

## SUPPLEMENTARY MATERIAL

### SUPPLEMENTARY RESULTS

#### **Alteration in EET Production Mediates High Glucose-Induced Podocyte Injury Through VEGF-A and NOX4 Dependent Mechanisms**

To further determine the role of CYP2C11-derived EETs in HG-induced podocyte injury and to confirm our *in vivo* data, rat and human podocytes were treated with 25 mmol/L D-glucose (HG) for 72 hours either in the presence or absence of sEH inhibitor, AUDA. Exposure of human and rat podocytes to HG significantly increased podocyte apoptosis as measured by cellular DNA fragmentation (**Suppl. Figures 3A and 5A**) and caspase 3 activity (**Suppl. Figures 3B and 5B**). This was paralleled by a significant reduction in CYP2C11 mRNA (**Suppl. Figures 3C and 5C**) and 14,15-EET levels (**Suppl. Figures 3D and 3E**). Besides, rat podocytes showed a significant increase in ROS production (**Suppl. Figure 3E**), NOX4 mRNA levels (**Suppl. Figure 3F**), protein expression (**Suppl. Figure 3G**), and NADPH oxidase activity (**Suppl. Figure 3H**). Treatment with AUDA significantly reversed these changes (**Suppl. Figures 3A-H and 5A-D**). Furthermore, a significant increase in VEGF-A mRNA levels was noted when rat and human podocytes were exposed to HG. Of note, treatment with AUDA markedly attenuated HG-induced VEGF-A expression (**Suppl. Figures 3I and 5E**). Collectively, these observations imply that decreased EET formation mediates the effect of HG on ROS production and exacerbates podocyte injury through a VEGF-A-dependent mechanism.

To further establish the role of VEGF-A in the observed podocyte injury, human and rat podocytes were transfected either with small interfering RNA (siVEGF-A) or with nontargeting siRNA (Scr; control) before exposure to 5 mmol/L D-glucose (NG) or 25 mmol/L D-glucose (HG) for 72 hours. Exposure of human or rat podocytes to HG results in a marked increase in VEGF-A protein

expression as compared to podocytes treated with NG, whereas transfection of rat podocytes with siVEGF-A significantly decreases VEGF-A mRNA levels in the HG milieu, while, and as expected, the use of Scr had no significant effect (**Suppl. Figures 4A and 5F**). These results were paralleled by an increase in podocyte apoptosis that was attenuated upon siVEGF-A transfection (**Suppl. Figures 4B-4C and 5G-5H**).

To confirm the crosstalk between CYP2C11-derived EET production and VEGF-A signaling pathway, 30 ng/mL of exogenous recombinant rat VEGF-164 (rVEGF-A) was added to cultured rat podocytes in NG for 72 hours. Exposure of rat podocytes to HG for 72 hours significantly increased their apoptosis, as measured by cellular DNA fragmentation (**Suppl. Figure 4B**) and caspase 3 activity (**Suppl. Figure 4C**). The use of 30 ng/mL of rVEGF-A for 72 hours mimicked the effect of HG (**Suppl. Figure 4B and 4C**). In addition, a significant increase in superoxide generation (**Suppl. Figure 4D**), NOX4 mRNA (**Suppl. Figure 4E**) and protein (**Suppl. Figure 4F**) levels and NADPH oxidase (**Suppl. Figure 4G**) activity was noted upon treating rat podocytes with either HG or exogenous VEGF-A. The use of siVEGF-A significantly reversed these observations (**Suppl. Figures 4D-G**). In our *in vitro* experiments, rats or human podocytes incubated in the presence of 25 mM of Mannitol show no changes in the measured parameters when compared to podocytes exposed to 5 mM glucose (data not shown). Taken together, our *in vitro* data indicate that HG-induced podocyte injury is mediated through the activation of VEGF-A signaling pathway, which acts as a mechanistic link between EET formation on one hand and Nox4 on the other.

**SUPPLEMENTARY FIGURE LEGENDS****Supplementary Figure 1:*****The effect of AUDA on Cyp2C11 mRNA levels and 14,15-EET formation***

(A) Relative mRNA levels of CYP2C11. (B) Histogram representing 14,15-EETs formation. It shows quantitation of the results from five different rats in each group. All values are the means  $\pm$  SE. \* $p < 0.05$  versus control; # $p < 0.05$  versus untreated diabetic rats.

**Supplementary Figure 2:*****Anti-VEGF and SU5416 regulate VEGF mRNA, protein and urine levels.***

(A) Relative mRNA levels of VEGF-A (B) Histograms showing quantitation of VEGF-A/GAPDH. (C) Urinary VEGF expressed in pg/24hr. All values are the means  $\pm$  SE. \* $p < 0.05$  versus control; # $p < 0.05$  versus untreated diabetic rats.

**Supplementary Figure 3:*****AUDA reverses HG-induced podocyte injury.***

Rat podocytes were exposed to either HG (25mmol/l) or HG with 50  $\mu$ M AUDA. Apoptosis assessed using (A) cellular DNA fragmentation and (B) caspase 3 activity. (C) Relative mRNA levels of CYP2C11. (D) Histogram representing 14,15-EETs formation. (E) HPLC used to evaluate superoxide generation. (F) Relative mRNA levels of Nox4. (G) Histogram showing quantitation of NOX4/GAPDH. (H) NADPH-dependent superoxide generation. (I) Relative mRNA levels of VEGF-A. All values are the means  $\pm$  SE. \* $p < 0.05$  versus control group; # $p < 0.05$  versus HG group.

**Supplementary Figure 4:*****VEGF-A signaling pathway mediates HG-induced podocyte injury.***

Rat podocytes were transfected with small interfering RNA (siVEGF-A) or with nontargeting siRNA (Scr) before exposure to HG (25mmol/l) or rVEGF-A (30 ng/ml) for 72hr. (A) Relative mRNA levels of VEGF-A. Apoptosis assessed using (B) cellular DNA fragmentation and (C) caspase 3 activity. (D) Superoxide generation assessed using HPLC. (E) Relative mRNA levels of Nox4. (F) Histogram showing quantitation of Nox4/GAPDH. (G) NADPH-dependent superoxide generation. All values are the means  $\pm$  SE. \* $p < 0.05$  versus NG + Scr group; # $p < 0.05$  versus NG + rVEGF-A group and \$ $p < 0.05$  versus HG + Scr group.

**Supplementary Figure 5:*****Increasing EETs bioavailability or inhibiting VEGF-A reverse HG-induced podocyte injury.***

Human podocytes were exposed to either HG (25mmol/l) or HG with 50  $\mu$ M AUDA. (A) cellular DNA fragmentation and (B) caspase 3 activity. (C) Relative mRNA levels of CYP2C11. (D) Histogram representing 14,15-EETs formation. (E) Relative mRNA levels of VEGF-A. All values are the means  $\pm$  SE. \* $p < 0.05$  versus control group; # $p < 0.05$  versus HG group.

In parallel experiments, human podocytes were transfected with small interfering RNA (siVEGF-A) or with nontargeting siRNA (Scr) before exposure to HG (25mmol/l) for 72hr. (F) Relative mRNA levels of VEGF-A. Apoptosis assessed using (G) cellular DNA fragmentation, and (H) caspase 3 activity. All values are the means  $\pm$  SE. \* $p < 0.05$  versus NG + Scr group; # $p < 0.05$  versus HG + Scr group.