Sal; p =not significant [n.s.]). However, 10 weeks after *UCn2* gene transfer, EF was increased (WD-Sal vs. WD-UCn2; $p<0.005$). **(B)** Velocity of circumferential fiber shortening corrected for heart rate (VCFc). Mice fed on the WD for 40 weeks showed no change in VCFc, an estimate of contractile function (NC-Con vs. WD-Sal; p =n.s.). However, 10 weeks after *UCn2* gene transfer, VCFc increased (WD-Sal vs. WD-UCn2; $p=0.0005$). **(C)** Heart rate. There were no group differences in heart rate. **(D and E)** LV end diastolic diameter (EDD) and end systolic diameter (ESD). Mice fed on the WD for 40 weeks showed no change in EF (NC-Con vs. WD-Sal; p =n.s.). However, 10 weeks after *UCn2* gene transfer, EDD and ESD were lower (WD-Sal vs. WD-UCn2; EDD, $p=0.01$; ESD, $p=0.004$). Symbols indicate mean values; bars indicate SE. p -Values are from unpaired Student's t -tests (two-tailed). Color images are available online.

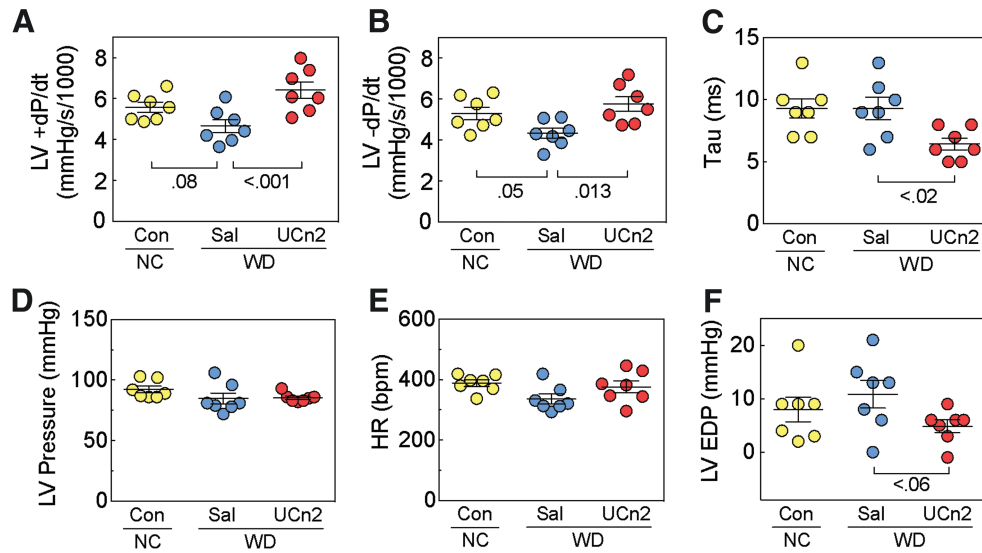


Figure 4. WD-fed mice: effects of *UCn2* gene transfer on LV function. **(A)** LV peak +dP/dt. The peak rate of LV pressure development (+dP/dt), a measure of contractile function, tended to be lower after 40 weeks on the WD (NC-Con vs. WD-Sal; $p=0.08$). However, 10 weeks after *UCn2* gene transfer, LV +dP/dt was higher and attained normal levels (WD-Sal vs. WD-UCn2; $p<0.001$). **(B)** LV peak -dP/dt. The peak rate of LV pressure decay (-dP/dt), a measure of LV relaxation, tended to be lower after 40 weeks on the WD (NC-Con vs. WD-Sal; $p=0.05$). However, 10 weeks after *UCn2* gene transfer, LV -dP/dt was higher and attained normal levels (WD-Sal vs. WD-UCn2; $p=0.013$). **(C)** Tau. The time constant of -dP/dt, Tau, was unchanged after 40 weeks on the WD (NC-Con vs. WD-Sal; $p=n.s.$) but was more rapid 10 weeks after *UCn2* gene transfer (WD-Sal vs. WD-UCn2; $p<0.02$). **(D and E)** LV pressure and heart rate. There were no group differences in LV developed pressure or heart rate after 40 weeks on the WD (NC-Con vs. WD-Sal; $p=n.s.$); 10 weeks after *UCn2* gene transfer, no group differences in LV pressure or heart rate were seen in WD-fed mice. **(F)** LV end diastolic pressure (EDP). LV EDP was unchanged after 40 weeks on the WD (NC-Con vs. WD-Sal; $p=n.s.$). However, 10 weeks after *UCn2* gene transfer, LV EDP tended to be lower, consistent with improved LV function (WD-Sal vs. WD-UCn2; $p<0.06$). Symbols indicate mean values; bars indicate SE. p -Values are from unpaired Student's t -tests (two-tailed), with Bonferroni correction **(A and B)**. Color images are available online.

tility and cellular metabolism (Fig. 5C and Table 1). Phosphorylation at T179 in the protein kinase A (PKA) catalytic domain was increased in LV samples from WD-fed mice that received *UCn2* gene transfer compared to WD-Sal mice (Fig. 5C and Table 1; $p=0.03$). Phosphorylation at T172 in the AMPK α subunit was reduced in WD-fed mice that received saline compared with NC-fed mice ($p=0.022$), and WD-fed mice that received *UCn2* gene transfer tended to show increased phosphorylation of AMPK α ($p=0.13$). No group differences were found in phospho-S2808 RYR2, SERCA2a, phospho-S16 PLB, phospho-S23/24 TnI, phospho-S473 AKT, phospho-S240 S6, or phospho-T374EBP1 (Fig. 5C).

DISCUSSION

The most important finding of this study was that a single injection of AAV8.*UCn2* increased LV function and glucose disposal in mice fed a higher-fat diet. The study tested whether *UCn2* gene transfer would have beneficial effects on diabetes-related LV dysfunction. LV dysfunction was achieved using a higher-fat diet—one that is similar in fat content to a standard WD.²¹ Mice that were fed this diet for 40 weeks (compared to NC with 10% fat)

showed increased body weight and liver weight, fasting hyperglycemia, and impaired glucose tolerance. WD-fed mice tended to exhibit impaired rates of LV pressure development (+dP/dt) and impaired rates of LV pressure decline (-dP/dt) compared to mice fed NC. These relatively load-independent measures are sensitive and accurate measures of LV systolic and diastolic function. A major finding of this study was that WD-fed mice that received *UCn2* gene transfer (compared to saline) had increased systolic and diastolic LV function 10 weeks later. This conclusion is based upon a blinded analysis of measures of glucose disposal and detailed analysis of cardiac function. These findings are important because the prevalence of diabetes-related LV dysfunction is increasing as the incidence of diabetes increases globally.⁵ A one-time administration of an agent with enduring benefits on glucose disposal and LV function is an attractive and unique therapeutic strategy.

WD: glucose homeostasis and LV function

WD has previously been used to induce LV dysfunction in mice,^{22,23} which was the rationale for using it. In the present study, mice fed WD for 40 weeks developed obesity, hyperglycemia, and im-

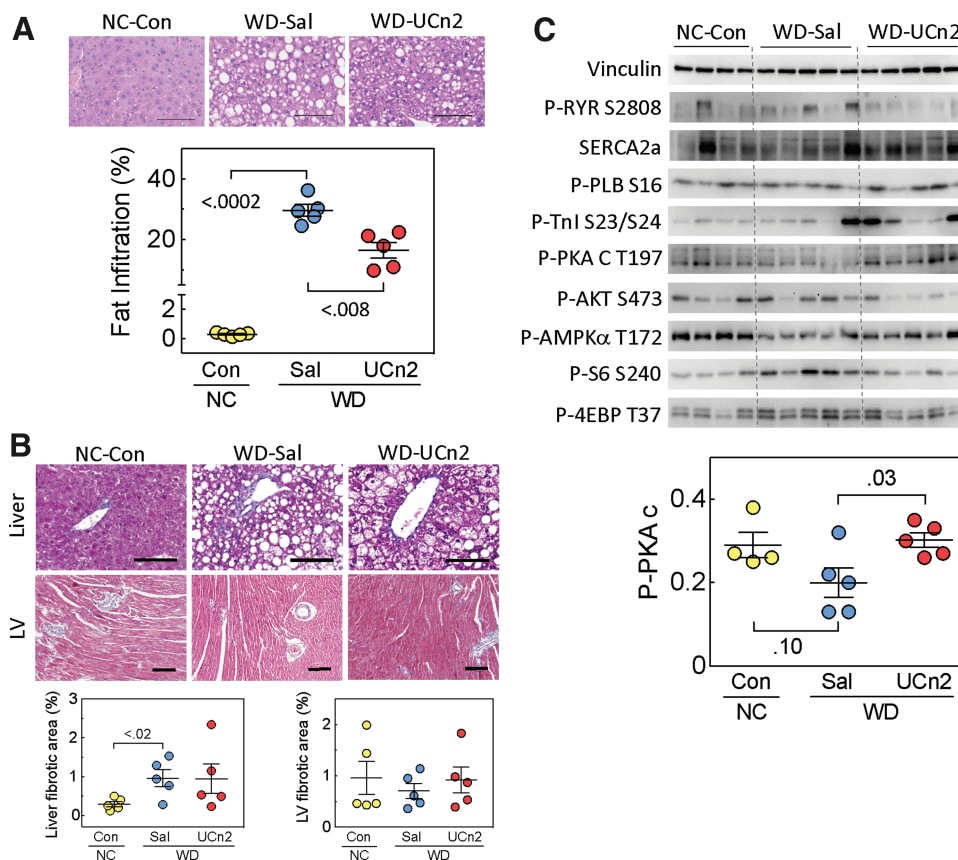


Figure 5. Histological analysis and immunoblotting. **(A)** Liver fatty infiltration. Area of lipid droplet in liver tissue sections stained with hematoxylin and eosin was measured. *UCn2* gene transfer in WD-fed mice decreased liver fatty infiltration compared to the WD-Sal group ($p < 0.008$). **(B)** Fibrosis in liver and LV. Liver and LV tissue sections were stained with Masson's trichrome. The fibrotic area in liver was increased in WD-fed mice receiving saline compared to NC-fed mice ($p = 0.02$), and *UCn2* gene transfer had no effect. No group differences were found in LV fibrosis. **(C)** Immunoblotting. The content of signaling proteins in LV homogenates was examined by immunoblotting. *UCn2* gene transfer increased phosphorylation of PKA catalytic subunit in WD-fed mice compared to WD-fed mice receiving saline. Scale bars: 100 μ m. Data are the mean \pm SE. p -Values are from unpaired Student's t -tests (two-tailed), with Bonferroni correction. Color images are available online.

paired glucose disposal (Fig. 2). WD-fed mice also exhibited LV hypertrophy (see LV–tibial length ratio; Fig. 2B). Although LV EF showed no NC-Con versus WD-Sal group difference (Fig. 3A), LV peak +dP/dt and LV peak –dP/dt tended to be reduced (Fig. 4A

Table 1. Immunoblotting quantification

	Normalized to vinculin		
	NC-Con (4)	WD-Sal (5)	WD-UCn2 (5)
p-RYR2 S2808	0.11 \pm 0.04	0.25 \pm 0.07	0.10 \pm 0.03
SERCA2a	0.94 \pm 0.28	0.84 \pm 0.17	0.94 \pm 0.16
p-PLB S16	0.63 \pm 0.12	0.49 \pm 0.08	0.46 \pm 0.07
p-TnI S23/24	0.11 \pm 0.02	0.53 \pm 0.39	1.17 \pm 0.47
p-PKA C T179	0.29 \pm 0.03	0.2 \pm 0.04	0.3 \pm 0.02 ^a
p-AKT S473	0.36 \pm 0.13	0.31 \pm 0.1	0.14 \pm 0.08
p-AMPKα T172	0.89 \pm 0.22	0.31 \pm 0.03 ^b	0.47 \pm 0.09
p-S6 S240/244	1.41 \pm 0.32	2.9 \pm 0.5	1.87 \pm 0.42
p-4EBP1 T37/46	0.28 \pm 0.06	0.41 \pm 0.07	0.25 \pm 0.06

Data are the mean \pm standard error. p -Values are from unpaired Student's t -tests (two-tailed).

^a $p = 0.03$ vs. WD-Sal.

^b $p = 0.022$ vs. NC-Con.

NC, normal chow; WD, Western diet; Con, control; Sal, saline; UCn2, urocortin 2.

and B). These less load-sensitive measures of LV function are superior to EF, an ejection phase index that is affected by loading conditions.²⁴

UCn2 gene transfer in WD-fed mice: glucose homeostasis

The mean plasma *UCn2* concentration was 21 ± 2 ng/mL 10 weeks after AAV8.*UCn2* delivery in WD-fed mice, a >20-fold increase compared to WD-fed mice that received saline (Fig. 1C). Increased plasma *UCn2* in WD-fed mice was associated with reduced fasting blood glucose levels compared to WD-Sal mice (Fig. 2D; $p = 0.012$), and also tended to show improved disposal in glucose tolerance testing (Fig. 2E; $p < 0.06$). Hyperinsulinemic-euglycemic clamp studies would be required for a quantitative assessment of insulin sensitivity.

UCn2 gene transfer in WD-fed mice: LV function

It was previously demonstrated that *UCn2* gene transfer increases LV function in normal mice¹⁷

and also increases function of the failing heart (induced by myocardial infarction).¹⁸ The present study found that *UCn2* gene transfer has beneficial effects on LV function in mice with diabetes-related LV dysfunction induced by WD. WD-fed mice that received *UCn2* gene transfer (WD-Sal) showed higher LV EF and VCFc (Fig. 3A and B). In addition, more detailed studies of LV function showed increased LV peak +dP/dt and LV peak -dP/dt in WD-fed mice that received *UCn2* gene transfer (Fig. 4A and B), indicating important benefits on systolic and diastolic LV function. The relative increases of 38% and 33% on peak +dP/dt and peak -dP/dt, respectively, would be expected to have important physiological effects on heart function in clinical settings. Tau, the time constant of LV pressure decline, was decreased (i.e., it was more rapid) in WD-fed mice that received *UCn2* gene transfer (Fig. 4C). These data indicate that *UCn2* gene transfer reverses the decline in LV systolic and diastolic function seen in WD-induced diabetes-related LV dysfunction (Fig. 4A and B). The extent of LV hypertrophy, however, was not reduced by *UCn2* gene transfer (Fig. 2B).

Diabetic-related LV dysfunction is associated with impaired insulin signaling, abnormal metabolic responses, and abnormalities in cardiac myocyte Ca²⁺ handling.^{25,26} *In vitro* assays using isolated cardiac myocytes or cell lines showed that *UCn2* peptide treatment increases cyclic adenosine monophosphate (cAMP) production, AMPK activity, phosphorylated phospholamban, and phosphorylated TnI via CRHR2 activation.^{10,27} It has previously been shown that *UCn2* gene transfer increases LV SERCA2a and improves Ca²⁺ handling in cardiac myocytes isolated from both normal and failing hearts of mice.^{17,18} These studies also showed increased β -adrenergic receptor-stimulated cAMP production and PKA activity in cardiac myocytes isolated from normal mice that had received AAV8.*UCn2*, but not cardiac myocytes isolated from failing hearts.^{17,18} The present study found that phosphorylation at T172 in the AMPK α subunit is decreased in LV homogenates from WD-fed mice that received saline compared to NC-Con mice, indicating reduced activation of AMPK signaling (Fig. 5C and Table 1). The phosphorylated PKA catalytic subunit was increased in LV homogenates from WD-fed mice that received *UCn2* gene transfer (Fig. 5C and Table 1). There was no group difference in SERCA2a content in this study (Fig. 5C). Taken together, increased PKA activation may have contributed to increased LV contractility in WD-fed mice that received *UCn2* gene transfer. Additional studies to determine the precise molec-

ular mechanisms underlying these beneficial effects are underway.

Limitations

The favorable effects of *UCn2* gene transfer on LV function in WD-fed mice likely require *UCn2* activation of CRHR2. It was previously shown that deletion of CRHR2 abrogates the beneficial effects of *UCn2* gene transfer on glucose disposal.²⁰ In that study, euglycemic clamps were used in insulin-resistant mice, and it was found that *UCn2* gene transfer increases skeletal muscle insulin sensitivity and reduces hepatic glucose production. *UCn2* increases Glut4 translocation in skeletal myotubes as effectively as insulin.²⁰ Whether the beneficial effects of *UCn2* on LV function reflect improved glucose disposal and consequently a reduction of the deleterious effects of sustained hyperglycemia on LV function or a direct effect of *UCn2* on Ca²⁺ handling in cardiac myocytes¹⁷⁻¹⁹ or both will require additional studies. However, there were additional benefits associated with reduced hyperglycemia following *UCn2* gene transfer in the present study, including a 44% reduction in fatty infiltration of the liver (Fig. 5A; $p < 0.008$). Finally, a one-time therapy that improves both glucose disposal and LV function would be a welcome addition to current clinical therapies.

In conclusion, *UCn2* gene transfer via a one-time i.v. injection of AAV8.*UCn2* increased LV systolic and diastolic function, reduced blood glucose, and reduced fatty infiltration of the liver in mice with diabetes-related LV dysfunction, indicating that *UCn2* gene transfer may be of potential therapeutic benefit in clinical settings.

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AUTHOR DISCLOSURE

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