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Original Article

The expressions of pigment epithelium-derived factor and adipose triglyceride lipase in human kidney and correlation with renal lipid accumulation

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Abstract: As one of the most severe complications of diabetes worldwide, diabetic nephropathy (DN) is the leading cause of end stage kidney disease. Studies have shown that renal lipid accumulation plays an important role in the pathogenesis of diabetic nephropathy. In animal and cell culture models of diabetes, PEDF plays a protective role in the development and progression of DN, PEDF is thought to exert its biologic actions by binding to a cell surface receptor. As the first identified receptor for PEDF, ATGL was revealed to have specific binding affinity for PEDF and activate its enzymatic lipase property by binding PEDF. Here, our study is the first to show the co-localization of PEDF and ATGL in human kidney on protein and tissue levels. PEDF and ATGL were significantly lower in human diabetic kidney. Simultaneously renal lipid accumulation was increased as compared to healthy kidney. Therefore, we speculate that PEDF regulates lipid metabolism of kidney through ATGL, and PEDF-ATGL nexus may be a therapeutic target for diabetic nephropathy.

Keywords: PEDF, ATGL, renal lipid accumulation

Introduction

One critical feature about the 7th edition of the IDF Diabetes Atlas is that the data shows a continued increase in the prevalence of diabetes [1]. Today, we know that 415 million people (one out of eleven adults) have diabetes and every six seconds a person dies with diabetes. Diabetic nephropathy is a leading cause of end-stage renal disease, and accounts for disabilities and the high mortality rates in patients with diabetes [1]. The pathogenesis of DN involves multiple processes, including inflammation, angiogenesis, oxidative injury, and podocytes structural and functional abnormalities. Despite major efforts to understand the pathogenesis of DN, the disease still progresses in spite of current therapeutic measures, such as control of blood pressure and blood glucose levels and other life style changes. Angiotensin converting enzyme (ACE) inhibitors (ACEi) are recognized as the standard care in DN, however, this therapy does not result in full reversal or even fully prevent the deterioration of renal function [2]. Thus, it is important to identify new therapeutics that will prevent or slow down the development and progression of diabetic kidney disease.

Pigment epithelial-derived factor (PEDF) is a 50-kDa secreted glycoprotein that belongs to the non-inhibitory serpin [3], PEDF is widely expressed throughout the human body and has multiple biological activities. Accumulating evidence suggests that PEDF can delay the progress of diabetic nephropathy [4]. PEDF has been shown to inhibit the secretion of vascular endothelial growth factor (VEGF) [5] and pro-inflammatory factors such as ICAM-1, MCP-1, TNF-alpha [6]. PEDF suppress renal fibrosis via inhibition of TGF-β1 and connective tissue growth factor [7]. He et al [8] showed that PEDF protect against renal cell apoptosis and damage via PPAR-γ activation. And PEDF inhibits proximal tubular cell injury in early diabetic nephropathy by suppressing advanced glycation end products (AGEs)-receptor (RAGE) axis [9]. Over the past two decades, understanding

the signaling pathways and mechanisms responsible for the multiple activities of PEDF has been compromised by the elusive nature of the receptor.

Adipose triglyceride lipase (ATGL, also called patatin-like phospholipase domain-containing protein 2) is the first identified receptor for PEDF [10], which has been demonstrated to catalyze the initial step of triglyceride hydrolysis by its triglyceride lipase activity in both adipocytes and non-adipocytes [11]. ATGL was revealed to have specific binding affinity for PEDF and activate its enzymatic lipase property by binding PEDF [10]. Abnormal lipid metabolism, renal lipid accumulation and lipotoxicity are associated with the pathological features of glomerulopathy. However, the mechanisms by which lipid accumulation leads to the development or progression of this disease have not been fully elucidated. The renal cortex in Atgl (-/-) mice demonstrated marked accumulation of lipid droplets, including the glomeruli, in contrast, there was little lipid accumulation in the glomeruli of Atgl (+/+) mice [12]. Renal PEDF levels were significantly reduced in genetic models of type 1 and type 2 diabetes (Akita and db/db, respectively) [13]. Previous reports showed that PEDF regulated triglyceride content of hepatocytes through activating ATGL lipase property in vitro [14]. Whether PEDF plays a role in the regulation of renal lipid metabolism via ATGL and prevents the development of DN? It is our research purpose, here we investigated if PEDF and ATGL were expressed in human kidney tissue. Furthermore we studied, the renal lipid accumulation, the expression pattern of PEDF and ATGL in human normal kidney and diabetic kidney.

Material and methods

The study was approved by the Medical Ethical Committee of Renmin Hospital of Wuhan University. All subjects provided their informed consent. Kidney samples were obtained from non-affected parts of tumor nephrectomy biopsies of patients with DM (n = 20) as a test group or patients without DM (n = 10) as a control group from the Department of Urology of Renmin Hospital of Wuhan University.

Clinical and biochemical assessments about the diabetic patients and controls were collected from the patients' files. Demographic and clinical data including age, sex, duration of diabetes, HbA1c, blood pressure, ACE/ARB medication, lipid-lowering medication, serum creatinine, blood urea nitrogen, lipids (LDL-c, HDL-c, TGs), β2-microglobulin, Cystatin C were recorded. Blood pressure was measured twice with a Hawksley sphygmomanometer after 10 min of supine rest.

Kidney tissue was stored at -80°C for protein isolation or embedded in paraffin for periodic acid-schiff, double immunofluorescence staining, The other part kidney biopsy tissue was immediately fixed in Karnovsky's fixative and then 1% buffered osmium tetroxide (method that preserved lipids) for Oil red O staining.

Renal histopathology

Kidney tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and 3-um sections were cut. Sections were stained with periodic acid-Schiff (PAS) stain. Each slice in each group randomly selected at least three of 200 times to take pictures. Using Image-Pro Plus 6.0 software to select 5 complete glomeruli and measure the glomerular area. Upper panel shows the representative microphotographs. Lower panel shows the quantitative data.

Double immunofluorescence staining

Double-labeling immunofluorescence was performed to detect the ATGL and PEDF localization in kidney. Kidney tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and 10 um sections were cut. Paraffin sections were deparaffinized and then were antigen repaired in EDTA buffer. The sections were washed 3 times with PBS (Phosphate Buffered Saline) and blocked for 30 min in hydrogen peroxide solution. Nonspecific binding sites on the samples were blocked for 20 min with 5% BSA. The polyclonal antibodies PEDF (1:150 dilution; rabbit, Abcam, ab180711) and ATGL (1:50 dilution; mouse, Santa, sc-365278) were applied to tissue and incubated overnight at 4°C. After washing, Cy3-conjugated goat anti-mouse or FITC-conjugated goat anti-rabbit IgG diluted 1:50 in 5% BSA/PBS was used as a secondary antibody to incubate at 37°C for 50 min respectively. The nuclei were counterstained with 50 ul DAPI (4,6-diamidino-2-phenylindole) for 5 min, then samples were mounted on glass slides using slowfade gold anti-fade reagent

Table 1. Clinical and biochemical characteristics of patients with DM and normal controls evaluated in this study

Baseline Parameters	Normal kidney (n = 10)	Diabetic kidney (n = 20)	*P* Value
Sex (male, %)	60	65	0.797
Age, y	56.3 ± 11.84	61.3 ± 10.84	0.258
DM duration, y	0	5.31 ± 3.64	< .001
HbA1c, %	5.31 ± 0.30	8.85 ± 1.95	< .001
SBP, mm Hg	140.80 ± 13.73	131.20 ± 15.55	0.109
DBP, mm Hg	82.20 ± 6.70	83.90 ± 8.62	0.590
HT, %	30	25	0.780
Anti-hypertensive drug, %	66.67	60	0.875
ACEI/ARB, %	66.67	40	0.537
Total cholesterol, mmol/L	4.35 ± 0.71	4.20 ± 0.67	0.590
TG, mmol/L	1.53 ± 0.58	1.35 ± 0.61	0.439
HDL-C, mmol/L	1.05 ± 0.28	1.11 ± 0.29	0.606
LDL-C, mmol/L	2.28 ± 0.66	2.59 ± 0.50	0.160
Lipid-lowering drug, %	20	25	0.770
Serum creatinine, mol/L	79.00 ± 24.29	82.85 ± 28.72	0.719
Serum urea nitrogen	5.08 ± 1.20	6.73 ± 2.59	0.068
Cystatin C, mg/L	1.20 ± 0.24	1.27 ± 0.28	0.470
β2-microglobulin, mg/L	2.43 ± 0.48	3.08 ± 1.04	0.074

Values are means ± SD, TG = triglyceride, HDL-c = high-density lipoprotein cholesterol, TC = total cholesterol, LDL-c = low-density lipoprotein cholesterol.

(Aspen Biological in Wuhan, AS1089, CA). Fluorescence labeling was evaluated via confocal microscopy (Micro Publisher; Q-IMAGING).

Western blotting

Kidney tissues were homogenized in lysis solution (0.1% Triton X-100) supplemented with protease inhibitor cocktail tablets (Wuhan goodbio technology). BCA protein assay (Wuhan goodbio technology) was used to determine protein concentration, 5 mg of kidney lysates were separated on a 10% SDS-PAGE and the separated proteins transferred onto a polyvinylidene fluoride membrane (millipore). After blocking using 5% non-fat dry milk, membranes were incubated with primary antibody overnight at 4°C, followed by incubation with appropriate secondary antibody for 1 hour at room temperature. Membranes were probed with primary antibodies to PEDF (1:1000, Snata, Mouse, Sc-390172) or ATGL (1:1000, Snata, Mouse, Sc-365278) or ACTIN (1:1000, Servicebio, Mouse, GB12001). The secondary antibody used for detection of ATGL and PEDF was anti-goat IgG-HRP (1:3000, Wuhan goodbio technology, goat, GB23301). Membranes were developed using enhanced chemiluminescence solutions (Wuhan goodbio technology) followed by exposure to X-ray film and densitometry performed using Alpha.

Lipid staining

To localize the renal accumulation of neutral fats, 5 um-thick kidney frozen sections were stained with oil red-O. Kidney frozen sections were fixed with 4% paraformaldehyde for 15 min, and stained for 10 min in the Oil Red O working solution (Wuhan goodbio technology, G1016), then differentiated for 1 min in 75% alcohol and returned to distilled water. The slides were counterstained with hematoxylin for 5 min and washed three times. The samples were mounted on glycerine gelatin. Representative photomicrographs were captured at 200× magnification using a system incorporated in the BX51 microscope (Olympus, Japan).

Statistical analysis

Values are shown as mean ± SD unless otherwise specified. Statistical analysis was performed using two-tailed Student's t-test for independent data. Pearson's correlations were calculated using SPSS software. $P < 0.05$ was considered significant.

Results

The general characteristics and clinical parameters of the cross-sectional study are summarized in **Table 1**. Compared with the control group, type 2 diabetic patients had higher levels of hemoglobin A1c (HbA1c). There was no significant difference with respect to sex, age, blood pressure, blood lipids and kidney function factors (include serum creatinine, serum urea nitrogen, Cystatin C, β2-microglobulin).

Histological assessment

The diabetic kidney tissue (**Figure 1B**) develop glomerulosclerosis, tubulointerstitial fibrosis.

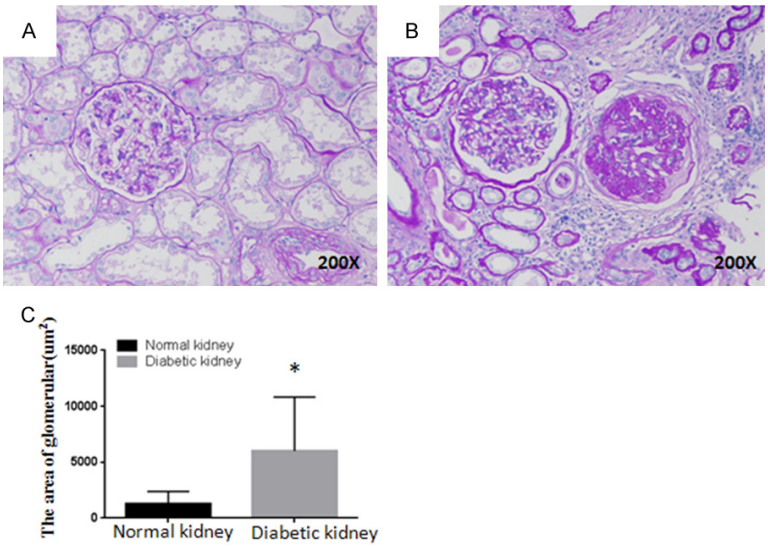

Figure 1. Sections were stained with Periodic Acid-Schiff (PAS), all glomeruli of (A and B) were graded individually at 200× magnification. Normal kidney tissue (A) has normal amounts of delicate mesangium supporting capillaries. In diabetic kidney tissue (B), PAS-positive matrix reveals lots of glycogen deposition, mesangial expansion, tubulointerstitial fibrosis. (C) Shows the quantitative data. *P < 0.05 vs. normal kidney.

Periodic acid Schiff staining clearly reveals mesangial expansion, and tubulointerstitial fibrosis when compared with age-and sex matched control kidney tissue (**Figure 1A** and **1B**).

PEDF co-localized with ATGL in human kidney

The precise localization of PEDF and ATGL in the kidney is not clear. To determine the localization of endogenous PEDF and ATGL in human kidney, kidney sections were determined using confocal microscopy imaging. Using a PEDF-specific antibody and ATGL-specific antibody, we identified PEDF is accumulated predominantly in the renal tubules and to a small extent in renal glomeruli (as shown in **Figure 2B**, **2F**). The expression of ATGL was evident in the renal tubules. As shown in **Figure 2D** and **2H**, the co-localization of PEDF and ATGL were widely appeared in cortical tubules, and the co-localization of PEDF and ATGL in diabetic kidney were obviously decreased compared with in normal kidney.

Decreased expression of PEDF and ATGL in the kidney of human diabetic

To determine if the PEDF and ATGL expression change is implicated in diabetic, we compared the PEDF and ATGL levels in the kidney of patients diagnosed with T2DM and age-matched control human kidney. Western blot analysis demonstrated that the PEDF and ATGL protein levels were significantly decreased in diabetic kidney, when compared with that in the non-diabetic control human kidney (**Figure 3**). The protein expression levels of PEDF and ATGL decreased simultaneously.

Lipid accumulation on diabetic kidney

Lipid storage droplets, analyzed by oil red-O staining, were rarely observed in control kidneys. In contrast, diabetic kidneys had abundant lipid storage droplets in the cortical tubules (**Figure 4B**). The lipid droplets were diffusely associated with cortical tubules (**Figure 4C**) and were less identified in the glomeruli and medulla (data not shown). The abundance of lipid storage droplets represented a major difference in the renal phenotype of diabetic and non-diabetic kidneys.

Discussion

In summary, the present study demonstrated for the first time that the co-localization of PEDF and ATGL are implicated in human kidney on protein and tissue levels. PEDF and ATGL were significantly lower in human diabetic kidney. These results are mostly consistent with the findings in animal models of diabetic kidney. Simultaneously, we showed evidence for lipid accumulation in human diabetic kidneys. Therefore, we hypothesized that lipid accumulation was significantly correlated with PEDF and ATGL expression in diabetic kidneys.

There is growing evidence that abnormal lipid metabolism and renal lipid accumulation play an important role in the pathogenesis of diabetic nephropathy. Evidence suggesting that renal lipid accumulation and lipotoxicity may lead to kidney dysfunction has mounted significantly in recent years [15, 16]. The most pronounced accumulation of TG (910-fold) occurred in the kidney compared with other non-

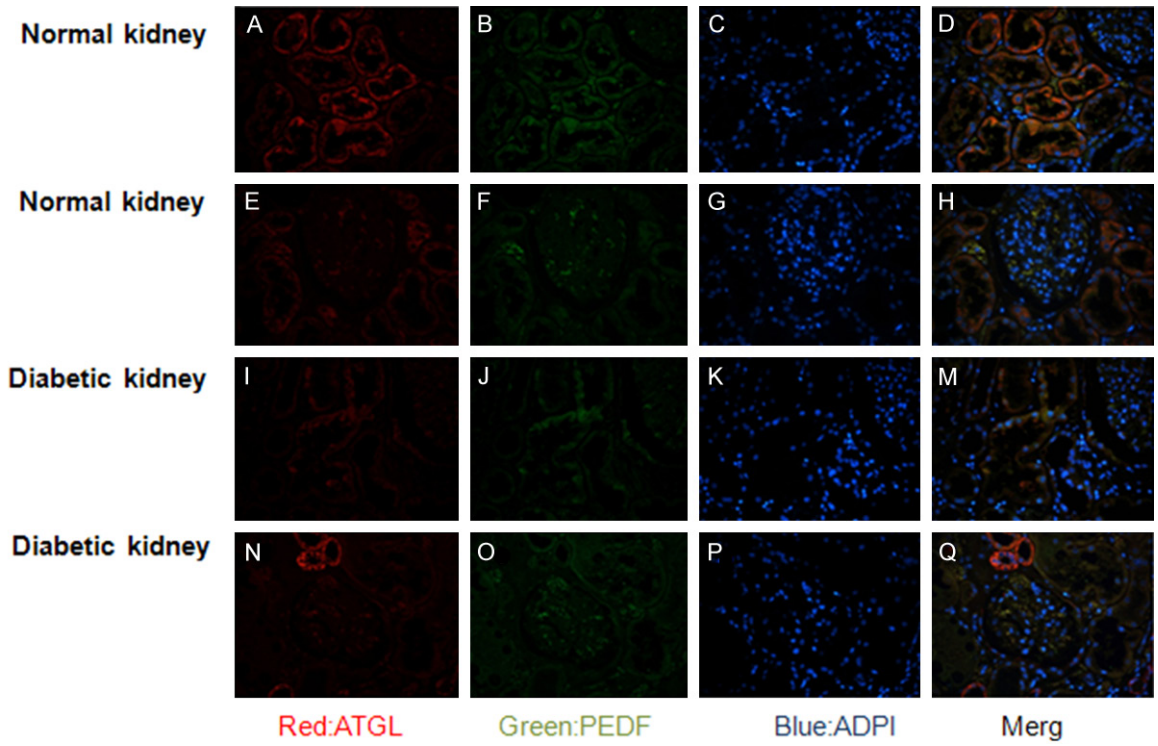

Figure 2. Immunofluorescence staining for PEDF and ATGL on a representative section in normal human kidney and diabetic kidney (200×); (A, I) ATGL is expressed in the renal tubules; (B, J) PEDF is expressed in the renal tubules; (C, G, K and P) are the nucleus of all cells; (D, M) Co-localization of PEDF and ATGL in renal tubules; (E, N) ATGL is expressed in the renal glomeruli; (F, O) PEDF is expressed in the renal glomeruli; (H, Q) Co-localization of PEDF and ATGL in renal glomeruli.

adipose tissues [17]. In this study, histological analysis of Oil red staining showed that the accumulating of lipid droplets increased in human diabetic kidney, which is consistent with the studies that renal TG and cholesterol metabolism results in lipid accumulation were altered in human diabetic nephropathy [16]. Ectopic lipid accumulation in the liver, heart, and pancreas is associated with lipotoxicity, inflammation, and fibrosis. Lipid accumulation in the kidney tissue may causes endothelial, vascular dysfunction and increase oxidative stress, insulin resistance eventually leading to renal dysfunction [18]. Schoenborn et al observed an association of two rare polymorphisms of the ATGL gene with glucose levels and type 2 diabetes, which altogether suggests an important role of this gene in the pathways of the metabolic syndrome [19]. Chen et al suggested that ATGL deficiency induced renal lipid accumulation, proteinuria and glomerular filtration barrier dysfunction [12]. Whether ATGL plays a role in the regulation of renal lipid metabolism and the development of DN has not been determined. In our present study, the ATGL protein level was reduced, while lipid accumulation of diabetic kidney was increased. We hypothesized that ATGL may mediate kidney lipid metabolism.

Pigment epithelium-derived factor (PEDF) is a multifunctional, pleiotropic secretory glycoprotein with anti-angiogenic, anti-oxidative, anti-inflammatory, anti-fibrotic properties. PEDF can protect against diabetic nephropathy in animal models. PEDF may be consumed in diabetic kidney to counteract the angiogenic and inflammatory responses of the endothelial cell, which would then lead to lower levels. Our studies also confirmed that the expression of PEDF was decreased in human diabetic kidney, which is similar to the results of another report, they demonstrated for the first time that the expression of PEDF was decreased at both the mRNA and protein levels in the kidney of diabetic rats [20]. A decreased PEDF expression in diabetic kidneys may contribute to the overproduction of extracellular matrix and the development of

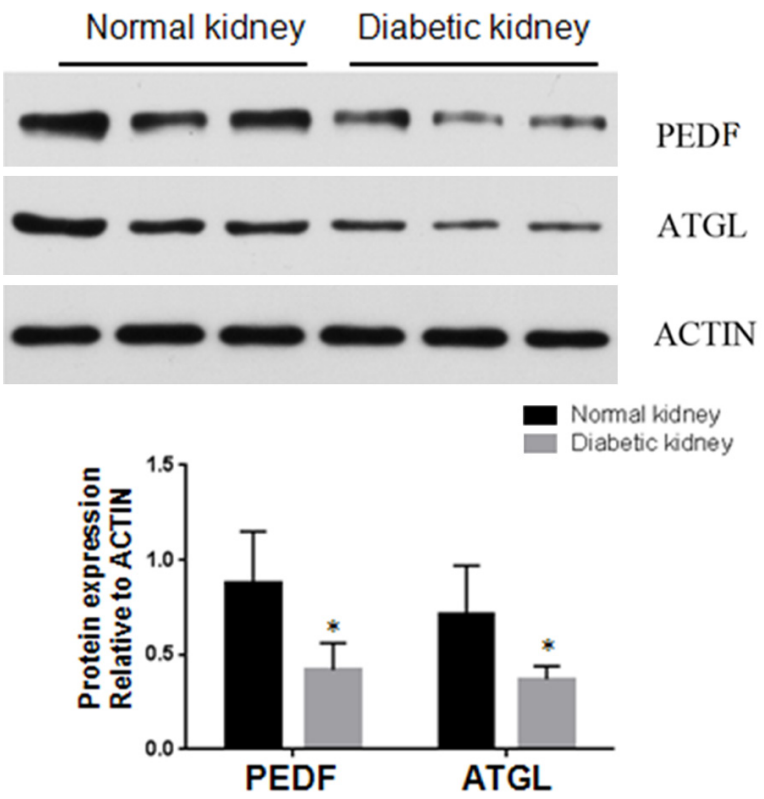

Figure 3. The Renal protein expression of PEDF and ATGL in the diabetic kidney and the normal kidney. *P < 0.05 vs. normal kidney.

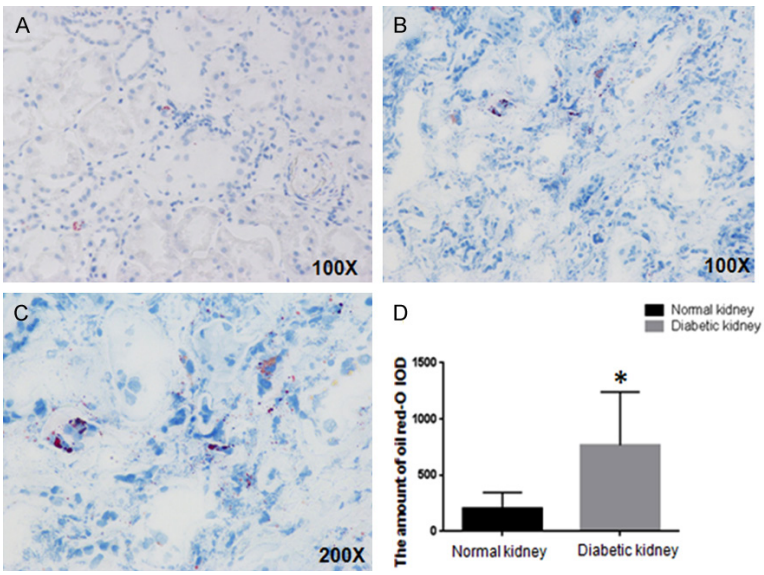

Figure 4. Lipid storage droplets accumulate in cortical tubules of human kidneys. A: Representative photomicrograph of oil red-O-positive lipid droplets in normal kidney tissue showed their small size and rare occurrence. B: Abundant larger droplets were observed in the cortical tubules of human diabetes kidneys. C: Magnified image of lipid storage droplets in human diabetic kidney illustrated their diffuse cytoplasmic localization. D: Each picture in each group randomly selected at least three 200× magnification. Image-Pro Plus measured the oil-red IOD value of the picture, *P < 0.05 vs. normal kidney.

DN. The latest research also showed that renal PEDF levels were significantly reduced in genetic models of type 1 and type 2 diabetes (Akita and db/db, respectively) [13] and PEDF plays a protective role in the development and progression of diabetic nephropathy [21]. In diabetic kidney tissue, PEDF levels were decreased, however circulating PEDF levels were increased [22]. In our study, the protein levels of ATGL and PEDF were simultaneously reduced in diabetic kidney compared with control kidney. But there were contradictions between our findings and the results published by Dai et al, who showed that PEDF attenuated ATGL protein accumulation via proteasome-mediated degradation in adipocytes and the negative correlation between PEDF and ATGL expression in adipocytes cannot be ignored [23]. Different tissue used in our and theirs studies may be a possible reason for the disparity.

As one of the receptors for PEDF, ATGL was a critical mediator of triacylglycerol lipolysis in multiple tissues. Immunoprecipitation assay was also performed to evaluate the binding between endogenous PEDF and ATGL in three immortalized cell lines [24]. The identification of ATGL as a putative receptor broadens the spectrum of potential PEDF targets. Previous studies have shown that PEDF and ATGL co-localized in hepatocytes and cardiomyocytes using double-labeling immunofluorescence [24, 25]. Chung C, et al showed that hepatocyte triglyceride metabolism was dependent on interactions between PEDF and ATGL, and the loss of PEDF would impair mobilization of triglycerides in the

liver [14]. These findings led this group to conclude, PEDF-ATGL nexus may be important in modulation of lipid metabolism by PEDF. Our study was the first to show co-localization of PEDF with ATGL in human kidney, which supported the hypothesis that PEDF may regulate the lipid accumulation through ATGL in the diabetic kidney. Studies showed that PEDF and 44 mer promoted TG degradation in cardiomyocytes after AMI via ATGL [25]. PEDF also regulated hepatic fat accumulation and loss of PEDF would impair mobilization of triglycerides in the liver [24]. We showed that PEDF and ATGL protein levels were significantly decreased in human diabetic kidney, while the renal lipid accumulation was increased. These findings showed the importance of PEDF-ATGL interaction in regulating the TG content of the diabetic kidney. The design and sample size of present study did not enable establishing a causal relationship among reduced PEDF, ATGL, and lipid accumulation in diabetic nephropathy. Further animal and cell experiments are needed to explore the mechanisms.

Our findings indicated that PEDF-ATGL interaction may play an important role in preventing lipid accumulation in the diabetic kidney. A recent study showed that PEDF treatment dependent on ATGL inhibited hepatic fat accumulation, oxidative stress and hepatic fibrosis in MCD-Fed Mice [26]. Further elucidation of the signaling mechanisms involved in PEDF-ATGL nexus are important for understanding the clinical association of PEDF deficiency and diabetic nephropathy, but also for developing PEDF and ATGL derivatives that can prevent renal lipid accumulation, inflammation, fibrosis of diabetic nephropathy.

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Disclosure of conflict of interest

None.

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