HHS Public Access

Author manuscript

*Biochimie*. Author manuscript; available in PMC 2020 August 01.

Published in final edited form as:

*Biochimie*. 2020 July ; 174: 74–83. doi:10.1016/j.biochi.2020.04.003.

# EDA2R Mediates Podocyte Injury in High Glucose Milieu

**Xiqian Lan**[1,*], **Vinod Kumar**[2], **Alok Jha**[2], **Rukhsana Aslam**[2], **Haichao Wang**[2], **Kehong Chen**[3], **Yueming Yu**[4], **Weimei He**[4], **Feilan Chen**[5], **Huairong Luo**[1], **Ashwani Malhotra**[2], **Pravin C Singhal**[2,*]

[1]Key Laboratory for Aging and Regenerative Medicine, School of Pharmacy, Southwest Medical University, Luzhou, Sichuan, China

[2]Feinstein Institute for Medical Research and Donald and Barbara Zucker School of Medicine at Hofstra/Northwell, Manhasset, NY, United States

[3]Department of Nephrology, Daping Hospital, Army Medical University, Chongqing, China

[4]Kunming Dongfang Hospital, Kunming, China

[5]Chongqing Engineering Research Center for Rodent Laboratory Animals, Chongqing Medical University, Chongqing, China

## Abstract

EDA2R is a member of the large family of tumor necrosis factor receptor (TNFR). Previous studies suggested that EDA2R expression might be increased in the kidneys of diabetic mice. However, its mRNA and protein expression in kidneys were not analyzed; moreover, its role in the development of diabetic kidney disease was not explored. Here we analyzed the mRNA and protein expressions of EDA2R in diabetic kidneys and examined its role in the podocyte injury in high glucose milieu. By analysis with real-time PCR, Western blotting, we found that both the mRNA and protein levels of EDA2R were increased in the kidneys of diabetic mice. Immunohistochemical studies revealed that EDA2R expression was enhanced in both glomerular and tubular cells of diabetic mice and humans. *In vitro* studies, high glucose increased EDA2R expression in cultured human podocytes. Overexpression of EDA2R in podocytes promoted podocyte apoptosis and decreased nephrin expression. Moreover, ED2AR increased ROS generation in podocytes, while inhibiting ROS generation attenuates EDA2R-mediated podocyte injury. In addition, EDA2R silencing partially suppressed high glucose-induced ROS generation, apoptosis, and nephrin decrease. Our study demonstrated that high glucose increases EDA2R expression in kidney cells and that EDA2R induces podocyte apoptosis and dedifferentiation in high glucose milieu partially through enhanced ROS generation.

[*]**Corresponding author:** lanxqwhx@hotmail.com, psinghal@northwell.edu.

Author contribution

Conception of the work: X.L.;

Collection of data: X.L., V.K., A.J., R.A., K.C., Y.Y., W.H., F.C.;

Analysis of data: X.L., H.W., F.C., H.L., A.M., P.C.S.

Writing of manuscript: XL, H.W., P.C.S.

All authors have approved the final article.

**Keywords**

Diabetic nephropathy; high glucose; EDA2R; podocyte; apoptosis; dedifferentiation

## 1. Introduction

Diabetic nephropathy (DN) is one of the most common complications in diabetic patients. Its clinical characteristics include progressive proteinuria and a decline in glomerular filtration rate. Histologically, DN is characterized by podocyte apoptosis, mesangial cell proliferation and sclerosis, glomerular basement membrane thickening, and tubulointerstitial fibrosis. In the United States, DN is the leading cause of the end-stage renal disease (ESRD) [1]. In developing countries such as China, DN is also emerging as a major contributing disease for the progression to ESRD [2]. For example, in Beijing and Shanghai, the percentage of patients with chronic kidney disease related to diabetes has exceeded the proportion of patients with chronic kidney disease related to glomerulonephritis [2]. However, detailed pathogenesis of the involved mechanisms of DN is still unclear; on that account, current therapeutic approaches are not effective in controlling the development and progression of DN, and the majority of DN patients continue to progress to ESRD [3]. To prevent or slow down the progression of DN, it is important to identify and validate unexplored pathogenic molecules related to DN and to exploit them as potential therapeutic targets.

Podocytes are highly specialized epithelial cells, constituting the critical molecular proteins required to maintain the selective permeability in the glomerular filtration barrier [4]. They are terminally differentiated cells which do not proliferate. Loss of the significant number of podocytes manifests in the form of proteinuria, mesangial expansion, and glomerulosclerosis. In diabetic milieu, podocytes are susceptible to injury [5–7]. High glucose-induced oxidative stress and generation of advanced glycation end products (AGEs) promote podocyte loss, predominantly via apoptosis. Additionally, these adverse factors may reduce the expression of slit diaphragm proteins such as nephrin, leading to podocyte dedifferentiation. Although the factors that cause podocyte injury in diabetes are actively investigated, little is known about the specific mechanisms.

EDA2R (Ectodysplasin A2 Receptor) is a member of the large family of tumor necrosis factor receptor (TNFR) that lacks a discernible death domain. It is also called an X-Linked Ectodysplasin-A2 Receptor (XEDAR) since its gene, *EDA2R*, is located on the X chromosome. The natural ligand of EDA2R is EDA-A2, a splicing isoform protein encoded by the ectodysplasin (EDA) gene. According to recent reports, stimulation of ED2AR causes apoptosis via the activation of caspase-3 in cancer cells, salivary gland epithelial cells, osteosarcoma cell lines, and hair follicle cells [8–16]. In addition, EDA2R expression in aging adipose, artery, heart, lung, muscle, and skin tissues is associated with cellular apoptosis [17, 18]. These studies suggest that EDA2R acts as an inducer of apoptosis.

Previous studies through microarray and RNA-seq analysis showed a possible higher expression of EDA2R in the kidneys of diabetic mice (both types 1 and 2) when compared to control mice [19, 20]. However, the mRNA and protein expressions of EDA2R in diabetic

kidneys have not been confirmed. Besides, the role of EDA2R in the progression of chronic kidney disease such as DN has not been explored. In the present study, we analyzed the expression of EDA2R and its distribution profile in diabetic kidneys, and further examined its role in high glucose-induced podocyte injury.

## 2. Materials and Methods

### 2.1. Reagents

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO) and was freshly dissolved in sterile citrate buffer (10 mM, pH 4.5). N-acetyl-L-cysteine (NAC) and Mito-TEMPO were from Sigma-Aldrich (St. Louis, MO) and were dissolved in DMSO. For cell treatment, 100 μM of NAC and 10 μM of Mito-TEMPO were added to the medium.

### 2.2. Animals and treatments

Unless otherwise mentioned, all animal administrations and treatments were performed following our previous publication [21].

To generate STZ-induced diabetic mouse model, 12-week-old female mice (20–25 g) in FVB/N background (purchased from Jackson Laboratory) were fasted but given water freely for 18 h, and then were intraperitoneally injected with STZ at a dose of 50 mg/kg bodyweight for 5 consecutive days. Normal control mice only received sterile citrate buffer in the same way. One week after the last injection, blood glucose levels of the mice were measured. Mice with glucose levels above 200 mg/dl were considered a successful diabetic model of hyperglycemia (diabetes mellitus, DM) and were used for further experiments. The mice were maintained for six weeks or five months after STZ injection. At the end of the treatments, urine and blood samples were collected, and then the mice were sacrificed with $CO_2$. The blood in the circulating vessels was expelled by perfusion with PBS, and the kidney samples were collected.

Blood urea nitrogen (BUN) was determined by using the Urea Nitrogen (BUN) Colorimetric Detection Kit (Thermo Fisher Scientific, Waltham, MA).

### 2.3. Human biopsies

Human renal biopsy samples were obtained from patients admitted in the nephrology departments of Daping Hospital (Chongqing, China) and Kunming Dongfang Hospital (Kunming, China), complied with the Helsinki declaration (Code of Ethics of the World Medical Association). The renal tissues were collected from 5 DN or 5 non-diabetic patients. DN patients were diagnosed according to typical DN characteristics such as hyperglycemia, proteinuria, and typical histological changes. Obtained renal tissues were fixed in formalin solution (10% buffered with PBS), embedded in paraffin, and then sectioned to 4-μm thickness. The studies were approved by the Research Ethics Boards of the Daping Hospital and Kunming Dongfang Hospital, and all patients provided informed consent.

### 2.4. EDA2R overexpression

Human podocytes were cultured and differentiated, as previously reported [21, 22]. EDA2R overexpression was conducted with the Effectene Transfection Reagent (Qiagen, Germantown, MD). By using 9 μl Effectene Transfection Reagent, differentiated podocytes ($2 \times 10^5$ in 60 mm dish) were transfected with 1 μg pCMV3-EDA2R or 1 μg control plasmid (Sino Biological, Wayne, PA).

### 2.5. EDA2R silence

siRNA specific for EDA2R (siEDA2R, Santa Cruz Biotechnology, Dallas, TX) was used to silence EDA2R expression in the cultured human podocyte. Sixty pmol siEDA2R or the same amount of control siRNA (siCon, Santa Cruz Biotechnology) was mixed with 18 μl Lipofectamine RNAiMAX Reagent (Invitrogen, Thermo Fisher Scientific, Waltham, US), and then the mixture was added to the differentiated podocytes ($2 \times 10^5$ in 60 mm dish), following the manufacturer's instruction. For 96-well plates ($1 \times 10^4$ cells per well), 10 pmol siEDA2R/siCon and 1.5 μl Lipofectamine RNAiMAX Reagent were used.

### 2.6. Real-time PCR

RNA preparation and real-time PCR were performed as our previous descriptions [21–23]. The primer sequences were listed in Table 1.

### 2.7. Western blotting analysis

Unless otherwise mentioned, Western blotting was performed as described in our previous publications [21–23]. Primary antibodies for EDA2R (ab203667, 1:1000) and nephrin (ab58968, 1:1000) were purchased from Abcam (Cambridge, MA), and antibodies for Bax (sc-526, 1:1000), Bcl2 (sc-492-g, 1:1000), Mcl-1 (sc-12756, 1:1000), and actin (sc-8432, 1:3000) were from Santa Cruz Biotechnology. The antibody for cleaved caspase 3 (AF835, 1:1000) was from R&D Systems (Minneapolis, MN).

### 2.8. Immunohistochemical studies

Immunohistochemical studies were performed following our established methodology in our previous publications [20, 21]. Primary antibodies for EDA2R and nephrin were purchased from Abcam (ab203667, 1:100) and from R&D System (AF3159, 1:100), respectively.

### 2.9. Apoptotic cell determination

In mouse tissues, apoptotic cells were assayed by using TUNEL staining with Click-iT™ TUNEL Colorimetric IHC Detection Kit (Thermo Fisher Scientific, C10625).

*In vitro* studies, cellular apoptosis was determined by using Hoechst33342 staining, as described in our previous publications [21, 22]. Cells with condensed and fragmented nucleus were identified as apoptotic cells.

### 2.10. Intracellular ROS measurement

Intracellular ROS was measured with 2, 7-dichlorofluorescein (CM-H2DCFDA) (Molecular probe, Carlsbad, CA), as described in our previous publications [22, 23]. Briefly,

differentiated human podocytes cultured in 96-well plates ($1\times10^4$ for each well) were transfected with pCMV-EDA2R/control plasmid (100 ng for each well in 0.75 μl Effectene Transfection Reagent) for 24 h, or were first transfected with siEDA2R/siCon (5 pmol for each well in 1.5 μl Lipofectamine RNAiMAX Reagent) for 24 h followed by a treatment with 5 or 30 mM glucose for another 24 h. After that, ROS was measured by analyzing the fluorescence intensity of CM-H2DCFDA, as per manufacturer's instructions.

### 2.11. Statistical analysis

Unless otherwise noted, statistical analysis was conducted following our previous reports [21–23]. All data were evaluated statistically by the analysis of variance (ANOVA), and then a software (Prism 4.0, GraphPad Software) was used to analyze the Newman-Keuls multiple comparison tests. Statistical significance was considered when p values $< 0.05$.

## 3. Results

### 3.1. Hyperglycemia causes kidney cell injury

In our previous study, we demonstrated that hyperglycemia was associated with podocyte apoptosis in $BTBR^{ob/ob}$ mice with type 2 diabetes [21]. In the present study, we generated a type 1 diabetes mouse model by injecting Streptozotocin (STZ). One week after the last injection, we determined the blood glucose concentrations (BGC) and selected those mice with BGC higher than 200 mg/dl as a diabetic group. The BGCs in the control group didn't change significantly in the following five months; while in the diabetic group, it increased quickly in the first six weeks, and then reached to a relatively stable level (Figure 1A).

Six weeks (6W) after STZ injection, the albumin/creatinine ratios of urine samples didn't show a significant difference between the control and the diabetic groups, but the BUN of diabetic mice was higher than that of control mice (Figure 1B). After five months (5M), both the BUN and albumin/creatinine ratios of diabetic mice were higher than those of control mice (Figure 1B). The diabetic mice also showed a greater percentage of apoptotic cells in the glomeruli (Figure 1C), and a lower level of nephrin expression (Figure 1D). These results indicated that kidney cell injury resulted in the malfunction of kidneys in STZ-induced hyperglycemic mice.

### 3.2. EDA2R expression is increased in the podocyte of diabetic kidneys

To determine the effect of hyperglycemia on EDA2R expression, we extracted kidney RNAs from the mice at six weeks and five months after STZ injection, and then performed real-time PCR analysis. Results showed that EDA2R mRNA in the kidneys of STZ-injected mice was much higher than that in control mice, in both groups (Figure 2A). We also determined EDA2R expression in the kidney of $BTBR^{ob/ob}$ mice and found that its mRNA was also higher than that in BTBR control mice (Figure 2A).

To examine the effect of hyperglycemia on EDA2R protein expression, we prepared tissue lysates from the mice kidneys and conducted the Western blotting analysis. The result showed that after five months of STZ injection, EDA2R protein expression was higher in the

kidneys of diabetic mice than that in control mice (Figure 2B). Similarly, its expression in the kidney of BTBR$^{ob/ob}$ mice was also higher than that in BTBR control mice (Figure 2C).

We further performed immunohistochemistry (IHC) to display the cellular expression of EDA2R in the kidneys of mice. Results showed that in both type 1 (5 months after STZ injection) and type 2 (BTBR$^{ob/ob}$) diabetic groups, the expressions of EDA2R in renal glomeruli and tubular cells were higher than that in their corresponding control mice (Figure 2DE).

We also performed co-labeling for nephrin and ED2AR to confirm their localization in podocytes. Here we selected STZ-injected mice as a representative, and found that enhanced EDA2R and decreased nephrin were co-localized in the hyperglycemic group (Fig. 2F), indicating that EDA2R is highly expressed in mouse glomerular podocytes in a high glucose milieu.

To validate the clinical relevance of the above findings, we examined the expression of EDA2R in diabetic patients' kidneys. Indeed, IHC showed an elevated expression of this protein in the glomeruli and tubular cells of diabetic patients as compared to those in non-diabetic patients (Fig. 3A). Immunofluorescence co-labeling showed that EDA2R expression was co-localized with nephrin, indicating that EDA2R is highly expressed in glomerular podocytes of diabetic patients (Fig. 3B). These results demonstrated that EDA2R expression was increased in podocytes of diabetic kidneys.

### 3.3. High glucose induces EDA2R expression in podocytes

To determine the induction of EDA2R by high glucose in podocytes, we performed *in vitro* studies. We used normal (5 mM) or high glucose (30 mM) to treat podocytes, and after 24 hours, we collected the cell lysate for Western blotting analysis, or collected total RNAs for real-time PCR analysis, to determine the protein or mRNA expressions of EDA2R. Results showed that both the mRNA and protein of EDA2R expression in podocytes treated by 30 mM glucose were higher than that treated by 5 mM glucose (Fig. 4). These results indicate that high glucose carries the potential to induce the expression of EDA2R in podocytes at both mRNA and protein levels.

### 3.4. Overexpression of EDA2R in podocytes promotes apoptosis and dedifferentiation

To evaluate the role of EDA2R on podocyte health, we overexpressed ED2AR in podocytes and then examined the induction of either apoptosis or dedifferentiation. The morphological apoptotic assay revealed that overexpression of EDA2R in podocytes enhanced the percentage of apoptotic cells (Figure 5A). Western blotting results showed that overexpression of EDA2R inhibited the expression of anti-apoptotic molecules such as Mcl-1 and Bcl2, but enhanced pro-apoptotic molecules such as Bax and cleaved caspase-3 (Figure 5B). These results confirmed that EDA2R promotes podocyte apoptosis.

The Western blotting analysis also demonstrated that overexpression of EDA2R down regulated the expression of nephrin (Fig. 5B); these findings indicate that EDA2R also promoted podocyte dedifferentiation.

### 3.5. EDA2R mediates podocyte injury through ROS generation

Reactive oxygen species (ROS) have been reported to be downstream products of TNFR-associated factors (TRAF)-mediated signal transduction [24]. However, as a member of the TNFR family, whether EDA2R mediates, ROS generation has not been reported yet. To address this issue, we overexpressed this gene in the podocyte and then examined the ROS generation. Overtly ED2AR-expressing podocytes displayed enhanced ROS generation (Fig. 6A), suggesting that ROS is also a downstream product of EDA2R.

To determine whether ROS plays an important role in EDA2R-mediated podocyte injury, we added its scavengers (N-acetyl-L-cysteine [NAC] and mito-TEMPO) to the medium of podocytes overexpressing EDA2R. After 48 h, the morphological apoptotic assay was carried out. ROS scavengers significantly attenuated the EDA2R-mediated apoptotic cell ratio (Figure 6B). In addition, Western blotting results demonstrated that these scavengers also partially restored EDA2R-decreased nephrin expression (Figure 6C). These results suggest that EDA2R mediates podocyte injury at least partially through ROS generation.

### 3.6. Confirmation of a causal relationship between EDA2R expression and induction of podocyte apoptosis and de-differentiation in high glucose milieu

To establish a causal relationship between high glucose-induced ED2AR expression and the induction of podocyte apoptosis and dedifferentiation, we used siRNA to knockdown EDA2R gene expression in podocytes. After 24 hours, we treated these cells with high glucose (30 mM), and then we stained them with CM-H2DCFDA. EDA2R-silenced podocytes displayed attenuated ROS production in high glucose milieu (Figure 7A). The morphologic assay showed a reduction in the occurrence of apoptosis in ED2AR-silenced cells in high glucose milieu (Figure 7B). Moreover, Western blotting assay revealed that the silencing of EDA2R expression partially reduced high glucose-induced cleaved caspase-3 expression, but restored high glucose-suppressed nephrin expression (Figure 7C). These results indicated that the silencing of EDA2R expression could partly attenuate high glucose-induced podocyte injury.

## 4. Discussion

Diabetes is growing to an epidemic proportion in modern society. The causes and molecular mechanisms of DN are still not clear, and the existing management of diabetic kidney patients constitutes a tremendous socioeconomic burden on society [1]. There is an urgent need to enhance the mechanistic understanding for developing new practical therapeutic approaches to prevent or slow the progression of DN. The present study demonstrated enhanced EDA2R expression in podocytes of both diabetic mice and human patients. *In vitro* studies revealed that high glucose induces EDA2R expression in podocytes. Overexpression of EDA2R in podocytes induced apoptosis and dedifferentiation, whereas, it's silencing partially attenuated high glucose-induced injuries in podocytes. These findings clearly establish a causal relationship between high glucose-induced EDA2R expression and podocyte injury. This is the first study to demonstrate the role of EDA2R in the development of DN.

Microarray and RNA-seq analysis are recently developed powerful methods for comparison of gene expression profiles between different groups. With these methods, previous studies revealed that many genes, including EDA2R, might be up-regulated by hyperglycemia in animal models [19, 20]. However, the mRNA and protein expression of EDA2R was not confirmed in those studies. In the present study, we validated the increase of both mRNA and protein of EDA2R in the kidneys of diabetic mice by using real-time PCR and Western blotting analysis. Notably, we found that EDA2R expression was elevated in the kidneys of both STZ-induced diabetic (six weeks and five months after STZ injection) and $BTBR^{ob/ob}$ mice, suggesting that EDA2R can be induced by both Type 1 and 2 diabetes, which is consistent with previous reports [19, 20]. We also performed IHC and IF staining, and confirmed that the expression of this gene was increased in the podocyte of both diabetic mice and human patients. In addition, *in vitro* studies showed that high glucose could directly induce EDA2R expression in human podocytes. These results confirmed that high glucose increased the expression of EDA2R in podocytes.

EDA2R has been reported to mediate apoptosis in several cell types [8, 9, 13–16]. Consistent with these reports, we also found that EDA2R induces apoptosis in podocytes. Moreover, the silencing of ED2AR in podocytes provided protection from high glucose milieu-induced apoptosis. In addition, EDA2R decreased the expression of nephrin, suggesting its role in podocyte dedifferentiation.

Enhanced ROS generation has been reported to play a vital role in podocyte injury during adverse conditions such as high glucose milieu [25–27]. The effect of increased EDA2R expression on ROS generation has not been reported yet. In the present study, overexpression of EDA2R increased ROS generation in podocytes, whereas, silencing of EDA2R attenuated high glucose-induced ROS generation, suggesting that EDA2R mediates ROS generation. This is consistent with the previous report that ROS is downstream products of TRAF-mediated signal transduction [24]. In addition, our study demonstrated that inhibition of ROS generation with its scavenger NAC and mito-TEMPO ameliorated EDA2R-mediated podocyte injury, suggesting that EDA2R induces podocyte apoptosis and de-differentiation, at least partly, through enhanced ROS generation.

EDA2R is downstream of the p53 gene, which is known for its tumor suppressor effect. NF-κB and JNK have been reported to the downstream signaling pathways for EDA2R [8, 9]. Interestingly, high glucose-mediated podocyte injury is also inflicted through these pathways [28–31]. On that account, there is a possibility that the expression of ED2AR in podocytes is being induced to activate these pathways in high glucose milieu.

In addition to podocytes, mesangial cells and tubular cells are involved in the progression of DN. For example, apoptosis of proximal tubular cells contributes to the progression of DN [32–34]. In the present study, IHC staining also revealed increased EDA2R expression in tubular cells. It will be worth investigating the effect of EDA2R on tubular cell injury in future studies.

Since knockout of the ED2AR did not result in significant phenotypic changes in kidneys [35], it appears that inhibition of EDA2R expression does not affect the normal renal

function. This would allow the modulation of EDA2R expression as an ideal target to prevent the onset or slow down the progression of DN. However, further studies needed to validate this notion.

In summary, the present study demonstrated the presence of EDA2R expression in podocytes, both *in vivo* and *in vitro*. Overexpression of EDA2R induced apoptosis and dedifferentiation in podocytes through enhanced ROS generation. The silencing of EDA2R expression prevented high glucose-induced podocyte injury.

## 5. Conclusion

In high glucose milieu, EDA2R mediates podocyte apoptosis and dedifferentiation through enhanced ROS generation. Our study revealed an important role of EDA2R in the development of DN, highlighting some new potential therapeutic targets for DN.

## Acknowledgment

P.C.S was supported by the NIH grants R01DK098074 and RO1DK118017; H.W. was supported by the National Institute of General Medical Sciences grants (R01GM063075.) and the National Center of Complementary and Alternative Medicine grant (R01AT005076).

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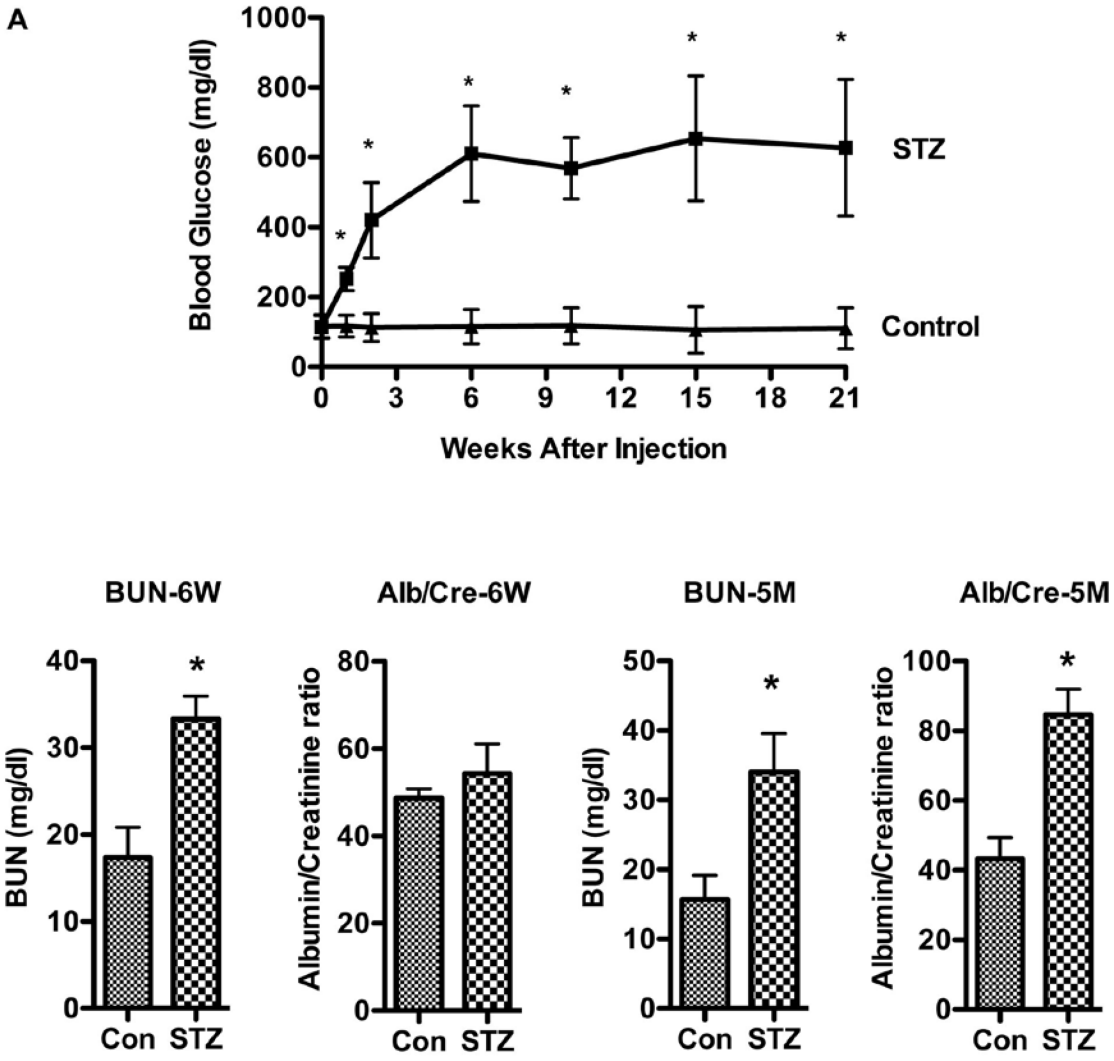


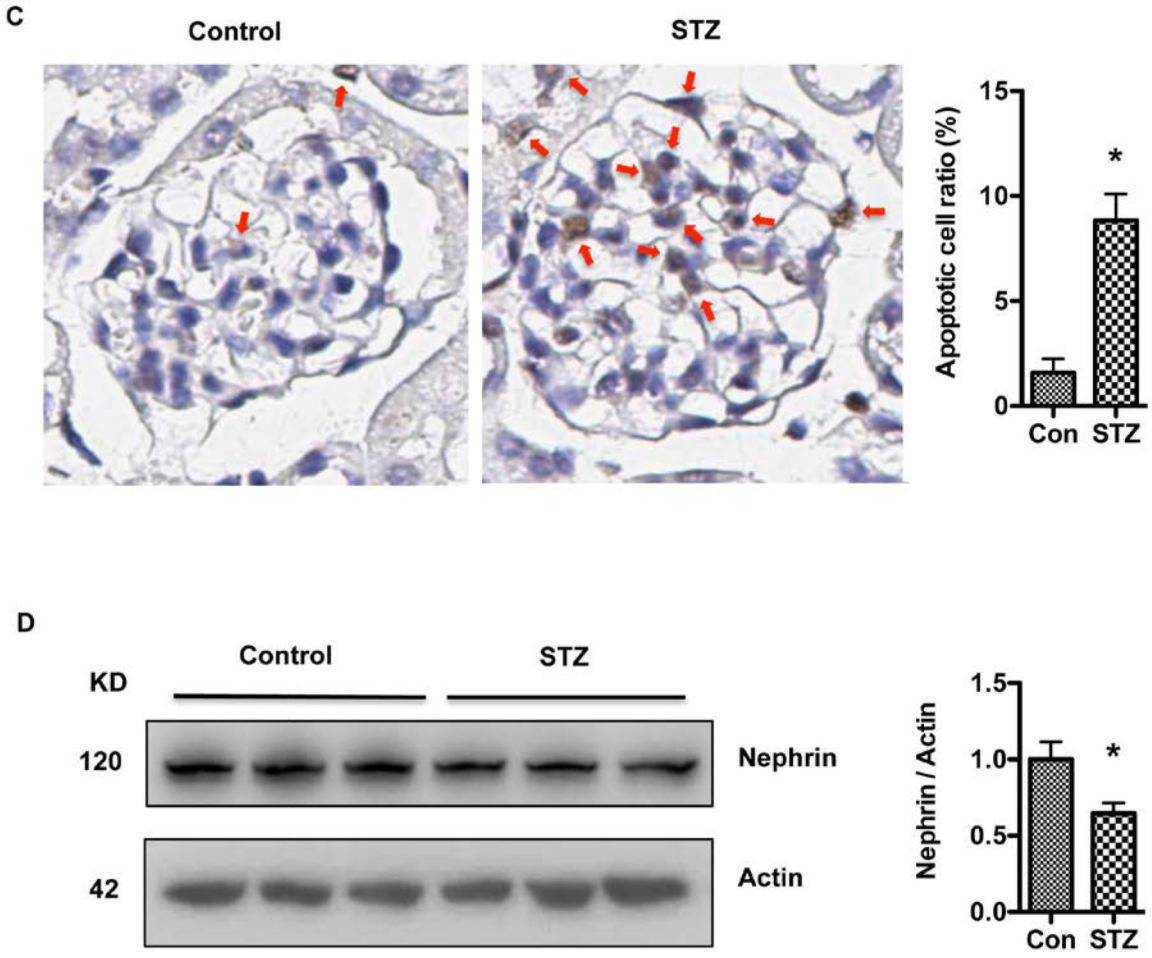
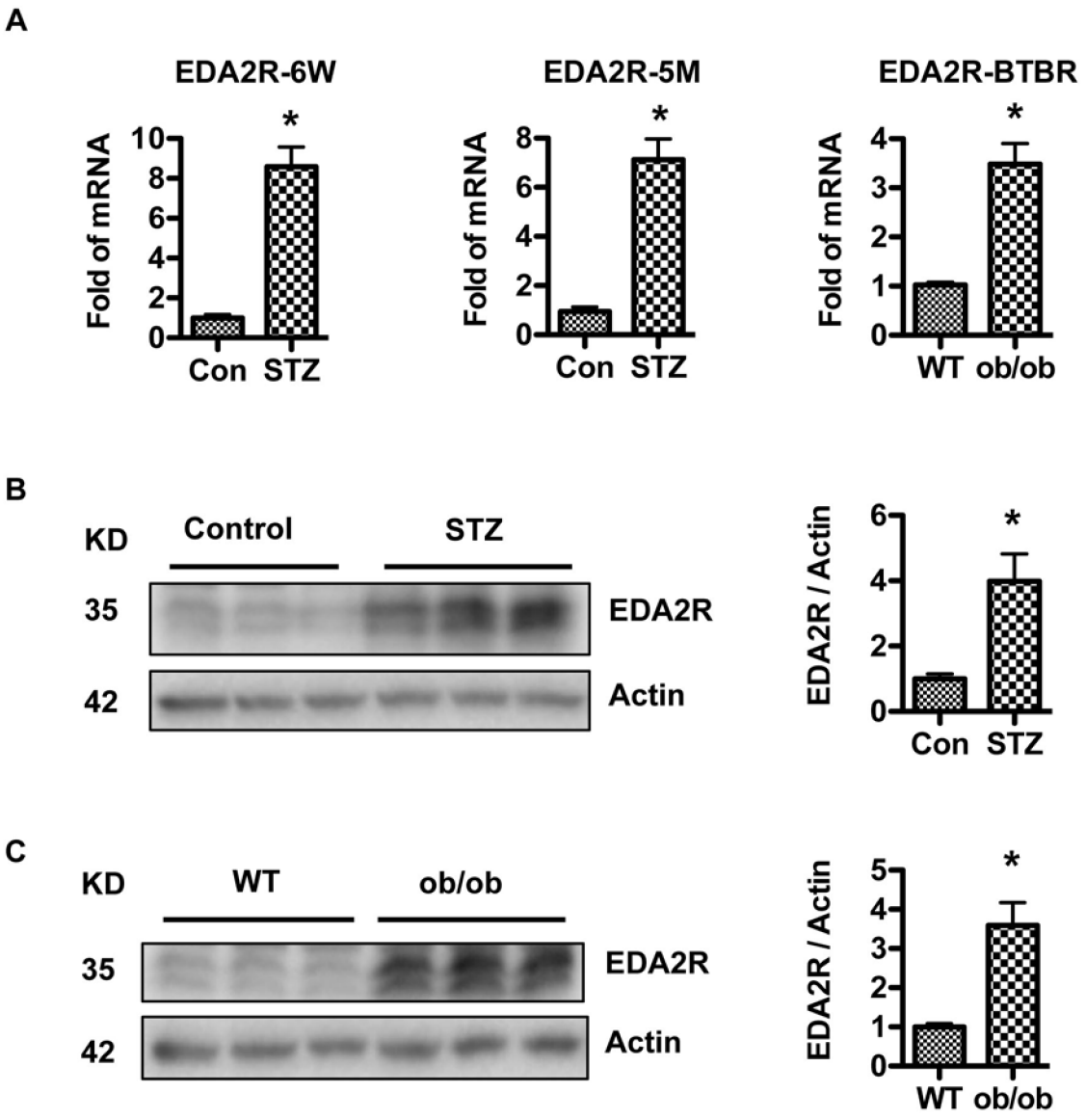



**Figure 1. Hyperglycemia causes severe kidney injury.**

Twelve-week-old mice were injected with STZ (50 mg/kg) or control buffer for 5 consecutive days. One week after the last injection, the blood glucose concentrations (BGC) were monitored (A). After 6 weeks and 5 months, blood samples were collected for BUN determination, and urine samples were collected for the assay of albumin-to-creatinine ratio (B). To examine the apoptotic cell ratio in the glomeruli, we sacrificed the mice after 5 months after injection and collected the kidney samples for TUNEL staining (C); apoptotic cell ratios in 10 randomly selected regions were calculated, and the statistical results (mean ± SD) were represented. The apoptotic cells were indicated with red arrows in the representative figures, and the magnifications were 400 x. We also collected the kidney tissue lysates for Western blotting to detect nephrin expression (D). The results (mean ± SD) from three independent samples were displayed. $P < 0.05$ (indicated with *) were regarded as statistically significant when compared with control mice (Con).



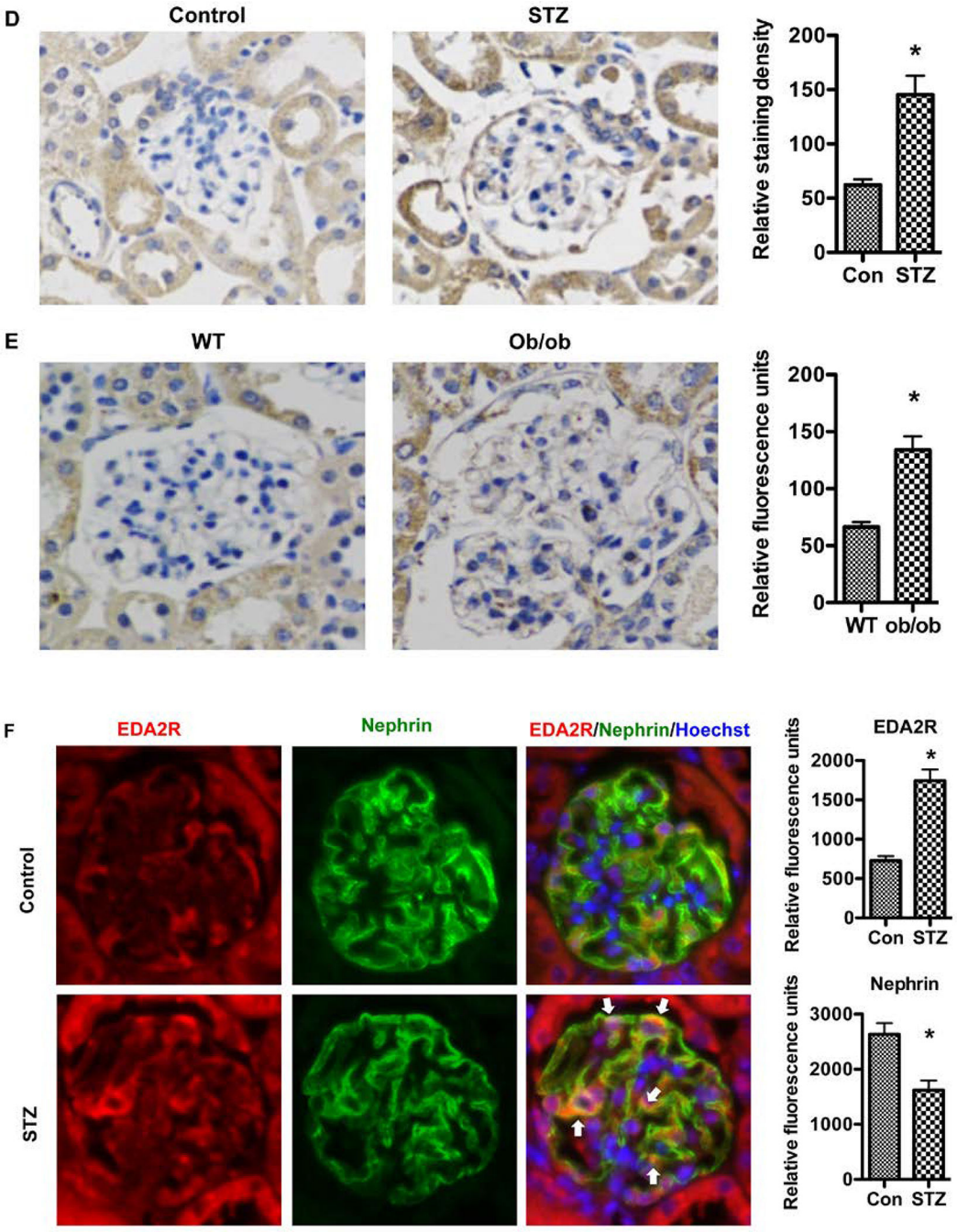



**Figure 2. Expression of EDA2R in mouse kidneys.**

**A**. Total RNAs were extracted from the kidneys of STZ/control buffer injected (6 weeks or 5 months after injection at 12 weeks old) and BTBR (wild type and diabetic ob/ob type at 14 weeks old) mice, and real-time PCR was performed to determine EDA2R mRNA expression. The results (mean ± SD) from three independent samples were displayed. **B-C**, part of the mouse kidneys obtained from **A** were homogenized, and the tissue lysate was subjected to Western blotting to determine EDA2R and nephrin protein expression. The STZ/control buffer-treated mice were 5 months after STZ injection (**B**). The results (mean ±

SD) from three independent samples were displayed. **D**-**E**, part of the mouse kidneys obtained from **A,** were used to prepare paraffin sections for immunohistochemistry to detect EDA2R expression. The STZ/control buffer-treated mice were 5 months after STZ injection (**D**). The average intensities of 10 randomly selected regions were used for the results (mean ± SD) calculation. The magnifications in the representative figures were 200 x. **F**, paraffin sections obtained from **D** were used for immunofluorescence staining to detect the expression of EDA2R and nephrin in mouse kidneys. The average intensities of 10 randomly selected regions were used for the results (mean ± SD) calculation. The co-localizations of EDA2R and nephrin were indicated with white arrows in the representative figures, and the magnifications were 200 x. For all groups, $p < 0.05$ (indicated with *) were regarded as statistically significant when compared with control mice (Con/WT).

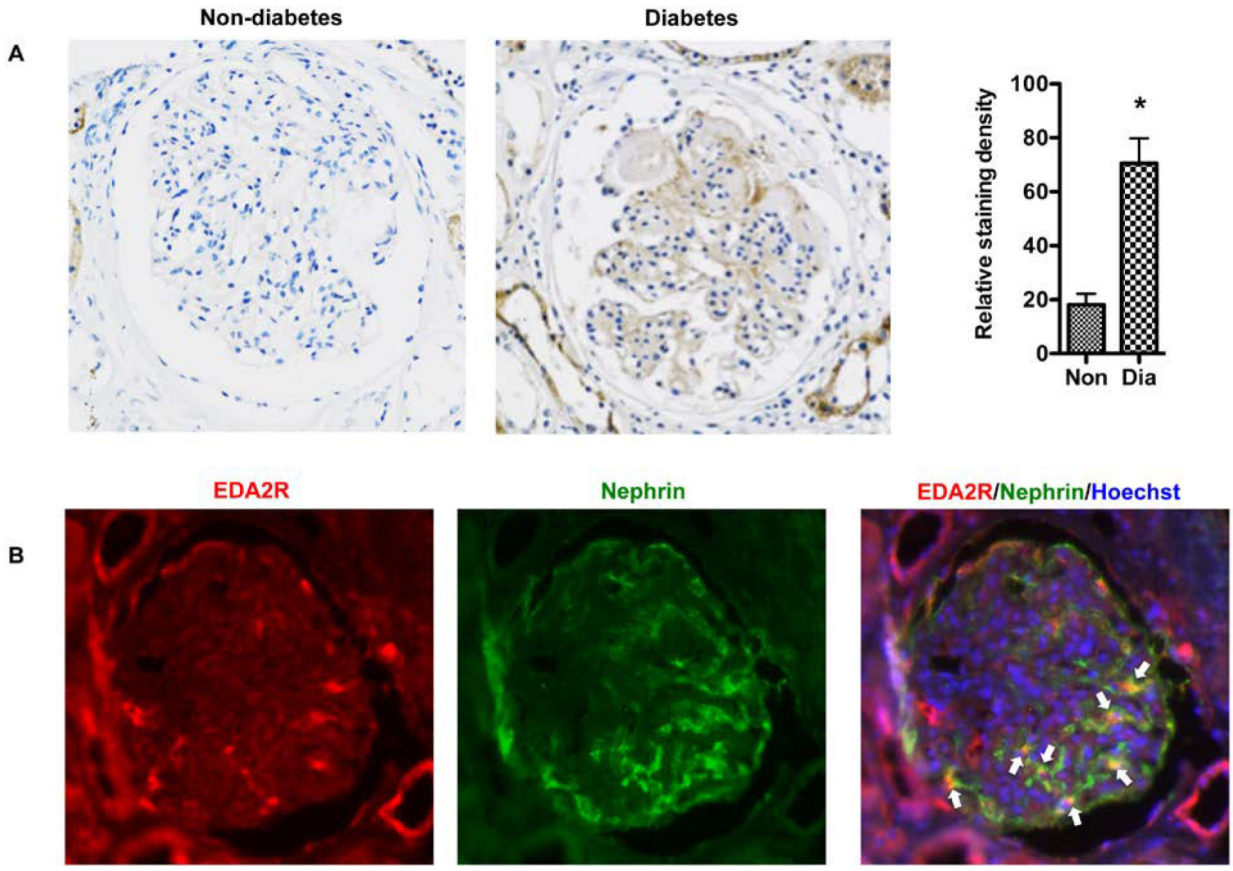



**Figure 3. Expression of EDA2R in human kidneys.**

**A.** Paraffin sections were prepared from the kidneys of diabetic or non-diabetic human patients and were used for immunohistochemistry to examine EDA2R expression in kidneys. The average intensities of 10 randomly selected regions were used for the results (mean ± SD) calculation. The magnifications in the representative figures were 400 x. $P < 0.05$ (indicated with *) were regarded as statistically significant when compared with non-diabetic patients. B, paraffin sections obtained from **A** were used for immunofluorescence staining to determine EDA2R and nephrin co-localization in kidneys. The co-localizations of EDA2R and nephrin were indicated with white arrows in the representative figures, and the magnifications are 200 x. Note: Non, non-diabetes; dia, diabetes.

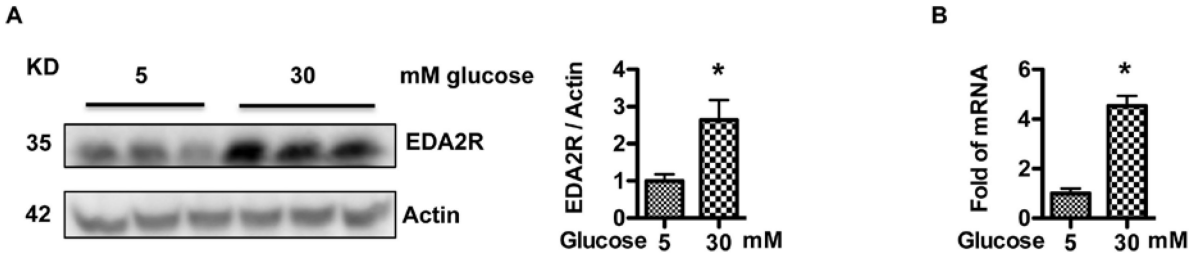



**Figure 4. EDA2R expression is increased by high glucose.**

Cultured human podocytes ($2\times10^5$ in 60 mm dishes) were treated with 5 mM or 30 mM glucose. After 24 h, the cellular lysates were collected and were subjected to Western blotting analysis to determine EDA2R protein expression (**A**). In parallel experiments, total RNAs were extracted and were used for real-time PCR analysis to determine EDA2R mRNA expression (**B**). The results (mean ± SD) from three independent samples were displayed, and $p < 0.05$ (indicated with *) were regarded as statistically significant when compared with control (5 mM glucose treatment).

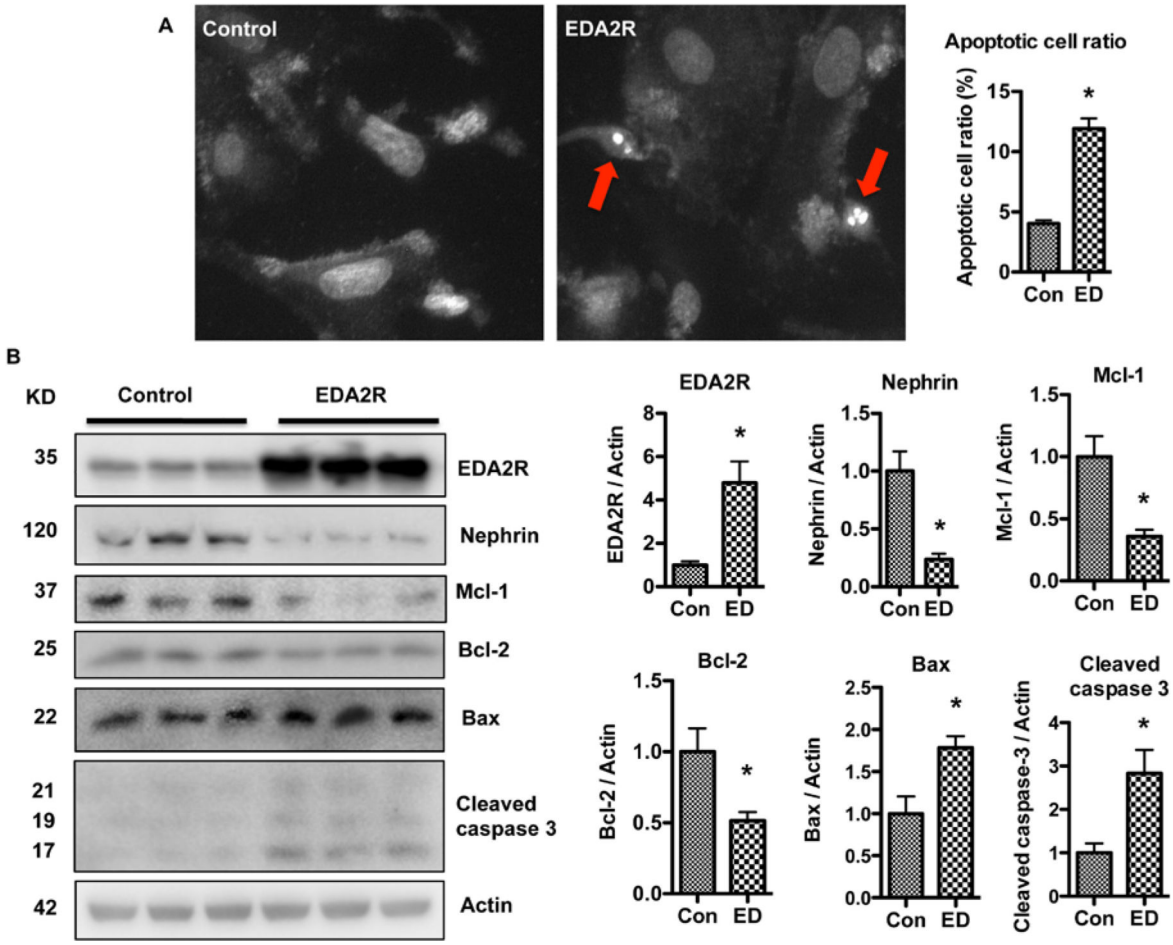



**Figure 5. Increased podocyte injuries are caused by EDA2R overexpression.**

Human podocytes ($2\times10^5$) were cultured in 60 mm dishes and were transfected with 1 μg of plasmid pCMV3-EDA2R or the same amount of control plasmid for 48 h. **A.** Hoechst33342 staining was conducted to determine apoptotic cells under a microscope. Apoptotic cell ratios in 10 randomly selected regions were calculated, and the statistical results (mean ± SD) were represented. The apoptotic cells (indicated with red arrows) were indicated with red arrows in the representative figures, and the magnifications were 200 x. **B**, cellular lysates were collected and were subjected to Western blotting analysis. The results (mean ± SD) from three independent samples were displayed. For both A and B, $p < 0.05$ (indicated with *), were regarded as statistically significant when compared with the control plasmid.

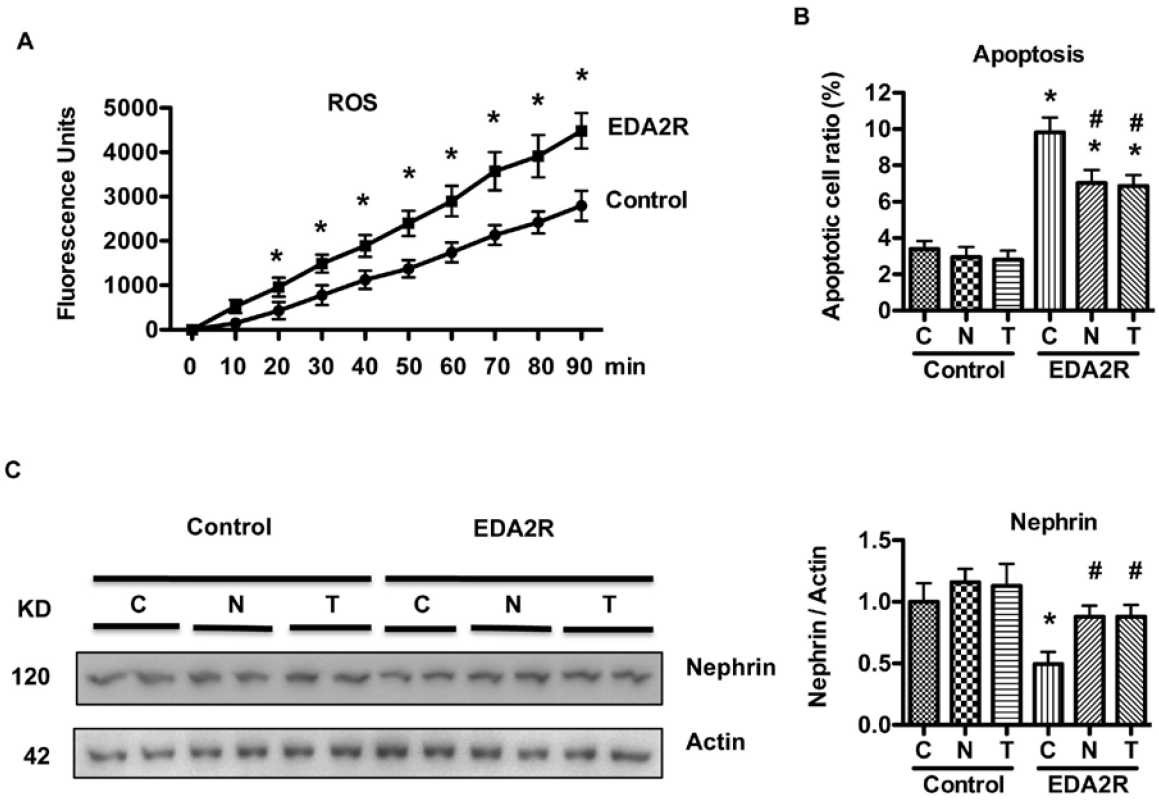



**Figure 6. EDA2R mediates podocyte injury through ROS generation.**

**A.** human podocytes in 96-well plates ($1\times10^4$ for each well) were transfected with pCMV-EDA2R/control plasmid (100 ng per well) for 24 h, and then ROS measurements were carried out by analyzing the fluorescence intensity of CM-H2DCFDA. **B.** human podocytes ($2\times10^5$) cultured in 60 mm dishes were transfected with 1 μg of plasmid pCMV3-EDA2R/control plasmid for 24 h, and then NAC (100 μM) or mito-TEMPO (10 μM) was added in the medium. After another 48 h, Hoechst 33342 staining was performed, and the number of apoptotic cells was counted under a microscope. Apoptotic cell ratios in 10 randomly selected regions were calculated, and the statistical results (mean ± SD) were represented. **C. A**fter the same treatment as B, cellular lysates were collected for Western blotting analysis. The results (mean ± SD) were from three independent samples. $P < 0.05$ were regarded as statistically significant when compared with the control plasmid (indicated with *), or when compared with EDA2R only (indicated with #). Note: C, control; N, NAC; T, mito-TEMPO.

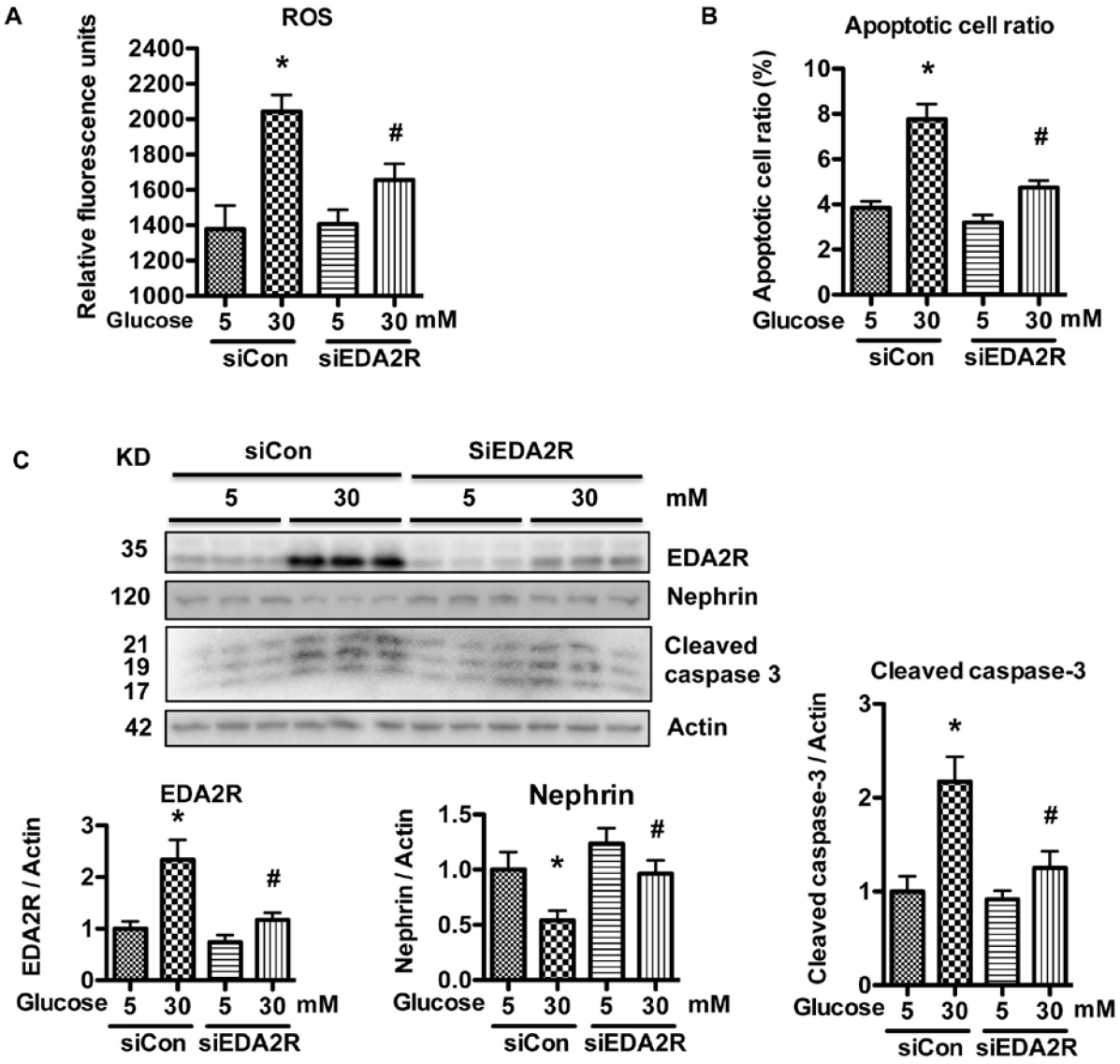



**Figure 7. High glucose-induced podocyte injury can be attenuated by EDA2R-silencing.**

**A.** cultured human podocytes ($1\times10^4$ in 96-well plate) were transfected with siEDA2R/siCon (10 pmol) for 24 h, and then treated with 5 mM or 30 mM glucose. After another 24 h, ROS generation was measured with CM-H2DCFDA. **B.** cultured human podocytes ($2\times10^5$ in 60 mm dishes) were transfected with siEDA2R/siCon (60 pmol) for 24 h and then treated with 5 mM or 30 mM glucose. After 24 h, Hoechst 33342 staining was performed, and apoptotic cells were counted under a microscope. Apoptotic cell ratios in 10 randomly selected regions were calculated, and the statistical results (mean ± SD) were represented. **C.** **A**fter the same treatment as **B**, cellular lysates were collected for Western blotting analysis. The results (mean ± SD) from three independent samples were displayed. $P < 0.05$ were regarded as statistically significant when compared with SiCon-5 mM (indicated with *) or when compared with SiCon-30 mM (indicated with #).

**Table 1.**

Primers for real-time PCR

| Target Gene | Forward/Reverse | Sequence |
|---|---|---|
| Mouse EDA2R | Forward | CCAGCTAATGAGGGCATCTTG |
| | Reverse | CCCATTGAGAATGGCTCTCTG |
| Human EDA2R | Forward | TTACCATGGCCTCCTGCAC |
| | Reverse | GGGCTGGGAACTTCAAAGG |
| Human / mouse GAPDH | Forward | GGGAAGCTCACTGGCATGGCCTTCC |
| | Reverse | CATGTGGGCCATGAGGTCCACCAC |