Ursodeoxycholic acid ameliorated diabetic nephropathy by attenuating hyperglycemia-mediated oxidative stress

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Abstract

Oxidative stress has a great role in diabetes and diabetes induced organ damage. Endoplasmic reticulum (ER) stress is involved in the onset of diabetic nephropathy. We hypothesize that ER stress inhibition could protect against kidney injury through anti-oxidative effects. To test whether block ER stress could attenuate oxidative stress and improve diabetic nephropathy in vivo and in vitro, the effect of ursodeoxycholic acid (UDCA), an ER stress inhibitor, on spontaneous diabetic nephropathy db/db mice, ER stress inducer or high glucose-triggered podocytes were studied. Mice were assigned to 3 groups (n=6 per group): control group (treated with vehicle), db/db group (treated with vehicle), and UDCA group (db/db mice treated with 40 mg/kg/day UDCA). After 8 weeks treatment, mice were sacrificed. Blood and kidneys were collected for the assessment of albumin/creatinine ratio, blood urea nitrogen (BUN), serum creatinine (SCr), insulin, total cholesterol, triglyceride, low density lipoprotein cholesterol (LDL-C), oxidized LDL-C, high density lipoprotein cholesterol (HDL-C), non-esterified fatty acid (NEFA), superoxide dismutase (SOD), catalase (CAT), methane dicarboxylic aldehyde (MDA), the expressions of SOD isoforms and glutathione peroxidase 1, as well as histopathological examination. In addition, generation of reactive oxygen species (ROS) was detected by 2’7’-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence. The results showed that UDCA alleviated renal ER stress-evoked cell death, oxidative stress, renal dysfunction, ROS production, upregulated the expression of Bcl-2 and suppressed Bax in vivo and in vitro. Hence, inhibition ER stress diminishes oxidative stress and exerts renoprotective effects.
Keywords

diabetic nephropathy; podocytes; oxidative stress; reactive oxygen species
Introduction

Diabetic mellitus is a global health problem in which blood glucose is persistently elevated and generates a cascade of events in organ including kidney.\textsuperscript{1,2}) The population with diabetes has been increasing worldwide and diabetic nephropathy (DN) now is a leading cause of end-stage renal failure.\textsuperscript{3})

The mechanisms by which hyperglycemia contributes to kidney remain limited due to various factors modulate the plasma glucose in the body. The management of DN is based on the control of plasma glucose levels.\textsuperscript{4}) Reviewing number of studies demonstrates that reactive oxygen species (ROS), associated with increased plasma glucose, has been implicated in the pathogenesis of DN, which results in the over-production of extracellular matrix proteins, mitochondrial damage and glomeruli injury.\textsuperscript{5-8}) Therefore, oxidative stress attenuation is an important pathway in DN prevention.\textsuperscript{9})

The endoplasmic reticulum (ER) regulates the folding of secretory and intracellular calcium.\textsuperscript{10}) Excessive unfolded proteins in the lumen of ER produces stress and contributed to the disorder of intracellular signal transduction pathways.\textsuperscript{11}) A number of pathophysiological conditions are associated with ER stress included diabetes. ER stress is a key mediator of $\beta$-cell apoptosis.\textsuperscript{12}) ER stress-related proteins were highly enhanced in the renal fibrosis of fibrotic kidney.\textsuperscript{13}) High glucose-induced apoptosis via ER stress in primary cultured rat mesangial cells.\textsuperscript{14}) Therefore, ER stress inhibitor seems to be a promising strategy for DN prevention.

Ursodeoxycholic acid (UDCA) is a chemical chaperone, known to improve ER folding capacity. It was reported that UDCA alleviated the pathogenesis of diabetic peripheral.\textsuperscript{15}) But
it is unknown whether it could protect against DN through oxidative stress. In this study, we hypothesized that UDCA protect against DN due to antioxidant effects. In order to verify the renal protective effect of UDCA, we evaluated the lipid-lowering and anti-oxidant effects using a spontaneous diabetic nephropathy model, ER stress inducer and high glucose-triggered podocytes.

**Materials and Methods**

**Drugs and Animals**

UDCA was obtained from Santa Cruz Biotechnology (Sigma-Aldrich, St. Louis, Missouri, USA). Before use, they were solubilized in DMSO (50 mM) for in vitro study and in 0.5 % carboxymethyl cellulose (CMC) solution for in vivo study. Six-week-old male non-diabetic db/m and diabetic db/db mice (C57BLKS/J-lepr<sup>db</sup>/lepr<sup>db</sup>) were obtained from Model Animal Research Center of Nanjing University. The experimental protocol was approved by the Ethics Committee of Putuo Hospital, Shanghai University of Traditional Chinese Medicine. The mice were given free access to food and water and maintained in conditions of 12/12 h light-dark cycle and controlled temperature (23 ± 2 °C) and humidity (55 ± 5 %).

**DN Model and Experimental Design**

The db/db mouse is the leading model of type 2 diabetes and DN. Db/db mice develop obesity, increased blood glucose levels and albumin since a genetic mutation in the leptin receptor gene. At 8 weeks of age, mice were divided into 3 groups. Group 1 consisted of non-diabetic control db/m mice (n=6), group 2 consisted of db/db mice as a diabetic
nephropathy group (n=6), group 3 consisted of db/db mice that were treated via intragastrically administration with 40 mg/kg /day UDCA (n=6) for eight weeks. Mice in group 1 and 2 were intragastrically administration with vehicle (0.5 % CMC). At the end of the experiment, mice were euthanized with pentobarbital sodium (50 mg/kg) and the kidneys were removed, washed in saline, decapsulated, blot dried. One kidney was fixed with 10 % formalin for histopathology examination and the left one was stored at -80 °C for kidney homogenate preparation. Blood samples were collected and centrifuged to obtain serum. Both urine and serum were stored at -80 °C for further study.

Histological examination

Hematoxylin and eosin (H&E) staining on the 3 μm paraffin embedded kidney sections was performed by routine histopathology procedures and observed through microscope to assess glomerular, tubular and vascular changes.

Immunohistochemistry was performed as previously reported.\textsuperscript{17} Paraffin-embedded sections of kidneys were incubated with GRP78 (ab28615, 1:1000 dilution, Abcam, Cambridge, UK). The integrated optical density was measured by computer analysis with Image-Pro Plus 6.0 (Media Cybernetics, Maryland, USA)

A TUNEL assay was performed with the In Situ Cell Death Detection kit (Millipore, Massachusetts, USA) according to the manufacturer’s instructions. Apoptotic cells with nuclei staining green were counted by fluorescence microscopy. All slides were observed without the observer knowing the experimental groups.
Biochemical assays

_Determination of blood glucose, BUN, SCr, insulin, total cholesterol, triglyceride, NEFA, HDL-C, LDL-C and oxidized LDL-C._

One touch blood glucose meter (OMRON, Kyoto, Japan) was used to assess fasting blood glucose. Automatic biochemical analyzer (Hitachi Model 7600, Japan) was employed to measure BUN, albumin/creatinine ratio, total cholesterol and triglyceride. Serum creatinine was determined using creatinine assay kit (ab65340, Abcam, Cambridge, UK). Insulin was detected using ELISA Kit (EZRMI-13K, Millipore, USA). NEFA (A042-2), HDL-C (A112-1), LDL-C (A113-1) and oxidized LDL-C (H248) were all obtained from Nanjin Jiancheng Bioengineering Institute (Nanjing, China) and performed according to the instructions of the manufacturer.

_Total protein_

Kidney cortex or cultured podocytes were lysed in RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) with a sonicator and then centrifuged at 12,000 g for 5 min at 4 °C. Total protein concentration from the supernatant was determined by the Bicinchoninic acid (BCA) protein assay (Boster Biotechnology, Wuhan, China).

_MDA, SOD and CAT_

MDA (10009055), SOD (706002) and CAT (707002) levels in kidney were assayed according to the instructions of the manufacturer (Cayman Chemical Company, Ann Arbor, MI, USA).
Cell culture and treatment

Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (Division of Nephrology, Massachusetts General Hospital, Harvard University) and were conducted as previously described. Podocytes (between passage 20 and 25) were incubated at 33 °C with 10 U/ml mouse recombinant interferon-γ (IFN-γ, Peprotech, Rocky Hill, NJ, USA), 10 % heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in RMPI 1640 for proliferation. At confluence podocytes were incubated at 37 °C for 14 days deprived of IFN-γ. Differentiated podocytes were cultured for 24 h in RMPI 1640 with 1 % FCS before various experimental conditions.

Cell viability assay

Podocytes were plated onto a 96-well dish in triplicate and incubated overnight. UDCA (50 μM) or Edaravone (Eda, 100 μM, ab120645, Abcam, Cambridge, UK) were pretreated for one hour then stimulated with 50 μM tunicamycin (Tu, Abcam, Cambridge, UK) for 24 h. At the end of the experiment, CCK8 (Dojindo, Maryland, MD) was added and incubated for 1 h at 37 °C. Absorbance was quantified at 450 nm on a multiwell fluorescence plate reader (Thermo Scientific Varioskan Flash, USA). Cell viability was calculated as as follows: \([1-A_{450 \text{ (experimental)}}/A_{450 \text{ (control)}}] \times 100\%\).

Immunofluorescence staining

Approximately 2×10⁵ cells were seeded in glass bottom cell culture dish. At the end of various experimental conditions, they were fixed and permeabilized and incubated with
primary antibody (GRP78, ab28615, 1:1000 dilution, Abcam, Cambridge, UK). To observe the expression of antibody, the plates were washed and stained with FITC-conjugated secondary antibodies. DAPI was added as a nuclear counterstain and visualized using a fluorescence microscope (Olympus, Japan).

ROS production.

ROS generation was measured according to previously described. Briefly, podocytes were seeded into 96-well plates, incubated for 24 h and subjected to different stimulation. Then podocytes were incubated for 45 min at 37 °C with 50 μM DCFH-DA (Invitrogen, California, USA) and detected at excitation wavelengths of 488 nm and emission wavelengths of 520 nm with a multiwell fluorescence plate reader (Thermo Scientific Varioskan Flash, USA).

Western blot

40-80 μg total protein samples were separated by 8-15% electrophoresis and electro-transferred to PVDF membranes. The membranes were blocked with 5% BSA for 1 h and incubated with Bax (2772, 1:1000 dilution, Cell Signaling Technology, USA), Bcl-2 (2870, 1:1000 dilution, Cell Signaling Technology, Massachusetts, USA), Bip (ab28615, 1:1000 dilution, Abcam, Cambridge, UK), phospho-IRE1α (NB100-2323, 1:1000 dilution, Novus Biological, Colorado, USA), cleaved ATF6 (ab11909, 1:1000 dilution, Abcam, Cambridge, UK), phospho-PERK (MA-15033, Thermo Fisher Scientific, Massachusetts, USA) and CHOP (2895, 1:1000 dilution, Cell Signaling Technology, Massachusetts, USA), SOD1
(ab13498, 1:1000 dilution, Abcam, Cambridge, UK), SOD2 (13141, 1:1000 dilution, Cell Signaling Technology, USA), SOD3 (SC-32220, 1:500 dilution, Santa Cruz Biotechnology, California, USA), glutathione peroxidase 1 (ab108427, 1:1000 dilution, Abcam, Cambridge, UK) and β-actin (ab8226, 1:1000 dilution, Abcam, Cambridge, UK) overnight at 4 °C. Then washed in TBST (Tris buffered saline containing 0.1% Tween 20) and incubated with horseradish peroxidase-conjugated secondary antibodies in 5 % BSA for 1 h. After washed, bands were detected by Bio-Rad VersDoc imaging system using chemiluminescence detection reagents (Thermo Fisher Scientific, USA) and analyzed with Bio-Rad Quantity One software.

Statistical Analysis

Data were analyzed by one-way ANOVA and subsequent Newman-Keuls testing for multiple comparisons using GraphPad Prism 5 software. Data were expressed as mean ± SD. Values of $P < 0.05$ were considered significant.

Results

UDCA ameliorated renal histopathology and function.

To evaluate the role played by UDCA in diabetic mice, we compared body weight and blood glucose levels in non-diabetic and diabetic mice. The baseline bodyweight, at the age of eight weeks, was no difference in both UDCA-treated and untreated mice. Baseline blood glucose level in db/m mice was significantly lower than diabetic mice. Untreated db/db mice increased gradually in body weight and blood glucose and insulin levels. UDCA treatment
decreased body weight and blood glucose and insulin (Table 1). Meanwhile, diabetic db/db mice presented significantly increased levels of Scr, BUN and albumin/creatinine ratio compare with non-diabetic db/m mice (Table 1). However, these parameters were all decreased in diabetic mice treated with UDCA (Table 1). In addition, as shown in Figure 1A, no histological alterations was observed in the kidney of non-diabetic db/m mice, stained with hematoxylin and eosin and examined microscopically. A variable degree of mesangial expansion and an increase in the total glomerular size in db/db mice were observed (glomerular size 4345±631 μm² vs 5892±428 μm², respectively). UDCA treatment significantly reduced the extent of mesangial expansion and total glomerular area (glomerular size 5204±321, Fig.1A). Thus, UDCA can attenuate the levels of renal function parameters.

**UDCA inhibited renal apoptosis**

To determine whether UDCA has an anti-apoptotic effect in vivo, we assessed apoptosis by TUNEL staining. In db/db mice, apoptotic cells were markedly increased compared with control group in kidney sections. When UDCA group was compared with vehicle-treated db/db group, the number of apoptotic cells was significantly decreased (Fig. 1B).

**UDCA attenuated ER stress in the diabetic kidney.**

To explore whether UDCA could inhibit ER stress in damaged diabetic kidney, we next observed the expression levels of ER stress marker using immunohistochemical staining. The activation of ER stress was assessed by measuring GRP78, a stress-induced molecular chaperone in the ER. Diabetic kidney displayed significantly increase in GRP78 when
compared to non-diabetic controls, in both the glomeruli and tubules (Figure 2A, B and C). UDCA attenuated the activation of GRP78 in the glomeruli and tubules. Meanwhile, molecules related to ER stress, including Bip, phospho-IRE1α, cleaved ATF6, phospho-PERK and CHOP, were also enhanced in db/db mice. Treatment with UDCA resulted in the decrease in these protein expressions (Figure 2D and E).

*Plasma lipid levels.*

The lipid levels of total cholesterol, triglycerides, NEFA, HDL-C, LDL-L and oxidized LDL-C in diabetic, non-diabetic mice and ER stress therapy mice were presented in Figure 3. The total cholesterol levels of UDCA-treated mice (3.59±0.63 mM) were lower by 24% than those of vehicle-treated db/db mice (4.47±0.72 mM). UDCA decreased triglycerides (1.45±0.19) by 43%, compared with db/db group (2.07±0.27 mM). In addition, NEFA, LDL-C and oxidized LDL-C was decreased from 1.45±0.65 mM, 3.62±0.71 mM, 0.48±0.03 μg/ml in db/db mice to 0.51±0.24 mM, 2.53± 0.67, 0.28±0.08 μg/ml in UDCA-treated mice, respectively. However, no significant changes were observed in HDL-C among the three groups.

*Effect of UDCA on renal anti-oxidation status.*

The activities of antioxidant enzymes and MDA reflect the state of oxidative stress. The effect of UDCA on activities of antioxidant enzymes in kidney are shown in Figure 4A&B. Renal SOD and CAT activities were decreased in untreated db/db mice. CAT activity increased from 13.97±0.58 U for diabetic control group to 17.43±1.87 U for UDCA treated
mice. SOD activity increased from 29.96±2.20 U for diabetic control group to 39.58±4.93 U. MDA, end product of lipid peroxidation, was decreased from 25.66±1.19 nM for diabetic control group to 22.16±1.15 nM (Figure 4C). Furthermore, western blot analysis among the three groups revealed remarkably reduced SOD1, SOD3 and glutathione peroxidase downregulation, which were observed in db/db mice. Reduction in SOD1, SOD3 and glutathione peroxidase were alleviated in UDCA-treated diabetic mice (Figure 4D, E and F). These data suggested the antioxidant capability of UDCA against oxidative stress in DN.

**Effect of UDCA on ER stress-evoked cell death and ROS generation in podocytes.**

As podocyte is the components of glomerular filtrating barrier and podocyte injury was the onset of DN, we chose podocyte in our in vitro study. As shown in Figure 5A, 50 μM Tu incubation, indicated prolonged ER stress stimulation, led to podocyte death. Pretreated with UDCA or a radical scavenger, Eda, partly prevented Tu-induced cell injury. ROS generation was also inhibited by UDCA or Eda (Figure 5B). In order to confirm these effects were associated with ER stress inhibition, western blot was performed and results showed both UDCA and Eda blocked the enhanced expression of ER stress markers, which was stimulated by Tu (Figure 5C and D).

**Effect of UDCA on high glucose-induced ROS production and ER stress in podocytes.**

High glucose was reported to generate ROS and trigger podocyte apoptosis. We observed the effects of UDCA on high glucose-induced ROS generation in podocytes. As displayed in Figure 5E, 30 mM high glucose exaggerated ROS production. Exposure to
mannitol for 24 h did not change the level of ROS. However, UDCA partially reduced HG-induced ROS production. Next, we explored the possibility that UDCA could inhibit ER stress induced by high glucose. As shown in Figure 5F, immunofluorescence analysis showed the activation of GRP78 in 30 mM d-glucose-incubated podocytes. UDCA significantly abolished high glucose-triggered GRP78 expression. In addition, high glucose-increased protein expressions of Bip, phospho-IRE1α, cleaved ATF6, phospho-PERK and CHOP were also inhibited by UDCA (Figure 5G and H).

*UDCA restored the balance of Bax and Bcl-2 expression in diabetic mice and podocytes.*

Having shown that UDCA was anti-oxidant in vivo, we next sought to determine the possible mechanism for this. The role of Bcl-2 and Bax protein were investigated. As shown in Figure 6A and B, high glucose triggered changes in Bax and Bcl-2 protein were prevented by UDCA. A striking reduction in Bcl-2 and enhancement in Bax expressions were detected in diabetic kidneys from untreated db/db mice. UDCA treatment restored the expressions of Bax and Bcl-2 (Figure 6).

**Discussion**

UDCA, clinical used in urea cycle disorders,\(^{21}\) has been shown to be a reliable ER stress inhibitor.\(^{22}\) However, the effect of UDCA on diabetic nephropathy has remained largely unknown. In the present study, we provide the verification for the protective actions of UDCA on diabetic nephropathy. We analyze db/db mice, a genetic model of type 2 diabetes with obesity and insulin resistance and diabetic nephropathy.\(^{23}\) We proved that UDCA has
beneficial effects in ameliorating lipid levels, renal function and its remarkable anti-oxidative role at least in part contributed to regulate these effects in db/db mice. In addition, we also demonstrated that UDCA reduced ER stress-evoked cell death, ROS production in podocytes. ROS inhibition also alleviated ER stress and podocyte injury. Furthermore, we found that the balance of Bcl-2 and Bax was regulated by UDCA in vivo and in vitro.

Intracellular and extracellular stimuli including hyperglycemia, hypoxia, oxidative stress, oxidized lipids, nutrient deprivation can trigger ER stress. Induction of ER stress is associated with diabetic and its complications. The db/db mouse is widely used in diabetes and its complications research. Suppressing ER stress alleviated obesity-induced hepatic insulin resistance, regulated β-cell death and inhibited MCP-1 expression in the kidneys of db/db mice. In this study, UDCA improved blood glucose and insulin level and attenuated the glomerular lesions in the kidney of db/db mice. To confirm these effects was due to ER stress, some important molecular indicators of ER stress were assessed. UDCA abolished the activation of key ER stress markers of diabetic mice in glomeruli and tubules. These results suggest UDCA improves the pathogenesis of DN by ER stress inhibition.

Recent evidence suggests inhibiting oxidative and lipid-mediated ER stress alleviated DN. Diabetes was associated with profound alterations in lipid and lipoprotein profiles. Oxidized lipids also play a vital role in the pathogenesis of diabetic nephropathy. We observed that the levels of blood glucose, total cholesterol and triglyceride in diabetic group were dramatically elevated when compared with the control group. Oral administration of UDCA significantly showed hypoglycemic, hypotriglyceridemic and hypocholesterolemic effects on db/db mice with a significant reduction in plasma blood glucose, total cholesterol
and triglyceride, NEFA, LDL-C and oxidized LDL-C. In addition, diabetic nephropathy is one of the major microvascular complications of diabetes mellitus. The development of DN in db/db mice was confirmed by significant elevation of serum creatinine, urea nitrogen, urinary albumin/creatinine ratio and kidney injury. UDCA alleviated the kidney function and morphological changes in db/db mice, suggesting prevention of diabetic complications by ER stress inhibitor.

Oxidative stress plays a vital role in the DN pathogenesis. Hyperglycemia induced the imbalance between the generation of ROS and the compensatory response from the endogenous antioxidant network. Prolonged ROS leads to renal dysfunction and histologic real injury. The histopathology result of this study confirmed the renal damage in diabetic mice as clear pathological changes were observed. UDCA managed to prevent this damage, confirming the renoprotective effect through ER stress blocker. Podocyte, one of the components of glomerular filtration barrier, was associated with the heavy proteinuria state. ER stress inducer, Tu, damaged podocyte and showed enhanced ROS production. However, Eda, an ROS inhibitor, prevented Tu-triggered cell death and ROS generation. The decreased expression of Bip, phospho-IRE1α, cleaved ATF6, phospho-PERK and CHOP in Eda-incubated podocyte indicated that ER stress-induced cell death was related to ROS production. In addition, significant decrease in ROS production and ER stress was observed in high glucose-triggered podocytes after UDCA treatment. These results suggested the ER stress activation was accompanied by ROS generation in podocytes.

Furthermore, oxidative damage, resulted from the accumulation of free radicals, was increased in DN. In our study, the level of renal MDA was increased in untreated diabetic
mice compared to normal control group. UDCA treatment demonstrated its anti-lipid peroxidation activity. Meanwhile, antioxidant enzymes are the first line of defense against oxidative damage. Superoxide radicals formed by DN are converted into hydrogen peroxide (H₂O₂) and then converted to water by CAT. Here, there was a decrease in SOD and CAT activities and glutathione peroxidase 1 expression in the diabetic mice compared with db/m mice. ER stress inhibitor, UDCA, increased the activities of CAT and SOD in kidney tissue.33) Interesting, the expression of SOD isoforms in the kidney was various in diabetic and non-diabetic mice. We observed down-regulation of SOD1 and SOD3 in the kidney of diabetic mice. UDCA treatment increased the expression of SOD1 and SOD3. Therefore, the preventive effects of UDCA on renal dysfunction are at least partly attributed to the inhibition of oxidative stress.

Mitochondria are the primary intracellular source of ROS and mitochondria injury leads to cell death including apoptosis and necrosis. Bcl-2 protein family, target genes of NF-κB, regulates cell death.10) Bax, a pro-apoptotic gene, induces apoptosis by the release of mitochondrial proapoptotic factors such as cytochrome C, leading to caspase activation and subsequent apoptosis. On the other hand, Bcl-2 prevents the release of mitochondrial proapoptotic factors.34) The imbalance between Bcl-2 and Bax were observed in high glucose-treated podocytes and diabetic db/db mice. UDCA, however, significantly restored these changes promising their potential anti-apoptotic property by suppressing mitochondrial pathway of apoptosis.

To conclude, the results of our study demonstrate that treatment with ER stress inhibitor, UDCA reduced the occurrence of oxidative stress and exhibits renal protection in diabetic
mice and in podocytes. Relief of ER stress could be the DN therapy in the future.

Acknowledgements

This study was supported by Innovation Program of Shanghai Municipal Education Commission (14ZZ118); Leading Academic Discipline Project of State Administration of Traditional Chinese Medicine of China, Talent Project of Integrative Medicine of Shanghai Municipal Health Bureau (ZYSNXD012-RC-ZXY); Key Medical Discipline Project of Shanghai Municipal Health Bureau (ZK2012A34) and the National Natural Science Foundation of China (81400728, 81473480).

Conflict of interest

The authors declare no conflict of interest.
References


8) Marrero MB, Banes-Berceli AK, Stern DM, Eaton DC. Role of the JAK/STAT


**Figure legends**

Fig. 1. UDCA ameliorated the renal histopathology. (A) Representative photomicrographs of kidney injury from db/m, vehicle-treated db/db, UDCA-treated db/db mice. Hematoxylin and eosin stain. Calibration bar=20 μm. Original magnification, ×400. (B) The effect of UDCA treatment on renal apoptosis. Representative pictures of kidney sections stained for TUNEL. Scale bars, 50 μm; original magnification, ×200 (n=6).

Fig. 2. UDCA inhibited ER stress in the kidneys of diabetic db/db mice. (A) The bioactivity of GRP78, an ER stress marker, was assessed by immunostaining. Diabetes induced a significant increase in the bioactivity of GRP78 in both the glomeruli and tubules of the kidney. (B) and (C) Quantification of integrated optical density (IOD) for GRP78 in kidney sections from db/m mice, db/db mice and db/db mice treated with UDCA. Scale bars, 20 μm; original magnification, ×400. ER stress markers were activated in db/db mice and down-regulated by UDCA treatment. (D) Representative immunoblots and quantification of key ER stress molecules (E) in Bip, phospho-IRE1α, cleaved ATF6, phospho-PERK and CHOP. **P<0.001, db/m compared to db/db mice, ##P<0.001, db/db compared to UDCA treated mice (n=6).

Fig. 3. Effect of UDCA treatment on plasma lipid levels in diabetic db/db mice. (A) Total cholesterol. (B) HDL-C. (C) Triglycerides. (D) LDL-C. (E) NEFA. (F) Oxidized LDL-C. *P<0.05, **P<0.001, db/m compared to db/db mice, #P<0.05, ##P<0.001, db/db compared to UDCA treated mice (n=6).
Fig. 4. Effect of UDCA on oxidative stress parameters. (A) Renal CAT activity. (B) Renal SOD activity. (C) Renal MDA activity. (D) Representative immunoblots and quantification of SOD1, SOD2, SOD3 and glutathione peroxidase 1 (E) among the three groups. *P<0.05, **P<0.001, db/m compared to db/db mice, #P<0.05, ##P<0.001, db/db compared to UDCA treated mice (n=6).

Fig. 5. UDCA improved ER stress-evoked injury and ROS generation in podocytes. (A) UDCA and Eda, a free radical scavenger, partly blocked Tu, an ER stress inducer, triggered cell death, (B) ROS production, and (C) ER stress activation. (D) Quantification of Bip, phospho-IRE1α, cleaved ATF6, phospho-PERK and CHOP. Cells were pre-incubated with 50 μM UDCA or 100 μM Eda and then stimulated with 50 μM Tu for 24 h. (E) UDCA reduced 30 mM d-glucose induced podocytes ROS generation. In addition, UDCA down-regulated high glucose-evoked ER stress as observed by the reduced GRP78 expression by immunofluorescence staining (F) and representative immunoblots (G) and quantification of key ER stress molecules (H) in Bip, phospho-IRE1α, cleaved ATF6, phospho-PERK and CHOP. Cells were divided into normal glucose group (5 mM d-glucose), mannitol group incubated in normal glucose group with 25 mM d-mannitol, high glucose (HG) group incubated in RPMI 1640 containing 30 mM d-glucose for 24 h. Scale bars, 100 μm; original magnification, ×200 (A) and (B) **P<0.001, compared with 50 μM tunicamycin for 24 h. (D) (E) and (H) **P<0.001, compared with control group, ##P<0.001, compared with Tu or 30 mM d-glucose group.
Fig. 6. Effect of UDCA on the expression of Bax and Bcl-2 in kidney and podocytes. (A) Representative western blot of Bax and Bcl-2 in 30 mM d-glucose-induced podocytes. (B) Relative protein expressions. **$P<0.001$, compare with control group, ##$p<0.001$, compared with 30 mM d-glucose group. (C) Representative western blot of Bax and Bcl-2 in db/m, db/db, UDCA-tread db/db mice. (D) Relative protein expressions. **$P<0.001$, db/m compared to db/db mice, ###$P<0.001$, db/db compared to UDCA treated mice (n=6).
Fig. 2

A negative control  db/m  db/db  UDCA

Glomeruli

Tabules

B

C

D

E

Bip(78 kDa)  phospho-IRE1α (130 kDa)  cleaved ATF6 (36 kDa)  phospho-PERK (170 kDa)  CHOP (27 kDa)  β-actin (42 kDa)

db/m  db/db  UDCA

Relative protein level

Biological and Pharmaceutical Bulletin Advance Publication
Fig. 3

A

Total cholesterol (mM)

B

HDL-C (mM)

C

Triglyceride (mM)

D

LDL-C (mM)

E

Non-esterified fatty acids (mM)

F

Oxidized LDL (μg/mL)
Fig. 4

A

CAT (U/mg protein)

B

SOD (U/mg protein)

C

MDA (μM/mg protein)

D

SOD1 (18 kDa)
SOD2 (22 kDa)
SOD3 (32 kDa)
Glutathione peroxidase 1 (32 kDa)
β-actin (42 kDa)

E

Relative protein level

F

Glutathione peroxidase 1/β-actin
Table 1. UDCA attenuated metabolism abnormalities

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<th>db/db (n=6)</th>
<th>UDCA (n=6)</th>
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<td>Baseline body weight (g)</td>
<td>22.70±1.08</td>
<td>24.08±0.79</td>
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<td>Baseline blood glucose (mM)</td>
<td>6.67±0.82</td>
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<td>Body weight after 8 weeks treatment (g)</td>
<td>26.70±1.37</td>
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<td>Blood glucose after 8 weeks treatment (mM)</td>
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<td>Scr (μM)</td>
<td>50.38±14.83</td>
<td>121.20±13.02**</td>
<td>66.91±35.80##</td>
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<td>BUN (mM)</td>
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<td>8.43±0.97**</td>
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<td>Albumin/Creatinine ratio (μg/mg)</td>
<td>17.03±15.67</td>
<td>173.22±68.74**</td>
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<td>Serum Insulin (ng/ml)</td>
<td>2.05±0.85</td>
<td>9.02±0.76**</td>
<td>4.87±0.35##</td>
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Diabetic db/db mice were treated with UDCA for 8 weeks between 8 and 16 weeks of age. Scr: serum creatinine. BUN: blood urea nitrogen. **P<0.001 vs. db/m; #P<0.05, ##P<0.001 vs. db/db (n=6).