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Effects of *Urocortin 2* Gene Transfer on Glucose Disposal in Insulin-Resistant db/db Mice on Metformin

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The study was designed to determine whether *urocortin 2* (*Ucn2*) gene transfer is as safe and effective as metformin in insulin-resistant mice. Four groups of insulin-resistant db/db mice and a nondiabetic group were studied: (1) metformin; (2) *Ucn2* gene transfer; (3) metformin + *Ucn2* gene transfer; (4) saline; and (5) nondiabetic mice. After completion of the 15-week protocol, glucose disposal was quantified, safety assessed, and gene expression documented. *Ucn2* gene transfer was superior to metformin, providing reductions in fasting glucose and glycated hemoglobin and enhanced glucose tolerance. The combination of metformin + *Ucn2* gene transfer provided no better glucose control than *Ucn2* gene transfer alone and was not associated with hypoglycemia. Metformin alone, *Ucn2* gene transfer alone, and metformin + *Ucn2* gene transfer together reduced fatty infiltration of the liver. Serum alanine transaminase concentration was elevated in all db/db groups (vs. nondiabetic controls), but the metformin + *Ucn2* gene transfer combined group had the lowest alanine transaminase levels. No group differences in fibrosis were detected. In a hepatoma cell line, activation of AMP kinase showed a rank order of combined metformin + Ucn2 peptide > Ucn2 peptide > metformin. We conclude (1) The combination of metformin + *Ucn2* gene transfer does not result in hypoglycemia. (2) *Ucn2* gene transfer alone provides superior glucose disposal versus metformin alone. (3) The combination of *Ucn2* gene transfer and metformin is safe and has additive effects in reducing serum alanine transaminase concentration, activating AMP kinase activity, and increasing *Ucn2* expression, but is no more efficacious than *Ucn2* gene transfer alone in reducing hyperglycemia. These data indicate that *Ucn2* gene transfer is more effective than metformin in the db/db model of insulin resistance and combined treatment with metformin + *Ucn2* gene transfer appears to have favorable effects on liver function and *Ucn2* expression.

Keywords: adeno-associated virus type 8, gene therapy, insulin sensitivity, metformin

INTRODUCTION

THE PREVALENCE OF type 2 diabetes in the United States in 2021, among people 20–79 years old, was 32–36 million, with 670,000 deaths attributable to diabetes annually. Worldwide the prevalence is 537 million, with 6.7 million deaths annually.[1] A recent study of 5047 patients with type 2 diabetes taking metformin combined with one of four additional hypoglycemic agents (insulin glargine; glimepiride, a sulfonylurea; liraglutide, a glucagon-like peptide-1 [GLP-1] receptor agonist; or sitagliptin, a dipeptidyl peptidase-4 [DPP-4] inhibitor) was reported.[2] After a mean follow-up of 5 years, only 29% reached or maintained the targeted glycated hemoglobin (HbA1c) level of <7%.

Randomized clinical trials have shown that tight glucose control (measured by HbA1c) reduces microvascular complications in type 1 diabetes[3] and appears to do so in patients with type 2 diabetes.[4] However, whether lowering abnormally high HbA1c levels is tightly linked with reductions in major adverse cardiovascular events (MACE: cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke) has been more difficult to

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demonstrate, in part because of the benefits of statin therapy, which is widely used in patients with diabetes and concomitant atherosclerotic cardiovascular disease.

Type 2 diabetes is a major risk factor for myocardial infarction, heart failure, stroke, peripheral vascular disease, amputation, kidney failure, blindness, neuropathy, and nonalcoholic fatty liver disease (NAFLD). The prevalence and dire consequences of type 2 diabetes and the difficulty of achieving guideline-directed medical targets indicate that new approaches are imperative. If body weight, low-density lipoprotein, HbA1c, and hypertension were well controlled, one might expect 10 years of life extension.[5]

An ideal therapy would have favorable effects on HbA1c, body weight, blood pressure, heart function, and NAFLD, a major cause of liver failure and mortality.[6] In addition, a therapy that requires infrequent administration is desirable because it would enhance compliance. Recent nonclinical publications indicate that these goals of an ideal therapy are achievable with a one-time intravenous injection of an adeno-associated virus vector 8 (AAV8) encoding *urocortin 2* (*Ucn2*).[7–12]

The incidence of metformin-induced severe hypoglycemic episodes is low in treating clinical type 2 diabetes, in part because metformin does not markedly alter insulin release or skeletal muscle insulin sensitivity.[13] In contrast, *Ucn2* gene transfer, when used as monotherapy in nonclinical models of diabetes, reduces hepatic glucose production, increases skeletal muscle insulin sensitivity, and increases insulin release.[7,8,10]

Despite these features of *Ucn2* gene transfer, which would predict substantial reductions in fasting glucose, we have not seen loss of consciousness or severe hypoglycemia in insulin-resistant mice that receive *Ucn2* gene transfer as monotherapy, although 12-h fasting glucose is often <100 mg/dL in insulin resistance models and as low as 60 mg/dL in normal mice.[7,8,10]

Therefore, it seemed plausible that the combination of metformin and *Ucn2* gene transfer might lead to unacceptably low glucose levels, which could thwart the development of *Ucn2* gene transfer for use in clinical diabetes. Finally, AAV8 vectors, used in high doses, are associated with elevation of alanine transaminase concentrations.[14] Given that db/db mice have increased alanine transaminase concentrations at baseline,[15] we were concerned that AAV8.m*Ucn2* might provoke increased liver inflammation.

To address these concerns, we used insulin-resistant db/db mice with fasting hyperglycemia, that were sufficiently young so that pancreatic release of insulin was still intact.[16] Our hypothesis was that *Ucn2* gene transfer in db/db mice, when added to metformin, would safely improve glycemic indices without causing hypoglycemia or increasing alanine aminotransferase. We also asked whether the deleterious effects associated with this model of insulin resistance (NAFLD and fibrosis of the liver or heart) would be affected by combination therapy.

METHODS

The Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011) was followed, and the Animal Use and Care Committee of the VA San Diego Healthcare System approved the studies.

Animals

Five-week-old, male, B6.BKS(D)-Lepr^{db}/J diabetic mice (db/db) were obtained from The Jackson Laboratory (Bar Harbor, ME). This line has insulin resistance, but maintains increased plasma insulin levels until late in life, providing a suitable type 2 diabetes model.[16] Four groups of db/db mice were studied, as described below and in Fig. 1. Studies of the four db/db groups were initiated when they were 5 weeks of age, and the in-life phase was completed 15 weeks later when mice were 20 weeks of age. Sixteen-week-old nondiabetic mice (male, C57BL/6, $n = 10$, 31.0 ± 0.6 g) were used as controls.

1. The metformin ($n = 5$) group received metformin (250 mg/[kg·day], po) for 15 weeks. Previous studies have shown a 25–29% reduction in blood glucose in db/db mice 14 days after daily metformin at a dose of 250 mg/(kg·day) by gavage.[17,18] One mouse in the metformin group had progressive weight loss and was removed from the study at week 7, resulting in a final group size of $n = 5$.
2. The *Ucn2* gene transfer ($n = 6$) group received AAV8.m*Ucn2* (2×10^{13} gc/kg, intravenously [i.v.]) in a one-time dose at 7–8 weeks.
3. The metformin + Ucn2 gene transfer ($n = 6$) group received metformin (250 mg/[kg·day], p.o.) daily for 15 weeks and received AAV8.m*Ucn2* (2×10^{13} gc/kg, i.v.) in a one-time dose at 7–8 weeks.
4. The saline ($n = 6$) group received i.v. saline in a one-time dose at 7–8 weeks.
5. Control mice (nondiabetic C57BL/6 mice) received no intervention ($n = 6$) or received AAV8.m*Ucn2* ($n = 4$; 2×10^{13} gc/kg, i.v.) in a one-time dose and were sacrificed 15 weeks later.

Mice were provided (*ad libitum*) a cereal-based diet (Harlan Teklad Laboratory) and housed (20–21°C) with lights off from 6 pm to 6 am daily.

AAV8.m*Ucn2* production and administration

An AAV8 vector encoding murine *Ucn2* with a chicken β-actin promoter was produced as detailed previously.[9] Plasmid pRep2/Cap8 was obtained from the University of Pennsylvania Vector Core. Under anesthesia (1.5% isoflurane through a nose cone), a small incision was made on the neck to expose the jugular vein for i.v. delivery. A 12–14-μL aliquot of AAV8.m*Ucn2* (depending on mouse weight) was brought to a final volume of 100 μL with 1 N

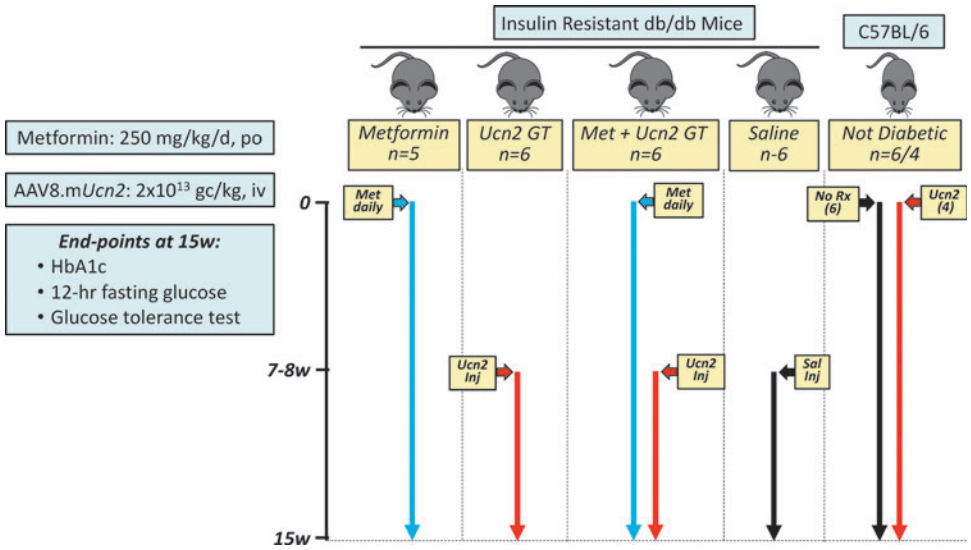

Figure 1. Experimental protocol. Our hypothesis was that *Ucn2* GT in db/db mice, when added to metformin, would safely improve glycemic indices without causing hypoglycemia. To do this, we used db/db mice (male, 5 weeks old at initiation of the study). These mice exhibit persistent insulin resistance and hyperglycemia. Insulin-resistant mice (db/db) were assigned to one of four following groups (as shown above): the metformin group ($n=5$) received metformin 250 mg/(kg·day) (p.o.) for 15 weeks; the *Ucn2* GT group ($n=6$) received AAV8.m*Ucn2* (2×10^{13} gc/kg, i.v.) in a one-time dose at 7–8 weeks; the metformin + *Ucn2* GT group ($n=6$) received metformin 250 mg/(kg·day) (p.o.) for 15 weeks and received AAV8.m*Ucn2* (2×10^{13} gc/kg, i.v.) in a one-time dose at 7–8 weeks; the saline group ($n=6$) received i.v. saline in a one-time dose at 7–8 weeks; and control group ($n=10$) comprised C57BL/6 male nondiabetic control mice ($n=10$; 16 weeks of age at initiation of the study). Six mice received no treatments, 4 received AAV8.m*Ucn2* (2×10^{13} gc/kg, i.v.) in a one-time dose, and all 10 were sacrificed 15 weeks later. *End points:* group differences in HbA1c, 12-h fasting glucose, and glucose tolerance levels at 15 weeks after study initiation (20 weeks of age). We also assessed serum lipid abnormalities, fatty infiltration of the liver, serum alanine transaminase concentrations, and fibrosis of the liver and heart for baseline and treatment effects. *Statistics:* one-way or two-way ANOVA; when ANOVA $p<0.05$, selected between-group comparisons were made using Sidak's multiple comparisons test. AAV8, adeno-associated virus vector 8; ANOVA, analysis of variance; GT, gene transfer; HbA1c, glycated hemoglobin; Met, metformin; *Ucn2* GT, *urocortin 2* gene transfer; Ucn2 Inj, *Ucn2* gene transfer; Sal Inj, one-time saline injection; No Rx, no intervention.

saline and subsequently delivered (2×10^{13} gc/kg, i.v.). Control mice received an equivalent volume of 1 N saline.

Glycemic indices

At 15 weeks, mice were fasted for 12 h and blood glucose levels were measured (Contour blood glucose meter through blood glucose test strips; Bayer) before and 30, 60, 90, and 120 min after glucose injection (2 g/kg, i.p.). Blood was collected through a small tail incision. HbA1c was determined using a Siemens DCA Vantage Analyzer and reagent kit (Tarrytown, NY, USA).

AMPK activity

Huh7 cells, a hepatoma cell line,[19] were stimulated with the following: (1) metformin (2 mM, 3 h); (2) Ucn2 peptide (100 nM, 15 min); (3) metformin (2 mM, 3 h) + Ucn2 peptide (100 nM, for final 15 min); and (4) saline. Cell lysates were used in immunoblotting to quantify phospho-5′ adenosine monophosphate-activated protein kinase, p-AMPK (T172), and total AMPK.

Plasma Ucn2 and liver *Ucn2* mRNA

Plasma Ucn2 was determined using a mouse Ucn2 enzyme immunoassay kit (Kamiya Biomedical, CA), as previously described.[7,9] Liver *Ucn2* mRNA was determined by reverse-transcription polymerase chain reaction, as previously described.[8]

Immunoblotting

Antibodies to phosphorylated and total AMPK were obtained from Cell Signaling Technology (Danvers, MA), and the antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from Sigma-Aldrich (Burlington, MA).

Blood chemistry

Serum alanine transaminase, albumin, cholesterol, high-density lipoprotein (HDL), and triglyceride levels were determined by Molecular Diagnostic Services (San Diego, CA, USA).

Histological analysis

Liver, transmural left ventricular (LV), and gastrocnemius samples were fixed in 10% formalin overnight, switched to 75% ethanol, and paraffin embedded. Tissue sections (5 μm) were mounted and counterstained with hematoxylin–eosin and Masson's trichrome. Stained tissue slides were scanned using an Axio slide scanner (Carl Zeiss Vision, Inc., San Diego, CA, USA), and images were quantified using ImageJ. To assess the area of lipid

droplets in the liver, frozen liver tissue was sectioned, mounted to slides, and stained with Oil Red O using a standard protocol at UCSD Histology Core.

The image was captured with a slide scanner (Axio Scan.Z1; Zeiss). Analysis was performed by converting the 8-bit red–green–blue image to a black–white binary image and thresholding for color saturation of the lipid droplet signal. After setting the scale of the image, the amount and size of lipid droplets were measured and displayed by ImageJ as surface area in square micrometers (μm^2).[20]

Statistical analyses

Power calculations were used to estimate the required group size, using a pooled mean coefficient of variation of the glycemic measures of 20%, based on our previous studies. A 33% relative mean difference (Ucn2 vs. saline in db/db mice) would require a group size of $n \geq 5$, assuming $p < 0.05$ and power of 80%. One-way analysis of variance (ANOVA) was used to detect overall group differences in the four db/db groups (saline, metformin, *Ucn2* gene transfer, and metformin + *Ucn2* gene transfer).

When the overall ANOVA had a $p < 0.05$, between-group comparisons were performed using Sidak's multiple comparisons test. In glucose tolerance tests (GTTs), the trapezoidal rule was applied to determine the area under the glucose concentration–time curve (AUC). Nondiabetic mice (C57BL/6) were included to establish the presence and degree of abnormal glucose disposal exhibited by db/db mice. These comparisons were made between nondiabetic control mice and saline-treated db/db mice using Student's *t*-test (unpaired, two tailed).

Group differences in body weight over time were tested for significance using two-way ANOVA, and between-group comparisons were performed using Sidak's multiple comparisons test. Comparisons were kept to a minimum to reduce type 1 statistical errors. Data represent mean ± standard error (SE). The null hypothesis was rejected when $p < 0.05$. Analyses were conducted using GraphPad Prism, version 9.0 (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Ucn2 expression

Plasma Ucn2 concentration increased in mice that received *Ucn2* gene transfer versus saline (saline: 2.5 ± .7 ng/mL, $n = 6$; *Ucn2* gene transfer: 44 ± .6.7 ng/mL, $n = 6$; $p = 0.0002$; Fig. 2A). There was a trend for higher plasma Ucn2 concentrations in mice that received metformin + *Ucn2* gene transfer (58 ± .4 ng/mL, $n = 6$; $p < 0.055$; versus *Ucn2* gene transfer alone; Fig. 2A).

Ucn2 gene transfer increased liver *Ucn2* mRNA to a mean > 25,000-fold versus saline ($p = 0.0002$; Fig. 2B), and mice that received *Ucn2* gene transfer + metformin showed an even higher liver mRNA expression versus *Ucn2* gene transfer alone (mean >196,000; $p = 0.03$; Fig. 2B).

These data confirm substantial *Ucn2* expression 7–8 weeks after a single intravenous injection of AAV8.*Ucn2* and suggest that the presence of metformin may augment the extent of *Ucn2* expression in the liver.

Glycemic indices

HbA1c. The ANOVA showed an overall group difference in HbA1c ($p = 0.002$; Fig. 2C). Diabetic db/db mice that received saline had a higher HbA1c level than nondiabetic control mice (control: 4.2 ± .05%, $n = 6$; db/db saline: 7.9 ± 1%, $n = 6$; $p = 0.004$; Fig. 2C), thus showing that db/db mice have substantial sustained hyperglycemia.

Metformin alone reduced HbA1c by 1.4 absolute percentage points, which did not achieve statistical significance ($p = 0.21$). *Ucn2* gene transfer alone reduced HbA1c by 3.1 percentage points versus saline ($p < 0.004$) reaching normal values (Fig. 2C). The combination of metformin + *Ucn2* gene transfer did not reduce HbA1c beyond what was achieved with *Ucn2* gene transfer alone.

Fasting glucose. ANOVA showed an overall group difference in 12-h fasting glucose levels ($p < 0.004$; Fig. 2D). Saline-treated db/db mice showed fasting hyperglycemia versus nondiabetic control mice (control: 86 ± 5 mg/dL, $n = 6$; saline db/db: 268 ± 52 mg/dL, $n = 6$; $p < 0.006$). Metformin alone reduced fasting glucose versus saline-treated db/db mice by a nonsignificant 19% (218 ± 34 mg/dL).

Ucn2 gene transfer alone reduced fasting glucose to 134 ± 12 mg/dL ($p = 0.01$). The combination of metformin + *Ucn2* gene transfer reduced fasting glucose to 95 ± 2 mg/dL (Fig. 2D), similar to nondiabetic control mice.

Glucose tolerance test. Examination of the glucose–time data (Fig. 2E) shows progressive improved glucose disposal in a rank order of metformin < *Ucn2* gene transfer < metformin + *Ucn2* gene transfer. Analysis of the area under the curve (Fig. 2F) indicates an overall group difference ($p = 0.005$, ANOVA), with between-group differences in saline versus *Ucn2* gene transfer ($p = 0.02$) and metformin + *Ucn2* gene transfer ($p = 0.04$).

We saw a reduction in AUC in db/db mice that received metformin alone, which did not achieve statistical significance.

Blood chemistry

Alanine transaminase showed a group difference among db/db mice ($p = 0.009$; Table 1). Alanine transaminase was 7.5-fold higher in db/db mice that received saline versus control (nondiabetic C57BL/6) mice. Mice that received the combination of *Ucn2* gene transfer + metformin exhibited lower serum alanine transaminase concentration versus saline-treated ($p = 0.036$) or metformin-treated db/db mice ($p < 0.007$).

No group differences were seen in serum concentrations of albumin, HDL, cholesterol, or triglyceride.

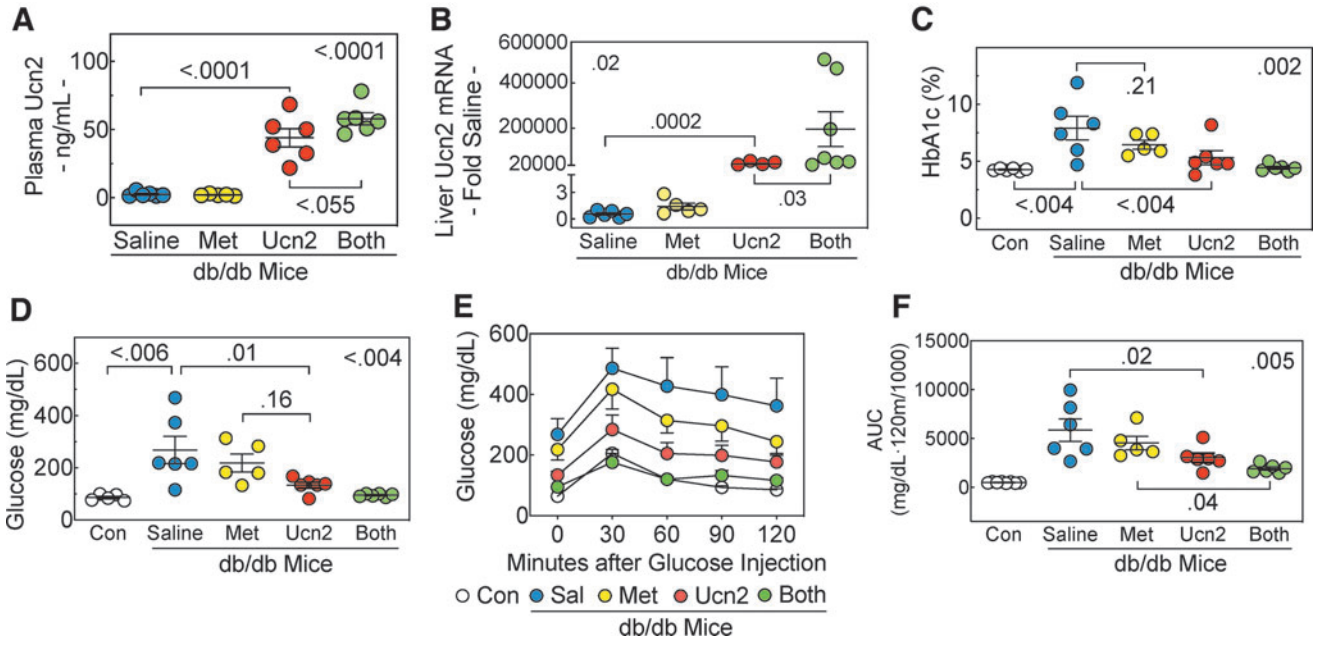

Figure 2. Ucn2 expression: effects of Ucn2 GT and metformin separately and when combined on glycemic indices in db/db mice. **(A)** *Plasma Ucn2.* A group difference in plasma Ucn2 among db/db mice was seen ($p<0.0001$; ANOVA). Seven–eight weeks after *Ucn2* GT treatment, plasma Ucn2 was >18-fold higher versus mice that received saline ($p<0.0001$). Plasma Ucn2 tended to be higher (>23-fold vs. saline) in mice that received *Ucn2* GT + metformin ($p<0.055$). **(B)** *Liver Ucn2 expression.* A group difference in liver expression of Ucn2 was seen among db/db mice ($p=0.02$; ANOVA). Seven–eight weeks after *Ucn2* GT treatment, liver *Ucn2* expression (mRNA) was higher versus db/db mice that received saline ($p=0.0002$). A higher liver *Ucn2* expression was seen in db/db mice that received *Ucn2* GT after having received 7–8 weeks of metformin ($p=0.03$). **(C)** HbA1c was higher in saline db/db mice versus nondiabetic control mice ($p<0.004$; Student's *t*-test, unpaired, two-tailed). A group difference in HbA1c levels among db/db mice was seen ($p=0.002$; ANOVA). Mice that received *Ucn2* GT showed lower HbA1c values versus db/db mice that received saline ($p<0.004$). Metformin + *Ucn2* GT did not further reduce HbA1c compared with *Ucn2* GT alone. **(D)** *Fasting glucose* was higher in saline db/db mice versus nondiabetic control mice ($p<0.006$; Student's *t*-test, unpaired, two-tailed). A group difference in fasting glucose among db/db mice was seen ($p<0.004$; ANOVA). Mice that received *Ucn2* GT had lower fasting glucose concentrations versus db/db mice that received saline ($p=0.01$). Metformin + *Ucn2* GT did not further reduce fasting glucose compared with *Ucn2* GT alone and did result in hypoglycemia (95 ± 2 mg/dL). **(E,F)** *Glucose tolerance testing.* **(E)** Concentration–time curves indicate a clear separation by group—with data from mice that received metformin + *Ucn2* GT nearly superimposable with data from nondiabetic control mice. **(F)** *Statistical testing* using area under the curve analysis showed a group difference in glucose tolerance among db/db mice ($p=0.005$; ANOVA). Mice that received *Ucn2* GT showed superior glucose clearance versus mice that received saline ($p=0.02$), and mice that received metformin + *Ucn2* GT showed superior glucose clearance versus mice that received metformin alone ($p=0.04$). One-way ANOVA was used to detect overall group differences in **(A–F)** (p value in *upper right* corner), and when $p<0.05$, Sidak's multiple comparisons test was used to detect selected between-group differences. Student's *t*-test (two tailed) was used when comparing db/db groups with nondiabetic control mice **(C, D)**.

Table 1. *Blood chemistry and body weight*

	db/db Mice					*Control (Nondiabetic)*		
	Saline (6)	*Metformin (5)*	*Ucn2 GT (6)*	*Both (6)*	p *ANOVA*	*Saline (6)*	*Ucn2 GT (4)*	p
Alanine transaminase (IU/L)	272 ± 48	333 ± 74	186 ± 24	$130\pm7^{a,b}$	0.009	36 ± 18	25 ± 2	0.6
Albumin (g/dL)	2.9 ± 0.2	3.4 ± 0.2	3.4 ± 0.2	3.4 ± 0.2	0.6	2.8 ± 0.1	2.6 ± 0.1	0.2
Cholesterol (mg/dL)	200 ± 31	200 ± 27	179 ± 17	179 ± 7	0.2	123 ± 2	135 ± 6	0.06
High-density lipoprotein (mg/dL)	84 ± 12	93 ± 9	81 ± 5	86 ± 3	0.7	73 ± 3	79 ± 3	0.2
Serum triglyceride (mg/dL)	80 ± 5	70 ± 6	68 ± 7	66 ± 5	0.2	60 ± 8	43 ± 2	0.13
Body weight, g (pre)	$31.5\pm.7^{c}$	33.4 ± 1.3	27.9 ± 1.6^{e}	30.8 ± 1.5^{f}	Group: 0.013	24 ± 0.5	24 ± 0.3	0.6
Body weight, g (final)	54.2 ± 3.4^{d}	58.8 ± 2.4	51.7 ± 2	$51.3\pm.8$	Time: <0.0001	30 ± 0.9	32 ± 0.5	0.13

Values denote mean ± SE. Blood samples obtained from db/db mice 15 weeks after continuous metformin treatment and 7–8 weeks after *Ucn2* GT. Blood samples obtained from C57BL/6 mice 6 weeks after saline treatment or *Ucn2* GT. No group differences before and after *Ucn2* GT were noted in the nondiabetic mice. Group sizes are presented in parentheses in column headings. One-way or two-way ANOVA was used to detect group differences. Serum alanine transaminase concentrations were 7.5-fold higher in db/db mice that received saline versus nondiabetic mice that received saline. In addition, there were overall group differences in serum alanine transaminase levels in db/db mice (ANOVA; $p=0.009$). Mice that received the combination of *Ucn2* GT and metformin exhibited lower serum alanine transaminase concentrations versus saline-treated db/db mice ($^{a}p=0.036$) or metformin-treated db/db mice ($^{b}p<0.007$). No group differences were seen in serum concentrations of albumin, high-density lipoprotein, cholesterol, or triglyceride. *Body weight in db/db* versus *nondiabetic mice*: pretreatment weight ($^{c}p<0.0001$) and final body weight ($^{d}p=0.0003$) were higher in saline db/db mice versus saline nondiabetic mice. *Body weight in db/db mice*: all db/db mice gained weight during the 15-week treatment protocol. A group difference ($p=0.013$) and time difference ($p<0.0001$) were found (two-way ANOVA). Between-group comparisons (Sidak's multiple comparisons test) showed that mice that received *Ucn2* GT weighed less than mice that received metformin ($^{e}p<0.016$). Mice that received metformin + *Ucn2* GT tended to weigh less than mice that received metformin alone ($^{f}p=0.08$).

ANOVA, analysis of variance; GT, gene transfer; SE, standard error.

Body weight

Pretreatment body weight was higher in db/db mice (saline db/db mice: 31.5 ± .7 g, $n=6$; saline nondiabetic mice: 24 ± .5 g, $n=6$; $p<0.0001$), and body weight at 15 weeks was higher in db/db mice (saline db/db mice: 54.2 ± 4.4 g, $n=6$; saline nondiabetic mice: 30 ± .9 g, $n=6$; $p=0.0003$). All db/db mice gained weight during the 15-week treatment protocol. A group difference ($p=0.013$) and a time difference ($p<0.0001$) were found among the four db/db groups (two-way ANOVA).

Between-group comparisons showed that mice that received *Ucn2* gene transfer weighed less than mice that received metformin ($p<0.016$). Mice that received metformin + *Ucn2* gene transfer tended to weigh less than mice that received metformin alone ($p=0.08$; Table 1).

Hepatocyte AMP kinase activity

To examine AMPK activity, we used stimulation of Huh7 cells (a hepatoma cell line[17]) with metformin, Ucn2 peptide, metformin + Ucn2 peptide, or saline (Con). Cell lysates were used in immunoblotting to quantify phospho-AMPK (T172) and total AMPK. Overall, the ANOVA showed group differences ($p=0.0004$; Fig. 3).

Compared with control, metformin alone tended to increase p-AMPK (24%; $p=0.10$), Ucn2 alone increased p-AMPK (33%; $p=0.025$), and their combination increased p-AMPK (76%; $p=0.0002$) versus control; $p=0.016$ versus Ucn2 gene transfer alone.

Histological analysis of the liver, left ventricle, and skeletal muscle

No group differences in fibrosis of the liver, left ventricle, or skeletal muscle were observed (Fig. 4A1–A3).

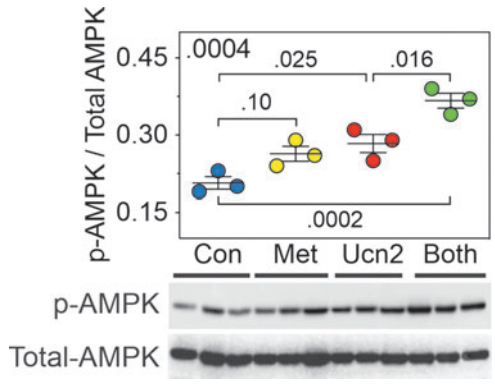

Figure 3. AMPK signaling. Huh7 cells, a hepatoma cell line,[19] were stimulated with the following: metformin (Met, 2 mM, 3 h); Ucn2 peptide (100 nM, 15 min); metformin and Ucn2 peptide (Both); or saline (Con). Cell lysates were used in immunoblotting to quantify phospho-AMPK (T172) and total AMPK. Overall group differences were seen ($p=0.0004$, ANOVA). Compared with control, the following increases in p-AMPK were observed: metformin (24%; $p=0.10$), Ucn2 (33%; $p=0.025$), and their combination (76%; $p=0.0002$). The Ucn2 peptide alone was not as effective as the combination of Ucn2 peptide and metformin ($p=0.016$). Experiments were performed in triplicate for each group.

Saline db/db mice showed 4.6 %± 0.9% liver fibrosis, similar to the other three groups of db/db mice. Liver samples from similarly aged normal mice show 0.9% ± 0.2% fibrosis.[12] Saline db/db mice showed 7.7% ± 0.3% LV fibrosis, similar to the other three groups of db/db mice. LV samples from similarly aged normal mice show 1.2% ± 0.4% fibrosis.[12]

Liver histology revealed increased lipid droplets in db/db mice that received saline when compared with the other groups (Fig. 4B). Quantitative histological analysis confirmed that db/db mice that received metformin, *Ucn2* gene transfer, or the combination of metformin + *Ucn2* gene transfer had reduced levels of fatty infiltration compared with untreated db/db mice (Fig. 4B1; ANOVA, $p=0.005$).

The reductions in fat infiltration in treated mice versus untreated db/db mice were as follows: metformin: 80% reduction, $p=0.01$; *Ucn2* gene transfer: 78% reduction, $p=0.01$; and metformin + Ucn2 gene transfer: 91% reduction, $p=0.005$.

DISCUSSION

There are three conclusions one can draw from the current study. First, the combination of *Ucn2* gene transfer + metformin does not result in hypoglycemia. Second, *Ucn2* gene transfer alone provides superior glucose disposal versus metformin alone. A third conclusion is that the combination of *Ucn2* gene transfer + metformin, while safe, is not more efficacious than *Ucn2* gene transfer alone in treating hyperglycemia in the db/db model of insulin resistance. Based on these data, there is a reason to be optimistic that both *Ucn2* gene transfer alone and in combination with metformin may be safe and effective in clinical settings.

Metformin's efficacy is primarily due to inhibition of hepatic gluconeogenesis.[13] Its tolerability, promotion of modest weight loss, low risk of hypoglycemia, and neutral or positive cardiovascular effects make it a consensus, preferred initial therapy for treating type 2 diabetes after lifestyle modifications (altered diet and increased activity) have failed.

Recently, the American Diabetes Association accepted other drugs as potential first-line therapies, including GLP-1 receptor agonists and sodium/glucose cotransporter 2 inhibitors, when diabetic patients have concomitant atherosclerotic cardiovascular disease, heart failure, or chronic kidney disease,[21] although the potential for better outcomes comes at high financial costs compared with metformin.[22]

In recent studies, we found that the Ucn2 peptide was as effective as insulin in Glut4 translocation in skeletal myotubes.[7] This insulin-like action of Ucn2 was also seen in a model of insulin deficiency, where *Ucn2* gene transfer alone normalized fasting glucose, insulin sensitivity, and insulin release *in vivo* and from isolated islets.[8]

This unanticipated success forewarned a possibility that *Ucn2* gene transfer might cause hypoglycemia when

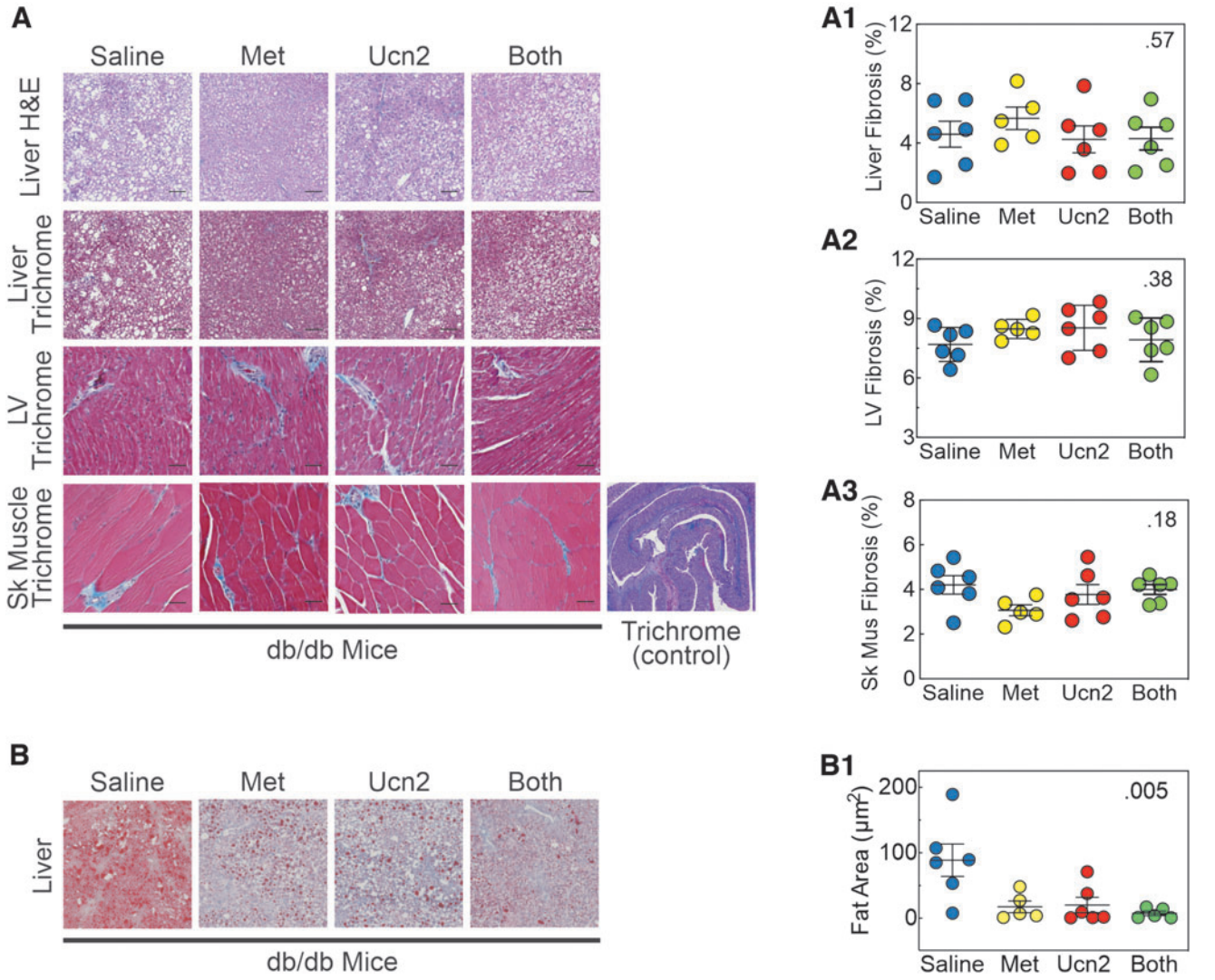

Figure 4. Effect of metformin and Ucn2 GT on fibrosis (liver, LV, and skeletal muscle) and liver lipid content. **(A)** H&E and Masson's trichrome staining showed no group differences in fibrosis in the liver, LV sample, or skeletal muscle (see graphs **A1, A2, and A3**). **(B)** Histology showed more lipid in saline-treated db/db mice than in other groups. Graph **B1** shows group differences in liver fat droplet area ($p=0.005$, ANOVA), with similar reductions in treated groups. Group size was $n=6$, except for the metformin group ($n=5$). H&E, hematoxylin–eosin; LV, left ventricular; Sk Mus, gastrocnemius muscle; Met, metformin; Ucn2, urocortin 2.

combined with other drugs used to treat diabetes, a concern that prompted the current study. We found that metformin + *Ucn2* gene transfer was not associated with fasting hypoglycemia. Future studies will also test insulin versus *Ucn2* gene transfer (and their combination) in a rodent model of insulin resistance.

Table 2 shows the key mechanisms by which metformin and *Ucn2* gene transfer act to improve glycemic control. In nonclinical studies, *Ucn2* gene transfer has been shown to increase both insulin release and insulin sensitivity to a greater degree than that seen with metformin and both reduce hepatic glucose production and stimulate hepatic AMPK in a dose-dependent manner. It therefore was a logical concern that combination therapy might cause hypoglycemia. This is important because clinical trials with *Ucn2* gene transfer are likely to enroll patients on metformin.

AMPK plays important roles in glucose metabolism and energy utilization. Metformin can directly or indirectly activate AMPK.[13] We previously reported that the Ucn2 peptide increases glucose uptake through increased activation of AMPK in skeletal myotubes.[7]

In the current study, we found no group differences in AMPK activation in liver homogenates. It is plausible that varying degrees of liver fibrosis and fatty infiltration precluded detection of group differences. Therefore, we performed *in vitro* studies (Fig. 3) where such interference was not present. We found that the Ucn2 peptide alone increased AMPK activity in Huh7 cells, a hepatoma cell line.[19] The combination of the Ucn2 peptide and metformin had synergistic effects on increasing phospho-AMPK (Fig. 3).

Comparisons of *Ucn2* gene transfer, metformin, and their combination provided useful insights. Glycemic indices

Table 2. *Glucose disposal effects of metformin and Ucn2 gene transfer on the liver and skeletal muscle*

	Metformin	*Ucn2 GT*	*Comments*	*Refs.*
Hepatic gluconeogenesis	↓	↓	Inhibition of hepatic gluconeogenesis is a key glucose-lowering mechanism for metformin.	7,8,13
Skeletal muscle insulin sensitivity	No Δ	↑	Determined by euglycemic clamps in mouse models of diabetes and in clinical type 2 diabetes.	7,8,25
Islet insulin release	↓	↑	Determined in isolated islet studies from mice.	8,26
AMPK activity (hepatocytes)	↑	↑	AMPK activation may play an important role in inhibition of hepatic gluconeogenesis.	13; Current study

Data from literature, including clinical and nonclinical data for metformin, and nonclinical data for *Ucn2* GT (*urocortin 2* gene transfer), including hyperinsulinemic euglycemic clamps.

AMPK, 5′ adenosine monophosphate-activated protein kinase; GT, gene transfer; *Ucn2* GT, *urocortin 2* gene transfer.

(HbA1c, fasting glucose, and GTT) tended to be reduced by metformin, but not as effectively as *Ucn2* gene transfer alone or metformin + *Ucn2* gene transfer. In previous studies of metformin treatment of db/db mice, reductions in mean fasting glucose of 25–29% were reported,[17,18] similar to the 19–23% reductions we saw in the current study.

Our db/db mice exhibited greater variations in 12-h fasting glucose than were seen in these previous reports, which may have impeded finding statistically significant metformin treatment effects. Previous studies had treatment durations of 2–4 weeks, much shorter than our 15-week treatment duration. This prolonged period would be anticipated to be associated with increased fatty infiltration of the liver and higher serum alanine transaminase concentration, which may have rendered metformin's beneficial effects on glycemic indices more variable.

Liver samples from db/db mice that received treatment with metformin alone showed an 80% reduction in liver fat versus saline db/db mice ($p = 0.01$). Mice that received *Ucn2* gene transfer alone showed a 78% reduction ($p = 0.01$), and mice that received combined treatment with metformin + *Ucn2* gene transfer showed a 91% reduction ($p = 0.005$).

In addition, mean values for serum alanine transaminase concentrations were 7.5-fold higher in saline db/db mice versus nondiabetic control mice. We saw a reduction in mean serum alanine transaminase concentrations in mice that received metformin + *Ucn2* gene transfer. These data indicate a possible synergy between the two therapies, which appears to reduce liver toxicity in this model of insulin resistance and obesity.

Despite favorable reductions in liver fatty infiltration and reduced alanine transaminase concentrations, liver fibrosis and cardiac fibrosis were unchanged. An attenuation of liver fibrosis was documented in 9-month-old db/db mice after 3 months of weekly treatment with FGF1.[23] Others report, in 4-month-old db/db mice, a >50% reduction in LV fibrosis 4 weeks after infusion of irisin peptide or gene transfer of fibronectin type III domain-containing-5 (Ad5.FNDC5 by direct cardiac injection).[24] Neither of these studies targeted hyperglycemia—the interventions were directed to offset inflammation.

Mice that received *Ucn2* gene transfer alone showed a >25,000-fold increase in *Ucn2* mRNA copies, consistent with what we have found in previous studies using the same vector dose.[8,11] Mice that received metformin + *Ucn2* gene transfer showed a >190,000-fold increase in liver *Ucn2* mRNA. Plasma Ucn2 concentration was increased in db/db mice that received *Ucn2* gene transfer versus saline, and there was a trend for higher plasma Ucn2 concentrations in db/db mice that received metformin + *Ucn2* gene transfer.

These data indicate a potential interaction between metformin and *Ucn2* expression. We speculate that metformin may increase stability of liver *Ucn2* mRNA. Alternatively, reduced liver fat and lower serum alanine transaminase levels may play roles in sustained *Ucn2* expression. The serendipitous finding that metformin may increase *Ucn2* expression could be a benefit in clinical trials, where enrolled patients would be treated with metformin when they are randomized to receive *Ucn2* gene transfer. Increased *Ucn2* transgene expression would enable selection of lower vector doses.

Limitations

The study was conducted on male mice only. We previously showed that both male and female db/db mice respond to *Ucn2* gene transfer,[7] but one cannot exclude the possibility of a sex–metformin-*Ucn2* gene transfer interaction. In addition, we examined glycemic indices only 7–8 weeks after *Ucn2* gene transfer treatment, so we cannot be certain that hypoglycemia or worsened hyperglycemia may occur at a later time.

However, we have previously published that the beneficial effects on glycemic indices are stable 4 months after *Ucn2* gene transfer treatment in diabetes models[8] and have documented sustained increases in plasma Ucn2 concentrations and favorable cardiac effects >20 months after one-time vector delivery.[12]

Conclusions

The combination of metformin + *Ucn2* gene transfer does not result in hypoglycemia. *Ucn2* gene transfer alone provides superior glucose disposal versus metformin alone. The combination of metformin + *Ucn2* gene transfer, while safe, is not more efficacious than *Ucn2* gene transfer alone in treating hyperglycemia in the db/db model of insulin resistance.

However, the combined treatment with metformin + *Ucn2* gene transfer appears to have beneficial effects in the liver, including reduced serum alanine transaminase concentrations and enhanced transgene *Ucn2* expression.

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AUTHORS' CONTRIBUTIONS

All authors have read and agree with the data presented in the article, which are not being considered elsewhere for publication. M.H.G. and N.C.L. were involved in design, collecting data, data analysis, graphs/tables, and editing; D.G. was involved in design, collecting data, and data analysis; T.G. was involved in collecting data and data analysis; and H.K.H. was involved in design, data analysis, graphs/tables, writing of the manuscript; supervision; and acquiring funding.

AUTHOR DISCLOSURE

H.K.H. is a founder, board member, and unpaid consultant of Renova Therapeutics. Renova played no financial, intellectual, or editorial role in the studies. None of the other authors have disclosures.

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