


CHOP-ASO Ameliorates Glomerular and Tubular Damage on Top of ACE Inhibition in Diabetic Kidney Disease

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ABSTRACT

Background Maladaptive endoplasmic reticulum stress signaling in diabetic kidney disease (DKD) is linked to increased glomerular and tubular expression of the cell-death-promoting transcription factor C/EBP homologous protein (CHOP). We demonstrated that targeting CHOP with locked nucleic acid (LNA)-modified antisense oligonucleotides (ASOs) ameliorates experimental 5 DKD.

Methods We determined the efficacy of CHOP-ASO in the early and late stages of experimental DKD (in 8- or 16-week-old db/db mice, respectively) alone or with an angiotensin-converting enzyme inhibitor (ACEi), after an *in vivo* dose-escalation study. Renal functional parameters and morphologic analyses assessed the effects and renal gene-expression profiling identified differentially regulated genes and pathways. Several human CHOP-ASOs were tested in hyperglycemia-exposed human kidney cells.

Results CHOP-ASOs efficiently GSH renal CHOP expression in diabetic mice and GSH markers of DKD at the early and late stages. Early combined intervention (CHOP-ASO and ACEi) efficiently prevented interstitial damage. Later, the combined treatment GSH indices of both glomerular and tubular damage more efficiently than either intervention alone. CHOP-ASO affected a significantly larger number of genes and disease pathways, including GSH sodium-glucose transport protein 2 (Slc5a2) and PROM1 (CD133). Human CHOP-ASOs efficiently GSH glucose-induced CHOP and prevented death of human kidney cells *in vitro*.

Conclusions The ASO-based approach efficiently GSH renal CHOP expression in a diabetic mouse model, providing an additional benefit to an ACEi, particularly at later timepoints. These studies demonstrate that ASO-based therapies efficiently reduce maladaptive CHOP 28 expression and ameliorate experimental DKD.

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Considering the increasing worldwide prevalence of type 2 diabetes, the incidence of diabetic kidney disease (DKD), the most common cause of terminal kidney failure, is expected to increase in future years.¹ Despite promising therapeutic developments over the last decades, DKD still lacks an efficient therapy that halts or reverses the disease.² In addition to optimized blood glucose control, inhibition of the renin-angiotensin aldosterone system is the mainstay of therapy. However, these therapies delay, but do not stop,

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disease progression.² SGLT2 inhibitors have emerged as promising drugs, showing renoprotective effects in clinical studies.³ However, their long-term efficacy and safety profile remain to be established.³ Hence, there remains an unmet need for new therapies to treat DKD.

The endoplasmic reticulum (ER) stress response constitutes a cellular response closely linked with metabolic diseases, such as diabetes mellitus and DKD.^{4,5} Physiologically, the ER folds proteins and manages Ca²⁺ homeostasis. On cellular stress, the ER launches a signaling response aiming to restore protein homeostasis and thus cellular function (adaptive ER stress response). To achieve this, three signaling arms may be engaged: PERK-eIF2 α , ATF6, and IRE1 α . The PERK-eIF2 α signaling arm attenuates general protein translation, thus alleviating the protein folding load, and induces cell cycle arrest. The transcription factor ATF6 is released from the ER, and after maturation (cleavage) in the Golgi, it translocates to the nucleus to regulate gene expression.^{4,5} IRE1 α splices XBP-1 RNA *via* its ribonuclease activity, promoting the expression of the primarily adaptive transcription factor XBP1 splice variant. Although the ER stress response primarily aims to restore cellular function, it will induce cell death in patients with persistent stress, largely through the transcription factor CHOP (maladaptive response).

The age-dependent induction of renal CHOP expression is accelerated in diabetic mice.⁶ CHOP induction is observed in murine models of diabetes and in humans with diabetes in the tubular and glomerular compartment.^{5,7–9} The concomitant induction of maladaptive transcription factor CHOP in both the glomerular and tubular compartments in DKD makes CHOP an attractive therapeutic target. Indeed, nonspecific approaches to restore ER function using chaperones, such as bile-acid derivatives (taurourso-deoxycholic acid, TUDCA; ursodeoxycholic acid) or 4-phenylbutyrate, demonstrated beneficial effects in experimental DKD.^{9,10} However, nonspecific ER chaperones may have additional effects, as demonstrated with TUDCA, which regulates FXR-dependent gene expression in the tubular compartment in addition to restoring ER function.⁹ Whether specific targeting of the maladaptive ER stress response by inhibiting CHOP conveys beneficial effects in DKD remains unknown.

Antisense oligonucleotides (ASOs) targeting specific genes have emerged as a powerful therapeutic for a number of diseases.^{11–13} Because ASOs reduce, but do not completely block, the expression of targeted genes, their effect is expected to differ from those seen in knockout mice. The partially sustained expression of genes targeted by ASOs may be advantageous, because a complete loss of function typically will not restore the physiologic situation. In this study, we tested ASOs targeting CHOP expression as a potential new approach for treating DKD. We used third-generation ASOs designed as gapmers with locked nucleic acid-modified nucleotides in the flanks, which

Significance Statement

The endoplasmic reticulum (ER) stress response and the maladaptive and cell-death-promoting transcription factor C/EBP CHOP have been linked with diabetic kidney disease (DKD). Specific therapies targeting maladaptive ER stress signaling are lacking. We show that an antisense oligonucleotide (ASO)-based approach reducing CHOP expression ameliorates DKD in mice, providing renal protection on top of ACE inhibition. CHOP inhibition improves both glomerular and tubular damage. ASO-based therapies are a potentially new approach to target maladaptive ER stress signaling and improve DKD.

increase the affinity of the ASO to the target RNA. In contrast to earlier generations, third-generation ASOs do not require transfection reagents or conjugations for efficient target knockdown *in vitro*.^{14,15} Furthermore, specific target knockdown can be achieved *in vivo* without the use of complex formulations.¹⁴ The efficacy of CHOP-ASO was determined alone and in combination with an ACE inhibitor in diabetic mice at early and late stages of DKD to investigate disease prevention or therapeutic intervention, respectively.

MATERIALS AND METHODS

For details, please see Supplemental Materials and Methods, the Major Resource Table, and the Supplemental References.

Mice

db/db (Lepr db/db) and nondiabetic db/m (Lepr db/+) mice (8- or 16-weeks-old) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Only male mice were used throughout this study in accordance with the approved procedures. All animal experiments were conducted according to standards and procedures approved by the local Animal Care and Use Committee (Landesverwaltungsamt Halle, Germany).

In Vivo Intervention Studies

Male db/db mice were randomly assigned to five experimental groups at 8 weeks (disease prevention model) or 16 weeks (disease intervention model) of age as follows: control (untreated); enalapril (E) treatment alone (ACE inhibitor, 50 mg/L administered orally *via* drinking water)⁹; CHOP-ASO alone (1 mg/kg, or 3 mg/kg on alternative days, administered intraperitoneally); nonspecific control oligonucleotide (CON, 1 mg/kg on alternative days, administered intraperitoneally); and combined E and CHOP-ASO treatment. A subset of db/db control mice received PBS as a vehicle control. Additionally, db/m (nondiabetic) mice were used as nondiabetic controls. After 8 (disease prevention model) or 6 (disease reversal model) weeks of treatment, mice were euthanized at 16 or 22 weeks of age,

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respectively, and perfused with ice-cold PBS, followed by 4% paraformaldehyde buffered in PBS. Kidneys were prepared as previously described.^{5,9,16,17}

Determination of Albuminuria

The day before tissue preparation, mice were individually placed in metabolic cages, and urine samples were collected over a 12-hour period. Urine albumin was determined using an ELISA for mouse albumin according to the manufacturer's instructions, and urine creatinine was determined using a commercially available assay of a modified version of the Jaffe method (X-Pand automated platform; Siemens).^{5,9,16,17}

Statistical Analysis

Statistical analyses were performed with ANOVA and *post hoc* comparison with the Tukey method. The Kolmogorov-Smirnov test or D'Agostino-Pearson normality test was used to determine whether the data were normally distributed. StatistixXL (www.statistixl.com), Prism 5 (www.graphpad.com) and R software¹⁸ were used for statistical analyses. To assess the different distributions shown in the Venn diagram, a chi-squared test was applied. Statistical significance was accepted at values of $P < 0.05$.

RESULTS

Inhibition of CHOP Prevents DKD in Mice as Efficiently as an ACEi

To evaluate the role of CHOP inhibition in DKD, we first conducted a dose-escalation study (1 and 3 mg/kg, on alternate days, for 8 weeks) of CHOP-ASO (ChA) using in db/db mice, in which CHOP levels have been reported to be increased.^{5,6,9} Compared with PBS-treated db/db mice or mice administered CON, CHOP-ASO-treated mice showed efficient reductions in the mRNA and protein levels of CHOP at 16 weeks (Supplemental Figure 1, A and B). No effect on body weight or blood glucose levels was observed at any tested dose (Supplemental Figure 1, C and D).

Using the lowest tested dose (1 mg/kg, on alternative days), we started a new study in db/db mice beginning at the age of 8 weeks (*i.e.*, before the establishment of albuminuria).¹⁷ Because the PBS and CON mice did not differ in the dose-finding study (Supplemental Figure 1 and data not shown), we only include a PBS group as control. We compared the efficacy of CHOP-ASO to that of the PBS-injected controls, the ACEi, E,⁹ or a combination of CHOP-ASO plus E (ChA+E, Figure 1A). The pharmacological interventions efficiently GSH renal CHOP expression in db/db mice (age 16 weeks), compared with the expression after treatment with PBS (Figure 1B), but had no effect on blood glucose levels or body weight

(Supplemental Figure 1, E and F). In parallel, we observed a marked reduction in albuminuria (reflected by the urine albumin to creatinine ratio, Figure 1C) and improvement in glomerular injury, as reflected by mesangial matrix accumulation (fractional mesangial area; Figure 1, D and E). Likewise, both the ACEi and CHOP-ASO GSH interstitial fibrosis and the expression of the kidney injury marker KIM-1, indicating tubular protection (Figure 1, D and F-H). With regard to KIM-1 expression, no additive effect of the combined ACEi and CHOP-ASO intervention was observed, the combined intervention GSH interstitial fibrosis slightly more efficiently than either therapy alone (Figure 1, D and F), suggesting an added value of the combination therapy. Indeed, although ACEi alone had no effect on tubular diameter (in agreement with previous findings⁹), this indicator of tubular damage was GSH by CHOP-ASO alone or CHOP-ASO+ACEi (Figure 1, D and G). These data demonstrate that therapeutic inhibition of CHOP ameliorates DKD.

Combined ACE and CHOP Inhibition Halts the Progression of DKD

We and others have previously shown that ACEi in rodents are less efficient when administered after established albuminuria.^{9,19} Given the efficient prevention of DKD by CHOP-ASO, we next asked whether CHOP inhibition provides an additive effect on top of ACEi in rodents with established albuminuria. We used the same interventions as above in db/db mice, but started the interventions in mice aged 16 weeks (*i.e.*, mice with established albuminuria and renal damage, Figure 2A).¹⁷ None of the interventions had an effect on blood glucose values, body weight, or parameters of liver function (plasma alanine aminotransferase, or aspartate aminotransferase, Figure 2B and Supplemental Figure 2, A-C). As expected, E normalized plasma levels of angiotensin II and systolic BP in db/db mice, whereas CHOP-ASO treatment had no effect on the plasma levels of angiotensin II or systolic BP in db/db mice (Figure 2C and Supplemental Figure 2D).

Compared with PBS-treated db/db mice or mice administered a nonspecific CON, CHOP-ASO (ChA), and E each GSH mesangial matrix accumulation and the combined intervention (ChA+E) conveyed an added effect (Figure 2, D and F). Likewise CHOP-ASO (ChA) and E markedly GSH albuminuria (Figure 2E) and the combined intervention (ChA+E) was slightly, although not significantly, better than either intervention alone ($P=0.09$ compared with ChA alone and $P=0.08$ compared with ACEi alone, Figure 2E). The combined intervention (ChA+E) was superior with regard to reducing the glomerular basement membrane thickness (Figure 2, D and G). Of note, only CHOP-ASO, either alone or in combination with ACEi, partially restored the density of podocyte foot processes (number of foot processes per μm , Figure 2, D and H).

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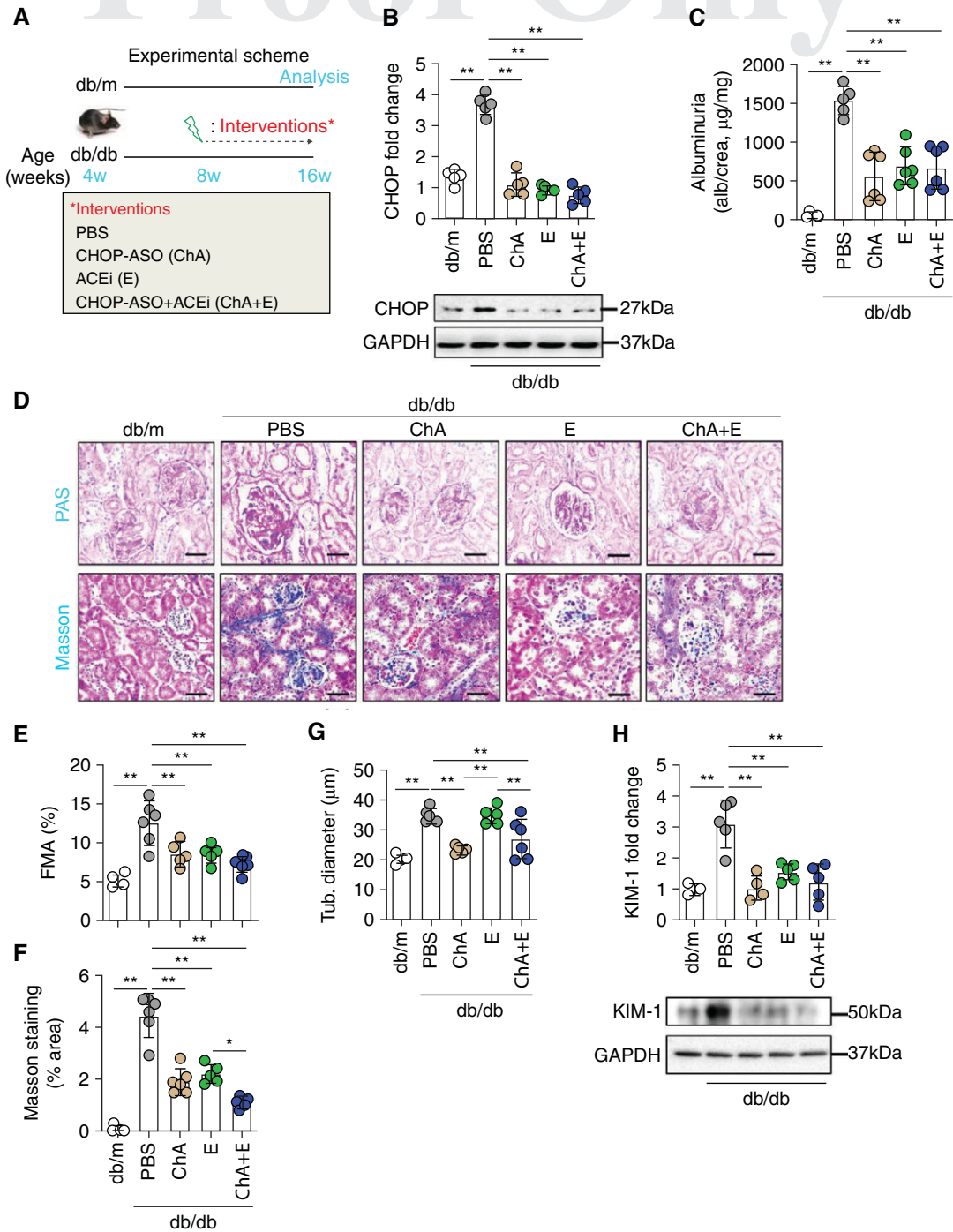


Figure 1. CHOP-ASO protects both glomerular and tubular compartments in db/db mice. (A) Experimental scheme. (B) Scatter plot with bars summarizing renal expression of CHOP (B, bottom: representative immunoblots, GAPDH was used for normalization). (C) Scatter plot with bars summarizing albuminuria levels (C, urine albumin-creatinine ratio). (D–H) Representative renal histology sections (D, periodic acid–Schiff [PAS] stain, upper panel, and Masson trichrome stain, lower panel) showing glomerular and tubular morphology. Scatter plot with bars summarizing glomerular extracellular matrix accumulation as indicated with PAS staining (E, fractional mesangial area, FMA). Scatter plot with bars summarizing renal collagen deposition using Masson’s trichrome stain (F, percentage of stained area) and tubular diameter (G, Tub. diameter). Scatter plot with bars summarizing renal expression of KIM-1 (H, top; bottom: representative immunoblots, GAPDH was used for normalization). Nondiabetic mice (db/m, 16 weeks old) and 16-week-old diabetic db/db mice treated for 8 weeks (starting at the age of 8 weeks) with PBS, ChA, ACEi, E, or ChA+E. Scale bar, 50 μm (D); * $P < 0.05$, ** $P < 0.01$, (B, C, and E–H: ANOVA); each dot in scatter plots represents one sample.

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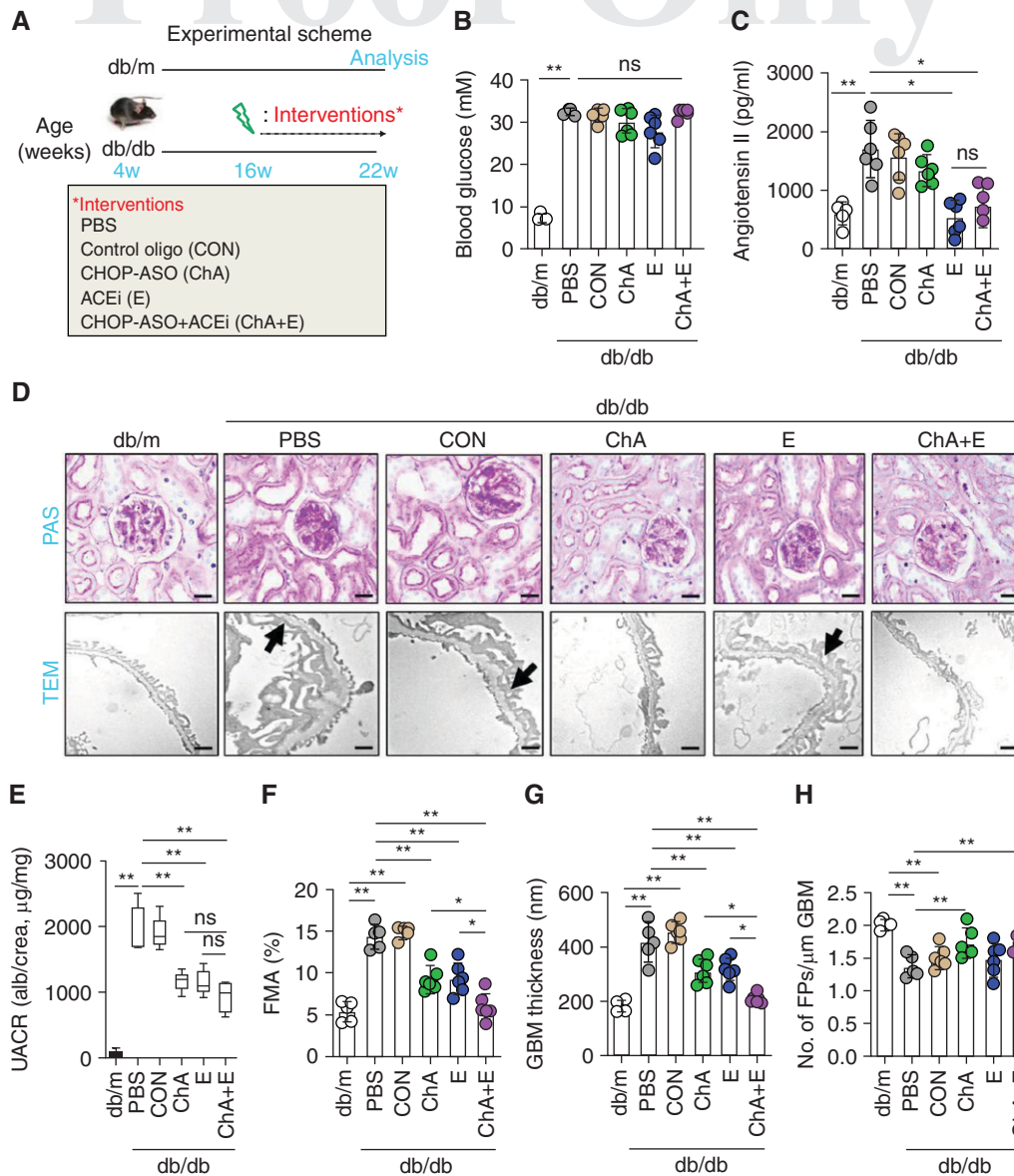


Figure 2. CHOP inhibition provides protection on top of E to halt the progression of DKD. (A) Experimental scheme. (B and C) Scatter plot with bars and box plots summarizing (B) blood glucose levels and (C) plasma angiotensin II levels, respectively. (D–H) Representative renal histologic sections showing (D, upper panel) PAS staining, (D, lower panel, the arrows indicate exemplary podocyte foot processes effacement) transmission electron microscopy (TEM). (E) Box plots summarizing glomerular extracellular matrix accumulation as indicated with (F) PAS staining (fractional mesangial area, FMA), (G) glomerular basement membrane thickness (GBM), and (H) number of podocyte foot processes per micrometer GBM as analyzed by TEM images. Nondiabetic mice (db/m, 22 weeks old) and 22-week-old diabetic db/db mice treated for 6 weeks (starting at the age of 16 weeks) with PBS, CON, ChA, ACEi, E, or ChA+E. Scale bar, (D, upper panel) 50 µm or (D, lower panel) 1 µm; * $P < 0.05$, ** $P < 0.01$, ns, nonsignificant (B, C, E–H: ANOVA); each dot in scatter plots represents one sample.

An additive effect of CHOP-ASO and ACEi was evident in the tubular compartment. Interstitial fibrosis was GSH by both CHOP-ASOs and ACEi and the combination of both was slightly superior to either intervention alone (Figure 3, A and C). Of note, although ACEi alone had no effect on the tubular diameter, CHOP-ASO alone or in

combination with ACEi efficiently GSH the tubular diameter (Figure 3, D). Improved protection from tubular damage by either CHOP-ASO alone or combined intervention with CHOP-ASO and ACEi was confirmed by GSH renal expression of KIM-1 by immunohistochemistry and immunoblotting (Figure 3, B and E–G).

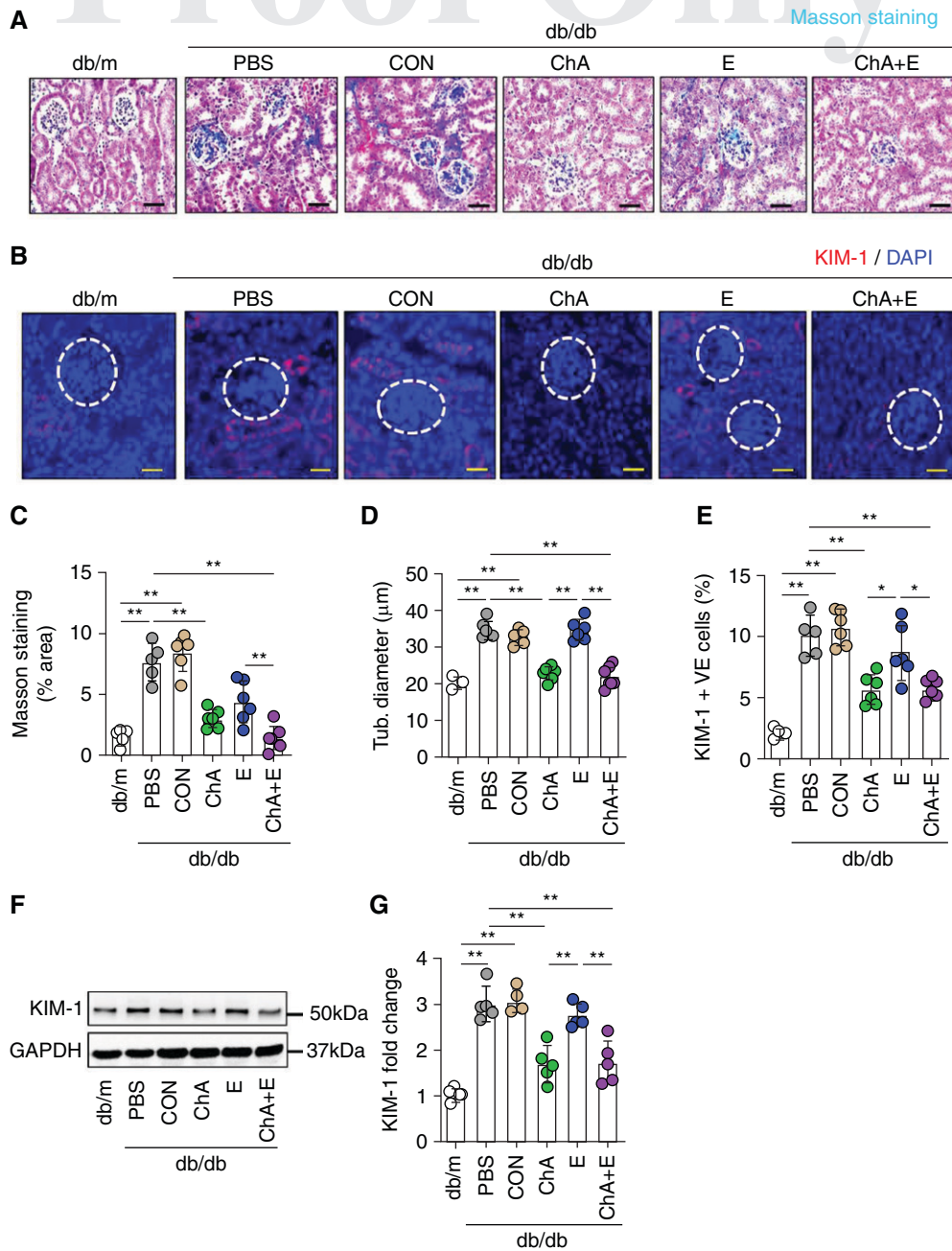


Figure 3. CHOP inhibition but not E halts the progression of hyperglycemia-induced renal damage. (A) Representative renal histologic sections showing Masson trichrome staining of glomerular and tubular compartments. (B) Representative immunofluorescence images of KIM-1 (glomeruli indicated by the white dashed circles) expression. (C–G) (C) Scatter plot with bars summarizing renal collagen deposition (Masson staining % area) using Masson’s trichrome stain and (D) renal tubular diameter. Scatter plot with bars summarizing the frequency of (E) KIM-1–positive renal cells using KIM-1 immunofluorescence staining. (F) Representative immunoblot images of renal KIM-1 expression (GAPDH was used for normalization). (G) Scatter plot with bars summarizing KIM-1 immunoblot data. Nondiabetic mice (db/m, 22 weeks old) and 22-week-old diabetic db/db mice treated for 6 weeks (starting at the age of 16 weeks) with PBS, nonspecific CON, ChA, ACEi, E, or ChA+E. Scale bar, (A) 50 μm and (B) 25 μm; * $P < 0.05$, ** $P < 0.01$ (C–G: ANOVA); each dot in scatter plots represent one sample.

Consistent with a protective effect of CHOP-ASO in the glomerular and tubular renal compartment, CHOP expression was induced and was associated with increased ATF6α expression in both compartments in diabetic mice (Figure 4

and Supplemental Figure 3). Both, increased CHOP and ATF6α expression were efficiently GSH by CHOP-ASO. Interestingly, unlike early intervention with ACEi (Figure 1B), later intervention with ACEi failed to suppress renal

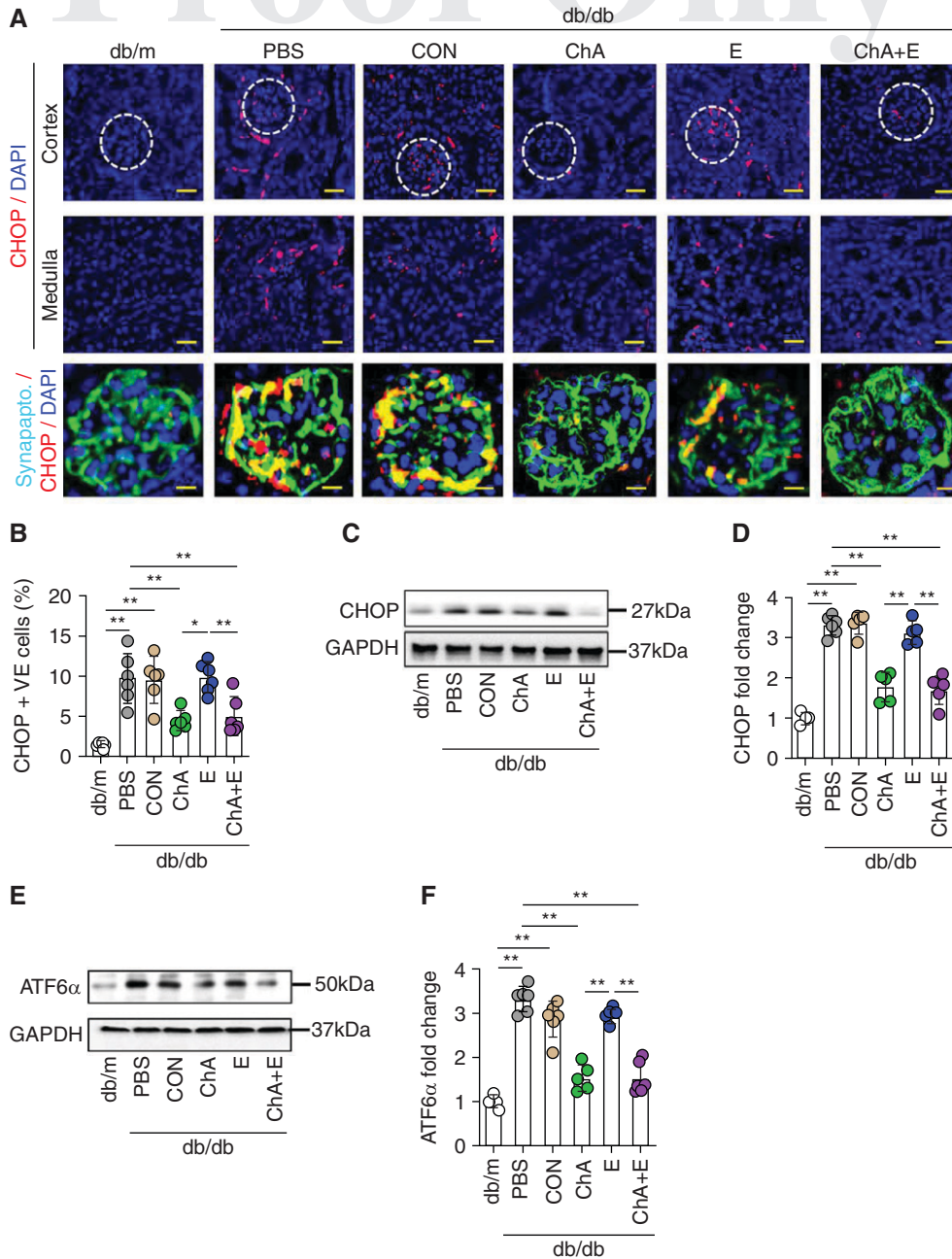


Figure 4. CHOP inhibition but not E reduces renal maladaptive UPR in diabetic mice. (A) Representative immunofluorescence images of CHOP (renal cortex; top panel, glomeruli indicated by white dashed circles; renal medulla; middle panel). (A, bottom panel) Representative co-immunofluorescence images of CHOP and podocytes marker, synaptopodin. CHOP-positive cells are indicated in red, synaptopodin in green and DAPI (blue) represents nuclear counterstaining. Scatter plot with bars summarizing the frequency of CHOP-positive (B) renal cells using CHOP immunofluorescence staining. Representative immunoblot images of (C) renal CHOP expression (GAPDH was used for normalization) and (D) scatter plot with bars summarizing CHOP immunoblot data. Representative immunoblot images of (E) renal ATF6 α expression (GAPDH was used for normalization) and (F) scatter plot with bars summarizing ATF6 α immunoblot data. Nondiabetic mice (db/m, 22 weeks old) and 22-week-old diabetic db/db mice treated for 6 weeks (starting at the age of 16 weeks) with PBS, CON, ChA, ACEi, E, or ChA+E. Scale bar, (A, upper and middle panels) 50 μ m and (A, bottom panel) 25 μ m; * P <0.05. ** P <0.01 (B, D, and F: ANOVA); each dot in scatter plots represent one sample.

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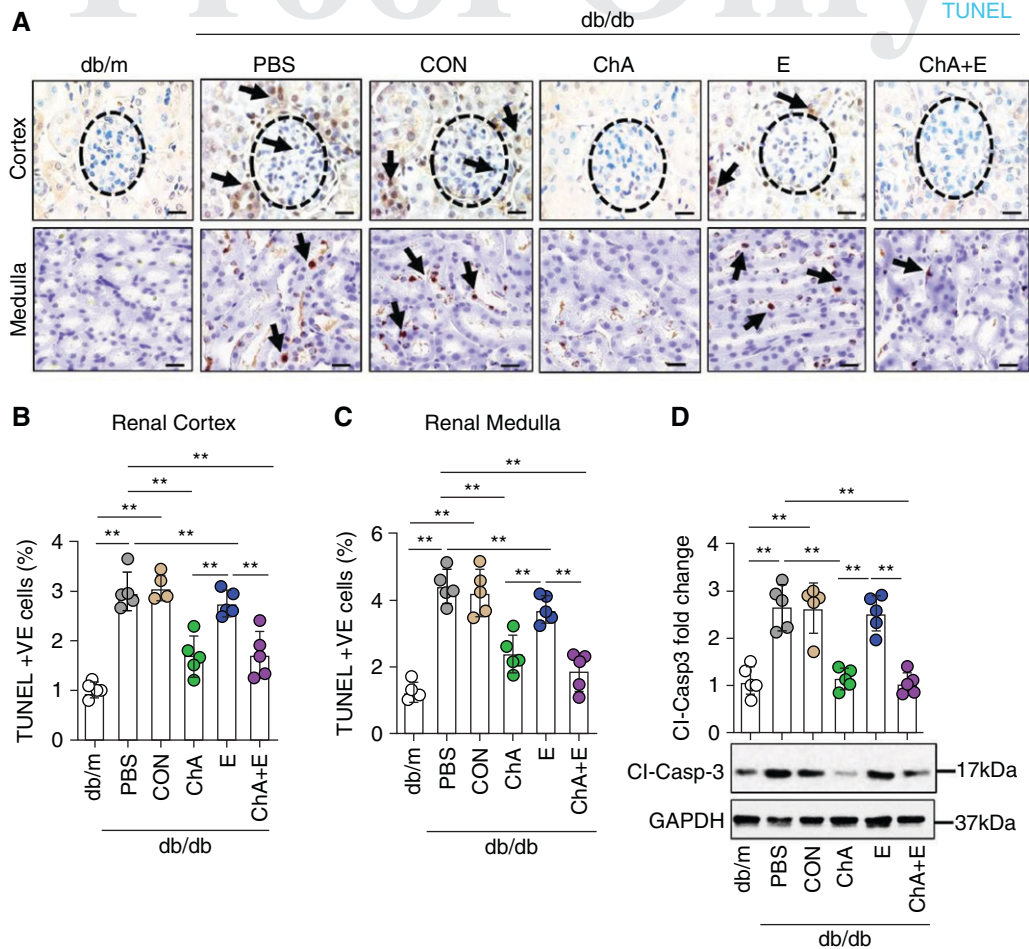


Figure 5. CHOP inhibition but not E ameliorates diabetes-induced cell death in renal glomerular and tubular compartments. (A) Representative renal histologic sections showing terminal deoxynucleotidyl transferase dUTP nick end labeling staining (TUNEL) (renal cortex, upper panel; renal medulla, bottom panel). TUNEL staining: brown, TUNEL-positive cells detected by HRP-DAB reaction; blue, hematoxylin counterstain. The arrows show exemplary TUNEL positive cells. Scatter plot with bars summarizing the frequency of TUNEL-positive cells in (B) renal cortex and (C) medullary areas. Representative immunoblot images of renal cleaved caspase-3 (D, bottom, GAPDH was used for normalization) and (D) scatter plot with bars summarizing cleaved caspase-3 immunoblot data. Nondiabetic mice (db/m, 22 weeks old) and 22-week-old diabetic db/db mice treated for 6 weeks (starting at the age of 16 weeks) with PBS, CON, ChA, ACEi, E, or ChA+E. Scale bar, 25 μ m (A); * P <0.05, ** P <0.01 (B–D: ANOVA); each dot in scatter plots represent one sample.

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CHOP and ATF6 α expression in glomerular and tubular compartments (immunofluorescence and immunoblotting, Figure 4 and Supplemental Figure 3), suggesting failure to suppress CHOP expression is linked to the impaired efficacy of ACEi in these mice. The persistent increase of ATF6 α and CHOP in ACEi-treated mice supports the notion that maladaptive ER stress signaling restricts the protective effect of ACEi, if initiated after the establishment of albuminuria.

Congruent with the suppression of CHOP in both renal compartments, CHOP-ASO, either alone or in combination with ACEi, was superior to ACEi alone in reducing the number of terminal deoxynucleotidyl transferase dUTP nick end labeling staining-positive cells within the renal glomerular and the tubular compartments (Figure 5, A–C

and Supplemental Figure 4). Suppression of renal cell death was confirmed by GSH levels of cleaved caspase-3 on CHOP-ASO, but not on ACEi intervention (Figure 5D).

Inhibition of CHOP Alters Gene Expression

CHOP promotes cell death and functions as a transcriptional regulator. Considering the latter, we next performed gene expression analyses (RNA sequencing) in the cohort of mice administered treatments starting at 16 weeks. Three samples, which were clearly outliers in the principal component analyses and did not pass internal quality standards, were excluded from the analyses (one each from the PBS, the control, and the CHOP-ASO+ACEi group,

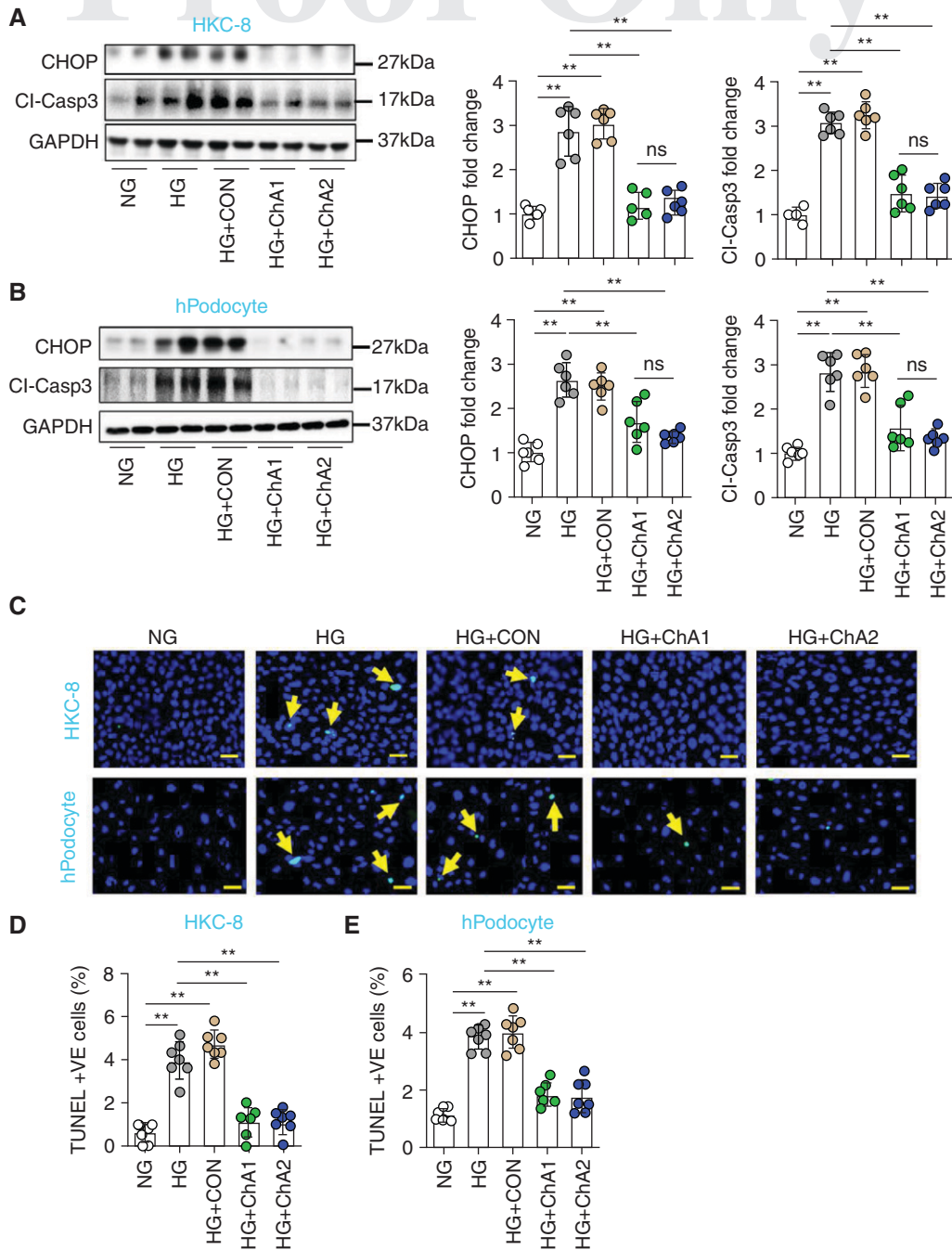


Figure 6. CHOP-ASO prevents glucose-induced cell damage in human renal cells. (A and B) Representative immunoblots of CHOP and cleaved caspase-3 expression in (A, left panel) HKC-8 cells or (B, left panel, three independent repeat experiments with two technical replicates each; GAPDH was used for normalization for both cell types) human podocytes and scatter plot with bars summarizing (A and B, middle panels) the CHOP and (A and B, right panels) cleaved caspase-3 immunoblot data. (C–E) Representative immunofluorescence images of TUNEL staining of (C, upper panel) HKC-8 cells and (C, lower panel) human podocytes. TUNEL-positive cells are indicated by green fluorescence, and nuclei were counterstained with DAPI (blue). Scatter plot with bars summarizing the frequency of TUNEL-positive (D) HKC-8 cells and (E) human podocytes. Each dot in the scatter plot with bars represents averaged data from six different visual fields of individual experiments (biological replicates). NG: normal glucose (5 mM glucose plus 20 mM mannitol); HG: high glucose (25 mM); HG+CON: (25 mM glucose+ 10 μ M CON); HG+ChA1: (25 mM glucose+ 10 μ M CHOP-ASO1); HG+ChA2: (25 mM glucose+ 10 μ M CHOP-ASO2). Scale bar, 20 μ m (C); ** P <0.01 (A, B, D and E; ANOVA). Each dot in the scatter plots represents one sample.

Supplemental Figure 5A). As expected, nondiabetic db/m mice were easily distinguishable from db/db mice in the principal component analyses, irrespective of the intervention (Supplemental Figure 5B). Three further samples, two from the CHOP-ASO+ACEi group and one from the ACEi-only group, were excluded from further analyses due to low quality (Supplemental Figure 5B). When only comparing db/db mice, mice receiving CHOP-ASO were clearly distinguishable from control db/db mice and db/db mice receiving CON or ACEi alone (Supplemental Figure 5C).

Due to the low number of mice ($n=2$) within the CHOP-ASO+ACEi group, and the high congruence in gene expression of this group and the CHOP-ASO group, this group was excluded from further *in silico* analyses of the expression data. In db/db mice receiving CHOP-ASO, 500 genes showed similar expression to those in db/m mice (298 genes with induced expression [261 + 37] and 202 genes with suppressed expression [182 + 20]), reflecting genes normalized by CHOP-ASO intervention (Supplemental Figure 6A). In comparison, 120 genes (77 induced genes [37 + 40] and 43 suppressed genes [20 + 23]) in ACEi-treated mice were similar to those in db/m mice (Supplemental Figure 6A). The number of genes specifically regulated by CHOP-ASO ($n=10,19,535$ upregulated; 274 + 261 that overlap with db/m, 484 downregulated, 302 + 182 that overlap with db/m) was significantly larger than the number regulated specifically in response to ACEi ($n=13,587$ upregulated [40 + 47], 48 downregulated [23 + 25]; chi-squared [11,328]=156.58, $P=1.59e-36$). The larger number of genes regulated in response to CHOP-ASO compared with ACEi is congruent with CHOP being a transcriptional regulator. Importantly, the pattern of gene expression in CHOP-ASO-treated mice reflects a change in the direction of gene expression in db/m mice (in the sense of normalized gene expression).

After correcting for multiple testing, we searched for disease-related pathways on the basis of the differentially expressed genes. Although only one gene set (Fibromyxosarcoma) showed significant enrichment in mice that were treated with ACEi alone (Supplemental Table 1), 16 gene sets were identified for genes regulated by CHOP-ASO (Supplemental Table 1). The top ranked disease-related pathways regulated by CHOP-ASO were hyperglycemia, tubulointerstitial fibrosis, hypertensive disease, malignant neoplasm of liver, and pancreatic carcinoma (Supplemental Figure 6B and Table 1). Thus, the top pathways related to CHOP-ASO-regulated genes are also related to kidney and fibrotic diseases.

We next chose the top-ranked gene set for cluster analyses (hyperglycemia, Supplemental Figure 6C, only differentially regulated genes with an adjusted $P<0.0005$ are shown, Supplemental Table 2). In these analyses, db/m mice were again clearly distinguishable from db/db mice, and mice receiving CHOP-ASO alone were distinguishable from control db/db mice (PBS control or CON) and from

db/db mice receiving ACEi (Supplemental Figure 6C). Among the top genes downregulated by CHOP-ASO were SGLT2 (Slc5a2), the therapeutic target of the increasingly used SGLT2 inhibitors, and PROM1 (CD133), linked with tubular dedifferentiation after acute renal injury (Supplemental Figure 6C and Table 2). Suppression of SGLT2 by CHOP-ASO was confirmed by immunoblotting and immunohistochemistry (Supplemental Figure 7). Thus, inhibiting CHOP expression conveys a strong additional effect on top of ACEi with regard to renal gene expression, corroborating the functional and morphologic results shown above (Figures 2 and 3).

CHOP-ASO Suppresses Glucose-induced Cell Damage in Human Renal Cells

To determine whether the corresponding CHOP-ASO convey a protective effect in human renal cells, we exposed HKC-8, podocytes, and embryonic kidney 293 cells to high glucose, resulting in increased CHOP expression (Figure 6, A and B, and Supplemental Figure 8A). Although CON had no effect on CHOP expression, two independent CHOP-ASO efficiently GSH CHOP expression (Figure 6, A and B and Supplemental Figure 8A). Both CHOP-ASOs markedly GSH glucose-induced cell death, as identified by cleaved caspase-3 immunoblotting (Figure 6, A and B and Supplemental Figure 8A) and terminal deoxynucleotidyl transferase dUTP nick end labeling staining (Figure 6, C–E and Supplemental Figure 8, B and C), whereas the control oligonucleotide had no effect. To validate the data obtained from the unbiased analyses, we exemplarily determined the expression of renal genes regulated by CHOP-ASO. In addition to SGLT2, we analyzed PROM1 (CD133). In HKC-8 cells exposed to high glucose, we observed increased SGLT2 and PROM1 expression, which returned to normal levels on CHOP suppression with CHOP-ASO, whereas CON had no effect (Supplemental Figures 4, 6, and 9). Thus, similar to what was observed in diabetic mice, CHOP suppression reversed glucose 47–induced expression of selected genes and protected human renal cells from glucose-induced 48 cellular damage.

DISCUSSION

Our key finding is the feasibility of targeting CHOP with CHOP-ASO, which ameliorates DKD in mice, providing an added effect on top of ACE inhibition. Although a pathogenic role of unfettered, maladaptive signaling *via* the ER stress response has been demonstrated in experimental and clinical studies in patients with diabetes, therapies specifically targeting the maladaptive ER stress response have not been established. In this study, we show that targeting the ER stress-dependent transcription factor CHOP, which has largely been linked with maladaptive ER stress responses,

may be a suitable approach to prevent or reduce the consequences of unfettered, maladaptive ER signaling.

Previous preclinical studies using nonspecific interventions acting as ER chaperones suggested that targeting the ER stress response conveys protective effects against DKD and other diabetic complications.^{5,10,20,21} However, the protective effects observed using these nonspecific interventions may in part reflect “off-target” effects. TUDCA, for example, conveys additional effects *via* FXR-dependent signaling in the tubular compartment, and may hence be associated with both additional beneficial and adverse effects.⁹ This study establishes that specifically targeting the ER stress response by reducing CHOP expression is sufficient to convey protective effects in DKD. We observed protective effects of CHOP inhibition in both renal compartments (glomerular and tubular compartments) and in human renal cells (podocytes, HKC-8 cells, HEK cells). The protective effects observed in both compartments are in agreement with (1) the protective effects mediated by ER stress chaperones, and (2) the induction of CHOP in both renal compartments in DKD.^{5,6,9}

ASOs, which are single-stranded, synthetic nucleic acids, are designed to hybridize to specific RNAs or pre-mRNAs and ultimately modulate the production of a specific protein.²² The discovery of chemical modifications that increase stability and affinity to the target RNA, such as phosphorothioated backbones and bridged nucleic acids (*e.g.*, locked nucleic acid and constrained ethyl modifications), has allowed the development of highly potent molecules to treat an increasing number of diseases. ASOs can be administered systemically, for example, by intravenous or subcutaneous injection.^{23–25} By applying stringent specificity criteria during the design of ASOs, the risk of side effects caused by down-regulated expression of off-target genes can be minimized and allows for the development of therapeutic ASOs with a favorable safety profile. This study is, to our knowledge, the first to show that an ASO-based therapy impairs a driver (CHOP) of maladaptive ER signaling, ameliorating DKD. Importantly, inhibiting renal CHOP expression provided a benefit on top of ACE inhibition. The synergistic effect of ACE-inhibition and CHOP-ASO does not reflect an additional lowering of BP by CHOP-ASO. We suspect the prevention of cell death and altered gene expression, such as GSH SGLT2 expression, contribute to the synergistic effects. Accordingly, ASOs specific for human CHOP GSH glucose-induced CHOP expression and cell death of human renal cells *in vitro*. Collectively, these data suggest inhibiting CHOP in humans to target DKD may be feasible, which needs to be elucidated in future studies.

CHOP-ASO inhibited the expression of genes linked to renal physiology. For example, we observed an induction of CD133 in diabetic mice, which was GSH on CHOP-ASO intervention. Because CD133 is induced on renal damage and reflects renal repair,²⁶ we suspect its GSH expression in CHOP-ASO-treated mice reflects attenuated renal injury. Likewise, CHOP regulated SGLT2 expression, which is

known to be expressed by proximal tubular cells and to be induced in DKD.³ SGLT2 inhibitors have emerged as a new therapeutic option that not only improves the metabolic profile in patients who are diabetic, but also conveys strong renoprotective effects.^{3,27} Intriguingly, SGLT2 inhibition reduces ER stress in renal tubular cells.²⁸ In this study, we show the reverse also holds true: inhibition of CHOP reduces SGLT2 expression. It appears possible but remains to be shown that CHOP and SGLT2 expression are linked by a positive regulatory mechanism that can be disrupted by inhibiting either CHOP or SGLT2 expression. Because the SGLT2 promoter does not contain a *bona fide* CHOP binding site, the interaction is likely indirect. Given the clinical experience, we would expect a combined intervention with SGLT2 and ACE inhibition would likewise show synergistic renoprotective effects in diabetic mice. Yet, we speculate that such combination therapy would not mimic the same changes in gene expression as observed with CHOP-ASO, considering CHOP is a transcriptional regulator, regulating in addition to SGLT2 other genes in this study. Further studies are needed to determine how CHOP regulates SGLT2 expression, to what extent the renoprotective effect of CHOP-ASO depends on the observed reduction in SGLT2 expression, and to which extent combination therapies with ACE inhibition plus CHOP-ASO or SGLT2 inhibition differ.

The efficacy of ACE inhibition varied according to the disease stage at which it was administered in this study. If administered at an early stage (8-week-old db/db mice; no albuminuria), ACE inhibition protected the mice from renal damage as efficiently as CHOP inhibition, whereas initiating ACE inhibition at a later timepoint (16-week-old db/db mice; after establishment of albuminuria) was only partially protective. This variable effect of ACE inhibition in mice is congruent with previously published data, suggesting initiation of ACE inhibition at a later time is less effective, particularly with regard to the tubular compartment.^{9,19} The time-dependent protective effect of ACE inhibition reflects the clinical observation that ACE inhibition is able to halt, or even reverse, newly detected microalbuminuria, although at advanced stages of DKD, ACE inhibition delays but does not halt the progression of DKD.²⁹ Of note, protection after early (age 8 weeks) but not later (age 16 weeks) intervention with ACE inhibition was associated with GSH renal CHOP expression in this study (Figures 1B and 3, A–D), suggesting early ACE inhibition may protect against DKD, in part by inhibiting renal CHOP expression.

This study, like any, has limitations. Thus, due to restriction in the permission for *in vivo* mouse work, we were only able to use male mice in this study. However, we have previously observed renal CHOP induction in both sexes in diabetic mice, and hence assume CHOP-inhibition will benefit both sexes. Furthermore, in this study we used a mouse model of type 2 diabetes mellitus, but we, and

others, have previously reported induction of renal CHOP expression in both type 1 and type 2 diabetic mice and humans.^{5,30} On the basis of the current observation and these previous studies, we speculate that CHOP-ASO-based therapy may be a feasible therapeutic approach for both patients with type 1 and type 2 diabetes, which needs to be evaluated in future clinical studies.

As with any therapy, targeting CHOP may potentially elicit additional effects. Genetic deletion of CHOP not only ameliorates the development of DKD or the age-dependent deterioration of renal function⁶ (as also observed in this study) but also improves β cell survival in two independent murine diabetic models (Akita and db/db mice) and alleviates atherosclerosis in mouse models.^{31–33} An enhanced ER stress response is associated with obesity and has been suggested to contribute to metabolic syndrome.³⁴ Complete CHOP deficiency has partially divergent effects on obesity and insulin sensitivity in mice: in some studies, CHOP deficiency promotes obesity, hepatic steatosis, and adipose tissue inflammation,^{35,36} whereas in others, CHOP deficiency protects against weight gain and maintains insulin sensitivity.^{37,38} In contrast, chemical chaperones convey beneficial metabolic effects,^{21,39} suggesting partial suppression of the UPR stress response and CHOP with the goal of restoring ER homeostasis may convey beneficial metabolic effects. In this study, we did not observe changes in body weight, plasma alanine aminotransferase and aspartate aminotransferase levels, or blood glucose levels after interventions, suggesting that partial suppression of CHOP using an ASO-based strategy has no major metabolic effects.

DISCLOSURES

B. Isermann reports receiving research funding from Secarna Pharmaceuticals GmbH, Munich, Germany. F. Jaschinski, R. Klar, and S. Michel are employed by Secarna Pharmaceuticals GmbH & Co. K. Shahzad reports receiving research funding from Secarna GmbH. M. Blüher reports having consultancy agreements with Amgen, AstraZeneca, Bayer, Boehringer-Ingelheim, Lilly, Novartis, Novo Nordisk, Pfizer, and Sanofi; and reports receiving honoraria from Amgen, AstraZeneca, Bayer, Boehringer-Ingelheim, Lilly, Novartis, Novo Nordisk, Pfizer, and Sanofi. M.M. Al-Dabet reports receiving honoraria from Deutscher Akademischer Austauschdienst. N. Klötting reports receiving research funding from DFG SFB1052 Project 209933838. P.R. Mertens reports receiving honoraria from Amgen, BMS, Boehringer Ingelheim, Genzyme, Novartis, and Roche; reports having patents and inventions including p18 protein as marker for inflammatory disease, Midkine as a marker for cardiovascular disease; and reports being a scientific advisor or member of Clinical Nephrology.

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M. Al-Dabet, S. Fatima, and I. Gadi performed the *in vivo* experiments. S. Ambreen assisted in histological analysis. A. ElWakiel and H. Khawaja assisted in *ex vivo* analyses. B. Isermann and K. Shahzad conceptually designed, interpreted the experimental work, and prepared the manuscript. F. Jaschinski conceptually designed the project. M. Blüher, N. Klötting, P. Mertens, and P. Nawroth provided reagents and critically discussed the data. R. Klar conceptually designed and assisted in manuscript preparation. S. Michel conducted RNA-sequencing data analysis and performed statistical analysis.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at <http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2021040431/-/DCSupplemental>.

Supplemental Material and Methods.

Supplemental Figure 1. Xxxxx.

Supplemental Figure 2. Xxxxx.

Supplemental Figure 3. Xxxxx.

Supplemental Figure 4. Xxxxx.

Supplemental Figure 5. Xxxxx.

Supplemental Figure 6. Xxxxx.

Supplemental Figure 7. Xxxxx.

Supplemental Figure 8. Xxxxx.

Supplemental Figure 9. Xxxxx.

Supplemental Table 1. Xxxxx.

Supplemental Table 2. Xxxxx.

Major Resource Table.

Supplemental References.

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