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Cytochrome P450 epoxygenase CYP2J2 attenuates nephropathy in streptozotocin-induced diabetic mice

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Abstract

Cytochrome P450 (CYP) epoxygenases metabolize arachidonic acid into epoxyeicosatrienoic acids (EETs), which play important and diverse roles in the cardiovascular system. The anti-inflammatory, anti-apoptotic, pro-angiogenic, and anti-hypertensive properties of EETs in the cardiovascular system suggest a beneficial role for EETs in diabetic nephropathy. This study investigated the effects of endothelial specific overexpression of the CYP2J2 epoxygenase on diabetic nephropathy in streptozotocin induced diabetic mice. Endothelial CYP2J2 overexpression attenuated renal damage as measured by urinary microalbumin and glomerulosclerosis. These effects were associated with inhibition of TGF- β /Smad signaling in the kidney. Indeed, overexpression of CYP2J2 prevented TGF- β 1 induced renal tubular epithelial-mesenchymal transition *in vitro*. These findings highlight the beneficial roles of the CYP epoxygenase-EET system in the pathogenesis of diabetic nephropathy.

Keywords

Arachidonic acids; cytochrome P450 epoxygenase; streptozotocin; diabetic nephropathy; tubular epithelial-mesenchymal transdifferentiation

1. Introduction

Approximately 20–30% of all diabetic patients develop evidence of diabetic nephropathy (DN), which represents a continuum from microalbuminuria, to overt nephropathy or macroalbuminuria, and finally end stage renal disease (ESRD) ¹. Indeed, DN is the main cause of ESRD requiring dialysis in developed countries ². DN is initiated by hyperglycemia and is characterized by mesangial expansion and thickening of the tubular and glomerular basement membranes due to excessive matrix accumulation, associated with progressive renal deterioration ³. However, a recent study has indicated that tubular injury and progressive interstitial fibrosis are more closely related to DN prognosis ⁴. TGF- β and its downstream signal transduction pathways play a major role in DN and have therefore been thoroughly studied ⁵. Among the features of the diabetic milieu, hyperglycemia, increased non-enzymatic glycation of proteins, de novo synthesis of diacylglycerol and subsequent activation of protein kinase C, increased intracellular glucosamine production, and enhanced

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renal production of vasoactive agents (angiotensin II, endothelins, thromboxane) have all been shown to increase the expression of TGF- β in cultured renal cells and in animal models of DN⁶. With the emerging evidence that TGF- β is involved in the pathogenesis and progression of DN, potential treatments with specific factors that interrupt the activity of TGF- β have become promising and attractive.

Epoxyeicosatrienoic acids (EETs), cytochrome P450 (CYP) epoxygenase metabolites of arachidonic acids (AA), have diverse biological effects within the cardiovascular system and have been identified as endothelium-derived hyperpolarizing factors, which activate calcium-sensitive potassium channels resulting in hyperpolarization of the resting membrane potential and the relaxation of vascular smooth muscle cells⁷. CYP epoxygenases are highly expressed in cardiomyocytes and vascular endothelial cells⁸. CYP epoxygenase overexpression, and the corresponding increase in EET levels, promotes an angiogenic phenotype, including endothelial cell proliferation, migration, and capillary tubule formation, as well as significantly protects endothelial cells from TNF- α induced apoptosis^{9, 10}. EETs upregulate eNOS in bovine aortic endothelial cells via activation of MAPK, protein kinase C, PI3-kinase/AKT, and MAPK signaling pathways^{11, 12}. 14,15-EET mediated mitogenic signaling pathway in a kidney epithelial cell line, which involved EET-dependent activation of metalloproteinases and release of the potent mitogenic EGFR ligand, HB-EGF¹³. EETs participate as second messengers in the angiogenic response initiated by VEGF and preventing the increase in CYP expression curtails the angiogenic response to VEGF¹⁴. Blocking PPAR γ by GW9662 abolished the EET mediated anti-inflammatory effect, which indicates that PPAR γ is an effector of EETs¹⁵. In the pancreas, CYP epoxygenase expression and EET production suggest that EETs also may be involved in pancreatic hormone secretion¹⁶. Indeed, 5,6-EET directly stimulates insulin release from isolated rat pancreatic islets *in vitro*¹⁷.

CYP, family 2, subfamily J, polypeptide 5 epoxygenase (CYP2J5) produces EETs and is highly expressed in the proximal tubule and collecting duct of mice¹⁸. The inhibition of soluble epoxide hydrolase 2 (EPHX2), the primary EET metabolizing enzyme, results in increased EET levels, which has been shown to attenuate the progression of renal damage associated with hypertension and type II diabetes¹⁹. The human ortholog of mouse CYP2J5 is CYP2J2²⁰. Our previous CYP2J2 gene therapy studies have demonstrated alleviation of insulin resistance and diabetic symptoms in db/db mice and fructose-induced insulin resistant rats²¹. We hypothesize that endothelial CYP2J2-overexpression and the resultant increase in EET production will attenuate DN in streptozotocin (STZ)-treated mice. Furthermore, we postulate that TGF- β 1 induced changes in cultured kidney cells will be attenuated by the administration of exogenous EETs.

2. Materials and Methods

2.1. Materials

Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories (Soham, UK). Enhanced chemiluminescence reagent was purchased from Pierce Chemical (Rockford, IL), pre-stained molecular weight standards were from Bio-Rad (Hercules, CA), and polyvinylidene difluoride membranes were from Schleicher and Schuell (Dassel, Germany). Antibodies against p-smad3, smad3, smad4, smad7 and α -SMA were purchased from Sigma-Aldrich (St. Louis, MO). Antibody against CYP2J2 was purchased from Abcam Inc. (Cambridge, MA). Antibodies against TGF- β 1, β -actin, E-cadherin were from Santa Cruz Biotechnology (Santa Cruz, CA). 14, 15-DHET ELISA Kit was from Detroit R&D (Detroit, MI). All other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Animal treatments

Experimental protocols complied with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Endothelial-specific CYP2J2 overexpression (Tie2-CYP2J2) transgenic mice on a pure C57BL/6 genetic background were generated in the Zeldin laboratory as previously described and genotyped using PCR-based methods²². All animals were housed (four per cage) in a temperature ($25\pm 3^{\circ}\text{C}$) and humidity ($50\pm 20\%$) controlled room with 12-h light, 12-h dark cycles and *ad lib* normal chow (9% fat, 20% protein, 53% starch, and 5% fiber) plus water. At 8-week-old, wild type (WT) and Tie2-CYP2J2 transgenic mice were injected with streptozotocin intraperitoneally (50 mg/kg, made freshly in 50mM sodium citrate buffer, pH 4.5) for 5 consecutive days, or with 50 mM sodium citrate solution only for control. Tail vein blood glucose levels were measured 1 week after the last STZ injection and mice with glucose levels > 250 mg/dl were considered diabetic. Then, wild type (WT) and Tie2-CYP2J2 transgenic mice were divided into control and diabetic groups (4 groups, 10 mice each). Systolic blood pressures were measured at end of experiment in conscious mice with a manometer-tachometer (Rat Tail NIBP System, ADI Instruments, Sydney, Australia) using the tail-cuff method as described previously²¹. All animals were euthanized 32 weeks later with pentobarbital (50 mg/kg body weight). Hearts, kidneys were collected, frozen with liquid nitrogen, and stored at -80°C .

2.3. Plasma and urine analysis

Blood samples were taken from the cavernous sinus with a capillary while under anesthesia. Plasma was prepared and stored at -80°C . Metabolic cages were used to collect 24-hour urine samples into brown bottles containing 500 μl toluene to prevent decay. Plasma glucose was measured in duplicate on an AEROSET Clinical Chemistry System (Abbott Laboratories, Chicago, IL). $\text{HbA}_{1\text{c}}$ (A1C) levels were determined using a commercially available analysis kit (DCA 2000; Bayer, Elkhart, IN) and blood urea nitrogen (BUN) was measured by BUN assay (Diagnostic Chemicals Ltd., Oxford, CT). We determined plasma creatinine levels by a HPLC-based method as described previously²³. Urinary microalbumin was measured a Murine Microalbuminuria ELISA kit (Exocell, Philadelphia, PA) to estimate albumin excretion rate as reported previously²⁴. Urine osmolarity was measured using a model 3D3 osmometer (Advanced Instruments Norwood, MA).

2.4. Evaluation of urine 14,15-DHET by ELISA

To assess *in vivo* EET production, an ELISA kit (Detroit R&D) was used to determine urinary concentrations of the stable EET metabolite 14, 15-dihydroxyeicosatrienoic acid (14,15-DHET), exactly according to the manufacturer's instructions.

2.5. Effects of EETs on TGF- β 1-induced TEMT

Human renal proximal tubular cells (HK-2 cell line) were purchased from ATCC (Number: CRL-2190) and cultured in DMEM/F12 with 10% fetal calf serum (5% CO_2 at 37°C). Tubular epithelial-mesenchymal transdifferentiation (TEMT) was induced in tubular cells by treating with TGF- β 1 (10 ng/ml) for 72 hours. In some studies, cells were simultaneously treated with either 11,12- or 14,15-EETs (1 μM) plus EGFR inhibitor (0.1nM AG1478) and PPAR- γ inhibitor (1nM GW9662). TEMT was assessed through morphological observation and analysis of E-Cadherin and α -SMA expression.

2.6. Western blotting

Proteins for western blotting were obtained from kidney according to method described previously²¹. Protein concentrations were estimated by the Bradford method²⁵. Equal amounts of protein sample were separated by electrophoresis on 10% SDS-PAGE gels. The resolved proteins were electrophoretically transferred to polyvinylidene difluoride

membranes using a transfer buffer containing 192 mM glycine, 20% (vol/vol) methanol, and 0.02% SDS. The membranes were incubated in 10 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 with 5% non-fat dried milk for 2 hours at room temperature and then incubated overnight at 4°C with the indicated primary antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence using horseradish-peroxidase-labeled anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, Soham, UK) and quantified by densitometry analysis using the Bio image system (Syngene, Cambridge, UK).

2.7. Analysis of renal morphology

A portion of kidney from each animal was preserved in 4% PBS-buffered formaldehyde solution and embedded in paraffin. Four 4µm paraffin sections were randomly chosen from one kidney from each animal and stained with periodic acid Schiff (PAS) reagent to evaluate morphological changes. In each section, six fields of kidney cortex were randomly selected at a magnification of 40X using an Olympus BH-2 light microscope (Olympus, Tokyo, Japan). Extracellular matrix (ECM) production was quantified as the ratio of collagen to non-collagen area (percentage of ECM) with the use of HMIAS (High Medical Image Analysis System 2000, Screen Imaging Technology Co., Wuhan, China). Integral optical density of α -SMA levels were quantified using the HAIPS Pathological Image Analysis System (Tongjiqianping Image Company, Wuhan, China). All sections were evaluated by investigators who were blinded to treatment group assignment. Severity of glomerular sclerosis was determined using the mesangial injury score (MIS) system previously described²⁶. Briefly, the severity was graded according to the percentage of glomeruli involved: 0 = no lesions; 1+ = 1%–25%; 2+ = 25%–50%; 3+ = 50%–75%; 4+ = 75%–100%. MIS was obtained by multiplying the severity score (0 to 4+) by the percentage of glomeruli which display the same degree of injury.

2.8. Immunohistochemistry and Immunocytochemistry

Process of immunohistochemistry and immunocytochemistry was performed as described previously²⁷ using α -SMA antibody. The ratio of α -SMA positive HK-2 cells was quantified using the HAIPS Pathological Image Analysis System (Tongjiqianping Image Company, Wuhan, China). Data from five experiments were expressed as the mean percentage.

2.9. Statistical analysis

Results are expressed as mean $\pm$ SEM. Multiple comparisons between two groups were performed with unpaired t tests; among three or more groups they were carried out with One-way ANOVA and Newman-Keuls tests for post hoc analyses. Values were considered significantly different if $P < 0.05$.

3. Results

3.1. Metabolic characteristics and renal function in streptozotocin-induced diabetic mice

Table 1 lists the biochemical and physiological parameters of WT and Tie2-CYP2J2 transgenic mice in both control and STZ-treated groups. Administration of STZ to mice significantly increased the ratio of kidney weight to body weight, blood glucose, HbA_{1c} (A1C), blood urea nitrogen, plasma creatinine, urinary microalbumin levels and decreased urinary osmolarity compared with control mice group ($P < 0.05$), indicating the characteristics of diabetic renal dysfunction and establishment of the early phase of diabetic nephropathy. However, the increase in the ratio of kidney weight to body weight, levels of blood glucose, HbA_{1c} (A1C), blood urea nitrogen, plasma creatinine, urinary microalbumin and the decrease urinary osmolarity were partially reversed in Tie2-CYP2J2-transgenic mice ($P < 0.05$). There was no significant difference in blood pressure among nondiabetic animals, diabetic

wide-type mice and diabetic transgenic mice. These results suggest that endothelial CYP2J2 overexpression significantly preserves renal function after induction of diabetes in mice.

3.2. Effect of CYP2J2 overexpression on renal expression of CYP2J2 and urinary excretion of 14, 15-DHET

Immunoblots performed with CYP2J2 antibodies revealed higher CYP2J2 protein expression compared to wild-type littermates in kidneys of Tie2-CYP2J2 transgenic mice, while wild-type littermate controls exhibited no expression (Figure 1A), which suggested Tie2-CYP2J2 transgenic mice had abundant endothelial-specific expression of the transgene in kidney. Induction of diabetes had no significant effect on CYP2J2 protein expression in Tie2-CYP2J2 transgenic mice. Compared to WT mice, Tie2-CYP2J2 transgenic mice exhibited an increase in urinary excretion of the stable 14,15-EET metabolite, 14,15-DHET (Figure 1B). Induction of diabetes with STZ treatment had no significant effect on urinary 14,15-DHET levels in WT mice; however, diabetic Tie2-CYP2J2 transgenic mice had increased urinary 14,15-DHET excretion compared to their non-diabetic transgenic controls. These results suggest that CYP2J2 overexpression is accompanied by enhanced CYP2J2 activity in Tie2-CYP2J2 transgenic mice.

3.3. Effect of CYP2J2 overexpression on kidney morphology in diabetic mice

We assessed kidney morphology by using three staining methods. Hematoxylin/eosin (Figure 2A) and PAS (Figure 2B) staining of WT diabetic mice revealed severe glomerular injury characterized by mesangial expansion, nodular glomerulosclerosis, arteriolar hyalinosis and increased mesangial matrix accompanied by sclerotic changes. Interestingly, these pathological changes were attenuated in Tie2-CYP2J2 transgenic diabetic mice. Likewise, immunohistochemical staining for α -SMA (Figure 2C and S1A) was significantly increased in glomeruli and peritubular capillaries of diabetic WT mice; however, the amount of α -SMA staining was significantly lower in diabetic Tie2-CYP2J2 transgenic mice ($P < 0.05$). Quantification of the degree of glomerular injury revealed that diabetic Tie2-CYP2J2 transgenic mice had significantly decreased area of positive PAS staining, glomerular cell number, and mesangial injury score (Figure 2D) compared to WT mice. Together, this data suggests that endothelial CYP2J2 overexpression partially protects against glomerular and tubulointerstitial injury in diabetic mice.

3.4. Effect of CYP2J2 overexpression on TGF- β /Smad signaling pathway

Diabetic WT mice had significantly increased levels of TGF- β 1 and p-Smad3, and decreased levels of Smad7 compared to WT control mice (Figure 3A, B and C). Interestingly, these differences were significantly attenuated in diabetic Tie2-CYP2J2 transgenic mice. These results suggest that endothelial overexpression of CYP2J2 significantly attenuates activation of the TGF- β /Smad signaling pathway in diabetic mice.

3.5. Synthetic EETs attenuate TGF- β 1-induced TEMT

HK-2 tubular cells incubated with TGF- β 1 for 72 hours displayed significant morphological changes, including a change from ellipse-shaped cells to spindle-shaped, fibroblast-like cells (Figure 4). Interestingly, addition of physiologically relevant concentrations of either 11,12-EET or 14,15-EET (1 μ M each) prevented these TGF- β 1 induced morphological changes. The effect of EETs could be blocked by addition of either an EGFR inhibitor (AG1478) or a PPAR γ inhibitor (GW9662) (Figure 4). Furthermore, we found that almost all cells were α -SMA-positive after treatment with TGF- β 1, suggesting induction of TEMT (Figure 5 and S1B). In contrast, the ratio of α -SMA positive cells induced by TGF- β 1 treatment was decreased by addition of either 11,12-EET or 14,15-EET. The effect of EETs was also

blocked by the addition of the EGFR inhibitor (AG1478) or PPAR γ inhibitor (GW9662) (Figure 5 and S1B).

Western blot analysis showed that TGF- β 1 treatment of HK-2 cells resulted in decreased E-cadherin expression and increased α -SMA and Smad4 expression (Figure 6A–C). Physiologically relevant concentrations of 11,12-EET or 14,15-EET attenuated these TGF- β 1-induced effects. Moreover, either EGFR inhibition with AG1478 or PPAR γ inhibition with GW9662 partially blocked the attenuating effect of EETs on expression of these proteins (Figure 6D–F). Together, these data suggest that TGF- β 1 inhibited E-cadherin expression, activated the Smad pathway, and induced renal tubular fibrosis and EETs significantly attenuated these effects of TGF- β 1 through a mechanism that involves activation of EGFR and PPAR γ .

4. Discussion

In this study, we injected one low dose STZ (50mg/kg body weight) daily by I.P. for 5 consecutive days to induce a diabetic state characterized by hyperglycemia and hypoinsulinemia in mice. We examined the effects of endothelial CYP2J2 overexpression on diabetic nephropathy, including renal function and morphology. We found that endothelial CYP2J2 overexpression attenuated the detrimental effects associated with DN. Specifically, CYP2J2 overexpression decreased hyperglycemia, improved renal function, and attenuated glomerular sclerosis. CYP2J2 overexpression also attenuated changes to the TGF- β /Smad signal transduction pathway which is typically activated in DN. Together; these data suggest that endothelial CYP2J2 overexpression protects the kidney against development and progression of chronic renal failure in this model.

TGF- β and its signaling pathways have been shown to play a major role in the pathogenesis of DN. TGF- β levels are elevated in the kidneys of insulin-dependent diabetic animals during both early and late stages of disease^{28, 29}. In the STZ-diabetic rat and mouse, increased TGF- β 1 expression in the renal cortex and glomeruli and upregulation of the TGF- β R2 mRNA and protein were noted early after the onset of diabetes. Short-term treatment of STZ-diabetic mice with a neutralizing monoclonal antibody against all three isoforms of TGF- β prevented glomerular hypertrophy, reduced the increment in kidney weight by 50%, and significantly attenuated the increase in TGF- β 1, α 1 (IV) collagen, and fibronectin mRNAs without affecting glycemic control³⁰. Administration of circular antisense TGF- β oligodeoxynucleotides was noted to have renoprotective potential by inhibiting the overexpression of TGF- β in the kidney of diabetic rats³¹. Hence, blockade of the TGF- β pathway is highly desirable and may represent a novel approach to the prevention and/or treatment of DN.

The Smad proteins are essential components of the intracellular signaling pathway activated by TGF- β 1, and participate in TGF- β 1-induced fibrosis. TGF- β 1 signals are transduced by transmembrane type I and type II receptors. Activation of the receptor initiates a signaling transduction cascade involving Smad proteins. Smad2 or Smad3 become phosphorylated and bind to Smad4, a common partner for all of the receptor-activated Smads. The Smad complex then moves into the nucleus and interacts with various transcription factors to regulate gene expression. During this process, Smad6 and Smad7 can inhibit the phosphorylation of Smad2 or Smad3 and blunt TGF- β signaling. Smad3 is thought to be the signaling arm that primarily mediates the auto-induction of TGF- β 1 and its effects on apoptosis and matrix expression^{32–36}. Interference with TGF- β signaling in Smad3 deficient mice limits diabetic glomerulosclerosis without affecting albuminuria³⁷. It was reported that bleomycin-induced pulmonary fibrosis³⁸ and radiation-induced cutaneous fibrosis³⁹, where enhanced TGF- β signaling seems to play an important role, were attenuated in mice lacking

Smad3. In the current study, we showed upregulation of TGF- β 1 expression and Smad3 phosphorylation, and downregulation of Smad7 expression in diabetic mice compared to WT controls. Furthermore, these changes in TGF- β /Smad signaling were attenuated in diabetic mice with endothelial CYP2J2 overexpression, an effect that is likely to be renoprotective during injury. Vascular endothelium regulates vascular tone and maintains free flow of blood in vessels, which is a multifunctional organ and regulates the release of endothelial derived relaxing factors, such as EETs and endothelial nitric oxide. Endothelial dysfunction plays an important role in the pathogenesis of diabetic nephropathy⁴⁰. Endothelial-specific CYP2J2 overexpression in transgenic mice was critical to the observed protective effects in our experiments.

Renal proximal tubular epithelial cells (PTEC) constitute the predominant cell type within the tubulointerstitium. In addition to the transport of fluid and electrolytes, there is increasing evidence that PTEC are involved in fibrotic processes, playing a pivotal role in the pathogenesis of tubulointerstitial disease in various renal diseases^{41, 42}. Tubulointerstitial fibrosis is the common pathway to end stage renal disease. Increasingly, studies show that EMT is a major contributor to the pathogenesis of renal fibrosis^{43–47}. EMT is a coordinated cellular response that involves several distinct processes, including disruption and disassembly of desmosomes and E-cadherin mediated adherens junctions, remodeling of the actin cytoskeleton, stress fiber formation, alteration of cell-matrix adhesion, and increased cell motility. EMT induced by TGF- β 1 plays a crucial role in renal fibrosis. Activation of TGF- β 1 signaling is sufficient to induce EMT in cultured epithelial cells, including a non-transformed mouse mammary cell line (NMuMG) and HK-2^{48, 49}. Advanced glycation end products (AGE) were found to induce EMT *in vitro* and in diabetic rats through activation of TGF- β 1 signaling, indicating an important role for this TGF- β 1-induced response in progression of DN⁵⁰. E-cadherin is a characteristic marker of tubular epithelial cells and is regulated by β -catenin. Loss of E-cadherin is associated with loss of renal epithelial cell function and is an early and important step in EMT^{51, 52}. On the other hand, α -SMA is the characteristic marker of myofibroblasts and is not abundant in renal tubular epithelial cells. α -SMA appearance in tubular cells suggests a transformation of cell phenotype to one that is fibroblast-like^{51, 52}. The present study found that TGF- β 1 induced transformation of the cellular phenotype from tubular epithelial cells to fibroblast-like cells. Smad4 is an important molecule of the TGF- β /Smad signaling pathway and TGF- β 1 induced the upregulation of Smad4, which is a marker of this signaling pathway. EETs significantly attenuated this transformation as seen by changes in the cellular expression of E-cadherin, α -SMA and Smad4, suggesting a role for these CYP epoxygenase products in protection against renal disease and EMT in this model of DN.

Previous studies demonstrated that EETs can increase heparin-binding EGF release via activation of matrix metalloproteinases and activation of EGF receptor to protect cells from apoptosis⁵³. EETs have also been shown to be involved in PPAR γ activation in endothelial cells¹⁵. In the current study, we showed that EET treatment of cultured HK-2 tubular cells reversed TGF- β 1 induced EMT and modulated TGF- β 1/Smad signaling. The use of EGFR inhibitor AG1478 and PPAR- γ inhibitor GW9662 attenuated the renal protection effects of EETs, indicated that EETs may impart their protective roles against DN by modulating TGF- β 1 signaling.

Previous studies also found that application of epoxygenases inhibitor MSPPOH or CYP2J2-selective inhibitor C26 abolished EETs production as well as effects of EETs in hypoxic pulmonary vessel model⁵⁴ and hematologic malignant disease⁵⁵, respectively. Our previous study demonstrated that epoxygenase overexpression increases EETs production and also attenuated insulin resistance, and in contrast, downregulation of epoxygenase induced a reduction in EETs production and subsequently enhanced dyslipidemia and

insulin resistance. In this study, CYP2J2 overexpression had attenuated induction of diabetes by STZ as evidenced by lower glucose and HbA1c levels *in vivo*, suggesting a lessening of the severity of diabetes by EETs. Synthetic EETs reversed tubular epithelial mesenchymal transdifferentiation by TGF- β 1 *in vitro*, indicating the role of EETS of attenuating renal injury directly. Thus the renal protective effects of EETs may involve both a lessening of the severity of diabetes and direct attenuation of renal injury. In summary, our data demonstrated that CYP2J2-derived EETs significantly attenuate hyperglycemia, and protected the kidney from morphologic changes (glomerular sclerosis) in STZ-induced diabetic mice. CYP2J2 overexpression downregulated TGF- β 1 and attenuated downstream signaling events *in vivo* (in kidneys of diabetic mice) and synthetic EETs prevented tubular epithelial mesenchymal transdifferentiation by TGF- β 1 *in vitro* (in cultured kidney cells). These results lend strong support to the potential application of CYP2J2 and EETs as a novel therapeutic approach in the prophylaxis and treatment of DN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AG1478	N-(3-Chlorophenyl)-6,7-dimethoxy-4-quinazolinamine
AA	arachidonic acid
α-SMA	alpha-smooth muscle actin
CYP	Cytochrome P450
DHET	Dihydroxyeicosatrienoic acid
DN	diabetic nephropathy
DMEM/F12	Dulbecco's modified Eagle's and Ham F12 medium
EETs	epoxyeicosatrienoic acids
EGFR	epithelial growth factor receptor
GW9662	2-Chloro-5-nitro-N-phenylbenzamide
HK-2	human renal proximal tubular cells
PAS	Periodic Acid-Schiff
PPARγ	peroxisome proliferator-activated receptor gamma
PTEC	renal proximal tubular epithelial cells
Smad7	Sma- and Mad-related protein7
STZ	streptozotocin
TEMT	tubular epithelial-mesenchymal transdifferentiation
TGF-β1	transforming growth factor beta1
Tie2-CYP2J2	endothelial-specific CYP2J2 overexpression

WT

wild type

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Fig. 1.

Effect of CYP2J2 overexpression on renal expression of CYP2J2 and urinary excretion of 14,15-DHET. (A) Representative western blots of CYP2J2 expression in kidneys. (B) Panel B shows urinary levels of 14,15-DHET, the stable 14,15-EET metabolite in the four treatment groups. Data are expressed as mean $\pm$ SEM (n=8 per group); *P<0.05 vs. WT control mice; #P<0.05 vs. non-diabetic transgenic control mice.

Fig. 2.

Endothelial CYP2J2 overexpression attenuates changes in kidney morphology associated with diabetic nephropathy. Paraffin sections of kidney cortex from the four treatment groups were stained with hematoxylin/eosin (A), PAS (B), and an antibody to α -SMA (C). Stained sections are shown at 200X magnification. (D) PAS stained sections were evaluated for the severity of glomerular sclerosis according to the MIS system established by Raij *et al*²⁶. Data are expressed as mean $\pm$ SEM (n=8 per group); *P<0.05 vs. WT control mice; # P<0.05 vs. WT diabetic mice.

Fig. 3.

Effects of endothelial CYP2J2 overexpression on TGF- β signaling pathways in diabetic mice. Representative western blots and densitometric quantification of at least three repeats for each experiment for the effects of endothelial CYP2J2 overexpression on TGF- β /Smad signaling pathways in mouse kidney are shown. (A) TGF- β expression, (B) phosphorylated Smad3 expression, and (C) Smad7 expression. Blots were scanned, and relative expression levels were normalized to either total Smad3 or β -actin. Data are expressed as means $\pm$ SE (n=8 per group); *P<0.05 vs. WT control mice; #P<0.05 vs. WT diabetic mice.

Fig. 4. Morphologic changes in HK-2 cells treated with EETs. HK-2 cells were incubated for 72 hours with TGF- β 1 (10 ng/ml) with or without synthetic EETs (1 μ M each) as indicated. For some experiments, the EGFR inhibitor (AG1478) or PPAR γ inhibitor (GW9662) were added concurrently with EETs. Photographs were taken using a Nikon microscope (phase contrast); magnification 100X.

Fig. 5.

EETs attenuate markers of TEMT induced by TGF-β1 in HK-2 cells. Anti-α-SMA staining of HK-2 cells treated with TGF-β1 (10 ng/ml) with or without synthetic EETs (1 μM each) as indicated. Control HK-2 cells are α-SMA negative, while TGF-β1 leads to TEMT and increased α-SMA staining, an effect that is attenuated by EETs (α-SMA staining positive cells are noted by a brown colored cell). Results shown are representative of three independent experiments. Photographs were taken using a Nikon microscope (phase contrast); magnification 200X.

Fig. 6.

EETs attenuate expression of TEMT markers induced by TGF- β 1 in HK-2 cells. HK-2 cells were incubated for 72 hrs with or without EETs (1 μ M in vehicle 1; 100% DMSO) and TGF- β 1 (10 ng/ml in vehicle 2; 4mM HCl with 1% BSA) as indicated. Representative western blots for E-cadherin (A and D), α -SMA (B and E), and Smad4 (C and F) are shown.

Densitometric quantification of western blots stained with E-cadherin, α -SMA, and smad4 antibody are normalized to β -actin. Data are expressed as mean $\pm$ SEM (n=8 per group);

*P<0.05 vs. all controls; **P<0.05 vs. TGF- β 1; #P<0.05 vs. TGF- β 1 + EETs.

Table 1

Physiological parameters of WT and endothelial CYP2J2 overexpression mice in control and diabetic cohorts

Genotype	WT	Tie2-CYP2J2	WT-DN	Tie2-CYP2J2-DN
Body weight (g)	35.2±0.7	35.1±0.8	24.8±0.6 [*]	25.1±0.7 [#]
Kidney(g/kg body wt)	7.1±0.2	7.0±0.3	11.8±0.6 [*]	8.7±0.4 [#]
Blood glucose (mg/dl)	149.4±8.1	144.8±9.2	506.6±34.8 [*]	395.7±28.7 [#]
HbA _{1c}	4.4±0.6	4.3±0.5	8.9±0.9 [*]	6.4±0.7 [#]
Plasma creatinine (mg/dl)	0.13±0.01	0.12±0.01	0.38±0.05 [*]	0.24±0.03 [#]
BUN (mg/dl)	15.6±1.2	14.5±1.3	39.7±2.2 [*]	22.3±1.6 [#]
Urinary microalbumin (μg/24h)	14.1±0.7	13.6±0.8	76.5±1.8 [*]	43.6±1.2 [#]
Urinary osmolarity (mOsm/kg.H ₂ O)	1350±115	1340±120	670±65 [*]	1096±98 [#]
Systolic blood pressure (mmHg)	104±4	102±5	99±4	98±3

Values are mean ± SEM.

^{*} P<0.05 vs. normal mice,[#] P<0.05 vs. wide-type diabetic mice.