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THE INVOLVEMENT OF RENAL CYTOCHROME P450 AND ARACHIDONIC ACID METABOLITES IN DIABETIC NEPHROPATHY

by
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AN ABSTRACT OF THE THESIS OF

Stephanie Atef Eid for Master of Science
Major: Physiology

Title: The Involvement of Renal Cytochrome P450 and Arachidonic Acid metabolites in Diabetic Nephropathy

Background: Diabetic nephropathy (DN), a serious complication of diabetes, is characterized by hypertrophy, matrix accumulation, and proteinuria leading to end stage renal disease. Proximal tubular cells contribute to the hypertrophic response of the kidney. Early stages of DN are also associated with alterations in renal sodium handling as well as hypertension; processes linked by involvement of the arachidonic acid (AA) metabolites 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs). Indeed, metabolism of AA to various intermediates is increased in a rat model of type 1diabetes. It is known that AA is metabolized by several cytochrome 450 (CYP) isoforms to produce20-HETE and EETs.

Aim: The present study aims to determine the specific AA-metabolizing CYP450 isoforms present in proximal tubules that are altered by high glucose in cultured mouse proximal tubular cells (MCT). It intends to investigate the effects of alterations in CYP isoforms and/or AA-metabolite levels in the pathogenic manifestation of DN in cultured MCTs. This work will investigate the mechanism of proximal tubular cell injury and the effect of inhibition of AA-metabolites *in vitro* and will also get more insight onto the cross-talk between CYP450 isoforms and other sources of Reactive Oxygen Species (ROS).

Methods: The peroxide-sensitive fluorescent probe 2′, 7′-dichlorodihydrofluorescin (DCF) diacetate was used to measure intracellular ROS production. Real-time Reverse Transcription –Polymerase Chain Reaction (RT-PCR) was performed to determine mRNA levels of fibronectin, Nox1 and Nox4. Western blot analysis was performed to study the protein expression of Cytochromes CYP4A, CYP2C11, and CYP4F3, Nox1 and Nox4, fibronectin, collagen IV, and AMPK. Apoptosis assay and Hoechst staining were performed to assess cell apoptosis and the assay of AA metabolism to measure 20-HETE and EET formation.

Results: Exposure of mouse proximal tubular cells to high glucose resulted in apoptosis. High glucose treatment increased ROS production and was associated with CYP4A and CYP2C11 upregulation, 20-HETE and EETs formation, and Nox oxidases upregulation. The effects of high glucose on Nox proteins and mRNA expression, matrix protein accumulation and apoptosis were blocked by HET0016, an inhibitor of CYP4A, and were mimicked by 20-HETE. Inhibition of EETs *in vitro* promoted the effects of HG on MCTs, thereby upregulating Nox1 and Nox4 and inducing apoptosis.

Conclusion: Our results indicate that hyperglycemia in diabetes has a significant effect on the expression of AA-metabolizing CYPs, manifested by increased AA metabolism, and might thus alter kidney function through alteration of type and amount of AA metabolites; this pathway is through an oxidative stress-dependent mechanism.

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LIST OF ABBREVIATIONS

DN:	Diabetic Nephropathy
20-HETE:	20-hydroxyeicosatetraenoic Acid
EET:	Epoxyeicosatrienoic Acids
AA:	Arachidonic Acid
ROS:	Reactive Oxygen Species
MCT:	Mouse Proximal Tubular Epithelial Cells
CYP450:	Cytochrome 450
HG:	High Glucose
HET0016:	N-hydroxy-N'-(4-butyl-2-methylphenol) Formamidine
MS-PPOH:	N-(methylsulfonyl) -2-(2-propynyloxy)–Benzenehexanamide

INTRODUCTION

Kidney injury is a serious life-threatening complication of type 1 and type 2 diabetes. Hyperglycemia induces a wide array of signaling dysfunction leading to organ damage. Although strict metabolic control prevents many of the burdens of the disease, it is often difficult to achieve. However, the mechanisms by which high glucose exerts its deleterious effects on the different types of cells in the body organs, particularly on renal cells, remain poorly understood.

A. Renal proximal tubular cells in diabetic nephropathy

Diabetes-related kidney disease or diabetic nephropathy (DN) is one of the most serious complications of diabetes worldwide, affecting up to 25% and 40% of all patients with type 1 and type 2 diabetes, respectively (Hall, 2006). It is a major risk factor for cardiovascular morbidity and mortality and is described in terms of vascular, glomerular, tubular and interstitial lesions that originally develop in the absence of assessable dysfunction (Gilbert and Cooper, 1999). Although DN has long been viewed as glomerulocentric, it is now widely accepted that the rate of deterioration of kidney function is intimately correlated with the degree of tubulointerstitial fibrosis (Bagby, 2007). An increase in tubular basement membrane mass is one of the earliest renal pathological changes in diabetes accompanied by the development of renal hypertrophy (Jones et al., 1999). Diabetes related kidney disease is also characterized by progressive accumulation of

extracellular matrix proteins including fibronectin, collagen and laminin in the tubular compartment. Fibronectin, a key matrix protein normally involved in diverse cellular functions including adhesion, migration, survival, differentiation and growth is accumulated in excess in DN (Young et al., 1995; Wang et al., 2008). The final pathological features of the disease include among others the development of tubulointerstitial fibrosis. Although the etiology of the tubulointerstitial pathology in DN is still unclear, much emphasis has been placed on the role of hyperglycemia *per se.* Proximal tubular epithelial cells are considered a primary target of hyperglycemia. And when exposed chronically to high blood glucose levels, they undergo tubulointerstitial changes seen in overt DN (Gilbert and Cooper, 1999; Jones et al., 1999). Most knowledge of the direct role of hyperglycemia on the tubulointerstitium comes from *in vitro* studies. In fact, proximal tubular epithelial cells exposed to high glucose concentrations (25 mM HG) have been shown to undergo hypertrophy and an increase in extracellular matrix protein accumulation. While the involvement of diabetes in the increased generation of extracellular proteins has been well established, the mechanisms by which hyperglycemia results in the accumulation of these proteins remain unclear.

B. Arachidonic acid metabolites in kidney function

Arachidonic acid (AA) can be metabolized along one of three pathways: the cyclooxygenase, the lipoxygenase or the cytochrome P450 (CYP) monooxygenase pathway. In the third pathway, AA is metabolized by several CYP450 isoforms to produce 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) among other eicosanoid products (Zeldin, 2001). 20-HETE is one of the main AA metabolites

produced in the renal vasculature and tubular compartment. It is synthesized by CYPs of the 4A and 4F subfamilies, which are highly expressed in the proximal tubules of the kidney (Powell et al., 1996; Schwartzman et al., 1996; Christmas et al., 2001; Kalsotra et al., 2005; Ito et al., 2006). 20-HETE exerts a wide range of regulatory and opposing functions depending on the location of its production. It is a potent vasoconstrictor known to play key roles in both tubular and vascular regulation of renal hemodynamics and extracellular fluid volume (Carroll, 2000). Also, 20-HETE has been shown to induce apoptosis in glomerular epithelial cells, through the upregulation of ROS production (Eid et al., 2009) and to increase endothelial cell proliferation through the stimulation of superoxide production (Guo et al., 2007). Thus, an aberrant increase in 20-HETE concentration is expected to have not only hemodynamic effects, but also a role in cellular proliferation and apoptosis, and can have a role in tissue hypertrophy.

EETs are mainly synthesized by CYPs of the 2B, 2C and 2J subfamilies. Studies have shown that CYPs 2C and 2J are the predominant isoforms of cytochromes expressed in the proximal tubules of the kidneys (Enayetallah et al., 2004; Imaoka et al., 2005). EETs have a multitude of potent biological activities that vary depending on the location of their production. In contrast to 20-HETE, EETs synthesized by the vascular endothelium are considered as potent vasodilators (Michaelis et al., 2003; Michaelis et al., 2005; Campbell et al., 2007). In parallel to their hemodynamic role, EETs have been shown to be involved in the regulation of cell proliferation (Harris et al., 1990; Sheu et al., 1995; Chen et al., 1998). On the other hand, EETs also play a critical role in inducing the upregulation of endothelial nitric oxide synthase (eNOS) expression and enhancing its phosphorylation (Jiang et al., 2007), thus leading to an increased nitric oxide (NO) production. NO, in turn,

will either manifest pro-apoptotic tendencies (Dimmeler and Zeiher, 1997; Nicotera et al., 1997) or will have effects on cell growth and proliferation (Sarkar and Webb, 1998; Villalobo, 2006; Metaxa et al., 2008). 11, 12-EET is the major epoxygenase metabolite produced in the kidney. Therefore, like 20-HETE, aberrant levels of EETs might be expected to alter cellular function.

C. Reactive oxygen species and diabetic nephropathy

Diabetes and hyperglycemia are accompanied by increased generation of reactive oxygen species (ROS) in the kidneys (Baynes and Thorpe, 1999). Antioxidant treatment prevents glomerular and renal hypertrophy as well as proteinuria, suggesting a pathogenic role of ROS in the pathology of DN (Ha and Lee, 2000; Brownlee, 2001; Kuroki et al., 2003; Koya et al., 2010). NADPH oxidases, mitochondrial electron transport chain, Angiotensin II are major sources of ROS (Brownlee, 2001; Harrison et al., 2003; Wolf, 2007). Other studies indicate that CYPs are significant sources of ROS in multiple organs (Puntarulo et al., 1998; Fleming et al.; 2001). ROS in DN play a key role in apoptosis, hypertrophy, fibrosis and other pathophysiological processes underlying renal dysfunction. ROS generation in the proximal tubules disrupts the structural integrity of the cell and its transport mechanisms (Paravicini et al., 2008).

D. NADPH in diabetic nephropathy

Recent studies indicate that NADPH-dependent ROS generation is increased in the renal cortex and glomeruli of mice with diabetes (Gorin et al., 2005; Satoh et al., 2005; Kim et al., 2006). Inhibition of Nox4 oxidase using an antisense oligonucleotide therapy

reduces diabetes-induced renal ROS generation, whole kidney and glomerular hypertrophy and fibronectin expression at two weeks of type 1 diabetes in the rat (Gorin et al., 2005). Six homologs of the cytochrome subunit of the phagocyte NADPH oxidase (Nox2/gp91phox) have been cloned. At least three different Nox isoforms, Nox1, Nox2, and Nox4 are expressed in the kidney cortex. (Bedard and Krause, 2007).

E. 20-HETE and EETs in diabetic nephropathy

The involvement of 20-HETE and EETs in hypertension has been extensively studied (Holla et al., 2001; Hoagland et al., 2003; Sarkis and Roman, 2004; Miyata and Roman, 2005; Wang et al., 2006). However, little is known about their involvement in other physical and physiological changes, especially their role in altering organ function in the diabetic milieu. The regulation of CYP450 is tissue and disease specific. Recent studies have reported alterations in CYP450 metabolites and implicated those changes as contributing to renal damage in obesity and diabetes (Enriquez et al., 1999; Dey et al., 2004). Eid et al. (2009) have shown that circulating levels of 20-HETE are increased while those of EETs are decreased in humans with renovascular disease. The role of 20-HETE and EETs in DN is yet to be elucidated. Microalbuminuria is one of the earliest detectable pathological abnormality in overt DN that eventually progresses to proteinuria, abnormal protein secretion, primarily albumin, into urine (Wolf and Ziyadeh, 2007). Controversial results have been reported regarding the exact role of 20-HETE in the progression of proteinuria. In recent studies, puromycin aminonucleoside (PAN) was used to induce albumin permeability in isolated glomeruli. Ex vivo experiments showed that 20-HETE protects glomeruli from this effect (McCarthy et al., 2005). Other investigators also showed

that the inhibition of the glomerular production of 20-HETE due to the increased levels of TGF-β9, early in hypertension, leads to proteinuria and glomerular injury (Dahly-Vernon et al., 2005). In contrast, a study by Eid et al. (2009) showed that the inhibition of 20-HETE production in type 1 diabetic mice results in the reduction of proteinuria and prevents glomerular epithelial cell depletion. These data clearly suggest the involvement of 20-HETE in proteinuric states. But the exact effect of 20-HETE and its mechanism of action need to be elucidated.

Comparably, the precise role of EETs in the progression of DN is still not well characterized. In a recent study, the inhibition of the enzyme soluble epoxide hydrolase, which causes an increase in the bioavailability of EETs, has been shown to contribute to albuminuria in mice models of progressive renal disease (Jung et al., 2010). Contrastingly, other researchers showed that the overexpression of CYP2J2 epoxygenase followed by increased EET generation in streptozotocin-induced diabetic mice attenuated microalbuminuria and glomerulosclerosis progression via inhibition of TGF-β/Smad signaling (Chen et al., 2011). Again, these data suggest that altered EET levels may be a general characteristic of proteinuric states, but the mechanism of action still needs to be elucidated.

F. Diabetic nephropathy and oxidative stress

Oxidative stress is thought to play a critical role in the development of DN. The overproduction of ROS occurs in the kidney concomitant with disease progression. Cytochromes P450 have been shown to be significant sources of oxidative stress in the kidneys, liver and coronary arteries (Puntarulo et al, 1998; Fleming et al, 2001; Eid et al,

2009). On the other hand, Eid et al. (2009) showed that 20-HETE increases ROS production in glomerular epithelial cells. Being heme-containing monooxygenases, CYPs activate oxygen prior to incorporating it into substrate. Futile or redox cycling of the enzyme, where activated oxygen "escapes" before incorporation into organic products leads to the formation of superoxide/H_2O_2. The involvement of CYPs family and their metabolites in the kidney to produce significant ROS, leading to DN remains poorly understood.

G. Hypothesis and aim of the study

Previous works in our laboratory suggest that the arachidonic acid metabolites of the cytochrome P450 monoxygenase pathway are involved not only in the regulation of vasculature, but also affect the filtration properties of the kidney, processes that are aberrant in DN. In this project, we hypothesize that diabetes-induced oxidative stress, secondary to alterations in the levels and activities of selected cytochromes P450 monooxygenases contribute to proximal tubular cell injury. We also hypothesize that CYPs are the sources of ROS in these cells in a hyperglycemic milieu. In order to test our hypothesis, the present study aims to:

- Determine the specific AA-metabolizing CYP450 isoforms present in proximal tubules that are altered by high glucose in cultured mouse proximal tubular cells (MCT).
- Investigate the effects of alterations in CYP isoforms and/or AA-metabolite levels in the pathogenic manifestation of DN in cultured MCTs.

- Investigate the mechanism of proximal tubular cell injury and the effect of inhibition of AA-metabolites *in vitro*.
- Identify the signal transduction pathway underlying the cross-talk between CYP450 isoforms and other sources of ROS.

Briefly, the goal of this project is to elucidate the mechanisms behind kidney damage during the onset and development of diabetes in order to identify new therapeutic approaches for the treatment of diabetic patients with renal disease.

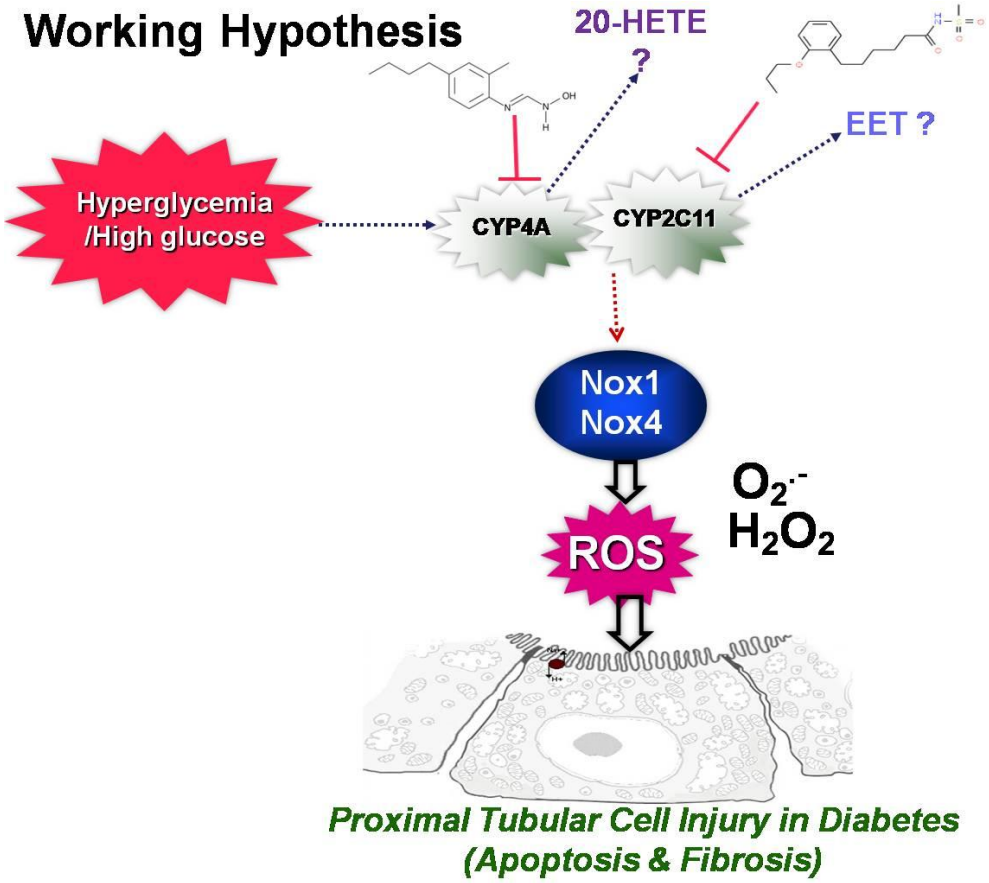


Figure 1. Proposed model of proximal tubular cell injury in diabetes

MATERIALS AND METHODS

A. Cell lines, culture conditions

Mouse proximal tubular epithelial cell line (MCT) was cultured regularly in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Steinheim, Germany) supplemented with 17% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich). Cultures were incubated under standard conditions at a temperature of 37ºC in a 5% CO_2 incubator. The cells were subcultured three times per week when complete confluence was reached. Cell treatment was performed with 25 mM glucose (HG) or 1.5 µM 20-hydroxyeicosatetraenoic acid (20-HETE). Cells were pretreated for an hour with 15 µM N-hydroxy-N'-(4-butyl-2-methylphenol) formamidine (HET0016), a specific inhibitor of CYP4A or N-(methylsulfonyl) -2-(2-propynyloxy) – benzenehexanamide (MS-PPOH), a selective inhibitor of 11, 12 EET.

B. Animal models

Male Sprague-Dawley rats weighing between 200 and 225 g were divided into three groups of four animals each. Rats in **group 1** were injected with sodium citrate buffer alone. **Group 2** rats were injected once intravenously via the tail vein with 55 mg/kg body wt streptozotocin (STZ) in sodium citrate buffer (0.01 M, pH 4.5) to induce diabetes. They were sacrificed after 6 weeks of treatment. **Group 3** rats were treated with insulin two days

following STZ injection. All of the rats were maintained in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center.

C. Microsome isolation

MCT cells in serum-free medium were homogenized in a 10 mM potassium phosphate buffer, pH 7.5, containing 250 mM sucrose, 1 mM EDTA, 2µg/ml aprotinin, 2µM leupeptin, and 1mM PMSF. Microsomes were obtained by differential centrifugation at 65000 rpm for 30 min at 4ºC, resuspended in a buffer consisting of 50 mM potassium phostphate, pH 7.5, containing 0.2mM EDTA and 20% glycerol. The microsomal pellet was used for Western blot and 20-HETE measurement by High-Performance Liquid Chromatography (HPLC).

D. Hoechst staining

To detect apoptotic nuclei, Hoechst 33258 (Invitrogen) staining was used, with a working concentration of 1 µg/ml. For live cell imaging, MCT cells were plated in Tek confocal dishes. When 60-70% confluence was reached, they were serum-deprived for 24 hours and then treated with 1.5 µM 20-hydroxyeicosatetraenoic acid (20-HETE) or with 25mM glucose (HG) in the presence or absence of HET0016 (15 µM) or MS-PPOH (15 µM) for 48 hours. MCTs were washed with phosphate buffered saline (PBS) and then stained with Hoechst for five minutes at room temperature. After storing the slides for 24 hours at 4ºC, Zeiss LSM710 confocal microscope (Carl Zeiss) was used for observations and analysis at 350-nm excitation and 460-nm emission. Images were taken from different fields and at

different magnifications. They were viewed and manually counted using the Zen Light Software.

E. Western blot analysis

MCT cells were grown to near confluence in 60-mm dishes and serum-deprived for 24 hours. All incubations were carried out in serum free DMEM at 37°C for a specific duration. The cells were lysed using RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS) ,0.5% sodium deoxylate, 150mM sodium chloride, 50mMTris-hydrochloride, 100 mM EDTA, 1% Tergitol (NP40), and 1% of the protease and phosphatase inhibitors. The cell lysates were centrifuged at 13,600 rpm for 30 minutes at 4°C. Protein in the supernatants was measured using the Bradford Protein Assay. For immunoblotting, 50µg of proteins were separated on 10% Polyacrylamide gel Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The membranes were blocked with 5% low fat milk in Tris-buffered saline and then incubated with rabbit polyclonal anti-Nox1(1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-Nox 4 (1:500, Santa Cruz Biotechnology), rabbit polyclonal phospho-AMPK α (Thr172) antibody (1:500,Cell Signaling), rabbit polyclonal anti-CYP4A(1:1000, abcam), rabbit polyclonal anti-CYP2C11(1:1000, abcam), rabbit polyclonal anti-CYP4F3(1:1000, abcam), rabbit polyclonal anti-collagen IV antibody(1:100, abcam) and rabbit polyclonal anti-fibronectin antibody(1:500, sigma-aldrich). The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:1000, Bio-Rad). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software.

F. Cellular DNA fragmentation ELISA

To investigate the mechanism of proximal tubular cell apoptosis *in vitro*, the cellular DNA fragmentation ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to detect BrdU-labeled DNA fragments in MCT cells. The cells were grown in 12-well tissue culture plates until 70-80% confluence. They were serum deprived for 24 hours and then treated for 48 hours according to the experimental conditions. In the first step of the apoptosis assay procedure, an anti-DNA coating solution containing anti-DNA antibody was adsorptively fixed in the wells of a microplate. Then, BrdU-labeled DNA fragments (15 µM), which bind to the immobilized anti-DNA antibody, were added to the cultured MCT cells 12 hours before stopping the treatment. In the third step, the immunocomplexed BrdU-labeled DNA fragments were denatured and fixed on the surface of the microplate by microwave irradiation, in order to improve the accessibility of the antigen BrdU for detection by the antibody. As a final step, anti-BrdU-peroxidase conjugate reacted with the BrdU incorporated into the DNA. The amount of peroxidase bound in the immune complex was photometrically determined, after the removal of unbound peroxidase conjugates and the addition of the substrate solution. Absorbance was measured at 450 nm against a reference wavelength of 650 nm using a microplate reader (Multiskan Ex).The mean of triplicate experiments was used +/- SD.

G. Detection of intracellular ROS

The peroxide-sensitive fluorescent probe 2', 7'-dichlorodihydrofluorescin (DCF) diacetate (Molecular Probes) was used to measure intracellular ROS in MCTs. Cells were grown in 12-well tissue culture plates until 70-80% confluence and serum-deprived for 24 hours.

They were treated with 1.5 µM 20-HETE or incubated in 25 mM glucose in the presence or absence of HET0016 (15µM). Immediately before the experiments, cells were washed with PBS containing $Ca^{2+}$and Mg^{2+} and then loaded with 5 µM DCF diacetate dissolved in PBS for 30 min at 37°C. DCF fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, and measured in a multiwell fluorescence plate reader (Fluoroskan Ascent, Thermo Scientific)

H. mRNA analysis

Real-time RT-PCR was performed to analyze mRNA using the ΔΔCT method. Total cellular RNA was isolated from MCTs using TRIzol reagent (Sigma Aldrich, Steinheim, Germany). mRNA was quantified using Icycler with SYBR green dye and mouse RT^2qPCR Primers (Bio-Rad Laboratory, CA, USA) for Nox1, Nox4 and fibronectin. GAPDH was used as internal reference gene.

Primer	**Sequence**	**Annealing TºC**
Nox1	F: GGATCCATGGCCTGGGTGGGAT R:GGATGCCTGCAACTCCCCTTATGG	55ºC
Nox4	F: TCCAAGCTCATTTCCCACAG R: CGGAGTTCCATTACATCAGAGG	55ºC
Fibronectin	F: ACGGTGTCAACTACAAGATCG R: GTCTTCCCATCGTCATAGCAC	55ºC

Table 1. Oligonucleotide primer sequences and conditions employed for real-time PCR

I. Assay of AA metabolism

Levels of 20-HETE and EETs were measured in isolated microsomes by HPLC. In short, [1-^{14}C] labeled arachidonic acid (50–100 µM) was dried down and resuspended in the reaction mix containing 50 µg microsomes, 30 mM isocitrate, and 0.2 unit isocitrate dehydrogenase in reaction buffer (100 mM potassium phosphate, pH 7.4, 5 mM magnesium chloride, and 1 mM EDTA). After incubation at 37°C for 5 min, the reaction was initiated by the addition of NADPH to a final concentration of 1 mM. Aliquots were removed at 30, 60, and 90 min, and the reaction was stopped by the addition of 100% methanol. The precipitated proteins were then pelleted by centrifugation (in a microcentrifuge), and the samples were stored at -20°C for later use. The metabolites were separated via HPLC on a C-18 column using an acetonitrile/H_2O gradient and identified by coelution with labeled standards (Eid et al.; 2009).

J. Statistical analysis

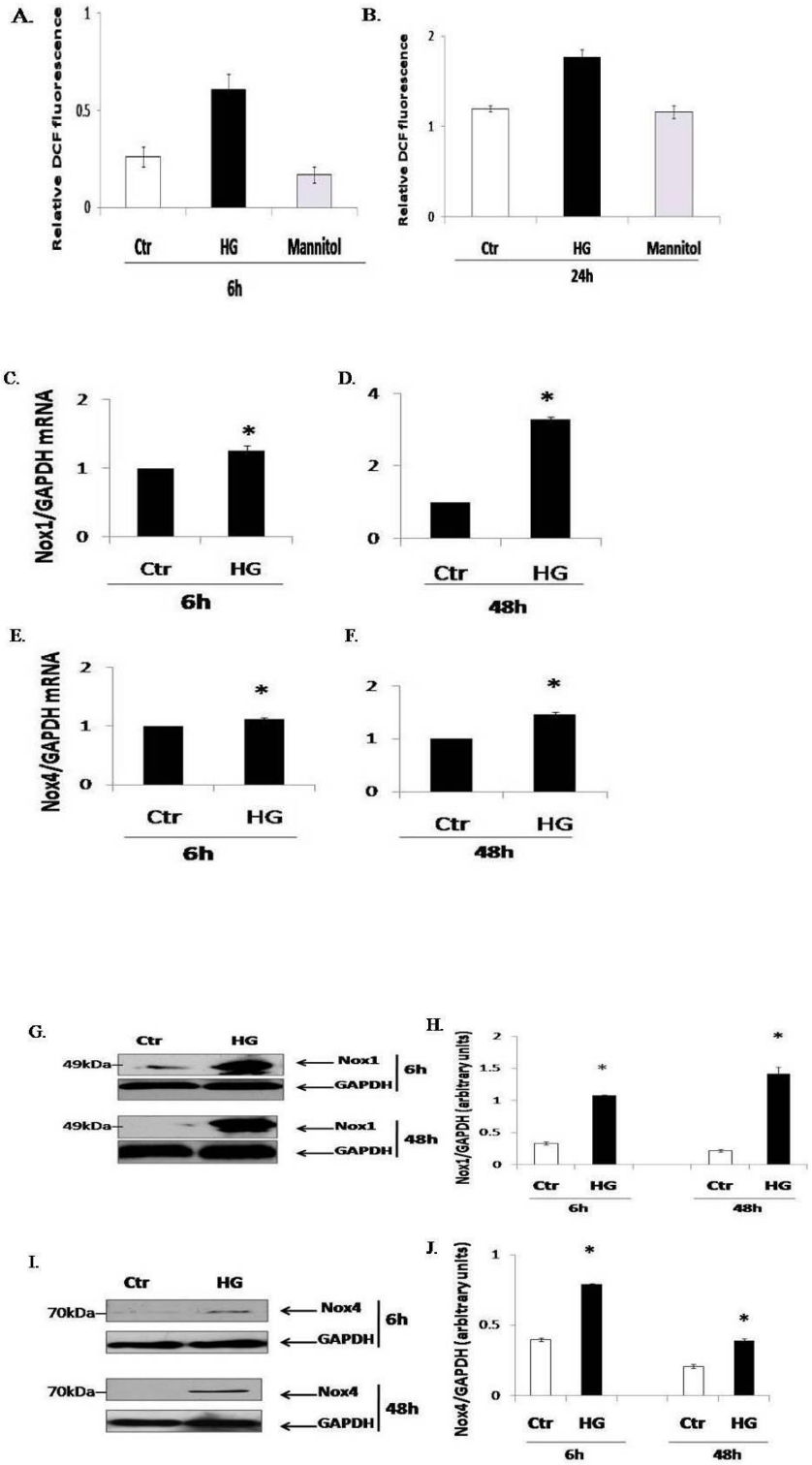
Results are expressed as mean ± SE from 3 to 4 independent experiments. Statistical significance is assessed by student's unpaired t-test. Significance is determined as probability (p) <0.05.

RESULTS

A. High glucose induces oxidative stress in mouse proximal tubules

Mouse proximal tubular epithelial cells (MCT) were exposed to 25 mM of glucose (HG) or to 5 mM of glucose (Ctr). HG results in an increase of ROS generation, as measured by DCF compared to cells incubated in 5 mM glucose. In these experiments, equimolar concentration of mannitol had no effect. ROS generation was detected after 6 hours, 24 hours of exposure to HG (Figure 2 A-B) and was sustained up to 48 hours (data not shown). The Nox family of NADPH oxidases are enzymes that can produce superoxide and other downstream ROS in cells and tissues (Bedard and Heinz Krause, 2007). Consistent with the DCF results, there was an increase in the mRNA levels of the two major Nox isoforms present in the kidney, Nox1 and Nox4 (Figure 2 C-F) as well as an increase in their protein expression in MCT cells exposed to HG, when compared to cells exposed to the control (Ctr) at 6 and 48 h of treatment with HG (Figure 2 G-J).

Figure 2.


A.
Relative DCF fluorescence
1
0.5
0
Ctr
HG
Mannitol
6h
B.
Relative DCF fluorescence
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Ctr
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Mannitol
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Nox1/GAPDH mRNA
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Nox4/GAPDH mRNA
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HG
Ctr
HG
6h
48h

Figure 2. Temporal effect of HG on ROS generation in mouse proximal tubular cells. MCTs were cultured for 6 (A) and 24 h (B) in either 5 mM glucose (ctr) or 25 mM glucose (HG). ROS generation was detected by DCF as described in MATERIALS AND METHODS section. Nox1 (C-D) and Nox4 (E-F) mRNA levels were increased in the presence of HG for the indicated time periods. The values represent the relative induction as measured by real-time RT-PCR relative to GAPDH mRNA levels. Representative western blots of Nox1 (G-H) and Nox4 (I-J) on homogenized MCTs following 5 mM (Ctr) or 25 mM (HG) glucose treatment for 6 and 48h are shown. The Western blots are quantified by densitometry and 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 GAPDH band, used as a loading control. For the statistical analysis, values are the means ± SE. *P<0.05, high glucose vs. control.

B. HG induces CYP4A and CYP2C11 protein expression and their corresponding AA metabolites 20-HETE and EET production

We next isolated microsomes from MCTs incubated in normal glucose (Ctr) or HG in the absence or presence of HET0016 (15 µM), a potent inhibitor of CYP4A (20-HETE synthesis). Our results showed that in MCT cells exposed to HG for 48 hours, CYP4A protein expression was increased. It was inhibited by the pre-treatment with HET0016 (Figure 3 A-B). This experiment was performed to test the efficiency of HET0016 as an inhibitor of CYP4A.

In parallel experiments, and in order to assess the efficiency of MS-PPOH on CYP2C11, microsomes were isolated from MCTs exposed to HG in the absence or presence of MS-PPOH (15 µM), a potent inhibitor of 11, 12-EET biosynthesis. Our results showed that HG upregulated CYP2C11 protein expression while MS-PPOH blocked the effect of HG-induced CYP2C11 upregulation.

Figure 3.

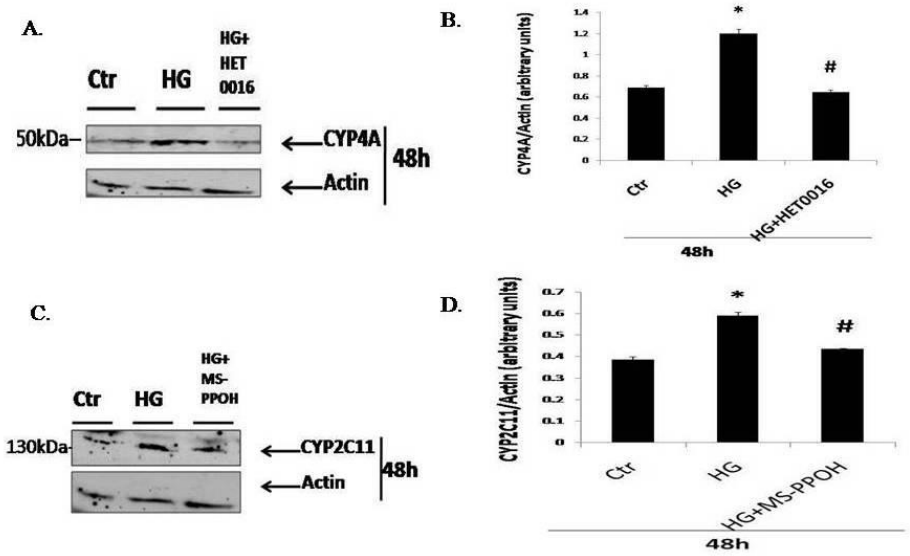
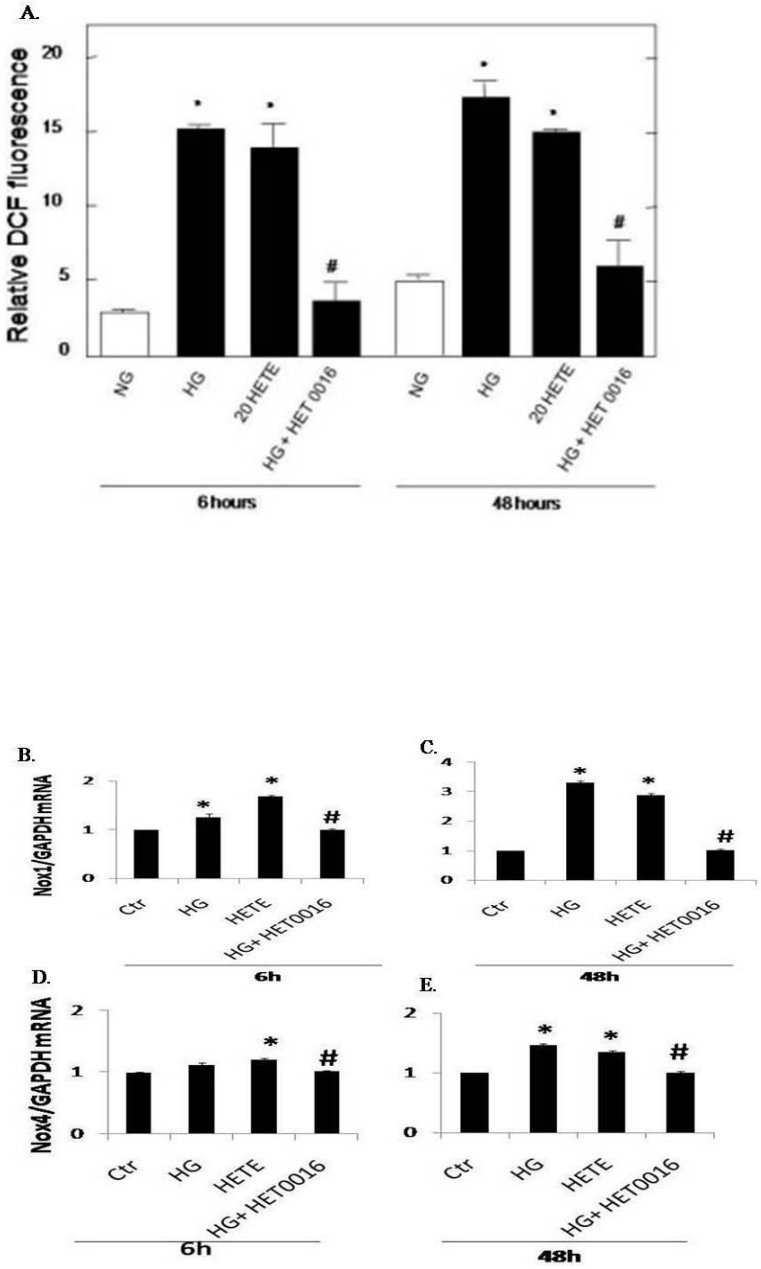


Figure 3. HG induces CYP4A and CYP2C11 protein expression.
Western blot analysis of microsomes derived from MCTs after 48 h treatment with HG in the presence or absence of HET0016 (15 µM) or MS-PPOH (15 µM) revealing the expression of CYP4A (A) along with their semi-quantifications (B), and CYP2C11 (C-D) Three independent experiments were performed (n=3). Values are the means ± SE. *P<0.05, high glucose vs. control; #P<0.05, high glucose + HET0016 or high glucose + MS-PPOH vs. high glucose.

C. 20-HETE generated by Cyp4a mediates the effect of HG on ROS generation.

In a new series of experiments, we analyzed the contribution of 20-HETE in mediating the effect of HG-induced ROS generation in proximal tubular epithelial cells. MCTs were exposed to HG in the presence or absence of HET0016 (15 μM, 1h pre-treatment) or treated with 20-HETE (1.5 μM).DCF fluorescence showed that the pre-treatment of MCT cells by HET0016 attenuated HG-induced ROS production. In contrast, 20-HETE mimicked the effect of HG on ROS generation (Figure 4 A). Concomitant with these observations, HG-induced Nox upregulation was inhibited upon pre-treatment with HET0016. However, mRNA and protein expression of Nox1 and Nox4 (Figure 4 B-K) was increased in MCT cells that were treated with 20-HETE, mimicking the effect of HG on these cells. Taken together, our data suggest that CYP4A, and its product 20-HETE mediated the effect of HG on ROS in proximal tubular cells.

Figure 4.


A.
Relative DCF fluorescence
20
15
10
5
0
NG
HG
20 HETE
HG + HET 0016
6 hours
48 hours
B.
Nox1/GAPDH mRNA
Ctr
HG
HETE
HG+ HET0016
6h
C.
48h
D.
Nox4/GAPDH mRNA
6h
E.
48h

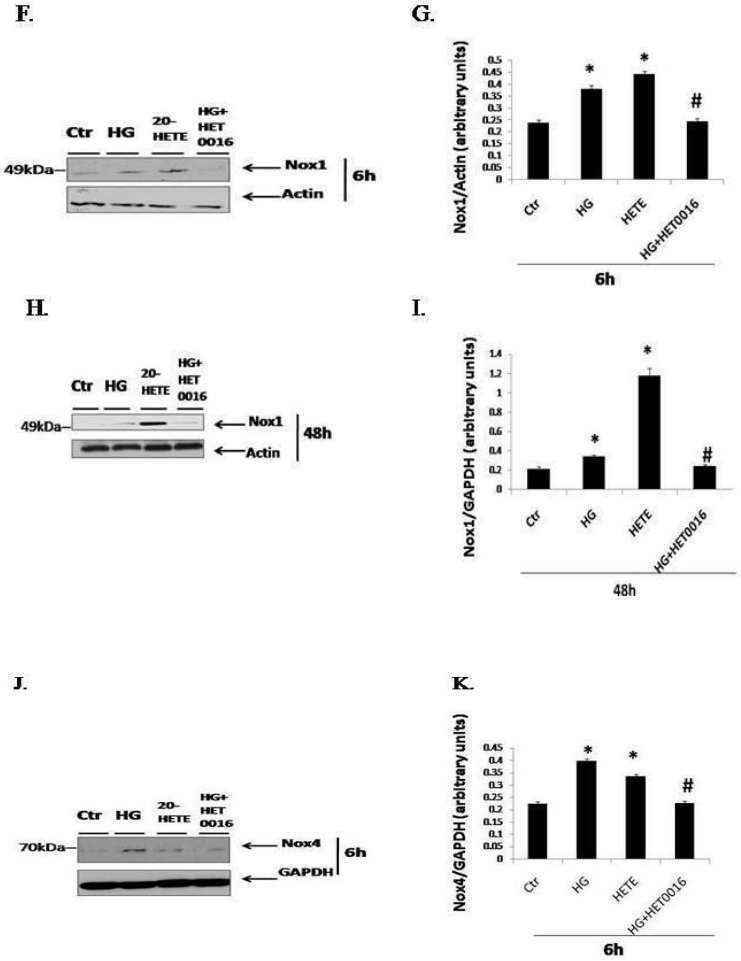


Figure 4. CYP4A dependent 20-HETE production mediates HG-induced ROS production in MCTs.

Serum-deprived MCTs were either treated with 20-HETE (1.5 µM) or with HG with or without pre-incubation with HET0016 (15 µM) for the indicated time periods. ROS generation was measured by DCF as described in MATERIAL AND METHODS section for 6 and 48 h (A). Values are the means ±SE of three independent experiments (n=3). *P < 0.05, high glucose or 20-HETE vs. control. #P<0.05, high glucose + HET0016 vs. high glucose. Nox1 (B-C) and Nox4 (D-E) mRNA levels. The values represent the relative induction as measured by real-time RT-PCR relative to GAPDH mRNA levels. Data represents the mean of three independent experiments (n=3) ± SE. Expression of Nox1 (F-I) and Nox4 (J-K) protein levels was determined by Western blotting analysis on MCT lysate for 6 and 48h. The Western blots are quantified by densitometry and are representative of three independent experiments (n=3). Each histogram represents the ratio of the intensity of Nox1 or Nox4 bands factored by the GAPDH or β-actin band, used as loading controls. Values are the means ± SE. *P<0.05, high glucose or 20-HETE vs. control; #P<0.05, high glucose + HET0016 vs. high glucose.

D. Blocking EET increases HG-induced ROS production in MCTs

Next, we assessed whether blocking 11, 12-EET mediates the effect of HG-induced oxidative stress by upregulation of Nox oxidases in MCT cells. Nox1 and Nox4 protein levels were analyzed by western blotting performed on MCTs incubated with HG in the absence or presence of MS-PPOH (15 μM, 1 h pre-treatment) for the indicated time periods. HG significantly increased Nox1 (Figure 5 A-B) and Nox4 (Figure 5 C-F) protein expression. Moreover, the upregulation of both oxidases culminated upon pretreatment of the cells with MS-PPOH followed by exposure to HG. These results indicate that the blockade of EETs worsens the effects of HG-induced NADPH oxidases Nox1 and Nox4 protein expression upregulation.

Figure 5.

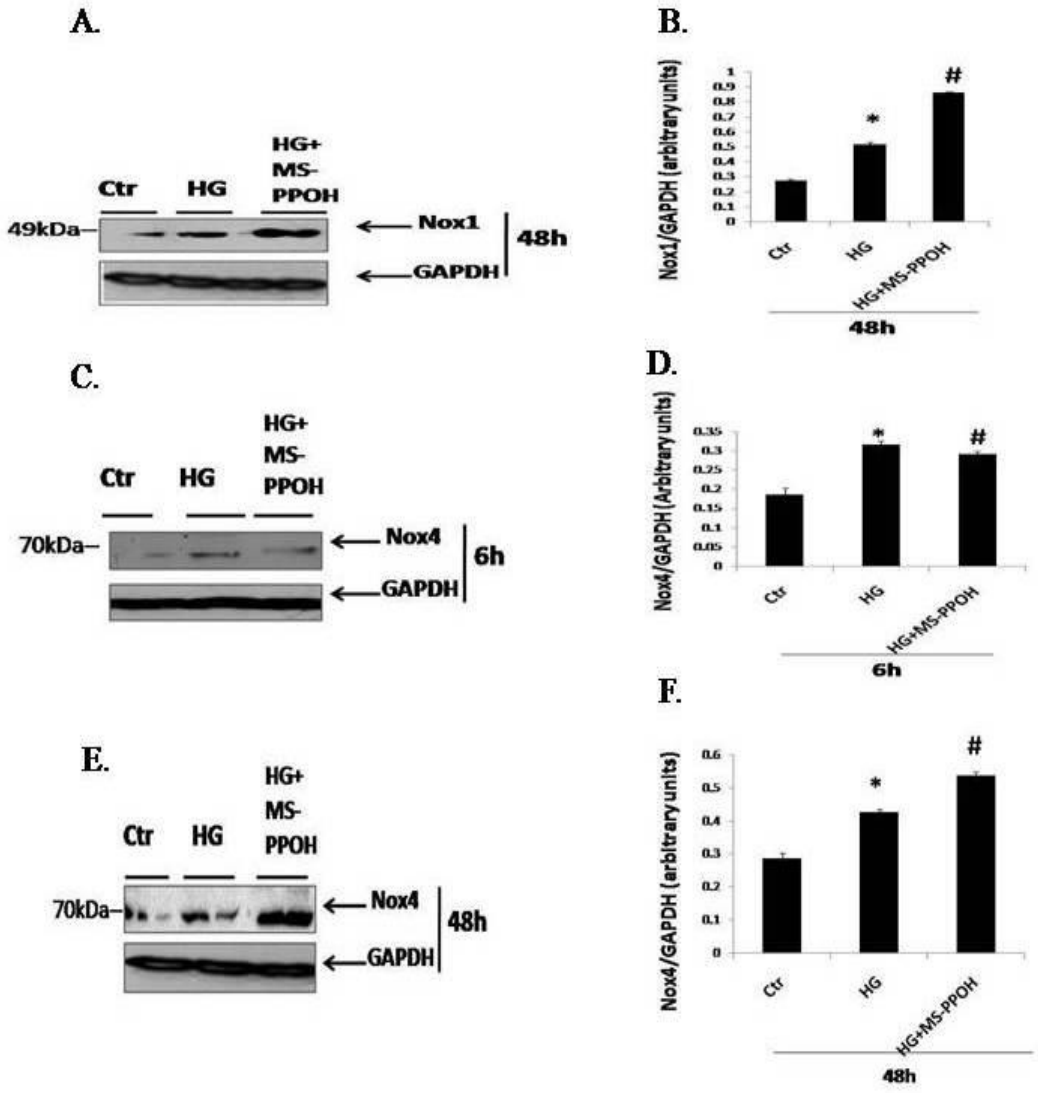


Figure 5. Effect of EET and its inhibitor MS-PPOH on renal expression of Nox oxidases.

Representative western blots of Nox1 after 48 h treatment (A) and Nox4 at 6 and 48 h treatment (B) were performed on MCT lysate exposed to HG (25 mM) in the absence or presence of MS-PPOH (15 μM).The Western blots are quantified by densitometry and 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 GAPDH used as a loading control. Values are the means ± SE. *P<0.05, high glucose vs. control; #P<0.05, high glucose + MS-PPOH vs. high glucose.

E. CYP4A-dependent 20-HETE production and EET inhibition mediate the effect of HG on matrix protein accumulation

In order to determine the role of 20-HETE and EETs to kidney injury in a hyperglycemic milieu in terms of matrix protein accumulation, we assessed the levels of fibronectin and collagen IV protein expression. MCTs were exposed to HG in the presence or absence of HET0016 (15 μM) or MS-PPOH (15 μM) or treated with 20-HETE (1.5 μM) for 6 and 48h. An increase in fibronectin mRNA levels (Figure 6 A-B) and protein (Figure 6 C-F) content was observed in MCTs treated with HG. However, when the cells were pre-treated with HET0016, the increased fibronectin mRNA and protein expression was inhibited. In addition, treatment of the cells with 1.5 μM 20-HETE resulted in an increase in fibronectin expression at the mRNA and protein levels, thereby mimicking the effect of HG. These effects were paralleled by similar changes in collagen IV protein expression (Figure 7 A-D).

Figure 6.

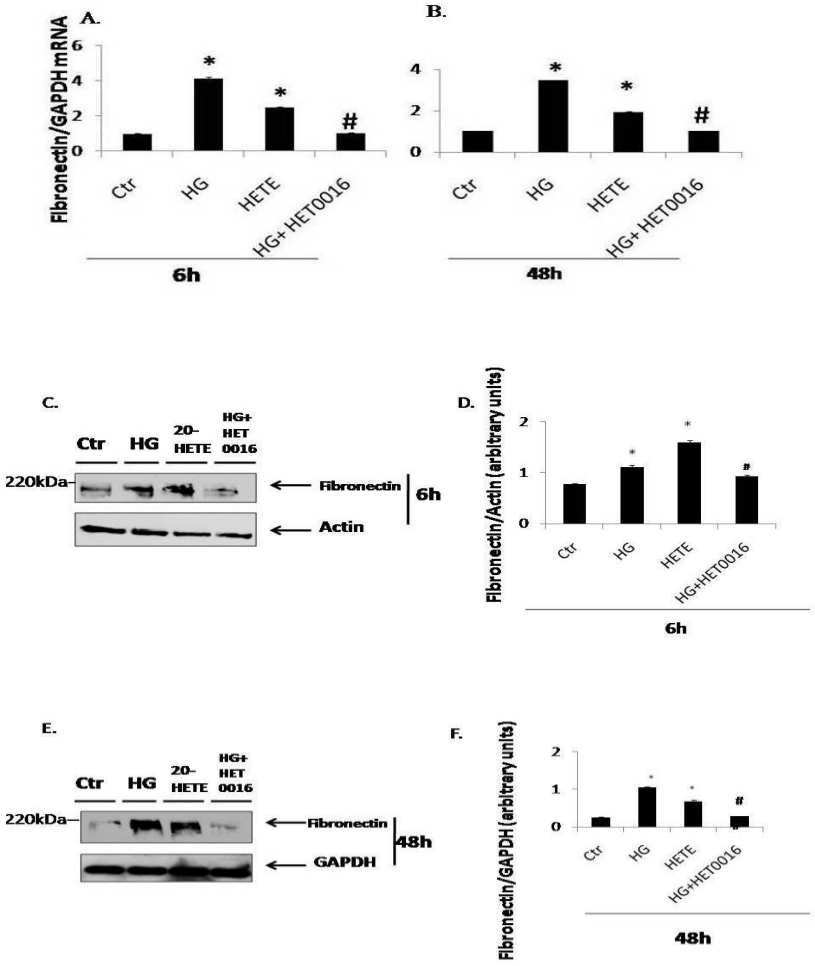


Figure 6. CYP4A-dependent 20-HETE production upregulates fibronectin expression. Serum-deprived MCTs were either treated with 20-HETE (1.5 µM) or with HG in the absence or presence of HET0016 (15 µM). Fibronectin mRNA levels were measured at 6 (A) and 48h (B) of treatment. The values represent the relative induction as measured by real-time RT-PCR relative to GAPDH mRNA levels. Data represents the mean of three independent experiments (n=3) ± SE.*P<0.05, high glucose or 20-HETE vs. control; #P<0.05, high glucose+HET0016 vs. high glucose.

Representative western blots of fibronectin protein expression on homogenized MCTs for 6 (C-D) and 48h (E-F) are shown. The Western blots are quantified by densitometry and are representative of three independent experiments (n=3). Each histogram represents the ratio of the intensity of fibronectin bands factored by the GAPDH or actin bands, used as loading controls. Values are the means ± SE. *P<0.05, high glucose or 20-HETE vs. control; #P<0.05, high glucose + HET0016 vs. high glucose.

Figure 7.

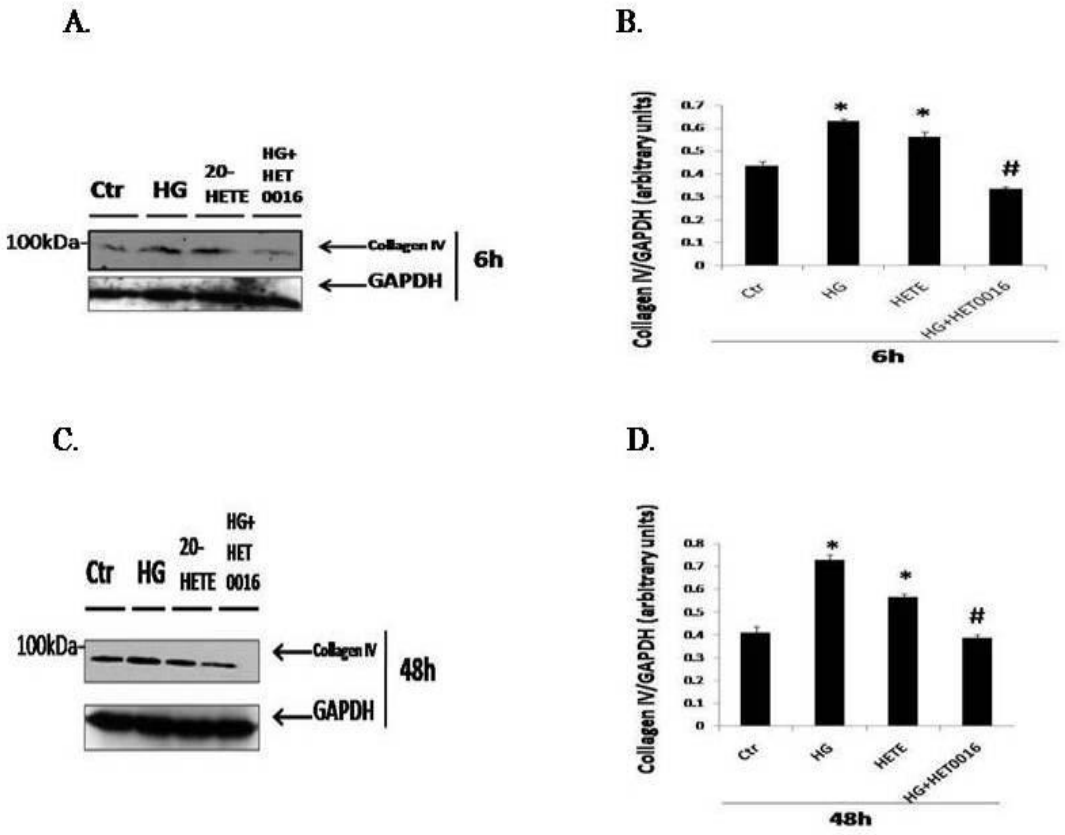


Figure 7. CYP4A-dependent 20-HETE production upregulates collagen IV expression. Serum-deprived MCTs were either treated with 20-HETE (1.5 μM) or with HG in the absence or presence of HET0016 (15 μM). Representative western blots of collagen IV protein expression on homogenized MCTs for 6 (A-B) and 48 h (C-D) are shown. The Western blots are quantified by densitometry and are representative of three independent experiments (n=3). Each histogram represents the ratio of the intensity of collagen IV bands factored by the GAPDH band, used as a loading control. Values are the means ± SE. *P<0.05, high glucose or 20-HETE vs. control; #P<0.05, high glucose+HET0016 vs. high glucose.

In contrast, HG-induced matrix protein accumulation was increased when MCT cells exposed to HG were pre-treated with MS-PPOH. Our results show that blockade of EETs in the presence of HG worsens proximal tubular cell injury by increasing the upregulation of fibronectin (figure 8 A-D) and collagen IV (Figure 9 A-D) protein expression. These observations may suggest that EETs play a critical role in decreasing matrix protein accumulation.

Figure 8.

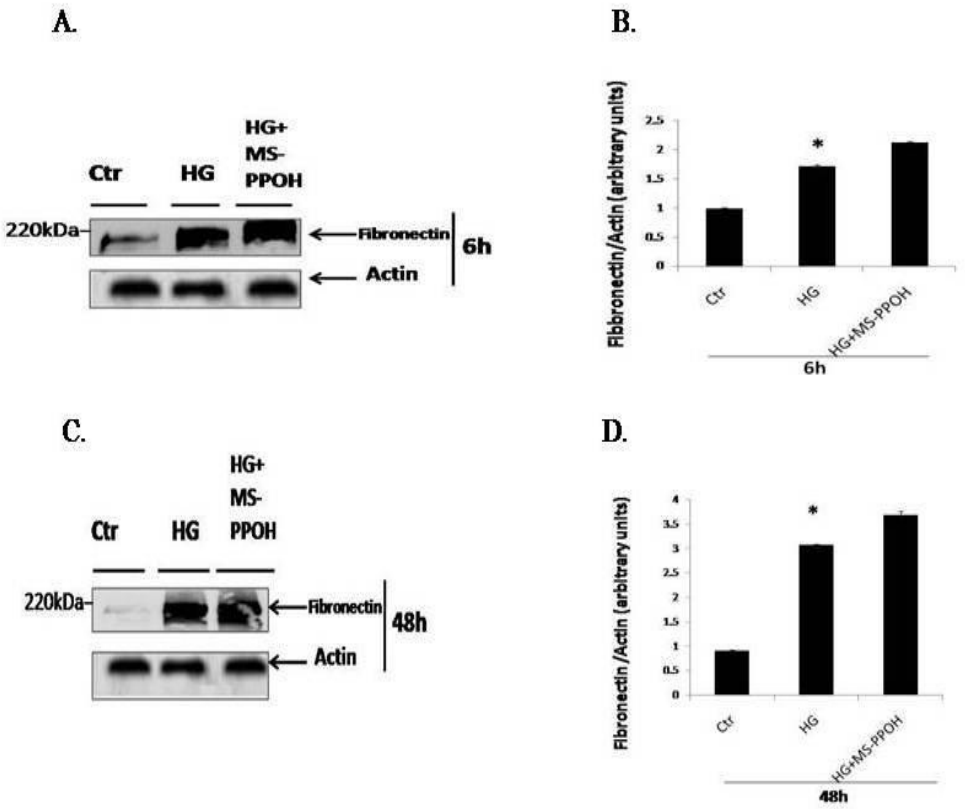


Figure 8. Inhibition of EET formation by MS-PPOH upregulates fibronectin expression.

Serum-deprived MCTs were treated with HG in the absence or presence of MS-PPOH (15 μM). Representative western blots of fibronectin protein expression on homogenized MCTs for 6 (A-B) and 48 h (C-D) are shown. The Western blots are quantified by densitometry and are representative of three independent experiments (n=3). Each histogram represents the ratio of the intensity of fibronectin bands factored by actin band, used as a loading control. Values are the means ± SE. *P<0.05, high glucose vs. control.

Figure 9.

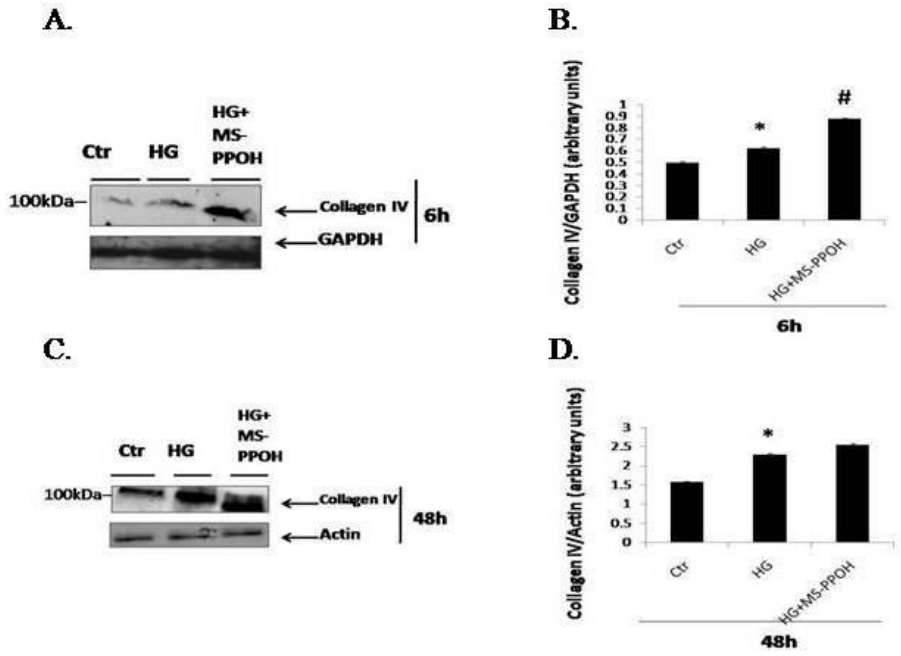


Figure 9. Inhibition of EET formation by MS-PPOH upregulates collagen IV expression.
Serum-deprived MCTs were treated with HG in the absence or presence of MS-PPOH (15 µM). Representative western blots of collagen IV protein expression on homogenized MCTs for 6 (A-B) and 48h (C-D) are shown. The Western blots are quantified by densitometry and are representative of three independent experiments (n=3). Each histogram represents the ratio of the intensity of collagen IV bands factored by GAPDH or actin bands, used as loading controls. Values are the means ± SE. *P<0.05, high glucose vs. control.

F. 20-HETE generation and EET inhibition mediate proximal tubular cell apoptosis induced by HG

We next investigated the effect of 48 h administration of 20-HETE or inhibition of EETs on proximal tubular cells apoptosis. Cellular DNA fragmentation ELISA and Hoeshst staining, 2 methods to assess cellular apoptosis, were performed on MCTs treated with 20-HETE (1.5 µM) or exposed to HG in the presence or absence of HET0016 (15 µM) or MS-PPOH (15 µM) for 48 h. Data in figure 10 A-B show that HG induced MCT cells apoptosis. This effect was inhibited by the pre-treatment with HET0016, suggesting that CYP4A mediated the apoptotic effects of HG on MCTs. Furthermore, 20-HETE was found to mimic the effect of HG on MCTs by inducing MCT cells apoptosis. Collectively, our results suggest that the elevated production of CYP4A-dependent 20-HETE increased ROS generation and resulted in MCT cells apoptosis.

On the other hand, HG-induced MCT cells apoptosis was further enhanced by the pre-treatment of the cells with MS-PPOH (Figure 10 A-B). These results indicate that blockade of EETs increases HG-induced apoptosis in proximal tubular cells.

Figure 10.

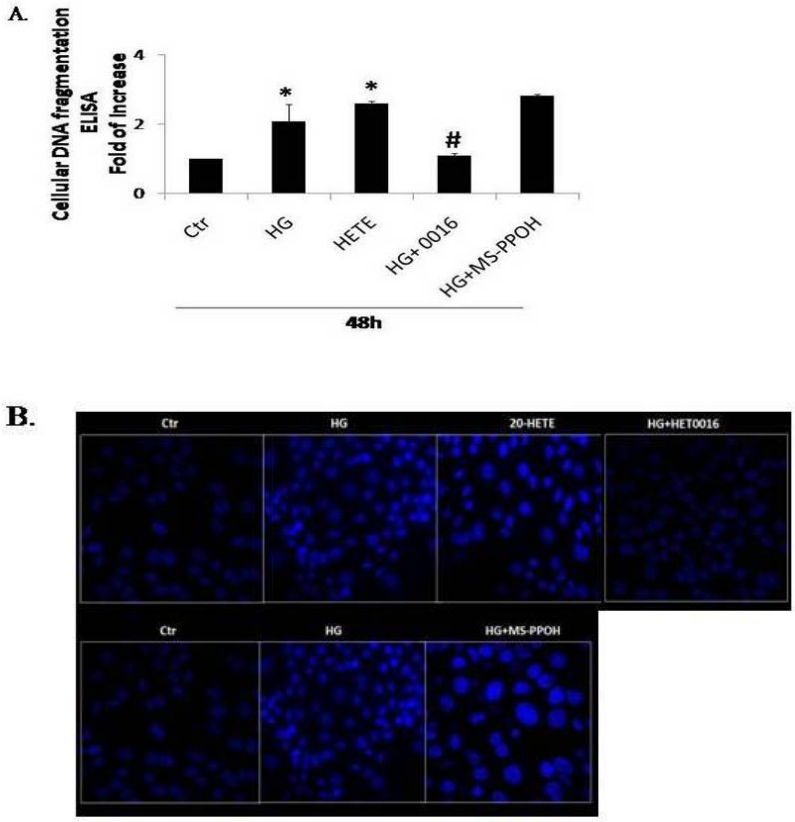


Figure 10. CYP4A-dependent 20-HETE production and EETs inhibition mediate HG-induced apoptosis in proximal tubular cells.
Serum-deprived MCTs were treated with 1.5 µM of 20-HETE or incubated in high glucose (HG; 25 mM) with or without HET0016 (15 µM) or MS-PPOH (15 µM) for 48 h. The cellular DNA fragmentation ELISA kit was used to detect BrdU-labeled DNA fragments in MCT cells as described in MATERIALS AND METHODS section (A). Data are means ± SE. *P<0.05, high glucose or 20-HETE vs. control; #P<0.05, high glucose + MS-PPOH or high glucose + HET0016 vs. high glucose. Apoptotic nuclei were detected using Hoechst 33258 (B), which stains cells that have already undergone apoptosis.

G. 20-HETE generation and EET inhibition decrease AMPK phosphorylation in MCT cells

AMP-activated protein kinase (AMPK) a serine/threonine kinase, is an energy sensor whose activity is regulated by glucose. AMPK has been shown to play a role in HG-induced ROS production and NADPH oxidases Nox1 and Nox4 protein expression (Eid et al., 2010). In order to elucidate the mechanisms by which the eicosanoid products regulate the onset and development of DN, we studied their effect on AMP-activated protein kinase (AMPK). We evaluated AMPK activity, assessed by p-AMPK protein expression, in MCTs treated with 20-HETE (1.5 μM) or exposed to HG in the presence or absence of HET0016 (15 μM) or MS-PPOH (15 μM) for 6 or 48 h. In cells incubated with HG, treatment with HET0016 restored the protein levels of AMPK α phosphorylation on its activating site Thr^{172} (Figure 11 A-D). In contrast, treatment of MCTs with 20-HETE mimicked the effect of HG and reduced AMPKα phosphorylation. As shown in figure 11, Pre-treatment of MCT cells with MS-PPOH mimicked the effect of HG and inhibited AMPK α phosphorylation (Figure 11 E-F).

Figure 11.

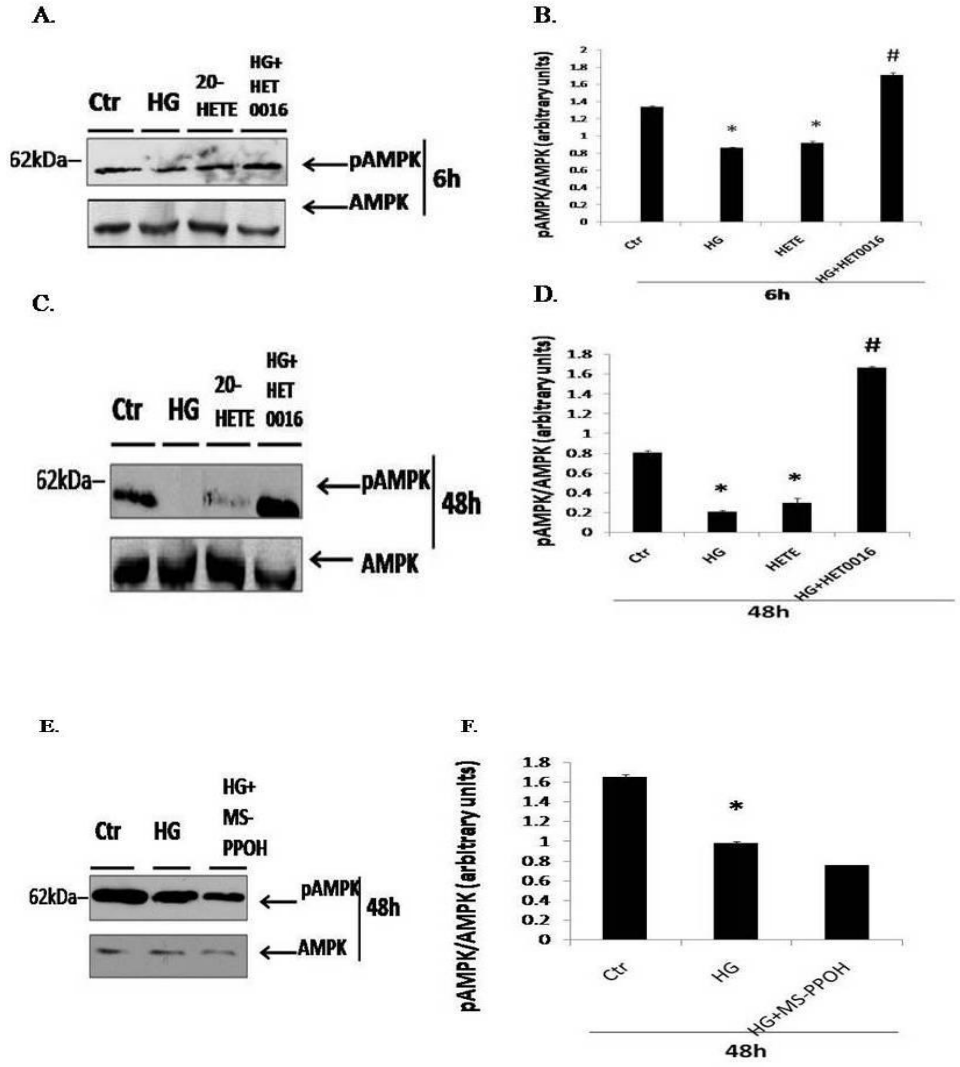


Figure 11. 20-HETE generation and EET inhibition decrease AMPK phosphorylation in MCT cells.

MCT cells were treated with 20-HETE (1.5 μM) or exposed to HG in the presence or absence of HET0016 (15 μM) or MS-PPOH (15 μM) for the indicated time periods. Representative western blots of phospho-Thr^{172} AMPKα and AMPKα are shown. The Western blots are quantified by densitometry and are representative of three independent experiments (n=3). Each histogram represents the ratio of the intensity of pAMPKα (Thr^{172}) bands factored by the AMPK band. Values are the means ± SE. *P<0.05, high glucose or 20-HETE vs. control; #P<0.05, high glucose + MS-PPOH or high glucose + HET0016 vs. high glucose.

H. High Glucose induces CYP 2B, 2C and 2F protein expression and 20-HETE and EET production in a rat model of type 1 diabetes

To determine the *in vivo* relevance of our findings in cultured cells, the levels of CYPs 2B, 2C, and 4F were assessed in the kidney cortex of a rat model of type 1 diabetes (rats injected with streptozotocin). Our data show an induction of the protein expression of CYP2B, 2C and 4Fand their corresponding activity, assessed by 20-HETE and EETs formation, in the kidney cortex of the diabetic rats when compared to normal rats (Figure 12 A-C).This induction was reduced in diabetic rats treated with insulin for 6 weeks. Insulin treatment was used to show that the changes observed in the diabetic state are not due to streptozotocin injection.

Figure 12.

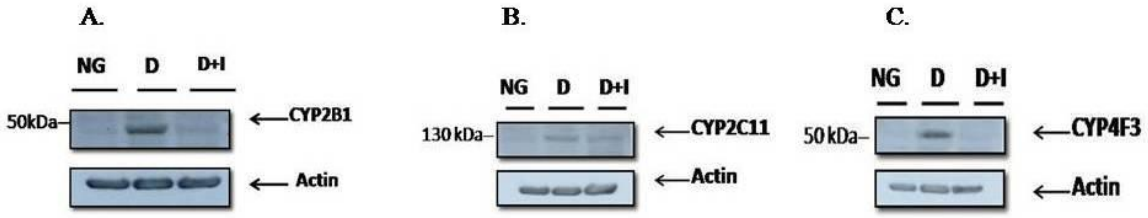


Figure 12. CYP 2B1, CYP 2C11 and CYP 4F3 Protein expression is induced in microsomes isolated from proximal tubules of diabetic rats.
Western blot analysis of microsomes derived from rat kidney cortex after 6 weeks of treatment with insulin; C=control animals; D=diabetic animals; D+I= STZ-induced type 1 diabetic animals treated with insulin. Significant induction of the CYPs is observed in the diabetic animal. Induction is decreased if the diabetic animal is treated with insulin.

Consistent with the induction of AA-metabolizing CYPs as demonstrated by western blot analysis, the metabolism of AA, 20-HETE (Figure 13 B) and EET (Figure 13 C) formation is increased in the diabetic animals.

More importantly, fibrosis assessed by fibronectin protein expression was increased in the kidney cortex isolated from diabetic rat when compared to kidney cortex isolated from control rats (C). Insulin treatment blocked the effect of diabetes induced kidney fibronectin accumulation (Figure 14).

Figure 13.

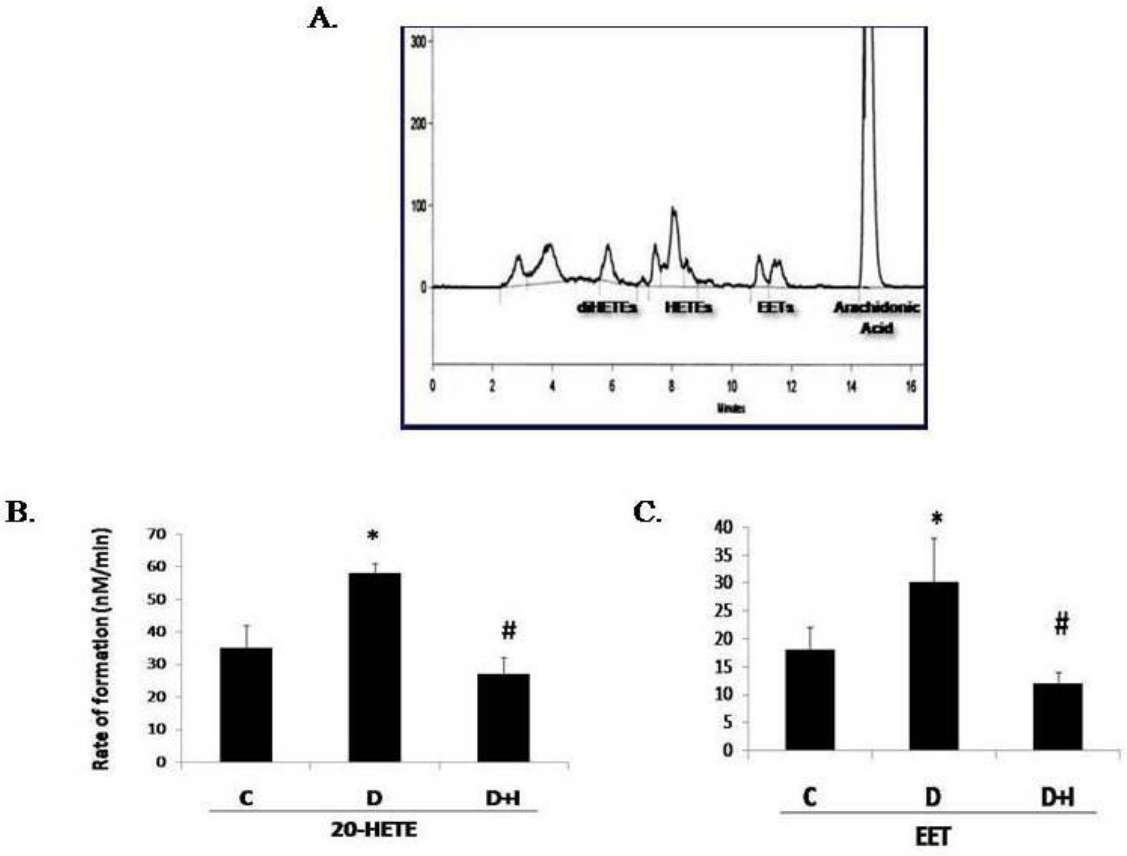


Figure 13. EETs and 20-HETE production is induced in microsomes isolated from proximal tubules of diabetic rats. A: Typical HPLC chromatogram showing arachidonic acid metabolites produced by rat kidney microsomes. B-C: Metabolism of AA by control and diabetic rat kidney microsomes. 50 μg of microsomes prepared from MCT cells were incubated with 50 μM ^{14}C-AA in the presence of NADPH. 20-HETE and EETs were separated by reverse-phase HPLC. Values are the means ± SE. *P<0.05, D vs. C; #P<0.05, D+I vs. D.

Figure 14.

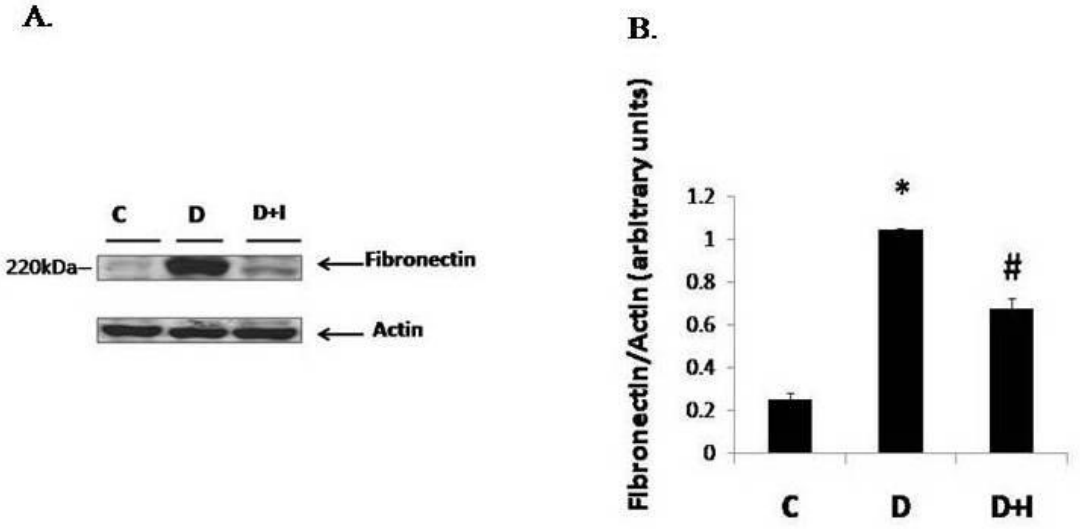


Figure 14. HG induces fibronectin expression in type 1 diabetic rat model. Representative western blot of fibronectin and actin are shown. The Western blots are quantified by densitometry and are representative of three independent experiments (n=3). Each histogram represents the ratio of the intensity of fibronectin bands factored by actin bands, used as a loading control. Values are the means ± SE. *P<0.05, D vs. C; #P<0.05, D+I vs. D.

Altogether, our data show that AA acid metabolites 20 HETE and EETS play a major role in diabetes induced kidney injury. Elucidating the mechanism of action of CYPs enzymes and their metabolites may introduce a new paradigm in the treatment of diabetes.

DISCUSSION

Proximal tubular epithelial cell apoptosis is an early feature of diabetic kidneys detected at the onset of diabetes (Ortiz et al., 1997). Oxidative stress plays a key role in the development and progression of diabetic complications, including proximal tubular cell apoptosis (Allen et al., 2003). In this study, we provide evidence that diabetic nephropathy is associated with a significant induction of expression and activity of cytochromes P450 and their AA metabolites, 20-HETE and EETs. We demonstrate that HG induces apoptosis of cultured proximal tubular epithelial cells through the production of ROS either by the upregulation of CYP4A or by the upregulation of Nox1 and Nox4. We show that 20-HETE, generated by CYP4A, induced ROS production, upregulated Nox1 and Nox4, increased extracellular matrix protein expression, and induced MCT cell apoptosis. Our data also indicate that 20-HETE decreased the phosphorylation of AMPK, an effect that occurs in a hyperglycemic milieu. Inhibition of CYP4A prevented oxidative stress, decreased extracellular matrix protein expression and reduced MCT cell apoptosis. Our *in vivo* data show that EET levels are increased at the onset of diabetes in the proximal tubules of type 1 diabetic rats. Inhibition of EETs *in vitro* promoted the effects of HG on MCTs, thereby upregulating Nox1 and Nox4, increasing extracellular matrix protein expression, inducing apoptosis and decreasing the phosphorylation of AMPK. It has been well documented that hyperglycemia leads to tubulointerstitial injury (Park et al., 2001; Hall, 2006). There is evidence that diabetes induces apoptosis in proximal tubular epithelial cells via ROS

generation (Allen et al., 2003; Liu et al., 2008). Previous reports showed that angiotensin II-induced proximal tubular cell apoptosis is mediated by oxidative stress (Bhaskaran et al., 2003).

Brezniceanu et al. (2007) found that limiting ROS production in the proximal tubules by overexpressing catalase, an enzyme that inactivates H_2O_2, reduces many of the diabetic complications including apoptosis. However, the molecular mechanisms of action of oxidative stress as well as the precise sources of ROS have not been fully characterized. NADPH oxidases are expressed in the kidney cortex (Bedard and Krause, 2007), with a prominent expression of Nox1 and Nox4 in the proximal tubules (Gill and Wilcox, 2006). When exposed to HG, different cell types rapidly generate ROS (Inoguchi et al., 2000; Eid et al., 2009). In this study, we demonstrated that HG results in a rapid generation of ROS in cultured MCTs. Mitochondrial electron transport chain, NADPH oxidases, and CYP450 are considered as potential sources of ROS in cells and tissues (Shah and Channon, 2004). However, CYP4A-dependent 20-HETE is highly produced in proximal tubules (Zhou et al., 2008) and exerts a wide range of regulatory and opposing functions depending on the location of its production (Natarajan et al., 2003). Here, we show that CYP4A protein expression and 20-HETE formation were increased in cells exposed to HG. We also show that HG-induced ROS generation, Nox oxidases upregulation, and cell apoptosis were inhibited when the cells were pre-treated with HET0016, a specific inhibitor of CYP4A. Furthermore, adding exogenous 20-HETE increased ROS generation and cell apoptosis. Therefore, our results demonstrate that CYP4A-dependent 20-HETE production induced proximal tubular cell apoptosis via enhanced ROS generation. Controversial data have been reported regarding the exact role of 20-HETE. Hoff et al. (2011) showed that 20-HETE is

overexpressed in ischemic kidney tissue and increases tubular epithelial cell apoptosis. Also, inhibition of CYP450 reduced apoptosis of LLC-PK1 cells treated with hydrogen peroxide (Baliga et al., 1996). Moreover, Zeng et al. (2010) demonstrated that 20-HETE increases NADPH-derived ROS production in cardiac myocytes. CYP4A-dependent 20-HETE production has also been associated with the tubular cell injury found in renal ischemia/reperfusion injury and that CYP4A-dependent ROS generation mediates tubular cell apoptosis (Nilakantan et al., 2008). However, 20-HETE mediates epithelial cell proliferation in cystic renal disease (Park et al., 2008). Dhanasekaran et al. (2009) have reported that 20-HETE inhibits apoptosis and protects pulmonary artery endothelial cells. Eid et al. have reported that HG decreases AMPKα phosphorylation at its activation site Thr^{172} and reduces AMPK activity (Eid et al., 2010). Our results indicate that AMPKα phosphorylation is restored upon pre-treatment with HET0016. In contrast, the addition of 20-HETE reduces AMPKα phosphorylation. Lieberthal et al. (2011) have demonstrated that activation of AMPK following acute metabolic stress inhibits apoptosis in cultured proximal tubular cells. Therefore, the role of AMPK inactivation in mediating the effects of 20-HETE in proximal tubular cell apoptosis, remains to be explored.

It is known that proximal tubular cells exposed to high glucose undergo increased matrix protein accumulation, mainly collagen IV and fibronectin (Gilbert and Cooper, 1999). Additionally, ROS play an important role in the progression of fibrosis (Barnes and Gorin, 2011). Recent studies demonstrated that the abnormal matrix production, induced by TGF-β1 requires ROS generation and subsequent activation of mitogen-activated protein kinase (MAPK) (Rhyu et al., 2012). New et al. have identified insulin-like growth factor I, as a mediator of fibronectin expression through Nox4-dependent ROS generation. However, the

precise source of ROS is yet to be determined. We have identified CYP4A-dependent 20-HETE as a major source of ROS in the proximal tubules. Our data showed that HG-induced matrix protein expression, fibronectin and collagen IV, is reduced when the cells were pre-treated with HET0016. Moreover, the addition of 20-HETE resulted in an increase in matrix protein expression within hours of the treatment. Collectively, these data indicate that 20-HETE formation induced matrix protein accumulation, and that this effect was likely mediated by enhanced ROS production. There is evidence that 20-HETE induces NADPH-dependent ROS production in cardiac myocytes and mediates fibrosis in ischemic injury (Zeng et al., 2010). On the other hand, Williams et al. (2007) have shown that 20-HETE has a renoprotective effect on the glomerular permeability barrier and may, thus slow the progression of matrix protein accumulation and renal fibrosis.

What about the epoxygenase pathway?

CYP2C and CYP2J, catalyzing the synthesis of 11, 12-EET are highly expressed in the proximal tubules (Huang et al., 2006). The regulation of EET production is tissue and disease specific (Natarajan et al., 2003). In the present study, we show that CYP2C11 protein expression and EET formation were increased in cells exposed to HG. We also show that HG-induced Nox oxidases upregulation, increased matrix protein expression and cell apoptosis were increased when the cells were pre-treated with MS-PPOH, a selective inhibitor of 11, 12 EET formation. Since blockade of EET promotes and worsens HG-induced apoptosis in proximal tubular cells, our data suggest a renoprotective role of EETs in the hyperglycemic milieu. Dhanasekaran et al. (2009) showed that EETs inhibited mouse cardiomyocyte apoptosis after exposure to hypoxia and reoxygenation. Pre-incubation of EETs with HG will enable the assessment of EETs effect in a hyperglycemic milieu.

Taken together, these observations may explain that in early diabetes, cytochrome enzymes are upregulated leading to an increased production in EETs and 20-HETE. However, as we showed, 20-HETE is injurious to the kidney and EETs appear to play a protective role. But it's not playing the needed role to neutralize the nocive effects of 20-HETE.

In conclusion, we identified, in the present study, CYP4A, 20-HETE and NAPDH oxidases as sources of ROS that lead to apoptosis when cultured proximal tubular cells are exposed to HG. We have identified 20-HETE as a mediator of proximal tubular cell injury in the diabetic milieu, and that CYP4A inhibitors may be useful in designing effective therapies in the treatment of DN. We also show that inhibition of EETs promotes the deleterious effects of HG suggesting a protective role of EETs in DN.

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