Klotho Deficiency Aggravated Diabetes-induced Podocyte injury

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Research

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Abstract

Background: Diabetic nephropathy (DN) is a progressive disease, the main pathogeny of which is podocyte injury inducing glomerular filtration barrier and proteinuria. The occurrence and development of DN could be partly attributed to the reactive oxygen species (ROS) generated by mitochondria. However, researches on how mitochondrial dysfunction (MtD) ultimately causes DNA damage is poor.

Methods: We generated streptozotocin (STZ)-induced diabetic mice with wild-type(C57BL/6J) or Klotho deficiency mice ($KL^{+/-}$) and treated podocytes with high glucose (HG) to investigated the function of Klotho on HG-induced podocyte injury *in vivo* and *in vitro*.

Results: The absence of Klotho aggravated diabetic phenotypes indicated by podocyte injury accompanied by elevated urea albumin creatinine ratio (UACR), creatinine, urea nitrogen. Then, Klotho deficiency could significantly aggravate DNA damage by increasing 8-OHdG and reducing OGG1. Finally, Klotho deficiency may promote MtD to promote 8-OHdG-induced podocyte injury.

Conclusions: Klotho deficiency may promote diabetes-induced podocytic MtD and aggravate 8-OHdG-induced DNA damage by affecting OOG1.

Background

The incidence and prevalence of diabetes mellitus (DM) have grown significantly throughout the world due to the overall increase in type 2 diabetes.

As one of the most frequent complications of DM, diabetic kidney disease (DKD) defined as a syndrome, is the most common metabolic disease in the world that is the leading cause of end-stage renal disease (ESRD) comprising about 40% of patients with CKD[1,2]. The onset of clinically overt DKD is defined as persistent proteinuria that is most closely associated with podocytopathies that results from damage to the glomerular filtration barrier at the level of the highly differentiated glomerular podocyte cells[3 – 5]. Despite the status quo, the factors that precipitate the development and progression of diabetic kidney disease (DKD) remain to be fully elucidated. Moreover, the molecular mechanisms leading to proteinuria and podocyte effacement are poorly understood.

DM is characterized by increased levels of reactive oxygen species (ROS) leading to high levels of adenosine triphosphate (ATP)[6]. Nevertheless, mitochondria are the energy powerhouses of cells by ATP synthesis through oxidative phosphorylation (OXPHOS) and play a key role in apoptosis. Mitochondria also attaches great importance to renal function, of which dysfunction is becoming increasingly recognized as contributing to renal glomerular and tubular diseases[7 – 11]. Besides, tubular epithelial cells are mitochondrial rich. Mitochondria dysfunction (MtD) and reactive oxygen species(ROS)-induced damage is also well reported in glomerular podocytes, as they are highly specialized and terminally differentiated epithelial cells[12]. As limited replicative capability of regeneration, podocytes injury may represent the major mechanism of progressive renal damage. Remarkably, mitochondrial electron

transport chain is identified as the major non-enzymatic source of diabetes-induced ROS in podocytes, that are believed to cause the onset of albuminuria followed by progression to renal damage through podocytes depletion[12]. MtD is usually accompanied by a reduction in the efficiency of the DNA repair capacity and antioxidant defense, consequently leading to the accumulation of cellular damage[13,14]. DNA continually exposed to exogenous and endogenous stressors will lead to DNA breaks, damage or improperly repair of which can activate pro-apoptotic pathways, or induce cellular senescence[14,16]. The most frequently formed oxidative DNA damage is 8-hydroxydeoxyguanosine (8-OHdG), that could be repaired by base-excision repair (BER) system. 8-oxo-deoxyguanosine DNA glycosylase 1 (OGG1), an enzyme that is involved in the process, is associated with the DNA repair activity and decreased risk for some oxidative stress-related diseases[17,18]. Cells have developed complex DNA damage signaling and repair mechanisms, that were collectively called DNA damage response (DDR)[15,16]. DNA damage also plays an important role in the development of DM and its complications[19,20], but deep molecular mechanisms remains to be studied.

α-Klotho, also known as Klotho, which predominantly produced in renal tubular epithelial cells, regulates ageing-related processes existing in membrane-bound and soluble forms[21]. Klotho may function as a humoral factor, can attenuate the development of hyperglycemia in mice challenged with DM[22]. Whether Klotho participates in regulating DNA damage to protect from MtD-induced injury of podocytes remains unknown.

In this study, our data showed that Klotho may aggravate MtD by affecting ROS and DNA damage by inhibiting OGG1 expression in diabetes-induced podocytes.

Materials

Animals

Male C57BL/6 mice were purchased from Beijing HFK Biologic Technology (Beijing, China); α-Klotho deficiency ($KL^{+/-}$, C57BL/6) mice generously provided by Army Military Medical University (Chongqing, China), were generated by mating pairs of heterozygous Klotho mice ($KL^{+/-}$) and their genotypes were verified by Mouse Direct PCR Kit (B40015, Bimake, Selleck). We used the following specific primers: wild-type, forward 5'-TTGTGGAGATTGGAAGTG GACGAAAGAG-3' and reverse 5'-CTGGAC CCCCTG-AAGCTGGA-GTTAC-3'; Klotho mutant, forward 5'-TTGTGGAGATTGGAAGTGGACGAAAGAG-3' and reverse 5'-CGCCCCGACCGGAGCTGAGAGTA-3'. These primers were expected to produce 815 bp (WT) and 419 bp (klotho-deficient) amplification products. The PCR conditions were as follows: denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 45 s, and a final extension at 72 °C for 10 min. All $KL^{+/-}$, or WT (C57BL/6) mice at 8 weeks of age were treated with a single daily intraperitoneal dose of 55 mg/kg streptozotocin (STZ) for 1 week. Non-diabetic mice were treated with citrate buffer for 7 days, i.p.. Mice were weekly monitored for body weight and blood glucose (Roche Glucose meter).

Podocyte Culture

Human podocytes were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS. To propagate podocytes, the culture medium was supplemented with 10 U/mL human recombinant γ-interferon (Sigma, St. Louis, MO, USA) to enhance expression of the T antigen, and cells were cultivated at 33℃ (permissive conditions). To induce differentiation, podocytes were cultured on type I collagen at 37℃ without γ-interferon for at least 14 days. Podocytes were cultured in 6-well plates and then preincubated with Klotho 48 h before HG induction. In parallel, podocytes incubated with mannitol (30 mmol/l) for same time were taken as negative control.

Histology

Histological and morphometric analysis was carried out on paraffin sections (2 μm thickness) cut on a rotation microtome (Microm) and stained with hematoxylin-eosin and periodic acid-schiff, respectively.

Urine Micro Albuminuria

Albumin concentration in spot urine samples was measured with a commercially available competitive enzyme-linked immunosorbent assay following the instructions of the manufacturer (TP0100-1KT, Sigma, USA) and was normalized to urine creatinine.

ELISA Assay of Serum Klotho

The blood samples from mice were collected and centrifuged for 10 minutes at 3000 rpm (4 °C), and the serum Klotho was measured in duplicate using a mouse Klotho ELISA kit according to the manufacturer's protocol (Cusabio, Cologne, Germany).

Detection of ROS Production

The detection of intracellular ROS was used an ROS–sensitive fluorescent probe 2',7'-Sdichlorodihydrofluorescein diacetate (DCFH-DA, HY-D0940, MCE). Podocytes were loaded with the fluoroprobe DCFH-DA (10 mmol/L) at 37 °C for 40 minutes in 200 ml serum-free STZEM. The fluorescent level was observed under an inverted fluorescence microscope, and the fluorescence intensity was measured at 480 nm excitation and 525 nm emission with a microplate reader (Thermo Fisher Scientific, Pittsburgh, PA).

Western Blotting Analyses

The proteins isolated from podocytes or kidney tissues was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA). The membranes were incubated at 4 °C overnight with the following primary antibodies: Rabbit polyclonal antibody to NPHS2(Podocin) (1:1000; ab50339 Abcam, Inc., Cambridge, UK), rabbit monoclonal to WT1 (1:1000; ab89901 Abcam), rabbit polyclonal to OGG1 (1:5000; bs-3687R, Bioss), Rabbit polyclonal to Caspase3 (1:1000; ab44976 Abcam).Then, the secondary antibodies were applied. The signals were developed with the ECL-Plus

Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK), and the densitometry analysis was performed with an image analysis system (Bio-Rad).

Immunofluorescence Assay

Immunofluorescent staining and images were obtained by a LSM780 laser scanning confocal microscope (ZEISS, Germany) system.

Transmission electron microscopy (TEM)

TEM images were analyzed using Image Pro plus 6.0. The GBM thickness, foot process width and the number of foot processes per μm of GBM were calculated using a curvimeter (SAKURAI CO., LTD, Tokyo, Japan).

Statistical Analyses

Data are expressed as means ± SE. Student's t-test was employed for comparisons between two groups. Multiple comparisons were performed using one-way ANOVA, followed by Bonferroni's post hoc test. P values < 0.05 were considered significant and are indicated in the figures by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001). Analyses were performed using Graph Pad Prism software (GraphPad Software Inc, version 7.0).

Results

Klotho deficiency exacerbated diabetic nephropathy

To analyze the relationship between Klotho and phenotypes of diabetic nephropathy. As high embryonic mortality and failure rate of diabetes model in the Klotho homozygous mice, we selected Klotho heterozygous mice. We generated STZ-induced diabetic mice with wild-type(C57BL/6J) or Klotho deficiency mice ($KL^{+/-}$) aged 8 to 10w. The concentration of Klotho in serum did decrease in diabetic mice, especially in STZ-induced $KL^{+/-}$ mice ($KL^{+/-}$ STZ) (Fig. 1a). Further compared with normal mice, all diabetic mice' concentration of blood sugar was higher than 13.8 mmol/l and no downregulation was observed, but the levels further increased in $KL^{+/-}$ STZ (Fig. 1b). Urine volume in diabetic WT group (WT STZ) was obviously higher than the WT group but Klotho deficiency aggravated the phenomenon when compared to WT STZ (Fig. 1c). Diabetes also induced kidney weight increase and increased ratio of kidney weight to body weight but Klotho deficiency induced greater increase of those (Fig. 1d and e). Meanwhile, we also measured serum creatinine, blood urea nitrogen (BUN) and UACR, respectively. We compared different groups and found the further rise of creatinine, BUN, as well as UACR in $KL^{+/-}$ STZ when compared to WT STZ (Fig. 1f-h). To analyze the difference of glomerular morphology in different groups, we used hematoxylin-eosin staining and found mesangial matrix expansion in WT STZ. However, $KL^{+/-}$ STZ also induced mesangial matrix expansion that had more expansion compared with WT STZ (Fig. 1i). Using periodic acid schiff (PAS) staining to observe glycogen deposition, we found there are glycogen deposition in glomerular of both WT STZ and $KL^{+/-}$ STZ. Moreover, compared with WT STZ,

glomerular accumulated more glycogen in $KL^{+/-}$ STZ (Fig. 1i). All these data indicated Klotho deficiency may aggravate diabetic renal dysfunction.

Klotho deficiency exacerbated STZ-induced podocyte injury

Whether Klotho deficiency induces podocyte injury, we examined the expression of WT1, podocyte's marker, by immunohistochemistry (IHC). We found diabetes inhibited the expression of WT1(Fig. 2a) and promoted expression of Caspase3 (Fig. 2a). It's worth noting that Klotho deficiency pushed the pathological process (Fig. 2a), as was further confirmed by immunofluorescence (Fig. 2b). Podocyte was injury as evidenced by glomerular basement membrane (GBM) thickening, podocyte foot process broadening or effacement in WT STZ, that were significantly lower than $KL^{+/-}$ STZ (Fig. 2c). Nephrin, another podocyte's marker, and Peroxiredoxin 2 (PRDX2), an antioxidant enzyme detoxifying reactive oxygen species, were also subjected to western blot analysis. We found Klotho deficiency further inhibited the expression of Nephrin and partially suppressed PRDX2 in $KL^{+/-}$ STZ as compared to WT STZ (Fig. 2d). The results suggested Klotho deficiency further exacerbated STZ-induced podocyte injury.

Klotho deficiency aggravated HG-induced MtD of podocytes

As glucose metabolism is related to energy metabolism, mitochondria play an important role in this process. To wonder whether Klotho deficiency affected MtD of podocytes, transmission electron microscope (TEM) was first performed. The results showed mitochondrial hypertrophy appeared in podocytes of WT STZ when compared to that of WT (Fig. 3a). Meanwhile, numerical density of damaged mitochondria increased significantly in WT STZ as compared to WT Veh (Fig. 3a). However, slight mitochondrial contraction was observed in non-diabetic $KL^{+/-}$ mice but this phenomenon was obviously aggravated in $KL^{+/-}$ STZ (Fig. 3a). Moreover, lower numerical density of damaged mitochondria was observed in $KL^{+/-}$ STZ when compared to WT STZ (Fig. 3a). Further analysis by immunofluorescence suggested 8-hydroxydeoxyguanosine (8-OHdG), a marker of DNA damage, increased with diabetes, especially in $KL^{+/-}$ STZ, which was inversely related to the expression of Nephrin (Fig. 3b). The results indicated Klotho deficiency may be related to MtD of podocyte.

Klotho deficiency affected OGG1 in HG-treated podocytes

Having found the relationship between Klotho and 8-OHdG, we wonder whether DNA damage repair enzymes were suffered interference. We first detected 8-oxoguanine DNA glycosylase (OGG1), a DNA repair enzyme, by westernblot and found the Klotho deficiency was accompanied with low expression of OGG1 in diabetic mice (Fig. 4a). Further experimental confirmation by immunofluorescence showed STZ-induced diabetes suppressed the expression of both synapotopodin,a marker of podocyte and OGG1. Klotho deficiency further inhibited the expression (Fig. 4b). Meanwhile, we analyzed the role of reactive oxygen species (ROS) in podocytes when treated with HG. We found a sharp rise of ROS in HG-induced podocytes (Fig. 4c). When applied with human Klotho recombinant protein or N-acetyl-L-cysteine (NAC), HG-treated ROS was ameliorated, respectively (Fig. 4c). We also demonstrated the expression of WT1, Podocin, OGG1 as well as Caspse3, respectively. Both Klotho and NAC could inhibit the reduction of WT1

and Podocin and reduce the expression of Caspase3 in HG-treated podocytes (Fig. 4d). Moreover, OGG1 was ameliorated by Klotho or NAC (Fig. 4d). The above results indicated that HG activated OGG1 which was disturbed by Klotho deficiency leading podocyte injury.

Discussion

Diabetic nephropathy (DN) is a progressive disease that affects about one third of diabetic patients, the main pathogeny of which is podocyte injury that leads to glomerular filtration barrier and proteinuria[23,24]. Klotho, anti-aging protein with critical roles in protecting kidney, is expressed predominantly in the kidney and secreted in the blood. Nonetheless, the mechanisms of Klotho ameliorating podocyte injury induced by reactive oxygen species (ROS), mainly generating in mitochondria, of which dysfunctions have been associated with apoptosis, aging, and a number of pathological conditions[25], are of insufficient coverage. Here, we investigated the function of Klotho dificiency on MtD of diabetes-induced podocytes. We found Klotho deficiency aggravated HG-treated MtD and podocyte injury resulting in proteinuria in vitro and in vivo experiments. Further experiments first showed Klotho deficiency promoted 8-OHdG to inducing DNA damage by affecting OGG1 expression in HG-treated podocytes.

Podocyte is nonrenewable and vulnerable to a variety of injuries as is highly specialized, terminally differentiated epithelial cells that line the outer surface of the glomerular basement membrane[26 – 28]. Enhanced production of reactive oxygen species (ROS) has been recognized as the major determinant of age-related endothelial dysfunction[29,30]. p66SHC induced by HG could mediate mitochondrial dysfunction[12,31,32]. However, our study focused on diabetes-induced DNA damage that may be the root cause of podocyte damage. As previously reported, we also confirmed that HG induced the production of ROS and leaded MtD. However, the correlation of researches between Klotho and ROS in diabetes mainly in ROS-induced podocyte apoptosis[12]. In this study, we studied the relationship between Klotho and DNA damage in diabetes nephropathy (DN). We found Klotho deficiency promoted 8-OHdG to induce DNA damage as inhibiting OGG1 expression in HG-treated podocytes.

As we have demonstrated the role of Klotho on diabetes-induced DNA damage of podocyte, how Klotho regulates OGG1 to inhibit 8-OHdG remains to be further studied. OGG1 is the major DNA glycosylase in human cells for removing 8-OHdG, one of the most frequent endogenous base lesions formed in the DNA of aerobic organisms. OGG1 could be phosphorylated in vivo on a serine residue and is subject to protein kinase C (PKC)-mediated phosphorylation in vitro, suggesting that PKC is responsible for the phosphorylation event[33]. Nevertheless, Protein kinase C (PKC) transducing signals is mediated by diacylglycerol (DG) or the second messengers Calcium ion[34]. Klotho as a regulator of calcium homeostasis inhibits transient receptor potential channel 6 (TRPC6)-mediated Ca^{2+} influx in cultured mouse podocytes and ameliorates albuminuria and renal fibrosis[35,36]. In summary, Klotho may be as a regulator of calcium to inhibit PKC and then activate OOG1, but further molecular mechanisms need further study.

Conclusions

Our results imply that Klotho deficiency may aggravate podocyte MtD by affecting ROS and 8-OHdG-induced DNA damage by affecting OOG1 in diabetes.

Declarations

Ethics approval and consent to participate

Experiments were performed in accordance with the Animal Research Institute Committee of University of Chinese Academy of Sciences for the Care and Use of Laboratory Animals.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Competing interests

The author declares that they have no competing interests.

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Authors' contributions

ZC wrote the article and edited the manuscript; QZ and CL were responsible for the statistical analysis; YPZ and SLY revised the manuscript. All authors read and approved the final manuscript.

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Figures

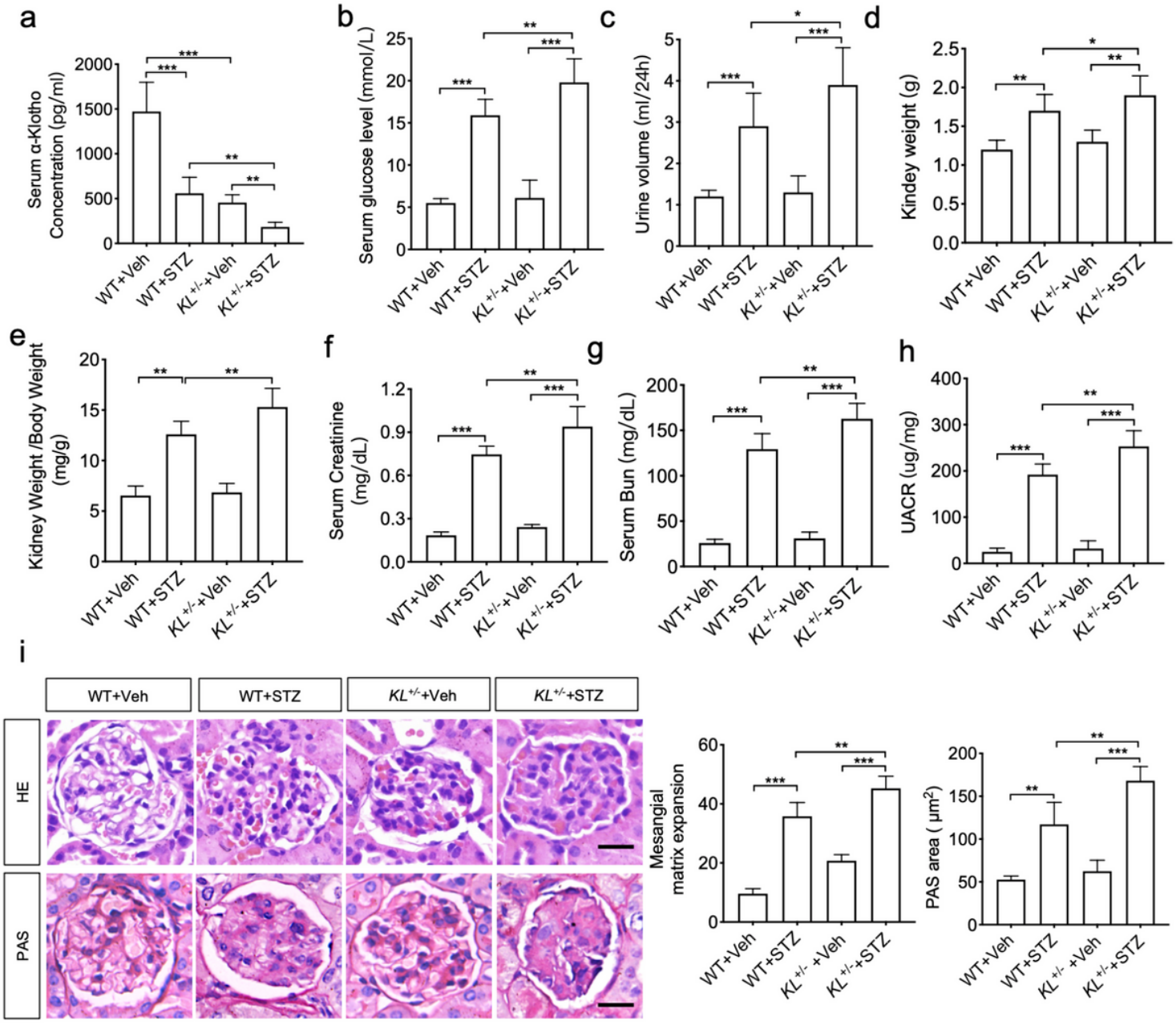

Figure 2

Klotho deficiency may aggravate diabetes-induced renal dysfunction. (a-d) Serum Klotho levels (Vehicle (injection of citrate buffer) =6, STZ (injection of 55mg/kg/d STZ) =6) (a), serum glucose levels (b), Urine volume (c) and kidney weight (d). (e) Ratio of kidney weight to body weight in STZ-induced diabetic mice and normal mice. Comparison of serum creatinine (f) and Bun (g) between vehicle mice and STZ mice. (h)UACR (urine albumin-to-creatinine ratio) of STZ-induced diabetic mice and normal mice. (i) Comparison of mesangial matrix expansion and glycogen deposition in glomerulus of vehicle mice and STZ mice by HE staining and PAS staining, respectively. Scale bar: 20μm. *P<0.05; **P<0.01; ***P<0.001. Veh, vehicle.

Figure 4

Klotho deficiency (KL+/-) promoted podocyte injury in STZ-induced mice. (a) Immunohistochemistry staining showing the expression of podocyte nuclear marker WT1 and Caspase3 related to apoptosis in WT Vehicle, WT STZ, KL+/- Vehicle and KL+/- STZ. Scale bar, red 50μm, black 20μm. (b)Immunofluorescence staining showing the expression of podocyte membrane marker Nephrin in WT Vehicle, WT STZ, KL+/- Vehicle and KL+/- STZ. Scale bar, white 20μm. (c) Representative photomicrographs and quantifications of mean glomerular basement membrane (GBM) thickness, mean foot process width, and the number of foot processes in different groups of mice by transmission electron microscopy (TEM) analyses. Scale bar, 200nm. (d) Representative western blot gel documents and summarized data showing the relative protein level of Nephrin and antioxidant related PRDX2 in the kidney from WT Vehicle, WT STZ, KL+/- Vehicle and KL+/- STZ. *P<0.05; **P<0.01; ***P<0.001.

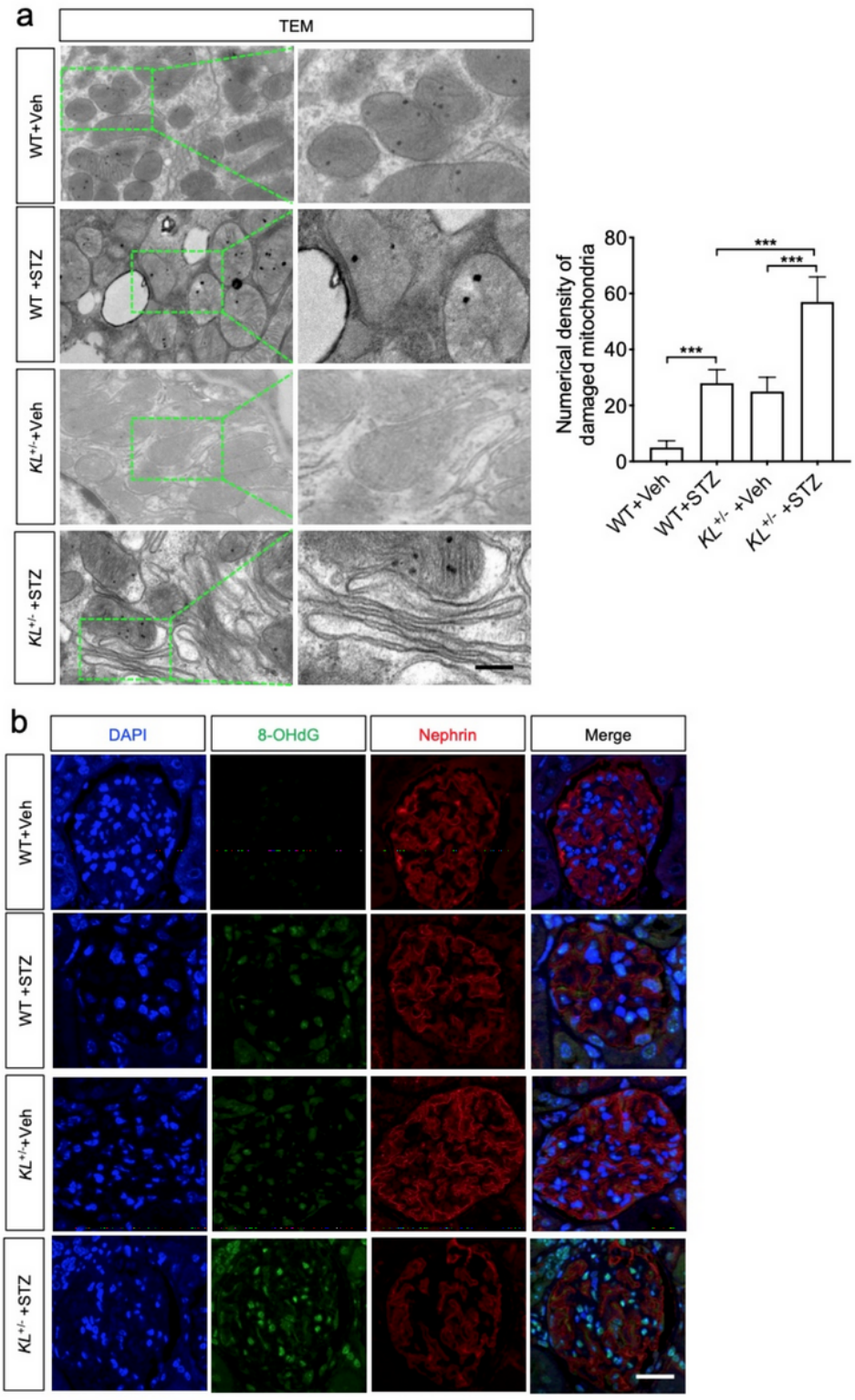

Figure 6

The effects of Klotho deficiency on mitochondrial morphology in diabetes-induced podocytes. (a) Changes of mitochondrial morphology in podocytes of each group. (b)Immunofluorescence staining showing the expression of DNA damage marker 8-OhdG as well as Nephrin in WT Vehicle, WT STZ, KL+/- Vehicle and KL+/- STZ. Scale bar, white 20μm.

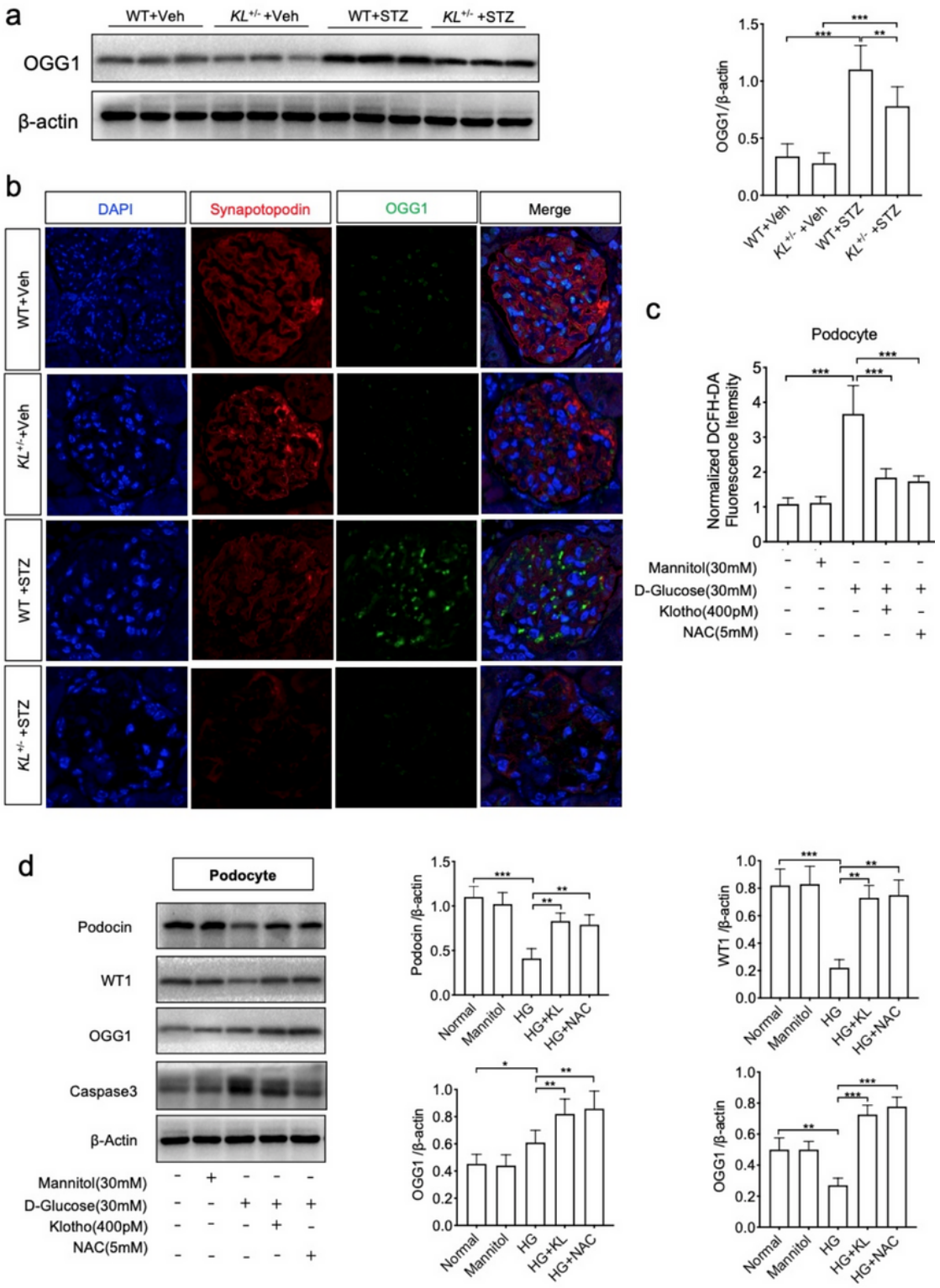

Figure 8

Klotho deficiency inhibited OGG1 aggravating podocytes injury in STZ-induced mice. (a) Representative western and summarized data showing the relative protein levels of OGG1 in the kidney from WT Vehicle, WT STZ, KL+/- Vehicle and KL+/- STZ. (b) Immunofluorescence staining showing the expression of OGG1 as well as podocyte membrane marker Synapotopodin in WT Vehicle, WT STZ, KL+/- Vehicle and KL+/- STZ. Scale bar, 20μm. (c) Representative western blot gel documents and summarized data

showing the relative protein level of Podocin, WT1, OGG1 and Caspase3 in podocytes. *P<0.05; **P<0.01; ***P<0.001.