Mechanisms of Podocyte Injury in Diabetes
Role of Cytochrome P450 and NADPH Oxidases

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OBJECTIVE—We investigated the role of cytochrome P450 of the 4A family (CYP4A), its metabolites, and NADPH oxidases both in reactive oxygen species (ROS) production and apoptosis of podocytes exposed to high glucose and in OVE26 mice, a model of type 1 diabetes.

RESEARCH DESIGN AND METHODS—Apoptosis, albuminuria, ROS generation, NADPH superoxide generation, CYP4A and Nox protein expression, and mRNA levels were measured in vitro and in vivo.

RESULTS—Exposure of mouse podocytes to high glucose resulted in apoptosis, with approximately one-third of the cells being apoptotic by 72 h. High-glucose treatment increased ROS generation and was associated with sequential upregulation of CYP4A and an increase in 20-hydroxyeicosatetraenoic acid (20-HETE) and Nox oxidases. This is consistent with the observation of delayed induction of NADPH oxidase activity by high glucose. The effects of high glucose on NADPH oxidase activity, Nox proteins and mRNA expression, and apoptosis were blocked by N'-hydroxy-N-(4-butyl-2-methylphenol) formamidine (HET0016), an inhibitor of CYP4A, and were mimicked by 20-HE. CYP4A and Nox oxidase expression was upregulated in glomeruli of type 1 diabetic OVE26 mice. Treatment of OVE26 mice with HET0016 decreased NADPH oxidase activity and Nox1 and Nox4 protein expression and ameliorated apoptosis and albuminuria.

CONCLUSIONS—Generation of ROS by CYP4A monooxygenases, 20-HETE, and Nox oxidases is involved in podocyte apoptosis in vitro and in vivo. Inhibition of selected cytochrome P450 isoforms prevented podocyte apoptosis and reduced proteinuria in diabetes. Diabetes 58:1201–1211, 2009

Diabetic nephropathy in humans is characterized by increased urinary albumin excretion (microalbuminuria), which often progresses to proteinuria, one of the most important prognostic risk factors for kidney disease progression (1). Glomerular visceral epithelial cells, or podocytes, play a critical role in maintaining the structure and function of the glomerular filtration barrier. Careful morphometric analyses of renal biopsy in subjects with type 1 and type 2 diabetes (2–4) demonstrate that the density of podocytes is reduced not only in individuals with diabetic nephropathy, but also in patients with short duration of diabetes before the onset of microalbuminuria (4,5). Studies in experimental models of type 1 and type 2 diabetes have also documented that podocyte depletion represents one of the earliest cellular lesions affecting the diabetic kidney (6,7). Among various morphologic characteristics, the decreased number of podocytes in glomeruli is the strongest predictor of progression of diabetic nephropathy, where fewer cells predict more rapid progression (3,4). Although these observations identify podocyte depletion as one of the earliest cellular features of diabetic kidney disease, the mechanisms that underlie the loss of podocytes in diabetic nephropathy remain poorly understood.

High glucose induces apoptosis (8), and there is evidence that podocyte apoptosis contributes to reduced podocyte number (9). High glucose, transforming growth factor-β (TGF-β), and angiotensin II (ANGII) induce apoptosis of cultured podocytes (9–12). ANGII appears to induce apoptosis in cultured rat glomerular epithelial cells at least partially via TGF-β because its apoptotic effect is attenuated by an anti-TGF-β antibody (12). There is also evidence that reactive oxygen species (ROS) contribute to podocyte apoptosis and depletion in cells exposed to high glucose and in experimental diabetic nephropathy (7). However, the sources of ROS and the kinetics of their generation have not been well characterized. We and others (13–15) have recently identified NADPH oxidases as major sources of ROS in kidney cortex and glomeruli of rats with type 1 diabetes. Six homologs of the cytochrome subunit of the phagocyte NADPH oxidase (Nox2/gp91phox) have been cloned (16). At least three different Nox isoforms are expressed in the kidney cortex: Nox1, Nox2, and Nox4 (16). Cytochromes P450 (CYP450s) are significant sources of ROS in many tissues (17,18). CYP450 metabolizes arachidonic acid into hydroxyeicosatetraenoic acids (20-HETEs) and EETs (epoxyeicosatrienoic acids). 20-HETE, the ω-hydroxylation product of arachidonic acid, is one of the major CYP eicosanoids produced in the kidney cortex (19–21). The predominant CYP450 in the kidney cortex that synthesizes 20-HETE is cytochrome P450 of the 4A family (CYP4A) (19–21). 20-HETE has multiple and opposing functions depending on the site of production and target cells/tissues (19,22–24).

In this study, we demonstrate that high glucose induces ROS production and apoptosis in cultured mouse podocytes through the upregulation of CYP4A with increased production of 20-HETE and upregulation of NADPH oxidases. Inhibition of 20-HETE production prevented podocyte apoptosis in vitro and decreased oxidative stress, podocyte apoptosis, and proteinuria in an in vivo model of type 1 diabetes.
RESEARCH DESIGN AND METHODS

Conditionally immortalized mouse podocytes, kindly provided by Dr. Peter Mundel (University of Florida, Miami, Florida), were cultured as previously described (20,25). In brief, cells were first grown under permissive conditions at 33°C in RPMI-1640 media containing 10% FCS, 50 units/ml interferon (IFN)-γ (IFN), and 100 units/ml of penicillin/streptomycin in collagen-coated flasks. Concentration of IFN-γ was then tapered to 10 units/ml, and cells were subcultured weekly under permissive conditions at 33°C in serum-free medium without IFN-γ. Near-confluent cells were grown-arrested in RPMI-1640 containing 0.2% BSA (fatty-acid free) and 5 mM/nl α-glucose for 24 h before the experiment.

Animal models. We used 7-month-old FVB and OVE26 mice on FVB background (Jackson Laboratories, Bar Harbor, ME). At 6 months of age, OVE26 mice were treated with N-hydroxy-N′-(4-butyl-2-methylphenyl) formamidine (HET0016), a specific inhibitor of CYP24A, for 3 weeks. Mice were placed in metabolic cages for urine collection, and before the treatment with HET0016, urine albumin was measured by an Alburell M test kit (Exocell, Philadelphia, PA) and expressed as micrograms of albumin per 24 h. Animals were killed by exsanguination under anesthesia. Both kidneys were removed and weighed. A slice of kidney cortex at the pole was embedded in paraffin or flash-frozen in liquid nitrogen for microscopy and image analyses. Cortical tissue was used for isolation of glomeruli by differential sieving with minor modification as previously described (20).

Detection of ROS. The peroxide-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescin (DCF) diacetate (Molecular Probes) was used to measure intracellular ROS as previously described (15). Cells were grown in 12- or 24-well plates and serum-deprived for 24 h. Immediately before the experiments, cells were washed with Hank’s buffered salt solution containing Ca²⁺ and Mg²⁺ and then loaded with 50 μM DCF diacetate dissolved in Hank’s buffered salt solution for 30 min at 37°C. Cells were then incubated with 15 min with HET0016 followed by the addition of the agonist or vehicle for various time periods. DCF fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, and measured in a multicolor flow cytometer (Wallac 1420 Victor, PerkinElmer).

NADPH oxidase activity. NADPH oxidase activity was measured in podocytes grown in serum-free medium or in glomeruli isolated from kidney cortex as previously described (13). Cultured podocytes were washed five times in ice-cold Hank’s buffer and incubated from the time of harvest until the time of centrifugation at 800 rpm, 4°C, for 10 min. The cell pellets were resuspended in lysis buffer (20 mM/nl KH₂PO₄, pH 7.0, 1 mM/nl EDTA, 1 mM/nl phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin). Cell suspensions or washed glomeruli were homogenized with 100 strokes in a Dounce homogenizer on ice. To start the assay, 20 μl of the supernatant was added to 50 mmol/l phosphate buffer, pH 7.0, containing 30 μM lucigenin, and 100 μM NADPH. Photon emission expressed as relative light units was measured every 20 or 30 s for 10 min in a luminometer. A buffer blank (45 s) was subtracted from each reading. Superoxide production was expressed as the average number of podocytes per glomerular section as previously described (31–33).

Annexin V and propidium iodide staining. An annexin V–fluorescein isothiocyanate (FITC) apoptosis detection kit (Calbiochem) was used for annexin V and propidium iodide staining according to the manufacturer’s protocol. Podocytes were cultured in 60 mm dishes to ~80% confluency. Cells were then preincubated with or without 10 μmol/l HET0016 before the addition of glucose (25 mM/l) or 1 mM/l of 20-HETE. Cells were then incubated for 24, 48, or 72 h at 37°C. The percentage of apoptotic and necrotic cells was assessed by fluorescence-activated cell sorting (San Antonio Cancer Institute core facility of the University of Texas Health Science Center San Antonio).

Electron microscopy. Kidney cortexes isolated from the FVB, OVE26, and OVE26 mice treated with HET0016 were cut into 0.5- to 1-mm² pieces and fixed overnight in cold 4% formaldehyde and 1% glutaraldehyde in phosphate buffer and then embedded in epon 812 resin. Then, 0.5-mm plastic sections were cut and stained with toluidine blue for identification of representative areas for subsequent sectioning by ultramicrotome. Ultrathin sections were stained with uranyl acetate and examined and photographed on a JEOL 100CX electron microscope. All electron microscope (EM) photomicrographs were printed on color high-quality paper. Capillary logs were examined and quantified in five glomeruli per group of animals (FVB, OVE26, and OVE26 mice treated with HET0016) for the degree of foot process effacement as described by Jo et al. (29).

Podocyte enumeration. Dual-label immunohistochemistry was used to identify and count glomerular epithelial cells relative to the glomerular basement membrane (GBM) using a modification of methods previously described (30). To identify podocytes, 3-μm frozen sections of kidney cortex on glass slides were stained with a goat anti-synaptopodin antibody (Santa Cruz Biotechnology) followed by Cy3-labeled donkey anti-goat IgG (Chemicon). After washing, and to identify the GBM, the sections were stained with a rabbit antibody directed against collagen type IV (Chemicon) followed by FITC-labeled donkey anti-rabbit IgG (Chemicon). After staining and washing, the sections were preserved on coverslips in Prolong gold antifade mounting medium with 4’,6-diamidino-2-phenylindole (Invitrogen) for the fluorescence detection of nuclei. Sections were examined by epifluorescence using excitation and band-pass filters optimal for FITC, Cy3, and 4’,6-diamidino-2-phenylindole. Digital images representing each fluorescence were taken of random glomeruli using an AX70 Research microscope and a DP70 digital camera (Olympus, Melville, NY). Then, 25–30 glomerular cross-sections per animal were photographed in each color channel providing a minimum of 100 composite images per experimental group. The images were merged and color balanced using Image-Pro Plus imaging software (Media Cybernetics, Silver Spring, MD), and podocytes were counted in projected images in a blind fashion by two individuals. Synaptopodin-positive cells on the outer aspect of the GBM were considered glomerular epithelial cells and counted. Synaptopodin-negative cells or those in the inner aspect of the GBM were not counted. The podocytes were then microdissected from each glomerulus containing the GBM using a plastic hypodermic needle, tracing the glomerular outline, encircling the area of interest, and calculating the surface area by computerized morphometry using MetaMorph 4.69. The results were expressed as the average number of podocytes per glomerular section as previously described (31–33).

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Values are the means ± SE of three independent experiments (n = 3). *P < 0.05, high glucose vs. control. B: Mouse podocytes were exposed to either high glucose (HG; 25 mmol/l) or normal glucose (NG; 5 mmol/l) for the indicated time periods. NADPH generation was measured by DCF with a multiwell fluorescence plate reader as described in Research Design and Methods. Values are the means ± SE of three independent experiments (n = 3). *P < 0.05, NG vs. HG. C: Expression of Nox1, Nox2, and Nox4 protein was determined by Western blotting analysis on homogenized podocytes. Actin was included as a control for loading and the specificity of change in protein expression. The Western blot is representative of three independent experiments (n = 3).

RESULTS

High glucose induces oxidative stress in podocytes. Exposure of podocytes to 25 mmol/l glucose (high glucose) results in a relatively rapid generation of ROS, as measured by DCF fluorescence, compared with cells incubated in 5 mmol/l glucose (normal glucose) or mannitol used as an osmotic control. ROS generation was detected after 6 h of exposure to high glucose and was sustained up to 72 h (Fig. 1A). NADPH oxidases of the Nox family are major sources of ROS in cells and tissue (13,15,16). However, there was no increase in NADPH-dependent superoxide generation 6 h after exposure of the cells to high glucose (Fig. 1C). The increase in NADPH oxidase activity was detectable only at 12 h and remained sustained up to 72 h (Fig. 1B), suggesting that other sources of ROS besides NADPH oxidases were involved in the ROS generation seen at early time points after treatment with high glucose. Consistent with these observations, we found that upregulation of the two major Nox isoforms, Nox1 and Nox4, was not seen before 12 and 24 h, respectively. Note that Nox2 isoform expression was not altered by high-glucose treatment.

High glucose induces CYP4A protein expression and 20-HETE production. We next examined whether CYP4A contributes to oxidative stress induced by high glucose in mouse podocytes. Microsomes were isolated from podocytes incubated in normal or high glucose. Our data show that CYP4A protein expression was increased in microsomes isolated from podocytes incubated in the presence of high glucose compared with podocytes incubated in normal glucose, with maximum expression seen at 6 and 12 h (Fig. 2A). The increase of CYP4A protein expression was accompanied by an increase in 20-HETE formation, the major biologically active metabolite produced by CYP4A (Fig. 2B).

20-HETE generated by CYP4A mediates the effect of high glucose on ROS generation. We next determined whether CYP4A-derived 20-HETE mediates the effect of high glucose on ROS generation in podocytes. DCF fluorescence was used to measure ROS in mouse podocytes exposed to high glucose, in the absence or presence of N-hydroxy-N’-(4-butyl-2-methylphenol) formamidine (HET0016; 10 μmol/l, 1 h), a potent inhibitor of CYP4A (20-HETE biosynthesis) (34). The increase in ROS generation induced by high glucose was inhibited by HET0016 (Fig. 2C). Moreover, we show that purified recombinant 20-HETE (1 μmol/l) mimicked the effect of high glucose on ROS production in podocytes (Fig. 2C). Collectively, these data indicate that 20-HETE, the major product of the CYP4A pathway, mediated the effect of high glucose on ROS in podocytes. Furthermore, the data indicate that the increase in ROS in podocytes was likely caused by sequential activation of CYP4A and activation of Nox oxidases.

Redox pathway implicating CYP4A-dependent generation of 20-HETE mediates the effect of high glucose on podocyte apoptosis. High glucose has been shown to induce podocyte apoptosis (7). Data in Fig. 3A and B show that high glucose induced podocyte apoptosis, as assessed by annexin V binding and Hoechst staining. This effect was blocked by the pretreatment of the cells with HET0016, suggesting that CYP4A contributed to the apoptotic effect of glucose in podocytes. This concept was further supported by the finding that 20-HETE mimicked the effect of glucose and induced podocyte apoptosis (Fig. 3A and B). These findings indicate that the increased CYP4A/20-HETE production induced ROS generation and resulted in podocyte apoptosis. Annexin V–binding assay detects the translocation of phosphatidylserine to the cell surface, an early event in cells undergoing apoptosis. Hoechst staining, on the other hand, shows the number of cells with chromatin condensation, i.e., that have already undergone apoptosis.

20-HETE generation by CYP4A mediates the effect of high glucose on NADPH oxidase activity and Nox protein expression and mRNA levels. The observation that upregulation of CYP4A expression preceded that of Nox1 and Nox4 oxidases suggests that CYP4A and its product 20-HETE are responsible for the increased expression of Nox oxidases and contribute to high-glucose–induced oxidative stress in podocytes. Nox1 and Nox4 protein and mRNA levels were measured in mouse podocytes exposed to high glucose in the absence or presence of 10 μmol/l HET0016. High glucose increased the expression of Nox1 and Nox4 proteins as well as mRNA levels of both oxidases. The increase in Nox1 and Nox4 protein and

![FIG. 1. Temporal effect of high glucose on ROS generation in mouse podocytes. A: Mouse podocytes were exposed to either high glucose (HG; 25 mmol/l) or normal glucose (NG; 5 mmol/l) for the indicated time periods. ROS generation was measured by DCF with a multiwell fluorescence plate reader as described in Research Design and Methods. Values are the means ± SE of three independent experiments (n = 3). *P < 0.05, high glucose vs. control. B: Mouse podocytes were exposed to either high glucose (HG; 25 mmol/l) or normal glucose (NG; 5 mmol/l) for the indicated time periods. NADPH oxidase-dependent superoxide generation was assessed by lucigenin-enhanced chemiluminescence. Superoxide anion was expressed as relative chemiluminescent light units (RLU) per milligram of protein per minute (n = 3). C: Expression of Nox1, Nox2, and Nox4 protein was determined by Western blotting analysis on homogenized podocytes. Actin was included as a control for loading and the specificity of change in protein expression. The Western blot is representative of three independent experiments (n = 3).](image-url)
mRNA levels in cells exposed to high glucose was inhibited by pretreatment of the cells with HET0016 (Fig. 4A–C). Moreover, treatment of the cells with 1 μmol/l 20-HETE mimicked the effect of high glucose and resulted in an increase in mRNA levels and protein expression of Nox1 and Nox4 (Fig. 4A–C). These effects were paralleled by changes in NADPH oxidase activity (Fig. 4D).

**CYP4A and 20-HETE mediate diabetes-induced podocyte apoptosis in OVE26 mice.** To determine the in vivo relevance of the findings in cultured cells, OVE26 mice were treated with HET0016 for 3 weeks. Mice were killed, and findings were compared with nontreated OVE26 mice and their FVB littermates. The protein expression of CYP4A was increased in microsomes isolated from glomeruli of OVE26 mice compared with control FVB mice (Fig. 5A). NADPH oxidase activity and Nox1/Nox4 protein expression were upregulated in the OVE26 mice and were significantly decreased in OVE26 mice treated with HET0016 (Fig. 5B and C). Treatment with HET0016 also resulted in a decrease in foot process effacement, podocyte apoptosis as observed by EM (Fig. 6A and B), and synaptopodyn protein expression as assessed by immunohistochemistry (Fig. 7A–C). OVE26 diabetic mice treated with HET0016 exhibited a significant decrease in albumin excretion. Values for urine protein are as follows: 0.035 ± 0.009 mg per 24 h for the FVB control mice, 4.55 ± 0.6 mg per 24 h for the OVE26 diabetic mice, and 2.37 ± 0.9 mg per 24 h for the OVE26 diabetic mice treated with HET0016 (Fig. 8). Altogether, these data show that ROS generation through CYP4A and Nox oxidases plays a critical role in podocyte injury and albuminuria in type 1 diabetes.

**DISCUSSION**

Podocyte apoptosis is an early glomerular phenotype that contributes to progressive podocyte depletion and albuminuria (7). Oxidative stress has been implicated in the pathogenesis of diabetes complications, including podocyte apoptosis (13,15,35). We demonstrate that high glucose induces apoptosis of cultured podocytes through the generation of ROS via sequential upregulation of CYP4A and the NADPH oxidases Nox1 and Nox4. We demonstrate, for the first time, that 20-HETE, the major product of CYP4A, enhanced NADPH-dependent superoxide anion generation, upregulated the expression of Nox1 and Nox4 proteins, and induced podocyte apoptosis. Inhibition of CYP4A prevented oxidative stress and podocyte apoptosis in vitro and reduced
FIG. 3. CYP4A-dependent 20-HETE production mediates high-glucose–induced podocyte apoptosis. Serum-deprived podocytes were preincubated with or without HET0016 (10 μmol/l) for 1 h and then treated with high glucose (HG; 25 mmol/l), normal glucose (NG; 5 mmol/l), or 1 μmol/l of recombinant 20-HETE for the indicated time periods. A: Representative photographs of annexin V and propidium iodide staining in different groups (n = 3 in each group). The number of apoptotic or necrotic cells was quantified by FACS analysis after staining with annexin V and propidium iodide. The cytograms show viable cells that did not bind annexin V or propidium iodide in the left lower quadrant. Cells at early stages of apoptosis that bound annexin V but that still had intact cell membranes and excluded propidium iodide are shown in the lower right quadrant. Cells with advanced stages of apoptosis or necrotic cells were both annexin V positive and propidium iodide positive and are shown in the upper right quadrant. B: Apoptotic nuclei were detected using Hoechst 33258. Chromatin condensation was examined by fluorescent microscopy. (A high-quality digital representation of this figure can be found in the online issue.)
FIG. 4. CYP4A-dependent 20-HETE production mediates high-glucose–induced Nox1 and Nox4 protein expression and NADPH oxidase activation. Serum-deprived podocytes were preincubated with or without HET0016 (10 μmol/l) for 1 h and treated with high glucose (HG; 25 mmol/l), normal glucose (NG; 5 mmol/l), or 1 μmol/l of pure recombinant 20-HETE for the indicated time periods. Expression of Nox1 (A) and Nox4 (B) protein was determined by Western blotting analysis on homogenized podocytes. The Western blots are representative of three independent experiments (n = 3). Each histogram represents the ratio of the intensity of the Nox1 or Nox4 bands factored by the actin band. Values are the means ± SE. *P < 0.05, high glucose or 20-HETE vs. normal glucose; #P < 0.05, high glucose vs. high glucose + HET0016. C: Nox1 and Nox4 mRNA levels. The values represent the relative induction as measured by real-time RT-PCR relative to GAPDH mRNA levels (n = 3). The values are the means ± SE. *P < 0.05, high glucose or 20-HETE vs. normal glucose; #P < 0.05, high glucose vs. high glucose + HET0016. D: NADPH-dependent ROS generation in podocyte homogenates was measured by lucigenin-enhanced chemiluminescence. Superoxide anion is expressed as relative chemiluminescent light units (RLU) per milligram of protein per minute. Values of three independent experiments (n = 3) are the means ± SE. *P < 0.05, high glucose or 20-HETE vs. normal glucose; #P < 0.05, high glucose vs. high glucose + HET0016.
albumin excretion and podocyte foot process effacement and depletion in OVE26 mice, a well-established model of type 1 diabetes.

There is good evidence that hyperglycemia and glucotoxicity contribute to podocyte injury (7). Diabetes induces oxidative stress, and podocytes are known to be...
particularly susceptible to oxidative injury (36,37). Previous studies demonstrated that intracellular ROS mediate apoptosis of podocytes in response to ANGII or high glucose (7,38). Susztak et al. (7) demonstrated that the apoptotic effect of glucose was inhibited by the superoxide dismutase mimetic tempol and inhibitors of mitochondrial respiratory chain and NADPH oxidases. However, the precise sources of ROS and the kinetics of the increase in NADPH-dependent superoxide generation have not yet been examined.

The kidney is known to express NADPH oxidase isoforms of the Nox family (13,15,35). At least three different Nox isoforms are expressed in the kidney cortex: Nox1, Nox2, and Nox4 (16). In many cells, glucose or ANGII elicit a rapid (within minutes) increase in ROS generation (7,15). We also found that high glucose rapidly increased ROS generation in cultured podocytes. However, there were no changes in NADPH oxidase activity or expression of Nox enzymes on short exposure of the cells to high glucose. This suggests that other sources of ROS may be responsible for ROS production at early time points after exposure of the cells to high glucose. There are multiple other sources of ROS in cells and tissues. ROS generation occurs as a byproduct of CYP450 enzyme activation. Mitochondria and peroxisomes are also important sources of ROS (16). CYP4A mRNA and protein are expressed in glomeruli of mice (20,25). The regulation of CYP4A and 20-HETE production is tissue- and disease specific (19). In this study, we show that CYP4A protein expression and 20-HETE production were increased within hours of expo-

![Figure 6: CYP4A contributes to podocyte apoptosis and foot process effacement in glomeruli of type 1 diabetic mice.](image)

A: Representative transmission electron micrographs of glomerular cross-section of FVB, OVE26, and OVE26 mice treated with HET0016. The images show a condensed podocyte nucleus, foot process effacement, cytoplasmic rarefaction, and basement membrane thickening of an OVE26 diabetic mouse. This effect was not seen in the OVE26 mice treated with HET0016. B: Semiquantitative analysis of foot process effacement of glomeruli from each group of animals (n = 5 per group). *P < 0.05, OVE26 mice vs. control FVB mice; #P < 0.05, decrease in the percentage of foot process effacement in OVE26 mice treated with HET0016 compared with OVE26 mice.
sure of the cells to high glucose, at time points where no changes in NADPH oxidase activity or Nox protein expression were observed. Importantly, we show that ROS production, Nox oxidases, and apoptosis in response to glucose were inhibited when the cells were treated with HET0016, a highly specific CYP4A inhibitor. Moreover, addition of exogenous 20-HETE enhanced ROS generation and induced podocyte apoptosis. Collectively, these data indicate that CYP4A and 20-HETE generation induced podocyte apoptosis, and this biological effect was likely mediated by enhanced ROS production. Nilakantam et al. (27) showed that CYP4A12 overexpression significantly exacerbates the tubular epithelial injury that is associated with renal ischemia/reperfusion injury and that the programmed cell death is partially dependent on enhanced generation of free radicals by CYP4A. On the other hand, 20-HETE appears to play a protective role in pulmonary artery smooth muscle cells by inhibiting apoptosis (28).

To explore the relevance to type 1 diabetes of our observations in cultured podocytes, we studied an established mouse model of type 1 diabetes, the OVE26 mouse. These mice develop morphologic and structural changes characteristic of human diabetic nephropathy (40,41). We also demonstrate that OVE26 mice exhibited thickening of the glomerular basement membrane, foot process effacement, podocyte apoptosis as seen by EM, and podocyte loss. Urinary albumin excretion in these mice was also much higher than that seen in the FVB control mice.

FIG. 7. CYP4A contributes to podocyte apoptosis and reduction in synaptopodin protein expression in glomeruli of type 1 diabetic mice. A: Representative immunofluorescence images of glomeruli stained with collagen IV (green), synaptopodin (red), and 4,6-diamidino-2-phenylindole (blue). B: Podocyte number per glomerular section was lower in OVE26 mice, with a significant increase in podocyte number in the OVE26 mice treated with HET0016 (n = 5 per group). C: Synaptopodin protein expression in OVE26 mice was decreased in the OVE26 mice and restored with the injection of HET0016. Values are the means ± SE. *P < 0.05, OVE26 mice vs. control FVB mice; #P < 0.05, decrease in albumin levels in HET0016-pretreated OVE26 mice vs. nontreated OVE26 mice (n = 5 per group). (A high-quality digital representation of this figure can be found in the online issue.)

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found an increase in the expression of CYP4A protein in microsomes isolated from glomeruli of OVE26 mice compared with glomeruli isolated from control FVB mice. Administration of a specific inhibitor of 20-HETE production, HET0016, to OVE26 mice attenuated albuminuria and reduced foot process effacement, apoptosis, and podocyte loss. Inhibition of CYP4A also resulted in downregulation of Nox1 and Nox4 protein and mRNA expression and markedly inhibited NADPH oxidase activity. These data indicate that 20-HETE plays an important role in the structural and functional changes of podocytes in type 1 diabetes. Whether the decreased expression of Nox1 or Nox4 contributes to the protective effect of CYP4A inhibition remains speculative. We recently demonstrated that NADPH-dependent ROS generation was increased in glomeruli of rats with streptozotocin-induced diabetes (13). Inhibition of Nox4 oxidase using antisense oligonucleotide therapy reduces glomerular ROS generation, glomerular hypertrophy, and fibronectin expression 2 weeks after the induction of type 1 diabetes (13). Urinary protein and albumin excretion were not measured in these studies. Sharma et al. (42) recently demonstrated that mouse podocytes expressed Nox4 and that adiponectin, which decreased podocyte permeability to albumin in vitro, reduced Nox4 expression. Therefore, the role of Nox proteins in mediating the effect of 20-HETE remains to be explored.

In conclusion, in the current study, we identified the sequential upregulation of CYP4A, 20-HETE, and Nox enzymes as sources of ROS generation that lead to apoptosis of cultured podocytes exposed to high glucose. We show that this redox pathway plays a key role in podocyte depletion and proteinuria in type 1 diabetes. These results suggest that enhanced release of 20-HETE in the diabetic state contributes to podocyte injury and that inhibitors of the CYP4A family may be therapeutically useful in the treatment of diabetic nephropathy.

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