# 東海大學生命科學系
# 博士論文

指導教授：謝明麗教授
Mingli Hsieh, Ph.D

## 分泌性 Cyclophilin A 於糖尿病腎病變之角色
## The role of secreted cyclophilin A in diabetic nephropathy

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中華民國一○五年十二月三十日

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# 東海大學生命科學系
# 博士論文學位考試審定書

生命科學系博士班研究生 蔡尚峰 君所撰寫之論文

（中文）

分泌型 Cyclophilin A 於糖尿病腎病變的角色

（英文）

The role of secreted Cyclophilin A in diabetic nephropathy

經本委員會審定通過，特此證明。

學位考試委員會　　召集人　　（簽名）

委　員

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**List of abbreviations**

8OHdG, 8-hydroxy-2'-deoxyguanosine

ACEi, Angiotensin converting enzyme inhibitor

ACR, albumin-creatinine ratio

AGE, advanced glycation end product

ARB, Angiotensin II Receptor Blocker

AUC, area under the curve

CAD, coronary artery disease

CKD, chronic kidney disease

Ccr, creatinine clearance rate

Cr, creatinine

CsA, cyclosporin A

CVD, cardiovascular disease

CypA, Cyclophilin A

DM, diabetes mellitus

DMEM, Dulbecco's Modified Eagle's medium

DMSO, Dimethyl Sulfoxide

DN, diabetic nephropathy

DPP4i, dipeptidyl peptidase 4 inhibitor

ELISA, enzyme linked immunosorbent assay

EMT, epithelial-mesenchymal transition

ESRD, end-stage renal disease

FBS, fetal bovine serum

GFR, glomerular filtration rate

GLP-1, glucagon-like peptide 1

HE stain, Hematoxylin and Eosin stain

IHC stain, immunohistochemical stain

IL, interleukin

JNC-7, Joint National Committee-7

L-FABP, liver-type fatty acid binding protein

MAPK, mitogen-activated protein kinase

MDRD, Modification of Diet in Renal Disease

PAS stain, Periodic acid – Schiff stain

PBS, phosphate buffered saline

PTEC, proximal tubule epithelial cell

ROC, receiver operating characteristic curve

ROS, reactive oxygen species

SAVOR, Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus

SCr, serum creatinine

sCypA, secreted cyclophilin A

SD, standard deviation

SEM, standard error of mean

TGFβ, transforming growth factor β

**摘要**

第二型糖尿病是最常見造成末期腎病變的原因，即使積極的用藥治療，很多病人仍然會進入末期腎病變而需要透析，因此，找尋更早診斷糖尿病腎病變的指標和治療方式就相當重要。Cyclophilin A (CypA)為 18-KD 的蛋白質，而其分泌型 (secreted CypA)已被了解與心血管疾病有關係：ERK1/2, Akt 以及 JAK 路徑。但糖尿病腎病變與 sCypA 的關係至今還沒有人探討過，我們想了解 sCypA 與糖尿病腎病變嚴重度的關係與可能的訊息傳導路徑。Dipeptidyl peptidase 4 inhibitors (DPP-4i)為口服降血糖藥，近幾年來被報導會有獨立於降血糖外保護腎臟的效果，我們也想了解此保護腎臟的效果機轉與 sCypA 是否有關係。

首先，我們先篩選五個不同嚴重度糖尿病腎病變的病人（各 20 人）以及正常腎功能的人，測定尿液 CypA 的量。相較於第一期，在第二期糖尿病腎病變時，可以偵測到尿液中的 CypA 的量有統計意義的上升(p=0.012)，此上升亦隨著疾病嚴重度而上升。因此，尿液中的 CypA 的量可以成為糖尿病腎病變嚴重度的指標，具有高敏度性(90.0%)與特異性(72.7%)。

CypA 既然可成為糖尿病腎病變的嚴重度指標，是否具有病生理上的角色？在 *db/db* 小鼠的模式上，尿液中的 CypA 於八週時亦可以

偵測到有意義的上升，而 Linagliptin 可以抑制在第 8 與 16 週抑制尿液中 8-hydroxy-2' –deoxyguanosine (8OHdG)的上升，但已無法抑制第 16 週 CypA 的上升。與基礎實驗中最佳的指標 8OHdG 相比 ，對於糖尿病腎病變，CypA 是更強、更早以及更敏感的指標。而在細胞模式上，氧化壓力與高糖刺激間膈細胞（MES-13 cell）與近端腎小管上皮細胞（HK-2 cell）下，細胞會分泌 CypA。高糖刺激 HK-2 細胞時，細胞會先分泌 TGFβ1，TGFβ1 會再刺激 HK-2 細胞分泌 CypA，分泌出來的 CypA，會刺激其胞內的受體 CD147 由細胞質往細胞膜移動，繼而影響胞內的 p38 MAPK 的上升。

根據人類、動物與細胞實驗，我們證實 CypA 除了是糖尿病腎病變嚴重度的指標，而且也與糖尿病腎病變病的致病機轉有關。而 Linagliptin 的護腎作用，也與此訊息傳導路徑有關。

關鍵字：分泌型 cyclophilin A，糖尿病腎病變，Linagliptin

**Abstract**

Type 2 diabetes mellitus (DM) is the most common single cause of end-stage renal disease (ESRD). In spite of many modern therapies of glycemic and blood pressure control for DM, many patients continue to experience progressive renal damage. Thus, researches involving new markers and a more detailed molecular pathway of DN are beneficial to treatment. Cyclophilin A (CypA) is an 18-kD protein and the secreted form of CypA (sCypA) was reported to correlate with cardiovascular diseases (CVD). Patients with CVD have more secretion of CypA, which stimulate the ERK1/2, Akt and JAK pathway. The connection between DN and sCypA has never been elucidated before this study, which aims to investigate sCypA's correlation with renal dysfunction and the associated molecular pathway. In addition, we examined closely at the renal protection effects of Linagliptin (BI-1356, Trajenta), a dipeptidyl peptidase 4 inhibitors (DPP-4i), and determined potential association between Linagliptin and sCypA.

In the human study, a total of 100 DN patients and 20 healthy control subjects were enrolled. The concentration of urinary CypA correlated well with the progression of renal function and a significant increase in urinary CypA was noted in stage 2 DN ($p=0.012$) and persisted in later stages. We could diagnose stage 2 DN using urinary CypA with a sensitivity of 90.0% and specificity of 72.7%. The area under curve was

up to 0.85, indicating a good discriminatory power. Thus, urinary CypA is a good marker for DN.

The mouse model exhibited a higher concentration of urinary sCypA and8-hydroxy-2' –deoxyguanosine (8OHdG) in *db/db* groups, both substances were detected as early as the 8th week up to the 16th week. Our results indicated that the sCypA is a better, stronger and more sensitive indicator for DN than 8OHdG. In the cell models, Hyperglycemia and oxidative stress both can stimulate mesangial cell (MES-13) and proximal tubular epithelial cells (HK-2) to secret CypA. Hyperglycemia stimulated HK-2 cells to secrete TGFβ1, which caused secretion of CypA. The sCypA further stimulated CD 147 to move outward from cytosol onto cell membrane in confocal microscopy, which was associated with the p38 MAPK pathway in the downstream.

According to human, mouse and cellular studies, we proved CypA is a good marker for DN. Secreted CypA is also associated with the pathogenesis of DN. Besides, renal protection of Linaglitpin is associated with this pathway.

Keywords: secreted cyclophilin A, diabetic nephropathy, Linagliptin

## 1. Background and introduction

Type 2 diabetes mellitus (DM) is the most common single cause of end-stage renal disease (ESRD)[1]. Almost half of the patients with ESRD are due to diabetic neprhopathy (DN). They suffer risks to cardiovascular disease (CVD), mineral bone disease, coagulopathy and immunocompromised conditions. Patient survival seen in diabetics undergoing dialysis is much lower than that in nondiabetic patients[2]. In addition, Taiwan has the world's highest ESRD prevalence by presenting with 68940 patients undergoing dialysis in 2010[3]. Such has become an enormous financial burden on the country[4] . In a 1990s study, DN developed in up to 50 percent of diabetic Pima Indians within 20 years, and 15 percent progressed to ESRD by this time[5]. In spite of many modern therapies of glycemic and blood pressure control for DM, many patients go on to experience progressive renal damage[6]. Since kidney injury by DM is inevitable, researches involving new markers and a more detailed molecular pathway of DN should not be delayed.

Currently, the stage of severity is determined according to the levels of albuminuria. Albuminuria is the most commonly used marker to predict onset and progression of DN clinically. However, this traditional marker for DN lacks both sensitivity and specificity to detect early stage of DN[7]. Furthermore, some DN patients with ESRD do not present with

significant albuminuria[8-10]. The lack of a strong association between glomerular filtration rate (GFR) and albuminuria suggests that an alternative to this albuminuria-based staging system is needed. Some studies have noted the existence of pathological change before microalbuminuria[8]. Therefore, even if microalbuminuria can be regarded as the earliest manifestation of DN, it is possible that a new biomarker for DN exists. Recently, different markers of DN were reviewed[8, 11, 12] including fibroblast growth factor 23[13], tubular markers[14] (kidney injury molecule1, neutrophil gelatinase-associated lipocalin, liver-type fatty acid-binding protein (L-FABP)[15]), inflammatory markers (interleukin 6 (IL-6), IL-8, monocyte chemoattractant protein 1, and interferon $\gamma$ –inducible protein )[16], urinary 8-hydroxy-20-deoxyguanosine[17], serum cystatin C[18], and so on. Among these, genetic susceptibility almost always leads to irreversible DN and detection of the clinical markers mostly occurs too late to diagnose and monitor the progression of DN. As such, it is crucial to find an earlier and reliable marker for DN. Earlier diagnosis and intervention may provide an opportunity to stop the permanent damage caused by DN.

Cyclophilin A (CypA) is an 18-kD protein with a highly conserved and ubiquitous character[19]. It is mostly distributed in the cytoplasm and is

capable of peptidyl-prolyl cis-trans isomerase activity. It facilitates protein folding and protein trafficking and acts as a cellular receptor for cyclosporine A (CsA). The expression of CypA is at relatively high level in the kidney[20], where proximal tubular epithelial cells are reported to contain distinctly more of CypA than the others[21]. For kidney diseases, the majority of the researches has been focusing on the cellular relationship between CypA and CsA, which was used as an immunosuppressant, and leaving behind its secreted form. Such secreted form of CypA (sCypA) was reported to correlate with CVD, asthma, rheumatoid arthritis, lung and liver injury[22]. Until now, sCypA has been thought to be a potential biomarker and mediator in CVD including vascular stenosis, atherosclerosis, and abdominal aortic aneurysms[23]. This possible character was shown by understanding how the reactive oxidative stress (ROS) in vascular smooth muscle cell could activate a pathway containing vesicles secretion of CypA, which stimulate the ERK1/2, Akt and JAK pathway[23].

The connection between DN and sCypA has never been elucidated before this study. Firstly, sCypA is associated with many inflammatory or infectious diseases such as rheumatoid arthritis, asthma and, periodontitis[24]. Since sCypA can be detected in diabetic patients' plasma, and is secreted by monocytes in response to hyperglycemia[25],

it could be a potential secretory marker of inflammation in type 2 DM[25]. Secondly, it is also reported that sCypA are related to solid organ diseases including CVD, acetaminophen-induced liver injury, and lung injury[24]. Hence, a relative high level of CypA expression in normal kidneys[20] leads to the rational that if an even higher level of sCypA existed in diabetic patients' kidneys and may play a pathogenic role. Being the direct product from kidney, urine content could be the best candidate when it comes to renal injury detection. Therefore, we believe that the sCypA level in urine is the most suitable indication for renal damage. Thirdly, in the microscopic level, there are three major histological changes in the glomeruli in DN: mesangial expansion, glomerular basement membrane thickening, and glomerular sclerosis[26]. The state of hyperglycemia stimulates mesangial cell matrix production[27] and mesangial cell apoptosis[28]. Hence, our study focuses on mesangial or other changes in relation to sCypA level. Finally, sCypA involving in the pathway of ERK/JNK has already been mentioned in CVD[23]. This pathway has also been found in mesangial cells in glomeruli under DN although it is associated with angiotensinogen mostly[29]. In the molecular level, our study looks at the extent to which sCypA is involved in the ERK/JNK pathways in DN, which is presented in both the cardiovascular and renal disease. In summary, we hypothesize that sCypA might be an important and novel maker for DN involved in mesangial cell

and it could also be an early target for the treatment of DN. If we can prove that the CypA is a good marker for DN, we will also find out whether sCypA is also a “maker” of DN or not.

Glucagon-like peptide-1 (GLP-1) including the dipeptidyl peptidase 4 inhibitors (DPP-4i) is a relatively new drug for hyperglycemia. GLP-1 acts as a gut incretin hormone as well as an effective therapeutic agent for type 2 DM. It is best known for its varies abilities such as cell proliferation, insulin secretion in a glucose-dependent manner, inhibition of glucagon, satiation, and gastric emptying delays, which together result in reduced circulating glucose[30]. GLP-1-based therapies also benefit patients with renal protection independent from glucose-lowering effects[31]. There are growing studies discussing the renal protection of DPP4i[32-35] but the mechanism is still unclear. The study of the saxagliptin assessment of vascular outcomes recorded in patients with diabetes mellitus-thrombolysis in myocardial infarction (SAVOR-TIMI) 53 is the largest prospective study of DPP4i up to now (16,500 patients with Type 2 DM)[34]. This study presents an improved ACR at 2 years (372 patients [11.1%] in the saxagliptin group vs. 295 patients [9.2%] in the placebo group), and is less likely to have a worsening ratio (414 patients [12.4%] in the saxagliptin group vs. 457 patients [14.2%] in the placebo group). As mentioned earlier that the renal protection is

independent from glucose lowering effect, Linagliptin (BI-1356, Trajenta) can lower albuminuria on top of recommended standard treatment in patients with type 2 DM and renal dysfunction[35]. It can reduce renal events by 16% (composite of 6 renal outcomes)[36]. Some other preclinical studies revealed the possible mechanism of the renal protection of DPP4i[32, 37, 38]. In our study, we also want to observe closely at the mechanism of the renal protection in Linagliptin and investigate the potential association between Linagliptin and sCypA.

**Specific aims**

In this thesis, we illustrate the followings:

I. The correlation between sCypA and renal dysfunction by examining urine from patients with type 2 DM and DN
II. The relationship between urinary CypA and the severity of diabetic nephropathy is diabetic mice.
III. The hyperglycemia and sCypA in mesangial and proximal tubular epithelial cells
IV. The molecular mechanism underlying the effects of sCypA on renal cells
V. The correlation of sCypA and protective function of Linagliptin

## 2. Materials and methods

### 2.1 Human studies

#### 2.1.1 Study Population

We recruited all the DM outpatients and healthy control groups with informed consent. In the group of DM patients, the different stages of DN were screened for the concentrations of urinary CypA. All subjects in this cross-sectional study were 20 years of age and older. Patients were free from infectious disease, inflammatory disease, liver disease, or malignancy, and all were non-smokers. Only metabolic syndrome and/or CVD were noted. Patients who took drugs for hypertension, DM, hyperlipidemia, hyperuricemia, CVD, hyperuricemia and gout were not excluded. Patients who took drugs for any other disease or condition were excluded. These data were collected in the outpatient department of metabolism and nephrology at Taichung Veterans General Hospital between January of 2014 and December of 2014. All of the study procedures were conducted in accordance with the ethical standards of Taichung Veterans General Hospital and were approved by the institutional review committee (CE14077, TCVGH).

#### 2.1.2 Data Collection

All DM patients were diagnosed according to the DM guidelines of the American Diabetes Association in 2013[39]. We collected the

participants' clinical parameters including gender, age (years old), and duration after diagnosis of DM (years). The stages of DN were categorized according to the previous literature[40] where stage 1 is associated with hyperfiltration and a measured GFR exceeding the upper limit of the normal range (120 mL/min per 1.73 $m^2$) or beyond +2 standard deviation (SD) from mean GFR. Stage 2 DN develops silently over many years and is characterized by morphologic lesions without signs of clinical disease. Thus, it is usually called the silent stage. Stage 3 DN is characterized by "microalbuminuria" where urinary albumin excretion is between 30 and 300 mg/day or between 30 and 300 mg/g creatinine on a spot urine sample. Patients with normal GFR (no > 2SD of GFR) and without microalbuminuria were defined as stage 2 DN. More importantly, some patients with normal GFR (no > 2SD of GFR) and without microalbuminuria do not have DN. Patients included in our study should fit the above criteria and should have increased GFR (>2 SD of GFR) before timing of recruitment (progression of stage 1 DN) to make sure they really had DN and they were in the stage 2 of DN. Stage 4 DN is defined by severely increased albuminuria, also known as the "macroalbuminuria" (urinary albumin excretion above 300 mg/day or above 300 mg/g creatinine on a spot urine sample). The final stage, stage 5, is known as end-stage renal disease (ESRD). Blood samples were tested for fasting sugar (mg/dl), glycated hemoglobin (HbA1c) (%), SCr

(mg/dl), GFR (ml/min per 1.73 $m^2$)[41], total cholesterol (mg/dl), triglyceride (mg/dl), and low density lipoprotein cholesterol (mg/dl). Spot urine test was used to measure the concentration of CypA (ng/ml) and ACR (mg/g). The index estimated glomerular filtrate rate (eGFR) was calculated using the modification of diet in renal disease (MDRD) equation[41]: eGFR (ml/min per 1.73 $m^2$) = $186*SCr^{-1.154}$ $*Age^{-0.203}*0.742$ (if female). Patients were screened for CVD (hypertension, stroke, coronary artery disease, heart failure, and aortic aneurysm). Hypertension was defined as an average home systolic blood pressure greater than 140mmHg and a diastolic blood pressure greater than 90mmHg before medication according to the definition for stage I/II hypertension set forth in the JNC-7 guidelines[42]. Patients currently receiving anti-hypertensive agents were deemed to have hypertension. Stroke was confirmed by neurologists or brain images. Coronary artery disease (CAD) was defined according to arterial angiography. Some were diagnosed according to cardiologists, who made diagnosis of CAD according to if patients with typical angina pectoris, myocardial infarction, or silent myocardial ischemia. They used electrocardiogram, cardiac enzyme, coronary calcium score and stress test to diagnose CAD. Heart failure was confirmed by cardiac sonography or the guidelines of the Framingham Study[43]. Drugs such as angiotensin-converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARB), dipeptidyl

peptidase 4 inhibitor (DPP4i), sulfonylurea, metformin, dipyridamole, pentoxifylline, and statin were also recorded to analyze possible correlations with urinary CypA concentration. We would also like to point out how we select patients. All data including medication, laboratory and clinical parameters are without significant changes within 6 months or between two-time outpatient department visits. We checked all parameters during the period of recruitment. If they remain similar to the previous data, we include them in the study. If there are significant changes, we will follow up this patient 3 months later and choose the data (the ones after 3 months) if it became similar to the previous data.

### 2.1.3 Urine collection and analysis

Urine was collected in the morning from the outpatient subjects and stored in an ice package immediately. Within 4 hours, it was then restocked under -80℃ until analysis. The expression of urinary CypA was examined using an enzyme-linked immunosorbent assay (ELISA) kit (SEA979Hu, Uscn Life Science Inc.). All data of urinary CypA were double-checked at least twice.

### 2.1.4 Statistical Analyses

Data were expressed as the mean±SD in continuous variables.

Mann-Whitney U test was used for continuous variables and the *Chi* Square test was used for categorical variables. A general linear model was used for categorical variables and simple linear regression was used for continuous variables. The results from Western blot were expressed as mean ± SEM and were analyzed by Student's *t* test. All statistical procedures were performed using the SPSS statistical software package, version 17.0

## 2.2. Animal studies

### 2.2.1 Type 2 DM mouse

All experimental protocols were approved by a named Taichung Veterans general hospital and licensing committee (Affidavit of Approval of Animal Use Protocol in TVGH, La-1031172) and all experiments were performed in accordance with relevant guidelines and regulations. Four-week-old male C57BLKS/J *db/db* and *db/m* mice were purchased from National Applied Research Laboratories (Taiwan, R.O.C.); *db/m* mice were used as controls in all experiments. All four groups of mice (n=10, respectively) were maintained on a 12-h light/dark cycle and were fed with standard laboratory diet and water *ad libitum* in a room with constant temperature (23±3°C) and humidity (55±15%). All mice were age- and gender-matched. Group one (*db/m*) and group 2 (*db/db*) were control groups without any treatment. Group 3 and 4 were treated with

Linagliptin (5mg/tab, BI-1356, film-coated tablet), dissolved in water, 3 or 15 mg/kg per day. The medication and water for untreated groups were administrated by oral gavages. They were fed from the age of 4 weeks, and were sacrificed at the age of 20 weeks. After sacrifice, the weight of the right kidney was measured and further histological analysis of the kidney tissue was conducted.

### 2.2.2 Body weight, kidney weight, food intake, water intake and daily urine collection

We weighed all mice once a week. The blood was drawn by the scissors tail method, and blood sugar was measured by the rapid blood assay (blood glucose test strip by glucose meter). The amounts of daily food intake and water consumption were measured and daily urine collection was recorded from the metabolic cages every four weeks.

### 2.2.3 24-hour urinary sCypA and 8-hydroxy-2'-deoxyguanosine (8-OHdG)

The amount of daily urine was collected from the metabolic cage and we used the ELISA kit (SEA979Mu, USCN Life Science Inc.) for determining sCypA. The amount of daily urine and concentration of sCypA in urine were calculated as the daily sCypA excretion amount. Urine was stored in an ice package immediately. Within 4 hours, it was

then restocked under -80℃ until analysis. All data of urinary CypA were double-checked at least twice. To determine the oxidative DNA damage in the kidney, we determined 24-h urinary 8-OH-dG concentrations using the ELISA kit (8-OH-dG Check; Institute for the Control of Aging, Shizuoka, Japan).

### 2.2.4 Renal function evaluation: serum creatinine, urine creatinine, albuminuria, daily urine amount

Blood were collected from the left ventricle and centrifuged after sacrifice at the 20th week. The plasma was stored at -70°C for subsequent analyses. At the 20th week, the concentration of plasma and urinary creatinine were measured using the autoanalyzer (TBA™-120FR, Toshiba). Creatinine clearance (Ccr) was calculated by (urine [Cr] X urine volume)/(plasma [Cr] X time) at the 20th week. Albuminuria was measured by the mouse albumin ELISA kit (E-90AL, Immunology Consultants Laboratory, Inc.) at the 8th and 20th week. All results from ELISA test were confirmed by performing the test twice.

### 2.2.5 Histological analysis: Light Microscopic Study

We selected 10 glomeruli from each mouse and there were 100 glomeruli from 10 mice in each group. The right kidney of each mouse was obtained for histological analysis. Histology was assessed after

hematoxylin and eosin (HE) staining as well as the periodic acid-Schiff staining (PAS). To examine the effects of Linagliptin on glomerular area and mesangial matrix area, we performed glomerular analysis on PAS–stained kidney sections. Mesangial matrix area and glomerular tuft area were quantified for each glomerular cross-section as previously reported[44]. More than 30 glomeruli dissected through the vascular pole were counted in each kidney, and the average number was used for analysis. We selected 10 glomeruli from each mouse and there were 100 glomeruli from 10 mice in each group. Quantitative histomorphometry to determine glomerulosclerosis was performed using the computer-aided image analysis system Image J and rated as described previously[45]. In brief, glomerulosclerosis was defined by the presence of PAS-positive material within the glomeruli. The level of glomerulosclerosis was assessed using a semiquantitative scoring method; two investigators scored the results in a blinded fashion.

### 2.2.6 Histological analysis: Immunohistochemical (IHC) stain for CypA and TGF-β1

We performed IHC staining for CypA and TGF-β1 at the 20th week. Small blocks of kidney tissues were fixed in 10% buffered formalin for 24 hours before being embedded in paraffin. Five-micrometer thick sections were de-paraffinized, washed with PBS, and incubated with

1.5% $H_2O_2$ in methanol to block endogenous peroxidase activity. Nonspecific binding was blocked with 10% normal goat serum in PBS. Sections were incubated overnight with the CypA (1:1000) (Upstate, Charlottesville, VA), and TGF-β1 (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) in a humidified chamber at 4°C. Sections were treated with an antigen-unmasking solution that consists of 10 mM Na citrate (pH 6.0) and 0.05% Tween 20. Antibodies were localized with the ABC technique (Vector Laboratories, Burlingame, CA) and 3,3-diaminobenzidine substrate solution with nickel chloride enhancement. Sections were then dehydrated in ethanol, cleared in xylene, and mounted without counterstaining. All of these sections were examined in a masked manner using light microscopy (Olympus BX-50; Olympus Optical, Tokyo, Japan). For the quantification of proportional area of staining, approximately 20 views (400 magnifications) were randomly located in the renal cortex and the corticomedullary junction of each slide (Scion Image Beta 4.0.2, Frederick, MD). We selected 10 glomeruli from each mouse. There were a total of 100 glomeruli from 10 mice in each group. Quantitative histomorphometry was conducted to determine positive staining for CypA and TGFβ1. Both were assessed using the computer-aided image analysis system Image J. The level of positive staining was assessed using a semiquantitative scoring method; two investigators scored the results in a blinded fashion.

## 2.3 Cell studies

### 2.3.1 Cell culture

MES-13 cells (glomerular mesangial cells from an SV40 transgenic mouse) were obtained from American Type Culture Collection (CRL-1927; Manassas, VA, USA). MES-13 were cultured in a 3:1 mixture of M199 (Invitrogen, Carlsbad, CA, USA) and Ham F-12 (Invitrogen), supplemented with 5% FBS, 1% penicillin-streptomycin, 1% L-glutamine, and 14 mM HEPES and maintained in an incubator at 37℃with 5% CO2. All culturing supplies were acquired from Life Technologies (Gaithersburg, MD, USA). Subsequently, the cell lysate and secreted cellular proteins were collected for Western blot analysis.

HK-2 cells (human proximal tubular epithelial cells) were obtained from the laboratory of Taichung Veterans General Hospital. HK-2 cells were maintained in DMEM/F12 and supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/amphotericin B, 1% glutamine (Invitrogen, Carlsbad, CA), and 1% Insulin-Transferrin-Selenium (Sigma, St. Louis, MO). Western blot analyses were used to determine the levels of endo-CypA, sCypA, p38, phosphorylated p38 and TGFβ1.

### 2.3.2 Chemicals and reagents

#### 2.3.2.1 $H_2O_2$ treatment on MES-13 cells

MES-13 cells were seeded in a 6 cm cell culture plate with 3 x $10^7$ cells/plate and incubated in M199:F12=3:1 complete medium for one day, then were replaced by serum-free medium (M199:F12=3:1) and incubated for another 2 Days. After 30 min of 0, 20 μM or 40 μM $H_2O_2$ stimulation, the secreted and cellular proteins were collected for immunoblotting analysis. To confirm the role of $H_2O_2$ in sCypA upregulation, mesangial cells were treated for 30 min with 20, and 40μM $H_2O_2$ and 300 U $ml^{-1}$ catalase (Sigma, St Louis, MO, USA) at the same time as described previously[46]. Catalase is the antagonist to $H_2O_2$ and this procedure aims at identifying the role of sCypA while mesangial cells are under free radical.

**2.3.2.2 Glucose treatment on MES-13 cells**

To test the response of high glucose concentration, MES-13 cells were seeded in a 6 cm cell culture plate with 3 x $10^7$ cells/plate and were incubated in M199:F12=3:1 complete medium for one day. Then they were replaced by serum-free medium (M199/low glucose: F12=3:1) for 2 Days. The cells were incubated in the serum-free mediums which were then supplemented with 10, 25, and 50 mM of glucose. The procedure was referenced from previous study[46]. After 0, 24 and 48 hr of treatment, the secreted cellular proteins were collected for immunoblotting analysis. We also repeated the 25 mM glucose treatment

procedure with and without Linagliptin management (1, 10, and 100 nM). Then the secreted cellular proteins were collected for immunoblotting analysis. This test was used to determine whether Linagliptin would counteract the effect of glucose. For time dependent examination of Linagliptin on MES-13 cells, we seeded MES-13 cells in 5% FBS medium for one day, then shifted to 0% FBS medium for two days, and shifted again to 25mM glucose + 0.5% FBS medium. Both the experimental (Linagliptin) and the control group (DMSO) were set to rest for 8 and 24 hours and we collected the protein for Western blotting. In order to verify a time dependent factor of Linagliptin, we treated MES-13 cells by 25mM glucose, with and without Linagliptin for 8 and 12 hours, and then collected the protein for Western blotting.

**2.3.2.3 Linagliptin effect on MES-13 cells**

For the study of sCypA expression in glucose treated MES-13 and rescued by Linagliptin, the protocols were as follows. Linagliptin (5mg/tab, BI-1356, film-coated tablet) and a pure powder (WO2013098775 A1, 1-[(4-Methyl-quinazolin-2-yl)methyl]-3-methyl-7-(2-butyn-1-yl)-8-(3-(R) -amino-piperidin-1-yl) xanthine) were sponsored by Eli Lilly Company. The film-coated tablets of Linagliptin were dissolved in water for animal experiments and the pure powder form was dissolved in Dimethyl

sulfoxide (DMSO) for cell experiments. Firstly, MES-13 cells were seeded in a 6 cm cell culture plate with 3 x $10^7$ cells/plate and were incubated in M199:F12=3:1 complete medium for one day. Then culture media were replaced by serum-free media (M199/low glucose: F12=3:1) for 2-day glucose starvation. Then on day 4, the cells were incubated in the media with 25 mM glucose and 0.5% FBS as high glucose condition for 16 hours and the procedure was performed according to the protocol described in a previous study[46]. After that, DMSO (control group), 1 nM Linagliptin, 10 nM Linagliptin, or 100 nM Linagliptin were applied to the cells for 8 hours to determine the effect of Linagliptin. The secreted cellular proteins were collected for immunoblotting analysis. After the preliminary data showed that 10 nM Linagliptin can block the secretion of CypA. For the time-dependent examination of Linagliptin on MES-13 cells, we seeded MES-13 cells in 5% FBS medium for one day, then shifted to 0% FBS medium for two days, and shifted again to 25mM glucose + 0.5% FBS medium. Cells were then treated with DMSO (as control group), and 10 nM Linagliptin for 12 and 24 hours. After the above treatments, the cell lysates and secreted cellular proteins were collected for Western blotting analysis.

#### 2.3.2.4 Glucose treatment on HK-2 cells

HK-2 cells were seeded in a 6 cm cell culture plate with 3 x $10^5$

cells/plate and were incubated in DMEM/F-12 1:11 medium (10% FBS) for one day. Then the medium was replaced with 0% FBS low glucose DMEM medium for 3-day glucose starvation. Initially, we tried to the expression of sCypA after glucose treatment. On the $5^{th}$ day, we treated HK-2 cells with 0, 10, 25, and 50 mM glucose and collected cell lysate for Western blotting. The Western blotting revealed the expression of sCypA.

After the above confirmation, on the $5^{th}$ day, HK-2 cells were treated with 35 mM glucose or 5 mM glucose for 30 minutes and intracellular proteins were collected to determine the expression of p-p38 by Western blotting analysis. We also treated HK-2 cells with 35 mM or 5 mM glucose for 12 hours and 24 hours to collect extracellular protein to determine the expressions of TGFβ1 and sCypA by Western blotting analysis.

**2.3.2.5 TGFβ1 treatment on HK-2 cells**

HK-2 cells were seeded in a 6 cm cell culture plate with 3 x $10^5$ cells/plate and were incubated in DMEM/F-12 1:11 medium (10% FBS) for one day. Then the medium was replaced with 0% FBS low glucose DMEM medium for 3-day glucose starvation. On the $5^{th}$ day, we treated HK-2 cells with or without 5 ng/ml TGFβ1 (Recombinant human TGFβ1

(R&D Systems, Minnesota, USA)). After 30-minute treatment, we collected intracellular proteins to determine the expression of p-p38 by Western blotting analysis; after a 24-hour treatment, we collect extracellular proteins to determine the expressions of sCypA by Western blotting analysis.

#### 2.3.2.6 CypA treatment on HK-2 cells

HK-2 cells were seeded in a 6 cm cell culture plate with 3 x $10^5$ cells/plate and were incubated in DMEM/F-12 1:11 medium (10% FBS) for one day. Then the medium was replaced with 0% FBS low glucose DMEM medium for 3-day glucose starvation. On the $5^{th}$ day, we treated HK-2 cells with 1 nM or 10 nM CypA. After 30-minute treatment, we collected intracellular proteins to determine the expression of p-p38 by Western blotting analysis; after a 24-hour treatment, we collect extracellular proteins to determine the expressions of TGFβ1 by Western blotting analysis.

#### 2.3.2.7 Western blotting

Western blot reagents were obtained from Pierce (Rockford, USA). Primary antibodies included polyclonal anti-cyclophilin A (1:5000, Millipore, MA, USA) , anti-TGFβ1 (1:1000, Gene Tex, Irvine, CA), anti-p38 MAPK (1:1000, cell signaling, Danvers, MA), and β-actin

(Sigma, MO, USA). All other chemical supplies were acquired from Sigma (St Louis, MO, USA). Protein extraction from HK-2 cells after treatment with different concentration of D-glucose (Sigma, St Louis, MO, USA), TGFβ1 (PeproTech, NJ, USA), CypA (Enzo Life Sciences, Inc) and Western blot analysis were performed as described previously[47].

### 2.3.2.8 Immunofluorescence staining for CD147

CD147 (also known as Basigin, or extracellular matrix metalloproteinase inducer (EMMPRIN)), is the membrane receptor for sCypA. To detect CD147, 80% confluent HK-2 cell was seeded on the cover glass coated with 1N HCl. The cells were exposed in CypA recombinant peptides (Enzo Life Sciences, Inc) for 10 minutes (0, 1, 10nM), then washed with PBS, fixed in 4% paraformaldehyde in PBS (pH=7.4) for 20 minutes at room temperature. After several washes with PBS, cells were blocked with 5% FBS, 20ug/ml RNase A in PBST (0.2% Triton X-100, PBS) for 1 hour at room temperature, the reacted overnight with anti-goat polyclone EMMPRIN antibody (1:100 dilution in PBST; Santa Cruz) at 4℃. After several washes with PBS to remove primary antibody, slides were incubated with 488-labeled secondary antibody and propidium iodide for 1 hour at room temperature. After several washes with PBS, slides were mounted with mounting solution and fluorescent

images were obtained using a Zeiss LSM 510 confocal microscope. In addition, in order to observe the expression of CD147 after the treatment of TGFβ1 (5 ng/ml), HK-2 cells were exposed in TGFβ1 protein for 10 minutes or 24 hours. HK-2 cells without TGFβ1 treatment were taken as a control.

### 2.4 Statistical analysis

The results from Western blot were expressed as mean ± SEM. The suitable cutoff value for the sCypA and 8-OHdG in urine at the 8th week were analyzed using ROC curve to determine the optimal sensitivity and specificity of the ROC curve. *Chi* Square test was used to differentiate the two examinations. All statistical procedures were performed using the SPSS statistical software package, version 17.0 (Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1 Human study and cellular study

#### 3.1.1 Baseline Characteristics of Cohorts

A total of 100 DN patients and 20 healthy control subjects were enrolled in this study (Table 1). The DN patients were categorized according to their stages of DN with matched basic variables. The control individuals were healthy subjects without any metabolic syndrome or

medical drug treatment. Among all 100 DN patients, there were no significant differences in gender distribution ($p$=0.553), age ($p$=0.469), fasting sugar ($p$=0.403), glycated hemoglobin ($p$=0.352), total cholesterol ($p$=0.447), triglyceride ($p$=0.324), or low density lipoprotein cholesterol ($p$=0.199). Prevalence rates of other metabolic syndromes and CVD were both similar, including hypertension ($p$=0.668), stroke ($p$=0.480), coronary artery disease ($p$=0.724), heart failure ($p$=0.712), aortic aneurysm ($p$=1.000), and hyperlipidemia ($p$=0.075). All included drugs were matched (except metformin) as well, such as ACEi or ARB($p$=0.144), insulin ($p$=0.625), DPP4i ($p$=0.710), sulfonylurea ($p$=0.276), dipyridamole ($p$=0.740), pentoxifylline ($p$=0.121), and statin ($p$=0.095). It was not possible to match the usage of metformin because it is contraindicated in advanced DN. Among all the basic characteristics, it was difficult to match duration of DM ($p$=0.009) because progression of DN is highly time-dependent. Patients with more severe DN had higher serum creatinine (SCr) ($p$<0.001), lower GFR ($p$<0.001), higher albumin creatinine ratio (ACR) ($p$<0.001), and higher urinary CypA ($p$<0.001). Taken together, all basic variables were matched (except metformin), and with later stage of DN, patients had worse renal function parameters, including SCr, GFR, and ACR. Importantly, the concentrations of urinary CypA were statistically different among the different stages of severity of DN.

### 3.1.2 Correlation between urinary CypA and other clinical variables

Because some variables are clinically associated with renal functions, we performed univariate analysis to verify the associations between these variables and urinary CypA (Table 2). Our analysis showed that if patients did not use metformin, the concentration of urinary CypA would increase by 3.281 ng/ml (Figure 1A). The concentration of urinary CypA increased by 0.395 ng/mL for each 1 mg/dl increase of SCr. With each 1 ml/min decrease in GFR, the concentration of urinary CypA increased by 0.030 ng/ml. Without proteinuria, the concentration of urinary CypA decreased by 3.095 ng/ml (Figure 1B). Even though there were no statistically significant differences among the different stages of CKD (Figure 1D), there seemed to be a trend of increasing urinary CypA in the later stages of CKD. Also, there was a trend of higher urinary CypA in the group with GFR less than 60 ml/min per 1.73 m2 as compared with the GFR group with more than 60 ml/min per 1.73 m2 (Figure 1C). For each 1mg/g increase in ACR, the concentration of urinary CypA increased by 0.001 ng/ml (Figure 2A and table 2). All of the abovementioned variables were renal function-related or renal function-dependent. In summary, the concentration of urinary CypA correlated well with the progression of renal function in DN patients, based on the albuminuria-based model.

### 3.1.3 Urinary CypA correlated with the severity of DN stages

The relationship between urinary CypA and ACR are summarized in figure 2A. The R square was 0.054 with a statistically significant correlation between urinary CypA and ACR. Moreover, we analyzed the correlation of urinary CypA among all 6 groups, including five stages of DN. The concentrations of urinary CypA were not different between the control group and stage 1 of DN ($p=0.117$) (Figure 2B). However, with progression of DN, urinary CypA significantly increased in stage 2 DN compared to that in stage 1 DN ($p=0.012$). Most importantly, the concentration of urinary CypA increased as DN stages progressed ($p=0.003$, $<0.0001$, and 0.005 between stage 2 and 3, stages 3 and 4, stages 4 and 5, respectively). Consistently, compared to patients with DN stage 1, the CypA concentrations in patients with DN stages 2 to 5 were significantly increased ($p=0.006$) (Figure 2C).

### 3.1.4 Diagnosis of silent stage of DN via urinary CypA

Since the concentration of urinary CypA significantly increased in stage 2 DN (silent stage) and it persistently increased significantly with the progression of DN, we performed an analysis of the receiver operating characteristic (ROC) curve (Figure 3). Our analysis demonstrated that when the concentration of urinary CypA was more than 0.7250 ng/ml, we could diagnose the silent stage of DN with a sensitivity of 90.0% and specificity of 72.7%. The area under curve (AUC) was up

to 0.85, indicating that the use of urinary CypA for diagnosis of silent stage of DN had a moderately good discriminatory power.

### 3.1.5. Secreted CypA in mesangial cells treated with high concentration of glucose or free radicals

At the microscopic level, there are three major histological changes in the glomeruli in DN: mesangial expansion, glomerular basement membrane thickening, and glomerular sclerosis[26]. A hyperglycemic state stimulates mesangial cell matrix production[27] and mesangial cell apoptosis[28]. Hence, we examined whether sCypA was secreted from mesangial cells following high glucose treatment. As shown in figure 4A, glucose increased-sCypA level in a dose-dependent manner (10, 25, and 50 mM). Statistical analysis showed that the increased expression of sCypA was found at 25 mM vs. control ($p=0.037$), and 50 mM vs. control ($p=0.037$). Expression of sCypA was much higher at 50 mM vs. 10 mM ($p=0.018$) (Figure 4B). Meanwhile, it is known that ROS also plays an important role in DN. NADPH oxidase-mediated renal ROS promotes mesangial expansion and albuminuria[48]. We found that the expression of sCypA was significantly increased after 20 or 40 μM $H_2O_2$ treatment for 30 minutes (Figure 4C). Quantitative assessment showed that either 20 μM or 40 μM $H_2O_2$ treatment significantly increased the expression of sCypA, which could be reversed by 300 U/ml of catalase (scavenger of

free radicals) (Figure 4D), which was used to counteract the effects of $H_2O_2$. It is worth noting that all the experiments were carefully performed with proper controls to eliminate CypA released from cell death. Taken together, free radicals or high concentrations of glucose stimulate the secretion of CypA from mesangial cells, suggesting that there is a link between sCypA and pathogenesis of DN.

### 3.1.6 Secreted CypA released from HK-2 cells upon high glucose or free radical treatment

Mesangial cell injury is the classical expression of DN, but recent studies suggested that DN is also a tubular disease. Early changes in tubular epithelial cells may be an essential factor in the development of progressive kidney diseases[49]. HK-2 cells, human PTEC, have been used as a cell model to study tubular diseases. Therefore, Western blotting was used to disclose the expression of sCypA whereby various concentrations of glucose and $H_2O_2$ were applied to HK-2 cells. As expected, different concentrations of glucose (10, 25, and 50 mM) could effectively increase the expression of sCypA (Figure 5A), indicating that hyperglycemia can also induce sCypA release from tubular cells. In addition, either 20 μM or 40 μM $H_2O_2$ treatment significantly increased the expression of sCypA, which could be reversed by 300 U/ml of catalase (Figure 5B).

## 3.2 Animal study

### 3.2.1 Effects of Linagliptin on the physiology of mice with DM

Since the *db/db (Lepr$^{db}$)* mouse model of leptin deficiency is currently the most reliable and widely used mouse for modeling type 2 DN[50], we treated both *db/db* and *db/m* mice with Linagliptin to observe the effects of Linagliptin on DN. All three groups of *db/db* mice exhibited the classical manifestations of DM: increased appetite (figure 6A), thirst (figure 6B), urinary frequency (6C), and weight (figure 1E). However, regardless of treatments (3 mg/kg/day or 15 mg/kg/day of Linagliptin), the blood sugar in all three groups remained the same (figure 6D). Therefore, we hypothesize all findings were independent from glucose-lowering.

### 3.2.2 Secreted CypA as an earlier indicator than 8-OHdG for DN and their associations with Linagliptin

Urinary **8-OHdG** is a reliable and early marker of reactive oxidative stress (ROS) and DN because it can represent DNA damage in early DN[51]. The expression of 8-OHdG in the urine at the 8th week in the *db/db* was increased significantly compared to that in *db/m* (p=0.026). This result could be suppressed by administering 3 and 15 mg/kg/day of Linagliptin (p=0.018 and p=0.028 respectively, figure 7A). The expression of 8-OHdG in the *db/db* at the 20th week increased

significantly compared to that in *db/m* (p=0.018), but it could only be suppressed by a high dose (p=0.047) rather than low (p=0.175) (figure 7B). In summary, we were able to detect the expression of 8-OHdG starting from the 8th week up to 20th. The receiver operating characteristic (ROC) curve is shown in supplementary data (figure 8A). On the other hand, the sCypA in the urine at the 8th week in the *db/db* increased significantly compared to that of *db/m* (p=0.006), and it could only be suppressed by high-dose Linagliptin (p=0.016) rather than low-dose (p=0.050) (figure 7C). The expression of sCypA in the urine from the 20th week in the *db/db* also increased significantly compared to that of *db/m* (p=0.019), however, the sCypA expression could not be suppressed regardless of high- or low-dose of Linagliptin (p=0.773 and p=0.149, respectively) (figure 7D). Similarly, we were able to detect the expression of sCypA starting from the 8th up to 20th. The ROC curve for sCypA is shown in supplementary data (figure 8B). In contrast to 8-OHdG, however, a much higher dose of Linagliptin was needed to suppress the expression of sCypA at the 8th week.

### 3.2.3 Histological evidence of CypA in DN at the 20th week

The IHC staining for CypA was significantly increased in the *db/db* (figure 9B) compared to *db/m* (figure 9A) in glomeruli, and the increased expression could be reversed by low-dose Linagliptin (figure 9C) and

further reversed by high-dose Linagliptin (figure 9D). All data were quantified in figure 9E. The data clearly indicate that a higher level of CypA exists in the mesangial area of glomeruli in DN compared to non-DN. In addition to IHC staining over glomeruli, there is increased IHC staining for CypA over peri-glomerrular tubules in the *db/db* (figure 10B) compared to *db/m* (s figure 10A).

### 3.2.4 Linagliptin's effects on clinical markers of DN

The hyperfiltration and albuminuria are landmarks for DN[40]. At the 20th week, the Ccr (creatinine clearance) increased in the *db/db* group compared to *db/m* (p=0.034)(figure 11A). The hyperfiltration could be inhibited by both doses of Linagliptin (p=0.021 and p=0.014 respectively) (figure 11A). On the other hand, albuminuria could be reduced at the 8th week at a low dose (p=0.045) or high (p=0.046) (figure 11B). Albuminuria was not reduced at the 20th week, even with a high dose of Linagliptin in the *db/db* (p=0.347)(figure 11C).

### 3.2.5 Linagliptin's effects on pathological findings of DN at the 20th week

TGFβ1 is a pivotal mediator in the pathogenesis of renal fibrosis[52]. Microscopically, the IHC staining for TGFβ1 in glomeruli increased in the *db/db* group compared to *db/m* (figure 12B vs. 12A). The increased

glomerular staining in the *db/db* (figure 12B) could be reversed by low- and high-dose Linagliptin (figure 12C and 12D). All data are quantified in figure 12E. These results suggest that Linagliptin can reduce the expression of TGFβ1 in glomeruli from DN. Increased TGFβ1 staining around peri-glomerular tubules can be detected in the *db/db* (figure 13 B) compared to *db/m* (figure 13A). However, the expression of TGFβ1 cannot be relieved by low (figure 13C) or high dose Linagliptin (figure 13D). These persistent increased stainings of TGFβ1 around tubules in all three *db/db* groups supported that very limited effect of Linagliptin on tubules because only 3-5% Linagliptin will enter tubular cells[53].

### 3.3 Cell study

#### 3.3.1 Effects of Linagliptin on expressions of sCypA on MES-13

In our previous cell studies, oxidative stress and hyperglycemia could stimulate MES-13 and HK-2 cells to secrete cyclophilin A[54]. To understand whether Linagliptin affects the expression of sCypA in the cellular model, MES-13 cells were treated with high glucose to stimulate sCypA under different concentrations of Linagliptin. Our results showed that Linagliptin successfully inhibited the expression of sCypA in cells treated with 25 mM glucose in all three different concentrations (1, 10, and 100 nM) (figure 14A). Under the same glucose concentration (25 mM), the 10 nM Linagliptin treatment was able to inhibit the expression

of sCypA for 8 hours with statistical significance ($p<0.001$) (Figure 14D). The effect could last for 12 hours (figure 14C), but a longer treatment time of up to 24 hours diminished the effect (figure 14B). These findings therefore indicate that Linagliptin certainly could act as a rescue reagent for MES-13 cells under hyperglycemia by reducing sCypA production. Since only 3-5% Linagliptin will enter tubular cells[53], we did not verify effects of Linagliptin on expressions of sCypA in HK-2 cells.

### 3.3.2 Molecular pathway of sCypA related DN

Since sCypA can regulate p38-MAPK signaling[55], we hypothesize that p38-MAPK is also involved in sCypA-related DN. Instead of MES-13, we chose HK-2 cells because of the following reasons. Firstly, p38 MAPK signaling pathway was associated with DN in HK-2 cells[56]. Secondly, receptors of sCypA, CD 147, are mostly distributed over HK-2 cells[57]. After treating by high glucose on HK-2 cells, the phosphorylated-p38 (p-p38) increased in Western blotting (figure 15A). The increased expression of TGFβ1 could be detected earlier (12 hours) compared to the increased CypA after a relatively longer duration (24 hours) (figure 15B). All were quantified in figure 15C for p-p38, figure 15D for TGFβ1 (12 hours) and figure 15E for sCypA (24 hours). After treating by TGFβ1, the expression of p-p38 and sCypA both increased (figure 16A). Nevertheless, after treating by CypA, the expression of

TGFβ1 did not increased (figure 16B), but the expression of p-p38 increased (figure 16C). Quantified data after the treatment of TGFβ1 are shown in figure 16D for p-p38 and figure 16E for sCypA. Quantified data of p-p38 after the treatment of CypA is shown in figure 16F. Taken together, our data indicate that hyperglycemia induced the secretion of TGFβ1 from HK-2 cells. Also, TGFβ1 stimulated the secretion of CypA, which may then result in the increment of p38-MAPK.

### 3.3.3 Secreted CypA and its receptor (CD147) on HK-2 cells via confocal microscopy

CD147 is a membrane receptor for sCypA and is mainly distributed in the cytoplasm[57]. CD147 is mostly concentrated in the PTEC[58]. Without any treatment, the CD147 is mostly distributed in the cytoplasm (figure 17A). After being treated with CypA (1 nM) for 10 minutes, the cytosolic CD147 moved toward cell membranes and the contours of HK-2 cells could be identified (figure 17B). After further high dose of CypA treatment (10 nM) for 10 minutes, almost all contours of HK-2 cells could be seen clearly (figure 17C). On the other hand, CD147 was mostly distributed in the cytoplasm (figure 17D) if there was no treatment of TGFβ1. After treatment of TGFβ1 for 10 minutes, the distribution of CD147 was mainly in the cytoplasm (figure 17E). However, after 24 hour- TGFβ1 treatment, the cytosolic CD147 moved toward cell

membranes (figure 17F). In summary, CypA was capable of immediately stimulating cytosolic CD147 of HK-2 cells to move toward the cell membrane while it would take 24 hours for TGFβ1 to do so.

## 4. Discussions

### 4.1 Current clinical marker for DN is not good enough

The current clinical markers for DN are GFR and microalbuminuria. SCr is routinely measured for GFR, which can be used to stage CKD regardless of DM association. Since all renal diseases will progress to CKD and the cause and progression of CKD are heterogeneous, every cause related to CKD should theoretically have its own staging or detection criteria. Specific markers allow physicians to target and treat the definite cause, thereby potentially preventing further renal function deterioration. Albuminuria or proteinuria is the typical marker used for staging DN progression. However, it has become evident that there exists a subpopulation of patients with discrepant classifications of DN (albuminuria-based) and CKD (GFR-based)[8-10]. Therefore, some committees are trying to develop a new classification of DN[59], combining both GFR and albuminuria systems.

### 4.2 Urinary CypA as a new marker for DN

In this study, we measured renal function parameters and

demonstrated that urinary CypA was significantly associated with SCr, GFR, proteinuria, ACR, stages of DN, and stages of CKD. In addition, either GFR-based or albuminuria-based classifications of DN correlated significantly with urinary CypA. When comparing different stages of DN or CKD, there was only a trend of higher CypA in higher CKD stages, but truly statistically significant difference existed among the different DN stages. This finding supports the notion that urinary CyA is better correlated using the albuminuria-based classification, which is the better and earlier detection method for monitoring DN compared with the GFR-based system in clinical practice. Although the albuminuria-based system is better than GFR, it is far from ideal for a number of reasons. First, increased albuminuria is actually a relatively late manifestation of early-stage DN, so it is not sensitive enough to detect early stages of DN. Second, only one-third of patients with microalbuminuria present with persistent macroalbuminuria according to one cohort study[60], indicating a poor predictive power for outcome of DN. Third, some patients have renal pathological changes without microalbuminuria [61]. Finally, albuminuria is not specific enough for DN because it can be detected in other non-DM related nephropathy, such as retinopathy and congestive heart failure[62]. Therefore, urinary CypA could have enormous value as an earlier marker than albuminuria for identifying DN.

### 4.3 Urinary CypA for diagnosing early DN with good sensitivity and specificity

In this well-matched cohort of DN patients, urinary CypA correlated well to the different severity of DN according to the albuminuria-based classification. Compared with the control group, urinary CypA indeed increased significantly in stage 2 DN and this increase persisted throughout the later stages. The increment was more significant with worsening DN stage. In stage 1 DN, kidneys become dilated and glomerular capillary hydrostatic pressure increased in DN[63]. There was a hemodynamic change without any ultrastructure abnormality. Stage 2 DN is a silent stage but, to date, no useful markers for detection have been identified. No microalbuminuria can be measured in clinical practice. However, hyperglycemic effects are initiated in this stage. The glomerular basement membrane becomes thicker, followed by an increase in mesangial volume, and interstitial expansion[40]. The above structural changes do not become significant until stage 3 DN. If stage 2 DN could be detected early, intensive blood sugar monitoring, timely diet restriction, and exercise education would be useful to avoid further silent deterioration of DN. In this study, we propose that urinary CypA can be used as an early marker for identifying stage 2 DN with a high sensitivity (90%) and high diagnostic power (AUC=0.885). Detection of urinary CypA is also very convenient because it is non-invasive. Now that urinary

CypA appears to be capable of identifying DN in the silent stage, perhaps the term “silent” can be considered redundant. In an extensive review conducted by Lee et al[64], urinary CypA was not mentioned as a potential biomarker for DN. This is the first study to use urinary CypA in early DN detection. CypA was mostly studied in CVD and lung or liver injury[22]. Asthma and RA are associated with this new marker[20]. According to an extensive review of CypA in human disease[24], its association with DM was only mentioned once by Ramachandran et al[25]. They examined proteomic profiling of high glucose primed monocytes, and found that CypA could be a potential secretory marker of inflammation in type 2 DM[65]. The present investigation is the third study to identify a correlation between CypA and DM. Furthermore, this is the first study to verify the association between urinary CypA and DN with strong statistical significance in this well-designed human cohort.

### 4.4 Urinary CypA for diagnosing DN compared to other markers in clinical practice

It has been noted that urinary podocalyxin[66] and podocalyxin-positive element[67], which increases after podocyte injury, could be useful as new biomarkers for early DN. However, podocalyxin also increases in other diseases with podocyte injury[66], including IgA nephropathy, focal segmental glomerulosclerosis, membranous

nephropathy, and lupus nephritis, indicating that urinary podocalyxin is not specific to DN. In addition, urinary podocalyxin level or podocalyxin-positive element were not reported for early detection of stage 2 DN. Moreover, another biomarker, urinary L-FABP, expressed in the proximal tubules of the human kidney, was recently found to be associated with DN[68]. L-FABP increased in a stepwise manner with progression of DN[11]. In a study of type 1 DM[69], urinary L-FABP was an independent predictor of progression of DN irrespective of disease stage. The AUC to predict the progression to stage 3 DN by measuring both urinary L-FABP and urinary albumin was up to 0.786. In another study of type 2 DM[70], when the urinary L-FABP level was more than 8.4 μg/g creatinine, clinicians could predict the progression of DN to stage 3 DN with a sensitivity of 0.700 and specificity of 0.781. Compared to L-FABP as a marker for predicting stage 3 DN, urinary CypA is the first marker to be proposed for predicting progression to stage 2 DN with a much higher sensitivity (0.900 vs. 0.700) and larger AUC (0.850 vs. 0.786). In a recent extensive review of urinary biomarkers for early DN beyond albuminuria[64], it was found that all of the studied biomarkers were limited to predicting microalbuminuria (stage 3 DN). Therefore, our data demonstrate that urinary CypA may have value as a novel biomarker for predicting DN as early as stage 2.

### 4.5 Origins in the kidney of urinary CypA: mesangial cells and PTEC

In kidneys, CypA is mostly distributed in tubules, followed by glomeruli[21]. Therefore, it is reasonable to hypothesize that urinary CypA could be secreted by tubular cells or mesangial cells. Because mesangial matrix expansion is a typical pathological finding of DN[26] and a high glucose state evokes an intrinsic proapoptotic signaling pathway in mesangial cells[28], we first studied the expression of sCypA from mesangial cells. As shown in figure 4, there was a significant release of CypA following glucose or free radical treatment. Even though DN has been traditionally considered as a glomerular disease, increasing evidence has shown that renal dysfunction correlates earlier and in association with the degree of tubular injury[71]. A novel mechanism for albuminuria from PTEC revealed that tubular epithelial cell injury occurs relatively earlier than glomerular injury. There are many chemokines released from PTEC which stimulate certain physiological signals and whose effects culminate in progressive tubular injury, interstitial inflammation, and fibrosis in DN[72]. Therefore, we next examined whether CypA can also be secreted by tubular epithelial cells as well. In figure 5, after treatment of HK-2 cells with various concentrations of glucose or free radicals, sCypA was clearly increased in the conditioned medium. In the cell study using MES-13 and HK-2 cells, our results demonstrated that CypA was secreted after glucose or free radical stimulation, indicating that CypA

could be secreted by either mesangial cell or PTEC into urine in early DN. Our results are consistent with those of previous studies that showed earlier renal dysfunction was associated with tubular change[71, 72] and the later but typical change was related to mesangial cell dysfunction in the glomerulus[27, 28]. Therefore, sCypA could be considered as both tubular and mesangial cell injury markers in DN.

### 4.6 Urinary CypA is a better, much stronger and earlier markers of DN compared to 8OHdG in animal models

In the DN animal model, our results demonstrate that although both sCypA and 8-OHdG are early indicators for DN, sCypA is a better indicator than 8-OHdG. Firstly, we compared the extent for which the value of the indicator has increased at the same time. A 12.7 folds of increase [(6656.1pg/day) / (523.1pg/day)] of sCypA concentration at the 8th week in the *db/db* compared to 1.7 folds [(11.62ng/day) / (6.83ng/day)] of 8-OHdG concentration also suggested that sCypA is a more sensitive and specific indicator than 8-OHdG. We used *Chi* square to examine the ROC curve for sCypA (figure 8B) and 8-OHdG (figure 8A), and they differed statistically ($p<0.0001$) for diagnostic power. Secondly, the sCypA detected at the 8th week is an early marker for DN since the blood sugar in the *db/db* began to rise slightly from the 4th week to the 8th (130 ± 4 mg/dL and 175 ± 29 mg/dL respectively) [73]. This

period of time corresponds to the duration of early-stage DN when mesangial matrix expansion is still not detectable microscopically. The early-stage DN is characterized by hyperfiltration, resulting in a mere increase in 23% of glomerular surface (eg. hypertrophy or hemodynamic hyperfiltration) [74]. Hence, urinary sCypA is an early DN marker because there is an obvious increase of sCypA at the 8th week when there are few pathological changes. On the other hand, the increased sCypA should not be considered as a general effect due to increased proteinuria in the *db/db* mice, because among the 4 groups of mice, there was no significant upsurge in albuminuria at the 16th week compared to that at the 8th (p=0.059, 0.064, 0.400, and 0.203 in numerical order). Our data are consistent with previous reports that albuminuria or proteinuria was not the variable to represent the severity of DN in the *db/db* and *db/m*[74-77]. According to the above reasons, we believe that urinary sCypA is a much earlier and stronger maker than 8-OHdG for DN. Consistently, the increased IHC staining for CypA was also detected in mesangial cells (figure 9B vs. 9A; 9E) and tubular cells (figure 10B vs. 10A) in DN. The above findings in animal models are all consistent with our previous human study that human urinary CypA can be detected since stage 2 DN[54]. Thus, we conclude that urinary sCypA could possibly be a much stronger and earlier factor involved in causing DN.

## 4.7 Renal protection of Linagliptin is associated with TGFβ1 and sCypA

Significant protective role of Linagliptin on renal function are similar to the previous report that renal protection of Linagliptin is associated with TGFβ1[78]: Linagliptin can interfere with the conversion of latent to active TGF-β1 and downstream fibrotic markers[78]. We also demonstrate that the increased staining for CypA in glomeruli of DN can be reduced by Linagliptin (figure 12), which suggests that renal protection of Linagliptin may be associated with CypA in glomeruli. Furthermore, it may be independent from tubular cells because less than 5% Linagliptin entered the tubules[53]. Besides, we postulate that sCypA may be a stronger pathological factor than that of 8-OHdG. At the 8th week, Linagliptin could suppress 8-OHdG at a low dose but suppression of sCypA required a high dose. Similarly, high dose of Linagliptin was able to suppress secretion of 8-OHdG but not sCypA at the 20th week. The more pathogenic marker, sCypA, could exist up to the 20th week and could not be suppressed by high dose Linagliptin. This is also an indirect evidence that sCypA has stronger pathogenic effects on DN than 8-OHdG. It is worth noting that renal protection of Linagliptin exists in this animal study independently from glucose lowering. Failure of glucose lowering by Linagliptin was similar to previous researches[33, 79, 80].

### 4.8 Renal protection of Linagliptin is independent from glucose-lowering effect and located on glomeruli

The control of blood sugar was sustained early with DPP4i in the animal model of *db/db*. Nonetheless, progression of insulin resistance (persisted increased body weight) appeared to block the improvement of glucose tolerance through DPP4i. Linagliptin is then effective in only the early stage of type 2 diabetes[79]. Other reason for the discrepancy of the blood sugar values obtained before and after 8-week Linagliptin treatments is that the db/db developed frank hyperglycemia (175 ± 29 mg/dL at the 8th week of age and 283 ± 77 mg/dL at the 10th)[73]. We highly suspect an unrestricted diet (figure 6A) leading to the increased weight gain (figure 6E) which also caused poor blood sugar control after the 8th week. We believe that one cannot rely merely on medication when treating diabetes. A restricted diet to prevent excessive weight gain is as important as prescription drugs, as reported in the study conducted by Ishibashi et al[81] where the *db/db* mice were fed with two feeding methods: standard chow twice a day and ad libitum. In Ishibashi's study[81], they raised mice with two feeding methods which gave 3.2 g/day or 5 g/day of food at the 12th week. The resulting body weight was 29.8±0.7g vs. 42.6±2.9 g respectively. DPP4i failed to control blood sugar in the db/db mice receiving chow ad libitum because of glucose toxicity and lipotoxicity[81]. In contrast, our study did not limit food

intake for all three *db/db* groups. Compared to the Ishibashi's study, all our 3 groups of db/db at the 12th week weighted more (49.9±0.64g, 50.2±0.47g, and 50.2±7.52g). As it was also observed in Ishibashi's study, the body weight in our db/db mice remained high regardless of DPP4i treatment. However, DPP4i can achieve fair blood sugar control in human because unlimited weight gain is less likely. Interestingly, we could observe the renal protection effect of Linagliptin independently from its glucose lowering effect. Moreover, the similar weight gain among all 3 db/db groups was consistent with the clinical finding that DPP4i plays a neutral role in body weight in diabetic patients[82].

In addition, our results are consistent with a recent study regarding DPP4-deficinecy in an animal model[83]. Firstly, our findings suggest that the main effects of DPP4i were on glomeruli, with less effect on tubules, which are similar to the effects of DPP4 deficiency on expansion of glomerular area and albuminuria reported by Matsui T *et al.*[83]. Secondly, Matsui T *et al.* also found that increased 8-OHdG levels in the kidneys were suppressed significantly in DDP4-deficient rats. Our study echoed their finding. Thirdly, Matsui T *et al.* demonstrated that decreased Advanced Glycation End Product (AGE)-Receptor for AGEs (RAGE) axis in the genetically DPP4 deficiency rats provided renal protection even though the fasting blood glucose was similar in DN rats with or

without DPP4 deficiency. In our study, Linagliptin reduced the increment of glucose-stimulated CypA without lowering fasting blood glucose. Both the internal (genetically DPP4 deficiency) and external (Linagliptin treatment) mechanisms resulted in less DN through less glucose toxicity (lower AGE-RAGE axis and lower glucose-stimulate CypA secretion, respectively), supporting the notion that the effects of renal protection from blocking DPP4 are the results from decreased glucose toxicity without lowering blood glucose,

**4.9 The interplay of sCypA may be a paracrine for MES-13 and an autocrine for HK-2 cells.**

We showed pathological evidence of strong positive staining for CypA over mesangial cells in glomeruli (figure 9B) and peri-glomerular tubules (Supplementary figure 10B vs. 10A). Typically, findings of DN are focused on mesangial cells in glomeruli. However, early changes in PTEC may be an essential factor in the development of progressive kidney diseases[72, 84, 85]. Based on our previous study[54], hyperglycemia stimulated both mesangial cells and PTEC to secret CypA. This finding is compatible with the distribution of CypA staining in the *db/db* mice. To this end, we propose that there is interplay between PTEC and mesangial cells, and sCypA is associated with this relationship. Secreted CypA is associated with inflammatory or infectious diseases[24],

especially in CVD[23, 65]. It is considered as a new promising target in cardiovascular therapy[23, 65]. ROS inducers, including angiotensin II, stimulate CypA secretion from vascular smooth muscle cells. The sCypA activates ERK1/2 and promotes ROS production, thus augmenting the full response[23]. In rheumatoid arthritis, CypA-CD147 interaction might cause the destruction of cartilage and bone by upregulating MMP-9 expression[86]. CypA also induced CD147-dependent chemotaxis of activated CD4+ T cells in asthma[87]. CypA expression correlated with MMP-1, MMP-2, and MMP-9 expression in periodontitis[88]. In our first published study[54], we detected increased urinary CypA since the silent stage of DN. We further examined the mechanism that sCypA is involved in DN by using the cellular model. It is known that released sCypA will bind to its receptors, CD147, in many different types of cells. Given the fact that there are different ligands for CD147 binding, it is worth noting that the movement of cytosolic CD147 to cell membrane immediately after cells is treated with CypA (figure 9). The above finding indicates that sCypA is indeed involved in cell surface localization of CD147. All the above findings indicated that the interplay of sCypA may be a paracrine for MES-13 and an autocrine for HK-2 cells.

### 4.10 Secreted CypA related pathway is associated with TGFβ1, CD147, and p38 MAPK in the pathogenesis of DN

We showed that hyperglycemia stimulated PTEC to secret TGFβ1, which is consistent with the previous reports that the synergism of high glucose concentrations with cytokines can stimulate TGFβ1 synthesis by PTEC[89, 90]. TGFβ1 is upstream to many fibrotic pathways and is a multifunctional regulator that modulates cell differentiation, proliferation, and migration and induces the production of extracellular matrix proteins[91]. All are pivotal processes that contribute to glomerulosclerosis[92]. In addition to the association of TGF-β1 with glomerular change, TGF-β1 has been shown to participate both directly and indirectly in tubule degeneration in DN[93]. The epithelial mesenchymal transition (EMT) is the mechanism in most studies[56, 94, 95]. TGF-β1 down-regulates the expression of epithelial cell adhesion molecules (E-cadherin and ZO-1), increases de novo α-SMA expression and actin reorganization, and finally enhances cell migration and invasion of the interstitium[94]. It is worth noting that TGF-β1 related EMT in PTEC had been recently studies by Zhi-Mei Lv et al[56]. It is about the p38 MAPK signaling pathway in hyperglycemia induced EMT in PTEC. However, how the TGFβ1 stimulate increased expression of p38 MAPK is still unknown. Our study provides further evidence to confirm that TGFβ1 stimulates secretion of CypA which may cause CD147 to move outward to the cell membrane. CD147 may serve as the membranous receptor for sCypA. Secreted CypA induced cell surface localization of

CD147 might cause increased expression of p38 MAPK, leading to a downstream reaction such as EMT[56].

The reasons that TGFβ1 is upstream to sCypA are as follows. Firstly, TGFβ1 can stimulate secretion of CypA (figure 16A) but not vice versa (figure 16B). Secondly, increased expressions of TGFβ1 can be detected at $12^{th}$ hour (figure 15B, and 15D) from hyperglycemia-treated HK-2 cells, but expressions of sCypA was not detected until 24 hours (figure 15B and 15E). Last but not the least, the surfacing of CD147 can be detected as soon as 10 minutes following treatment of CypA (figure 17B and 17C) but 24 hours after TGFβ1 (figure 17F). Based on the above findings, in addition to functioning as a marker for DN, sCypA may also have a pathological role for DN. Taken together, our study is the first one to point out the association of sCypA and DN. We propose that sCypA is involved in the cross-talk between mesangial cells and PTEC through TGFβ1, CD147, and p38 MAPK (figure18).

## CONCLUSION

Based on human, animal and cell studies, sCypA was shown to be not only a marker of DN but also appeared to play a pathological role for DN. The renal protective effect of Linagliptin may be associated with blockage of sCypA in glomeruli. The sCypA may have potential as a

treatment target and thus further study is needed in the future.

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TABLE 1. Demography of Different Stages of Diabetic Nephropathy

| Diabetic Nephropathy Stage | Healthy Control Groups | 1 | 2 | 3 | 4 | 5 | $P^*$ Value | Total of DN Patients |
|---|---|---|---|---|---|---|---|---|
| Number | 20 | 20 | 20 | 20 | 20 | 20 | | 100 |
| Gender (male) (n, %) | 10, 50.0% | 10, 50.0% | 13, 65.0% | 12, 60.0% | 10, 50.0% | 10, 50.0% | 0.469 | 55, 55.0% |
| Age (y/o) | 45.8 ± 3.2 | 42.9 ± 13.1 | 59.2 ± 11.1 | 56.6 ± 11.2 | 59.3 ± 9.9 | 62.7 ± 8.1 | 0.469 | 57.3 ± 11.6 |
| Duration, years | – | 3.4 ± 2.7 | 5.7 ± 4.2 | 9.6 ± 8.7 | 10.6 ± 7.6 | 17.0 ± 9.3 | 0.009 | 7.9 ± 7.4 |
| Fasting sugar, mg/dL | 89.6 ± 5.4 | 147.8 ± 57.3 | 144.8 ± 57.3 | 151.0 ± 65.5 | 152.3 ± 47.4 | 121.4 ± 21.0 | 0.403 | 142.9 ± 50.5 |
| HbA1c, %[†] | 5.6 ± 1.3 | 7.2 ± 2.3 | 7.8 ± 2.8 | 7.6 ± 1.7 | 7.7 ± 2.6 | 6.4 ± 0.8 | 0.352 | 7.4 ± 2.3 |
| Creatinine, mg/dL | 0.70 ± 0.36 | 0.60 ± 0.10 | 1.09 ± 0.49 | 1.13 ± 0.49 | 3.28 ± 3.25 | 7.54 ± 4.12 | <0.001 | 2.41 ± 3.11 |
| GFR[‡], mL/min per 1.73 $m^2$ | 142.1 ± 10.0 | 133.6 ± 19.3 | 77.4 ± 28.2 | 78.2 ± 38.9 | 37.7 ± 30.1 | 7.4 ± 4.12 | <0.001 | 69.1 ± 48.2 |
| Total cholesterol, mg/dL | 190.2 ± 15.2 | 145.2 ± 50.0 | 149.2 ± 24 | 166.7 ± 43.6 | 180.9 ± 60.5 | 154.6 ± 42.1 | 0.447 | 162.7 ± 47.2 |
| Triglyceride, mg/dL | 100.8 ± 30.3 | 156.6 ± 55.1 | 133.6 ± 69.3 | 191.8 ± 103.5 | 162.7 ± 104.6 | 179.7 ± 134.7 | 0.324 | 161.0 ± 96.3 |
| Low density lipoprotein cholesterol, mg/dL | 77.3 ± 10.2 | 86 ± 29.8 | 88.0 ± 17.4 | 86.2 ± 31.6 | 119.5 ± 52.6 | 87.9 ± 34.8 | 0.199 | 96.4 ± 39.2 |
| ACR, mg/g[§] | 10.3 ± 5.6 | 11.7 ± 18.1 | 11.0 ± 27.3 | 142.6 ± 82.2 | 2426.0 ± 2409 | 4893 ± 3485 | <0.001 | 1238.4 ± 237.7 |
| CypA[‖], ng/mL | 0.7354 ± 0.4510 | 0.6088 ± 0.3795 | 1.8684 ± 2.0116 | 3.0157 ± 9.4954 | 4.11580 ± 3.6078 | 7.3818 ± 04.4710 | <0.001 | 3.1638 ± 5.3630 |
| Hypertension (n, %) | 0, 0% | 13, 65.0% | 12, 60% | 13, 65% | 12,60% | 13, 65% | 0.668 | 65, 65.0% |
| Stroke (n, %) | 0, 0% | 1, 5% | 2, 10% | 2, 10% | 2, 10% | 1, 5.0% | 0.480 | 8,8.0% |
| Coronary artery disease (n, %) | 0, 0% | 0, 0% | 2, 10% | 2, 10% | 2, 10% | 2, 10% | 0.724 | 8, 8% |
| Heart failure (n, %) | 0, 0% | 0, 0% | 2, 10% | 1, 5% | 3, 15% | 1,5.0% | 0.712 | 7, 7.0% |
| Aortic aneurysm (n, %) | 0, 0% | 0, 0% | 0, 0% | 0, 0% | 0, 0% | 0, 0% | 1.000 | 0, 0% |
| ACEi[¶] or ARB[#] (n, %) | 0, 0% | 12, 60.0% | 9, 45% | 9, 45% | 13, 65% | 18, 90.0% | **0.144** | 61, 61.0% |
| Insulin (n, %) | 0, 0% | 2, 10.0% | 6, 30% | 5, 25% | 7, 35% | 7, 35.0% | 0.625 | 27, 27.0% |
| DPP4i[$] (n, %) | 0, 0% | 10, 50.0% | 6, 30% | 9, 45% | 10, 50% | 10, 50.0% | 0.710 | 45, 45.0% |
| Sulfonylurea (n, %) | 0, 0% | 4, 20.0% | 4, 20% | 8, 40% | 9, 45% | 10, 50.0% | 0.276 | 35, 35.0% |
| Metformin (n, %) | 0, 0% | 18, 90.0% | 17, 85% | 10, 50% | 8, 35% | 0, 0% | **<0.001** | 53, 53.0% |
| Dipyridamole (n, %) | 0, 0% | 0, 0% | 1, 5% | 0, 0% | 1,5% | 0, 0% | 0.740 | 2, 2.0% |
| Pentoxifylline (n, %) | 0, 0% | 0, 0% | 1,5% | 3, 15% | 6, 30% | 6, 30.0% | 0.121 | 16, 16.0% |
| Statin (n, %) | 0, 0% | 20, 100% | 14, 70% | 11, 55% | 17, 85% | 12, 60.0% | 0.095 | 74, 74.0% |
| Hyperlipidemia (n, %) | 0, 0% | 20, 100% | 16, 80% | 11, 55% | 19, 95% | 18, 90.0% | 0.075 | 84, 84.0% |
| Dialysis (n, %) | 0, 0% | 0, 0% | 0, 0% | 0, 0% | 0, 0% | 20, 100% | **<0.001** | 20, 20% |
| GFR[‡] >60 mL/min per 1.73 $m^2$ | 6, 100% | 20, 100% | 16, 80% | 12, 60% | 5, 25% | 0, 0% | **<0.001** | 53, 53.0% |
| CKD stage | 0, 0% | 1.0 ± 0.0 | 1.9 ± 0.9 | 2.1 ± 0.9 | 3.5 ± 1.2 | 4.5 ± 0.9 | <0.001 | 2.5 ± 1.5 |

DN = diabetic nephropathy.
[*] Kruskal Wallis test between 5 stages of diabetic nephropathy.
[†] Glycated hemoglobin.
[‡] Glomerular filtration rate.
[§] Urine albumin creatinine ratio.
[‖] Cyclophilin A.
[¶] Angiotensin-converting-enzyme inhibitor.
[#] Angiotensin II receptor blockers.
[$] Inhibitors of dipeptidyl peptidase 4.

TABLE 2. Univariate Analysis and Multivariate Analysis

| Univariate Analysis* | *P* Value | $R^2$ |
|---|---|---|
| Age | 0.605 | |
| Gender | 0.305 | |
| Hypertension | 0.997 | |
| Stroke | 0.664 | |
| Coronary arterial disease | 0.470 | |
| Heart failure | 0.570 | |
| ACEi† or ARB‡ | 0.773 | |
| Sulfonylurea | 0.507 | |
| Insulin | 0.973 | |
| DPP4i§ | 0.952 | |
| Metformin | 0.004 | 0.095 |
| CypA$ = 1.581 + metformin (use)*0 or metformin (no use)*3.281 | | |
| Dipyridamole | 0.794 | |
| Pentoxifylline | 0.648 | |
| Statin | 0.177 | |
| DM | 0.425 | |
| Hyperlipidemia | 0.255 | |
| Proteinuria | 0.008 | 0.082 |
| CypA$ = 4.438 + proteinuria(yes)*0 or no proteinuria*(−3.095) | | |
| Dialysis | 0.389 | |
| GFR ≥ 60 mL/min per 1.73 $m^2$ | 0.060 | |
| Duration | 0.957 | |
| Fasting sugar | 0.898 | |
| HbA1c‖ | 0.686 | |
| Creatinine | 0.037 | 0.052 |
| CypA$ = 2.241 + Cr*0.395 | | |
| Glomerular filtration rate | 0.013 | 0.052 |
| CypA$ = 5.270 + GFR*(−0.030) | | |
| Total cholesterol | 0.133 | |
| Triglyceride | 0.567 | |
| Low density lipoprotein cholesterol | 0.796 | |
| ACR¶ | 0.034 | 0.054 |
| CypA$ = 2.461 + ACR*0.001 | | |
| Multivariate analysis | *P* value | $R^2$ |
| ACR | 0.414 | |
| GFR# | 0.547 | |
| Metformin | 0.081 | |

*General linear model for categorical variables and simple linear regression for continuous variables.
†Angiotensin-converting-enzyme inhibitor.
‡Angiotensin II receptor blockers.
§Inhibitors of dipeptidyl peptidase 4.
‖Glycated hemoglobin.
¶Urine albumin creatinine ratio.
#Glomerular filtration rate.
$Cyclophilin A.

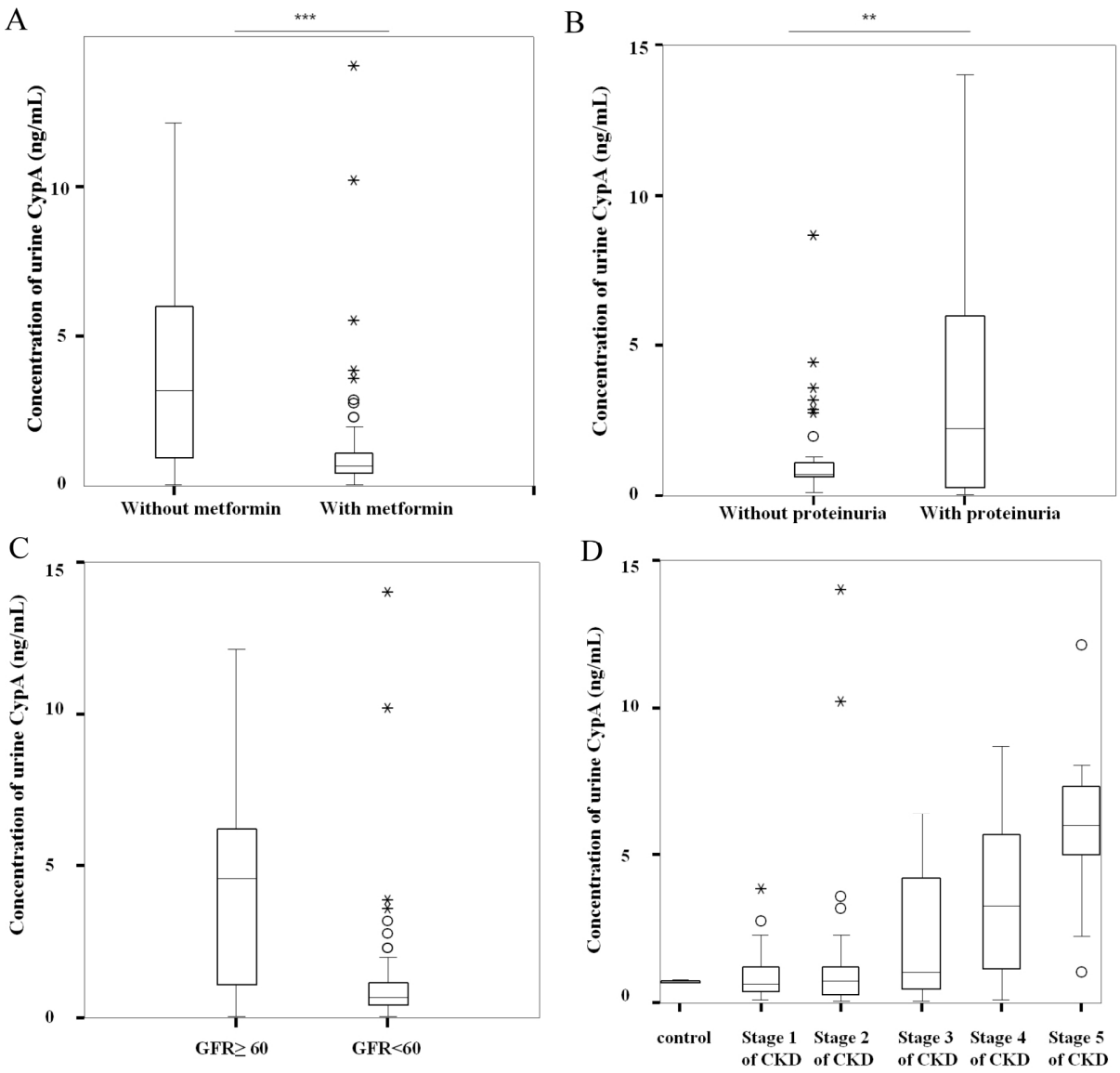
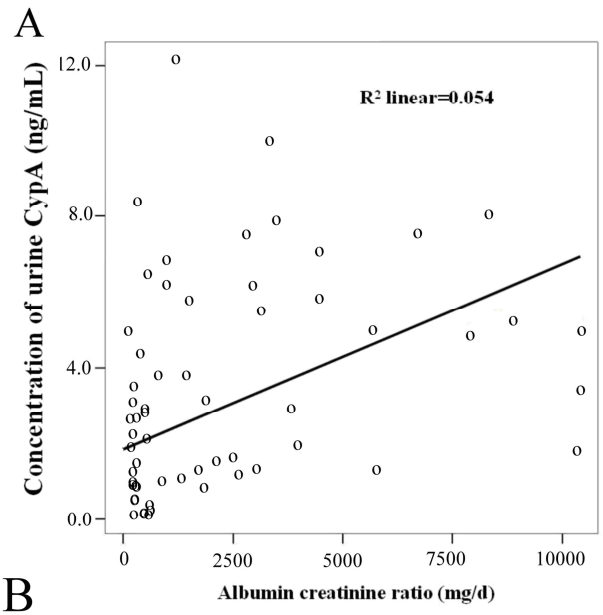
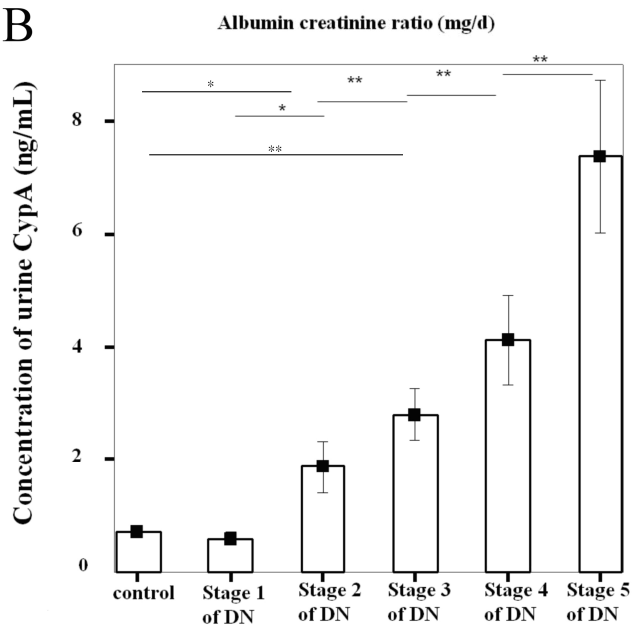



**Figure 1. Univariate analysis between clinical parameters and urinary CypA.**

*P* value for A was <0.001, for B was 0.007, and for C was <0.060. *P* value between stages 1 and 2, 2 and 3, 3 and 4, 4 and 5 of CKD were 0.511, 0.633, 0.365, 0.203 and 0.061, respectively.


A
Concentration of urine CypA (ng/mL)
12.0
8.0
4.0
0.0
R² linear=0.054
0
2500
5000
7500
10000
Albumin creatinine ratio (mg/d)


B
Concentration of urine CypA (ng/mL)
8
6
4
2
0
*
**
*
**
**
**
control
Stage 1 of DN
Stage 2 of DN
Stage 3 of DN
Stage 4 of DN
Stage 5 of DN

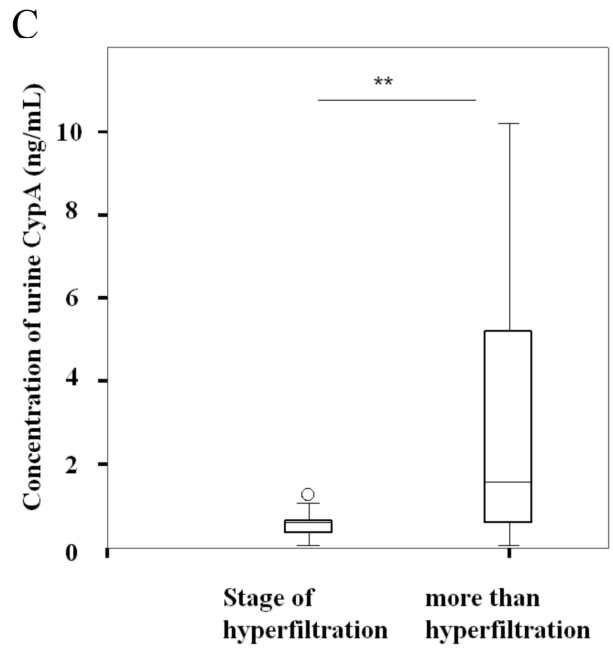

C
Concentration of urine CypA (ng/mL)
10
8
6
4
2
0
**
Stage of hyperfiltration
more than hyperfiltration

**Figure 2. Concentrations of urinary CypA in different stages of DN.**

(A) Concentration of urinary CypA and ACR were plotted. When ACR increased by 1mg/g, the concentration of urinary CypA increased 0.030 ng/mL (CypA=2.461+ACR*0.001). $R^2$ linear was 0.054. (B) No difference in concentration of urinary CypA was found between stage 1 DN and healthy control groups ($p$=0.117). However, there were statistically significant differences between stages 1 and 2, stages 2 and 3, stages 3 and 4, and stages 4 and 5 DN ($p$=0.012, 0.003, <0.001, and 0.005, respectively). (C). The differences between stage 1 DN and stages 2-5 DN were statistically significant ($p$=0.006).

*$p$<0.05, **$p$<0.01, ***$p$<0.001

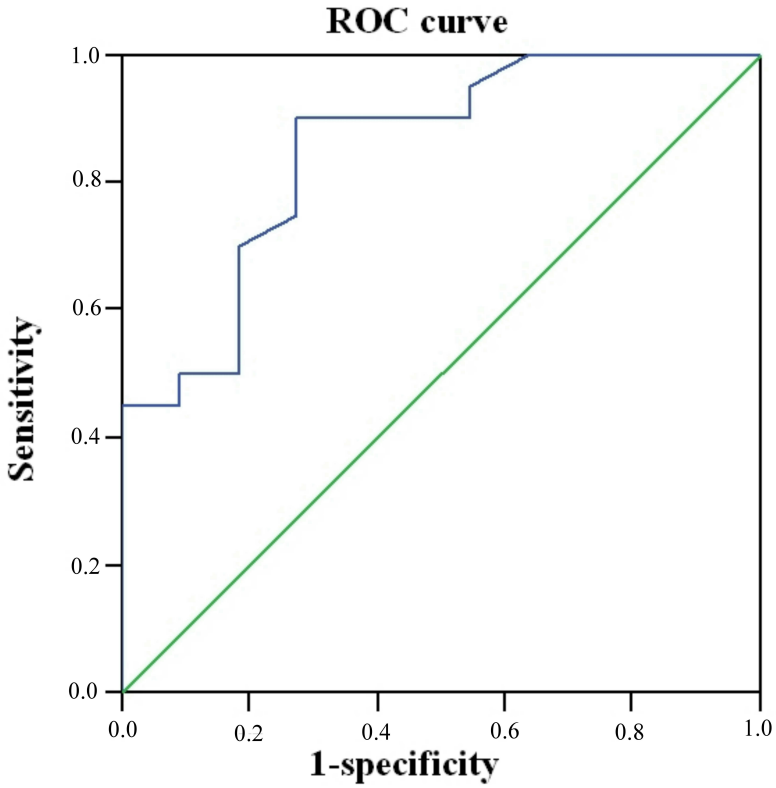



**Figure 3. ROC curve for diagnosing silent stage of DN via urinary CypA.**

The concentration of urinary CypA to diagnose silent stage of DN was 0.7250 ng/mL with a sensitivity of 0.900 and a specificity of 0.727. The area under the ROC curve was 0.850.

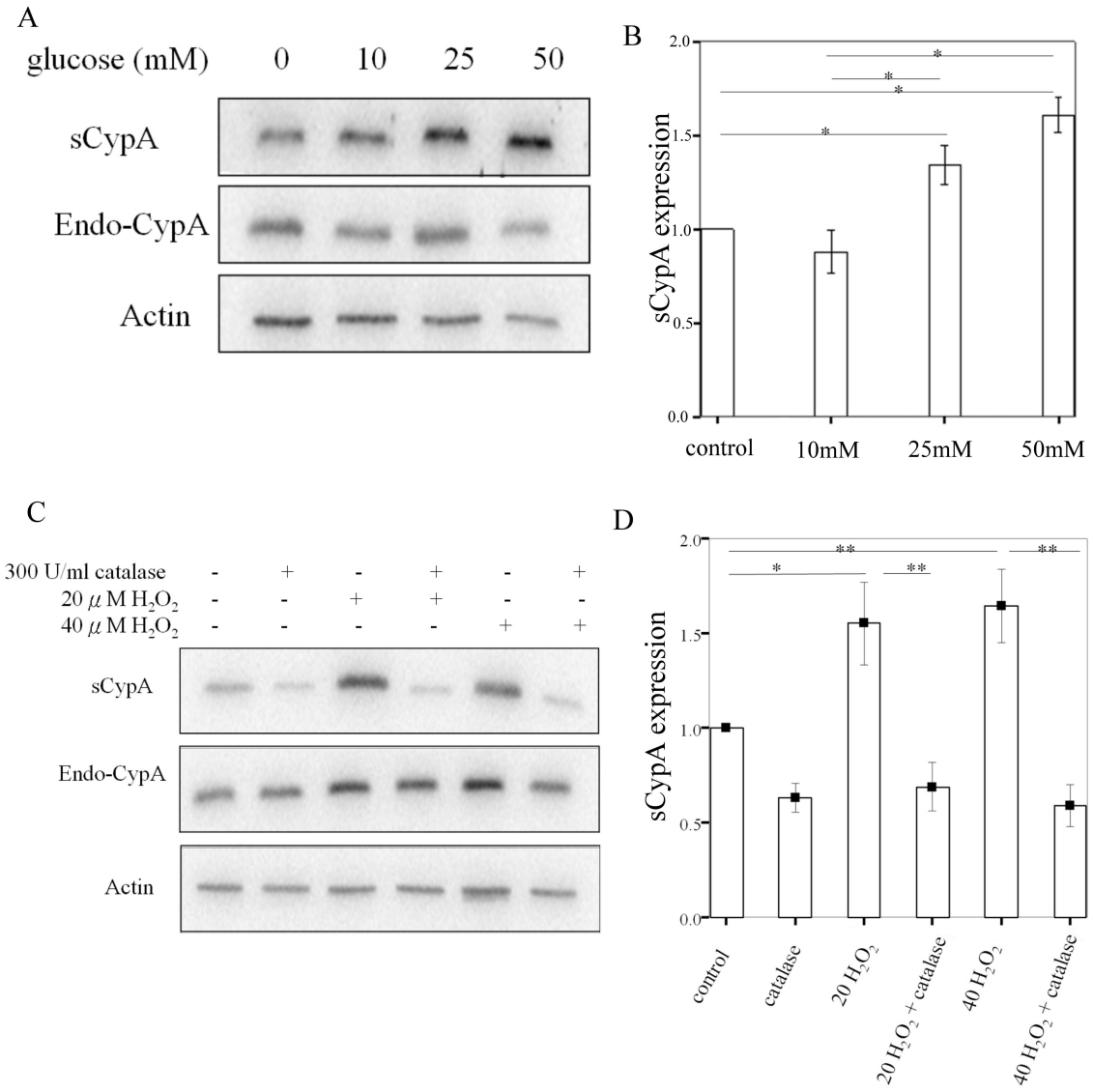



**Figure 4. Western blotting of sCypA expression in MES-13 cells treated with different concentrations of glucose and $H_2O_2$.**

(A) Glucose increased the expression of sCypA, which was dose-dependent (10, 25, and 50mM). (B) Statistical analysis showed that the increased expression of sCypA was found at 25mM vs. control ($p$=0.037), and 50mM vs. control ($p$=0.037). Increased expression of sCypA was observed at 50mM vs. 10mM ($p$=0.018). (C) The expression

of sCypA was increased in cells with 20 or 40 $\mu$M $H_2O_2$ treatments. They could both be counteracted by 300 U/ml catalase. (D) Quantitative assessment showed that 20 $\mu$M $H_2O_2$ increased the expression of sCypA ($p$<0.05) (n=6), which could be reversed by 300 U/ml catalase ($p$<0.01) (n=6). The sCypA expression was also stimulated by 40 $\mu$M $H_2O_2$ ($p$<0.01) (n=6), which could be counteracted by 300 U/ml catalase (p<0.01) (n=6). (n=4)

*p<0.05, **p<0.01.

Endo-CypA: endogenous cyclophilin A.

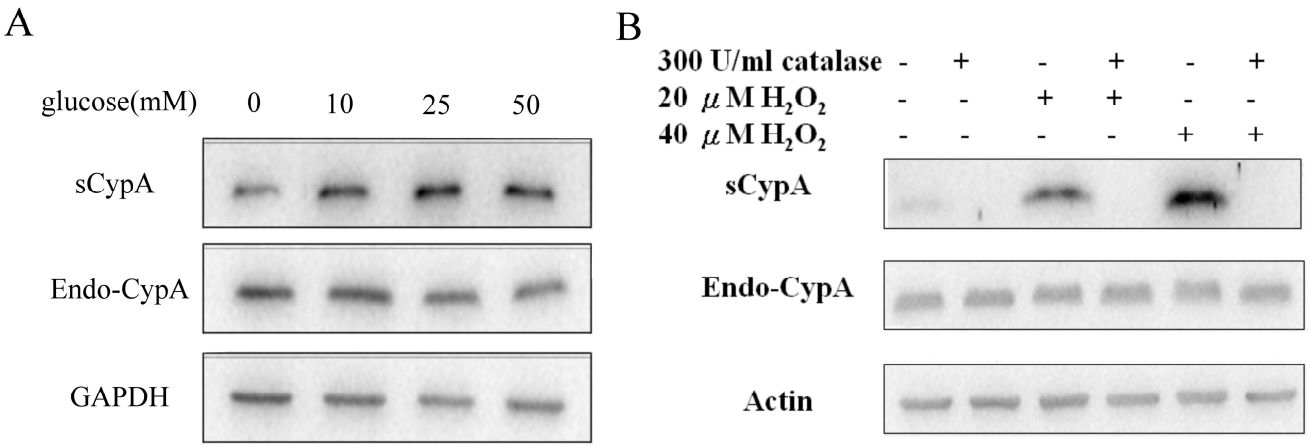



**Figure 5. Western blotting of sCypA expression in HK-2 cells treated with different concentrations of glucose and $H_2O_2$.**

**(A)** HK-2 cells were treated with different concentrations (10, 25, 50 mM) of glucose. All concentrations of glucose increased the expression of sCypA. (B) The expression of sCypA was increased in cells with 20 or 40μM H2O2 treatments. They could both be counteracted by 300 U/ml catalase.

Endo-CypA: endogenous cyclophilin A.

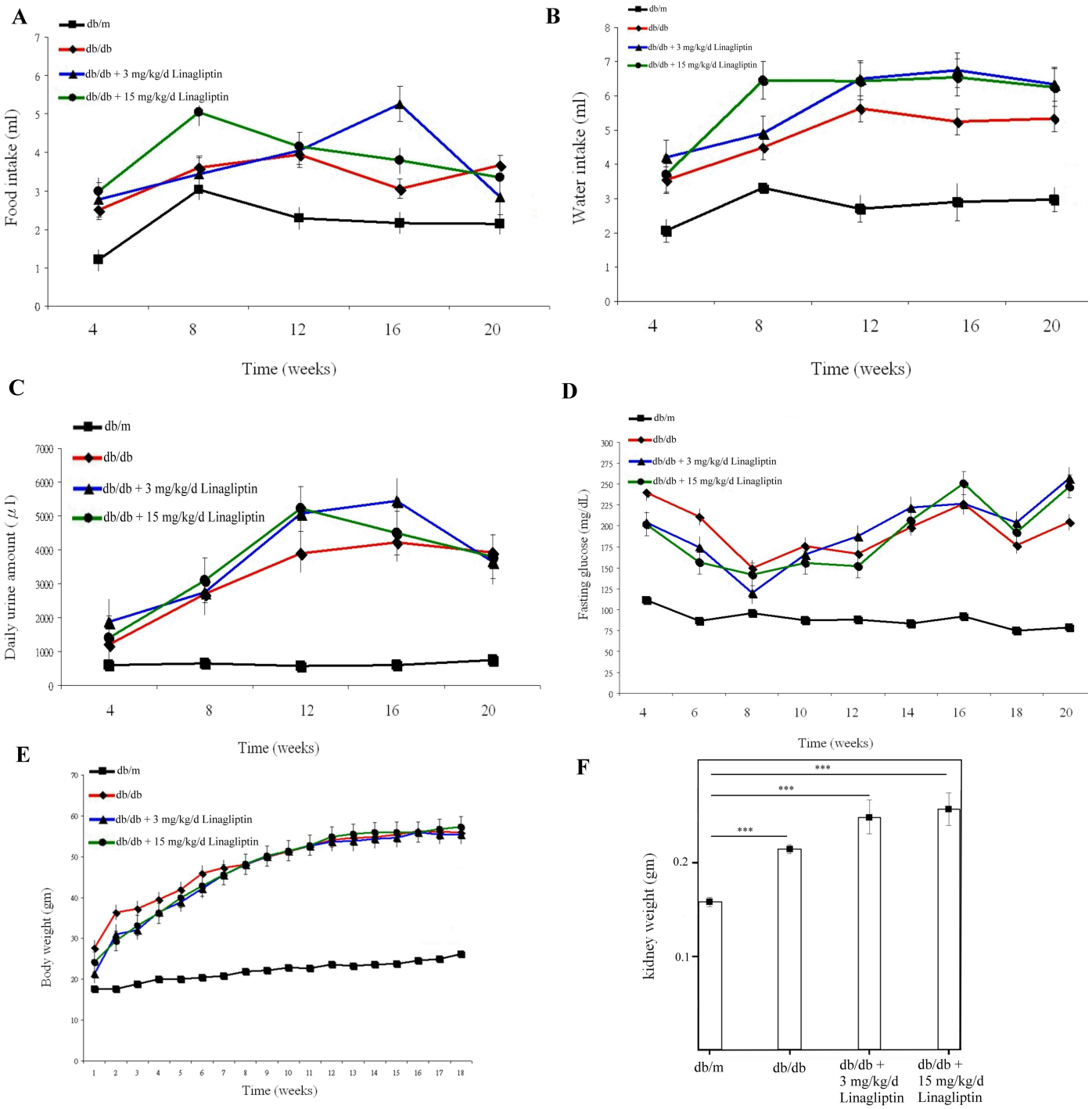



**Figure 6. Physical data of mice.**

(A) Three groups of mice with diabetic nephropathy (*db/db*) ate more food than mice without diabetic nephropathy (*db/m*). However, there was no difference among all 3 *db/db* groups. (n=10)

(B) Three groups of mice with diabetic nephropathy *(db/db*) drank more

water than mice without diabetic nephropathy (*db/m*). However, there was no difference between all 3 *db/db* groups. (n=10)

(C) Three groups of mice with diabetic nephropathy *(db/db*) produced a greater urinary volume than mice without diabetic nephropathy (*db/m*). However, there was no difference between all 3 *db/db* groups. (n=10)

(D) Mice with diabetic nephropathy had significantly higher blood glucose than *db/m*. However, there was no difference between all 3 *db/db* groups. (n=10)

(E) Three groups of mice with diabetic nephropathy *(db/db*) had much higher body weight than mice without diabetic nephropathy (*db/m*). However, there was no difference between all 3 *db/db* groups. (n=10)

(F) Three groups of mice with diabetic nephropathy *(db/db*) had heavier kidneys than mice without diabetic nephropathy (*db/m*) did. However, there was no difference between all 3 *db/db* groups. (n=10)

***$p<0.001$

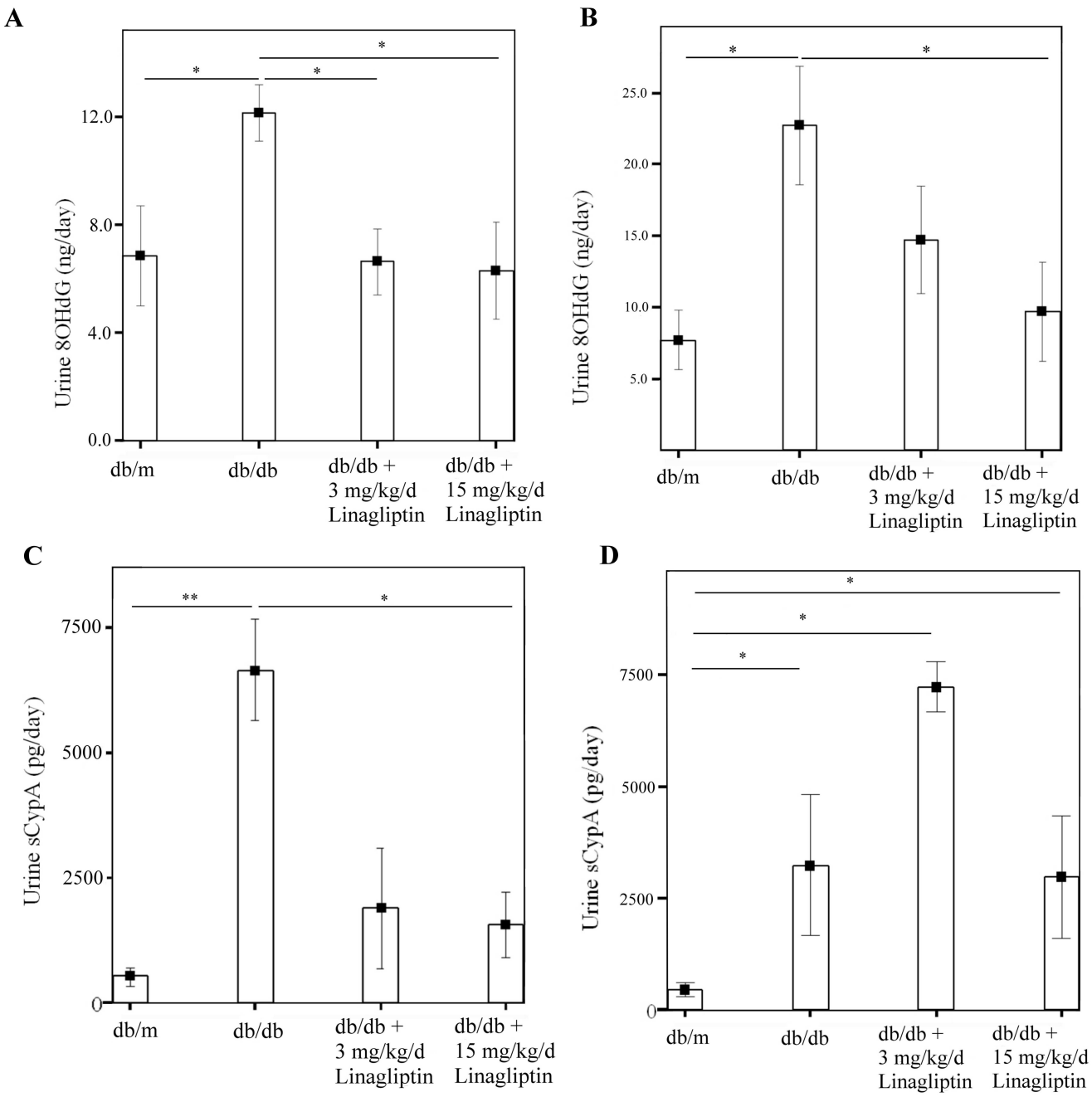



**Figure 7. Expressions of 8-OHdG and CypA from mice's urine at the 8th week and 20th week.**

(A) The expression of 8-OHdG in *db/db* from the urine at the 8th week was increased significantly compared to *db/m* (p=0.026). This could be suppressed by administering both 3 and 15 mg/kg/day Linagliptin

(p=0.018 and p=0.028 respectively).

(B) The expression of 8-OHdG in *db/db* from urine at the 20th week was increased significantly compared to *db/m* (p=0.018), but this could only be suppressed by higher dose (15 mg/kg/day) of Linagliptin (p=0.047) instead of lower dose (3 mg/kg/day) ( p=0.175)

(C) The expression of sCypA in *db/db* from urine at the 8th week was increased significantly compared to *db/m* (p=0.006), and it could only be suppressed by higher dose (15 mg/kg/day) of Linagliptin (p=0.016) instead of lower dose (3 mg/kg/day) (p=0.050).

(D) The expression of sCypA in *db/db* from urine at the 20th week was also increased significantly compared to *db/m* (p=0.019), but could not be suppressed by either high or low dose of Linagliptin (p=0.773 and p=0.149 respectively).

(n=6 in *db/m*, n=5 in *db/db*, n=6 in *db/db* +3 mg/kg/day Linagliptin, and n=5 in *db/db* + 15 mg/kg/day Linagliptin)

*p<0.05, **p<0.01

**A.** **B.**

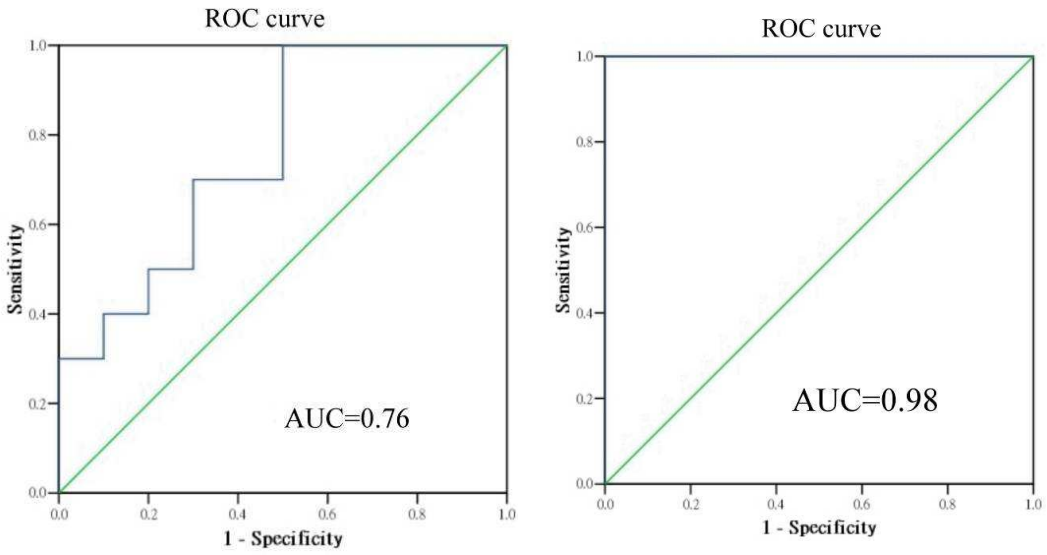



**Figure 8. ROC curve of 8OHdG and urinary CypA**

(A) At the 8th week, *db/db* mice had higher daily urinary 8OHdG (> 9.1254 ng/day) with the sensitivity of 70% and the specificity of 70% (AUC=0.76)

(B) At the 8th week, *db/db* mice had higher daily urinary CypA (> 1287.9806 pg/day) with the sensitivity of 98% and the specificity of 99% (AUC=0.98).

(n=6 in *db/m*, n=5 in *db/db*, n=6 in *db/db*+3mg/kg/day Linagliptin, and n=5 in *db/db*+15mg/kg/day Linagliptin).

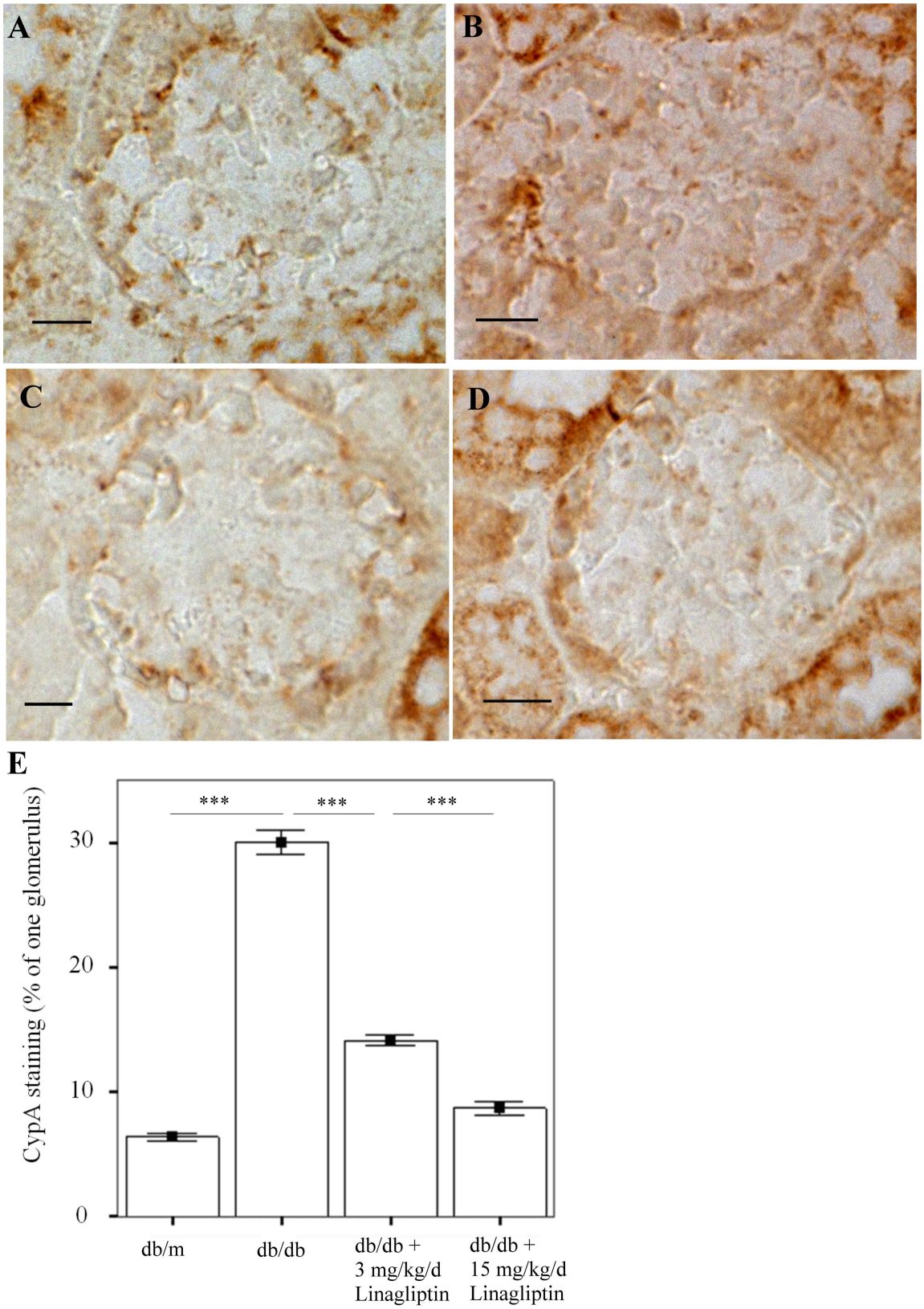


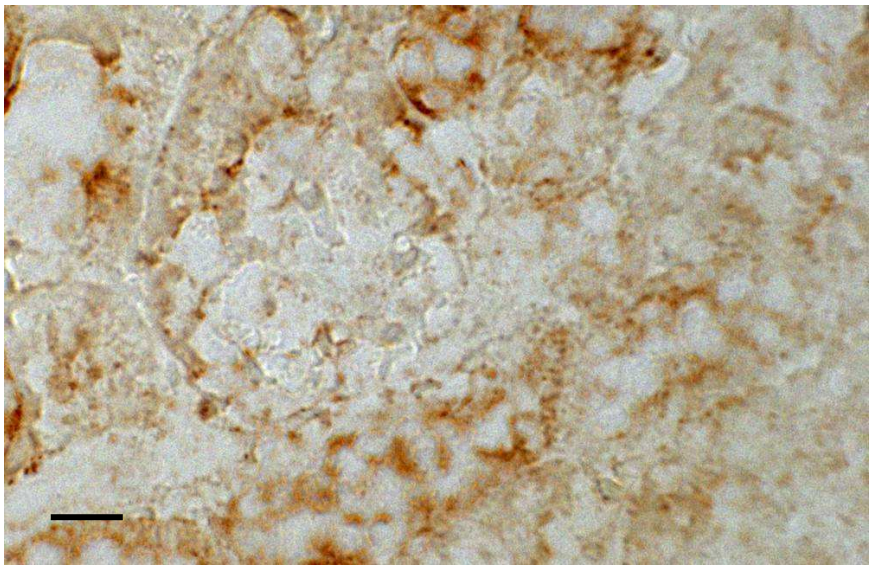

**Figure 9. IHC staining of glomeruli for CypA in *db/db* diabetic compared to *db/m* nondiabetic kidneys at 20th week.**

(A)(B)(C)(D) IHC staining for CypA (brown staining) was increased in the *db/db* group compared to *db/m* (B vs. A). The increased staining for CypA in *db/db* (B) could not be observed in high dose (C) or low dose (D) Linagliptin treatment.

(E) Quantitative data (n=100 in each group) for IHC staining for CypA. Increased staining for CypA in *db/db* could be reversed by both dose of Linagliptin treatment significantly.

***p<0.001. scale bar, 50 $\mu$ m

**A.**



**B.**

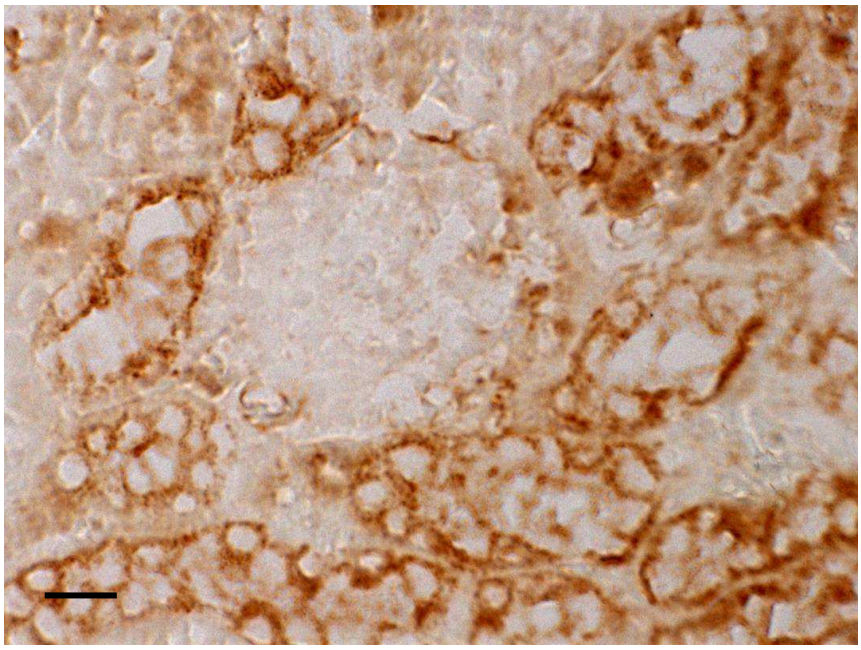
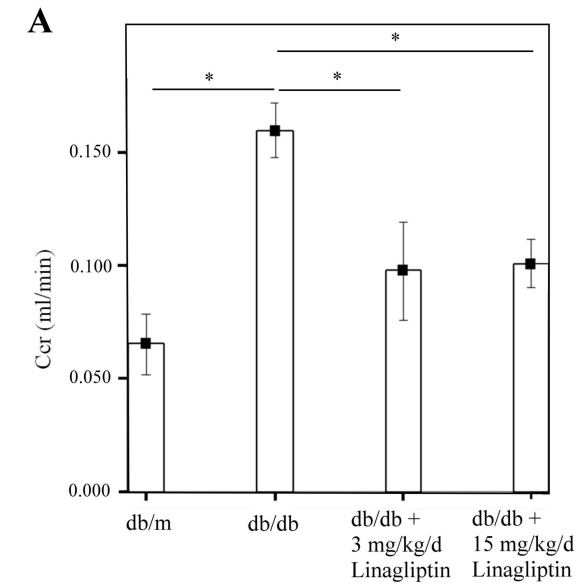


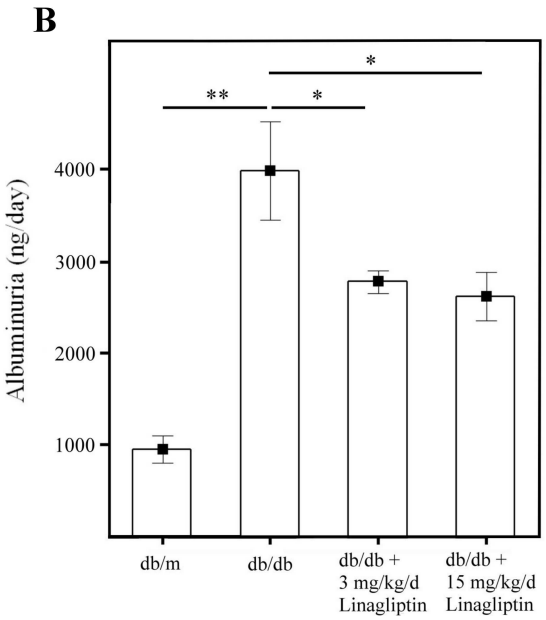
**Figure 10. IHC staining for CypA around peri-glomerular tubules**

(A) IHC staining for CypA around peri-glomerular tubules in *db/m* non-diabetic kidneys at 20th week.

(B) IHC staining for CypA around peri-glomerular tubules for CypA in *db/db* diabetic kidneys at 20th week.

scale bar, 50 $\mu$ m


A
*
*
*
0.150
0.100
0.050
0.000
Cer (ml/min)
db/m
db/db
db/db +
3 mg/kg/d
Linagliptin
db/db +
15 mg/kg/d
Linagliptin


B
**
*
*
4000
3000
2000
1000
Albuminuria (ng/day)
db/m
db/db
db/db +
3 mg/kg/d
Linagliptin
db/db +
15 mg/kg/d
Linagliptin

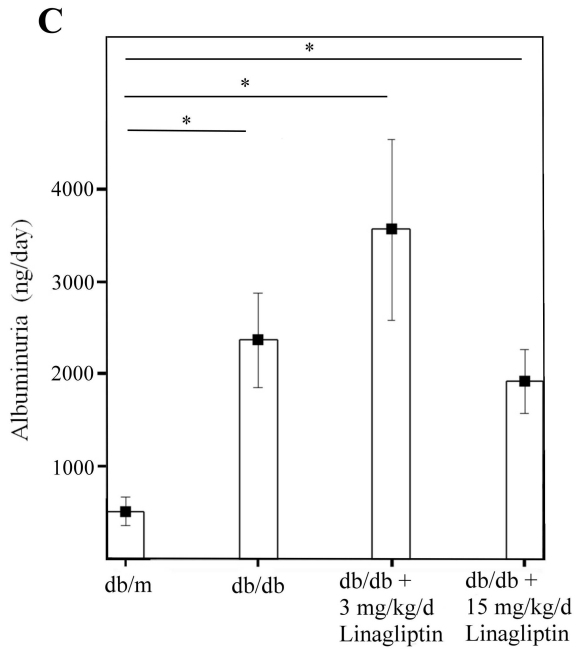

C
*
*
*
4000
3000
2000
1000
Albuminuria (ng/day)
db/m
db/db
db/db +
3 mg/kg/d
Linagliptin
db/db +
15 mg/kg/d
Linagliptin

**Figure 11. Renal function evaluations of mice, including creatinine clearance and daily albuminuria.**

(A) The Ccr was increased at the 20th week in the *db/db* group when compared to the *db/m* (p=0.034). The increased hyperfiltration could be inhibited by high- or low-dose Linagliptin (p=0.021 and p=0.014 respectively).

(B) Daily albuminuria at the 8th week was increased in *db/db* group (p=0.006), which could be inhibited by 3 mg/kg/day-Linagliptin (p=0.045) or 15 mg/kg/day-Linagliptin (p=0.046).

(C) Daily albuminuria at the 20th week was also increased in the *db/db* group (p=0.028). The increased albuminuria could not be inhibited by either high- or low-dose Linagliptin (p=0.327 and p=0.142 respectively).

(n=6 in *db/m*, n=5 in *db/db*, n=6 in *db/db* +3 mg/kg/day Linagliptin, and n=5 in *db/db* + 15 mg/kg/day Linagliptin)

*p<0.05, **p<0.01

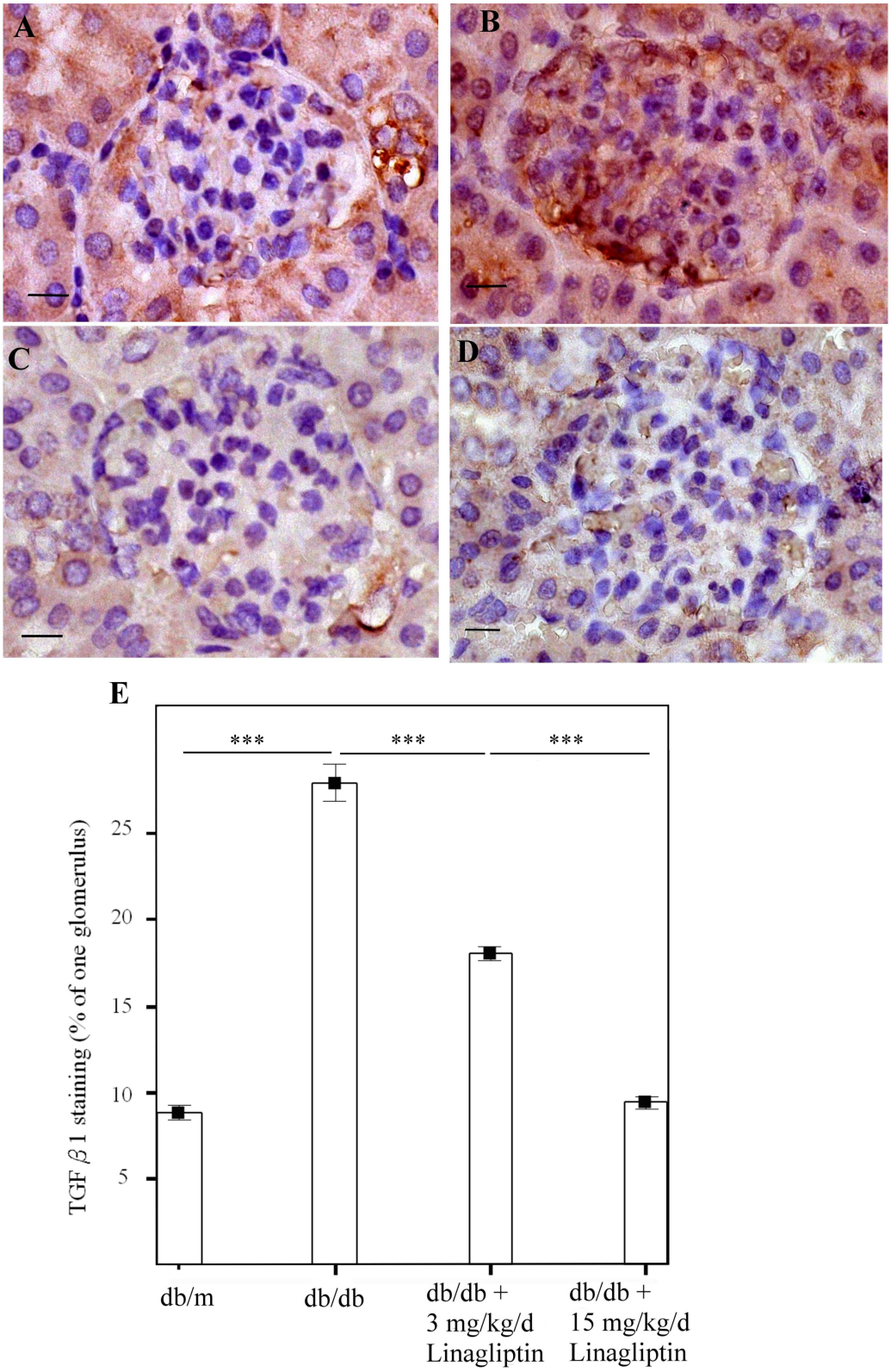



**Figure 12. IHC staining for TGF $\beta$1 in *db/db* diabetic compared to *db/m* nondiabetic kidneys at 20th week.**

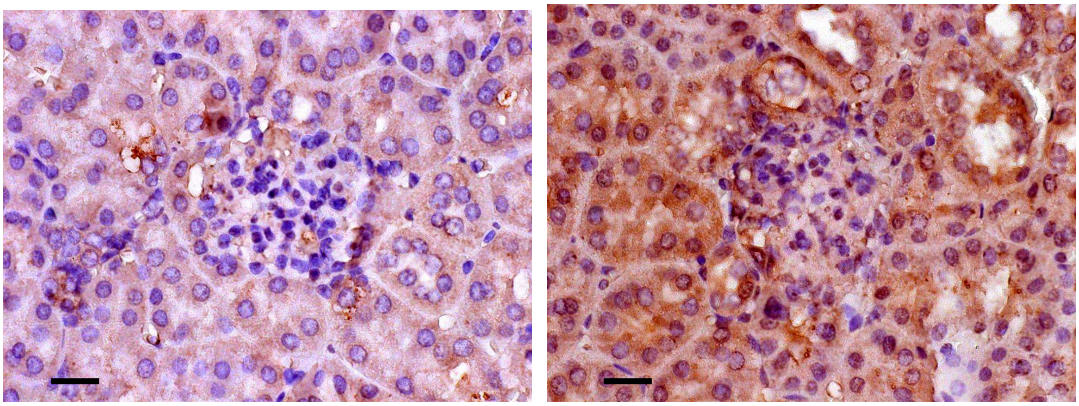
(A)(B)(C)(D) IHC staining for TGFβ1 (brown staining) in glomeruli was

increased in the *db/db* group compared to *db/m* (B vs. A). The increased glomerular staining in *db/db* (B) could not be observed in either low-dose (C) or high-dose (D) Linagliptin treatment.

(E) Quantitative data (n=100 in each group) for IHC staining for TGF $\beta$ 1 in glomeruli. Increased staining for TGFβ1 in *db/db* could be reversed by both doses of Linagliptin treatment significantly.

***p<0.001.  scale bar, 50 $\mu$ m

A. B.



C. D.

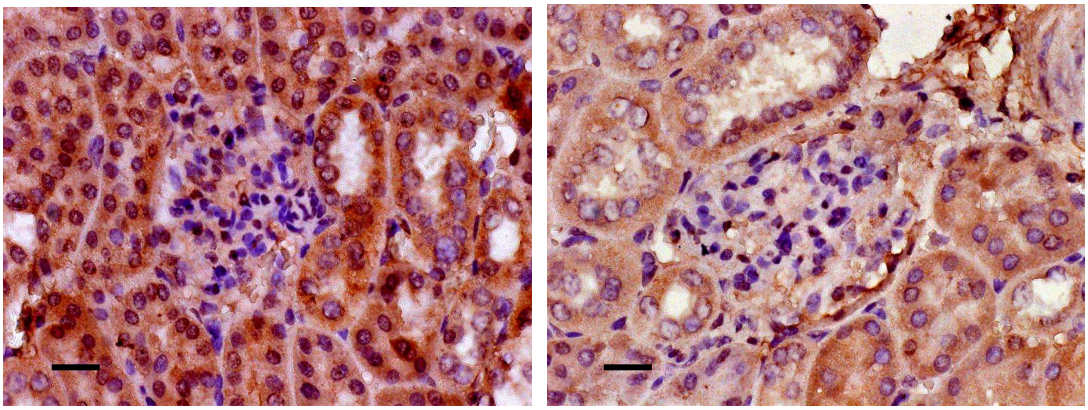


**Figure 13. IHC staining of peri-glomerular tubules for TGF $\beta$ 1**

(A) IHC staining of peri-glomerular tubules for TGF $\beta$ 1 in *db/m* diabetic kidneys at 20th week.

(B) IHC staining of peri-glomerular tubules for TGF $\beta$ 1 in *db/db* diabetic kidneys at 20th week.

(C) IHC staining of peri-glomerular tubules for TGF $\beta$ 1 in *db/db* diabetic kidneys treated by low dose Linagliptin at 20th week.

(D)IHC staining of peri-glomerular tubules for TGF $\beta$ 1 in *db/db* diabetic kidneys treated by high dose Linagliptin at 20th week.

scale bar, 50 $\mu$ m

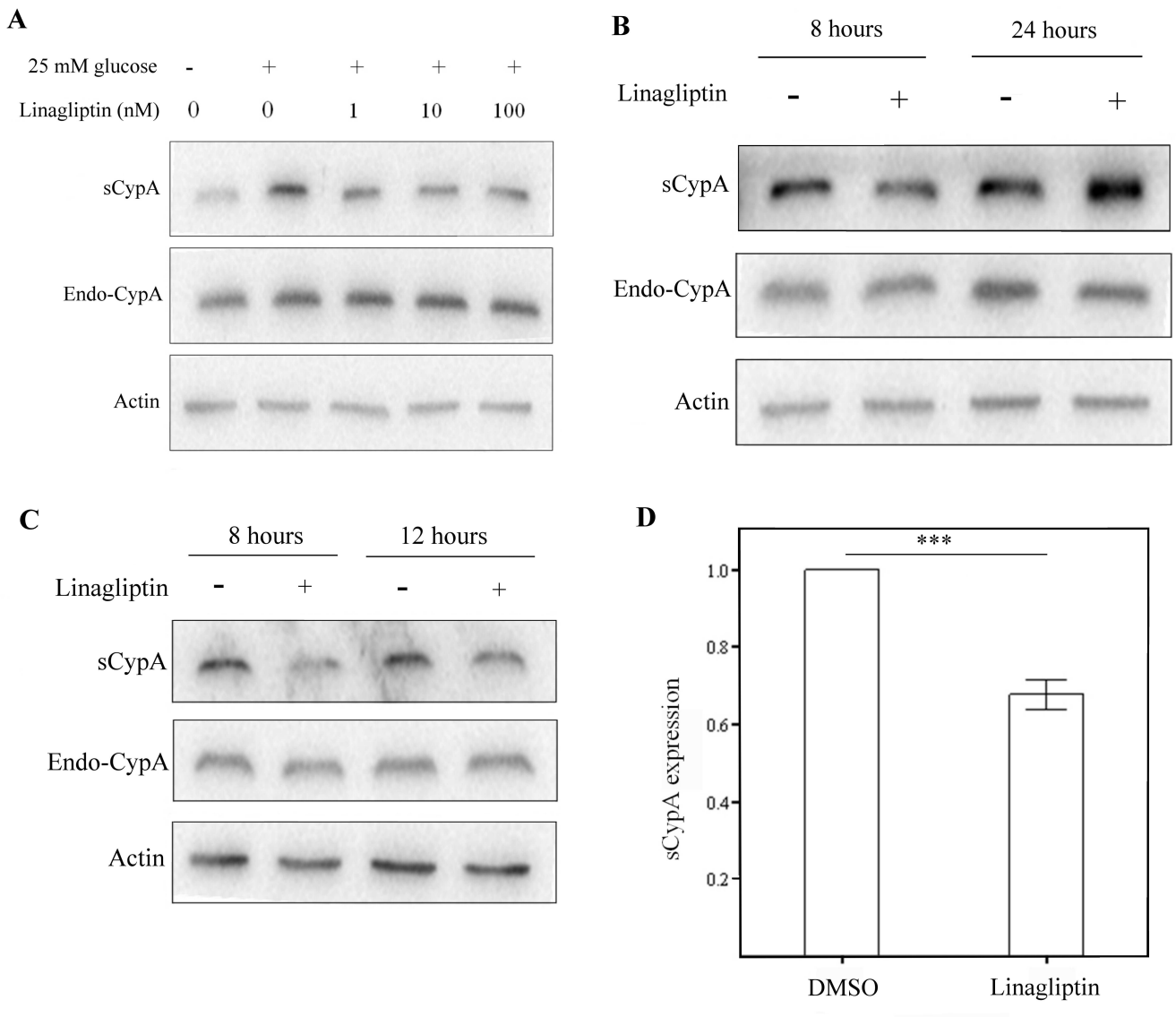



**Figure 14. Western blotting of sCypA expression in glucose treated MES-13 cells and rescued by Linagliptin.**

(A) The expression of sCypA treated by 25 mM glucose was inhibited by Linagliptin in all three different concentrations (1, 10, and 100 nM) for 8 hours.

(B) Under high glucose condition (25 mM), the 10nM Linagliptin treatment was able to inhibit the expression of sCypA for 8 hours. A longer treatment time for up to 24 hours diminished the effect.

(C) The effect of Linagliptin on the inhibition of sCypA could last for 12 hours.

(D) Statistical analysis showed that the inhibition could be sustained for at least 8 hours with statistical significance ($p<0.001$) ($n=4$).

***$p<0.001$; Endo-CypA: endogenous Cyclophilin A

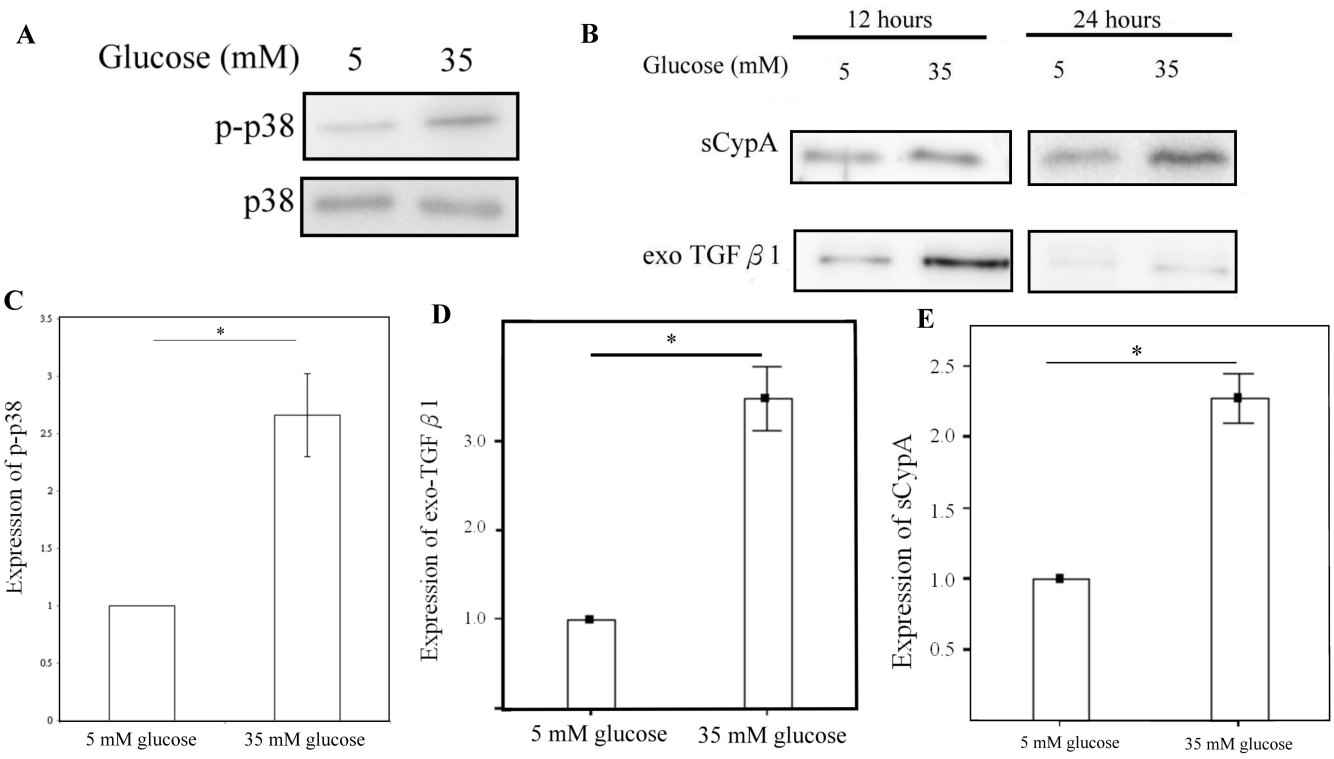



**Figure 15. Expression of phosphorylated p38, sCypA and TGFβ1 after high glucose treatment of HK-2 cells in Western blotting.**

(A) After high glucose (35 mM) treatment for 30 minutes, the expression of p-p38 increased.

(B) After high glucose (35 mM) treatment for 12 hours, the expression of TGFβ1 increased but not for sCypA. After high glucose (35mM) treatment for 24 hours, the expression of sCypA increased but not for TGF $\beta$ 1.

(C) Quantitative data for the expression of p-p38 in (A). ($p<0.05$) (n=3)

(D) Quantitative data for the expression of TGFβ1 after 12 hours treatment of high glucose. ($p<0.05$) (n=3)

(E) Quantitative data for the expression of sCypA after 24 hours-treatment of high glucose. ($p<0.05$) ($n=3$)

*$p<0.05$

Exo- TGFβ1: exogenous TGFβ1

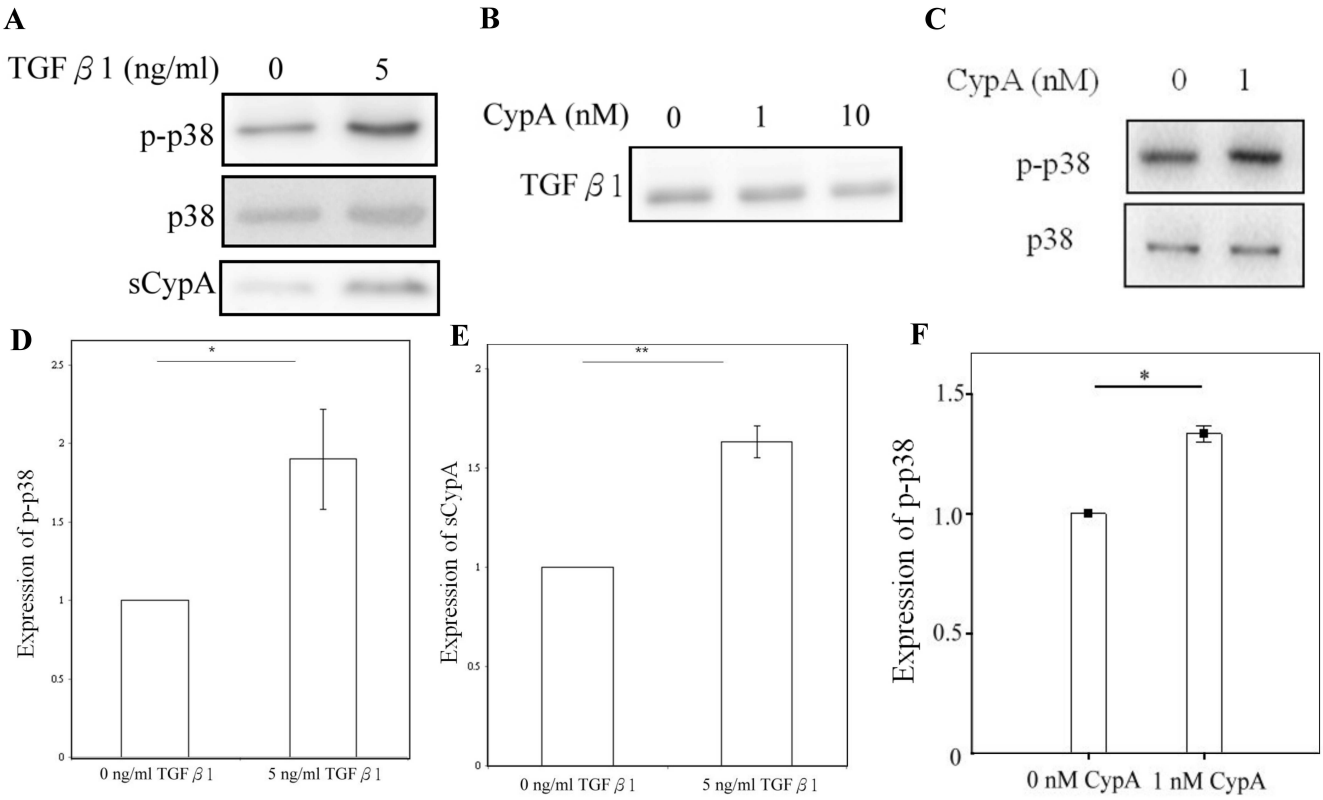



**Figure 16. Expression of phosphorylated p38 and sCypA by TGFβ1 treatment and expression of TGFβ1 by sCypA treatment in HK-2 cells.**

(A) After treatment of TGFβ1 (5ng/ml) for 30 minutes and 24 hours, the expression of p-p38 and sCypA both increased, respectively

(B) After treatment of CypA (1 and 10 nM) for 24 hours, the expression of TGFβ1 did not increase.

(C) After treatment of CypA (1 nM) for 30 minutes, the expression of p-p38 increased.

(D) P-p38 increased significantly after treatment with TGFβ1 ($p<0.05$) (n=3)

(E) SCypA increased significantly after treatment with TGFβ1 ($p<0.01$)

(n=3)

(F) P-p38 increased significantly after treatment with CypA ($p<0.05$) (n=3)

*$p<0.05$, **$p<0.01$

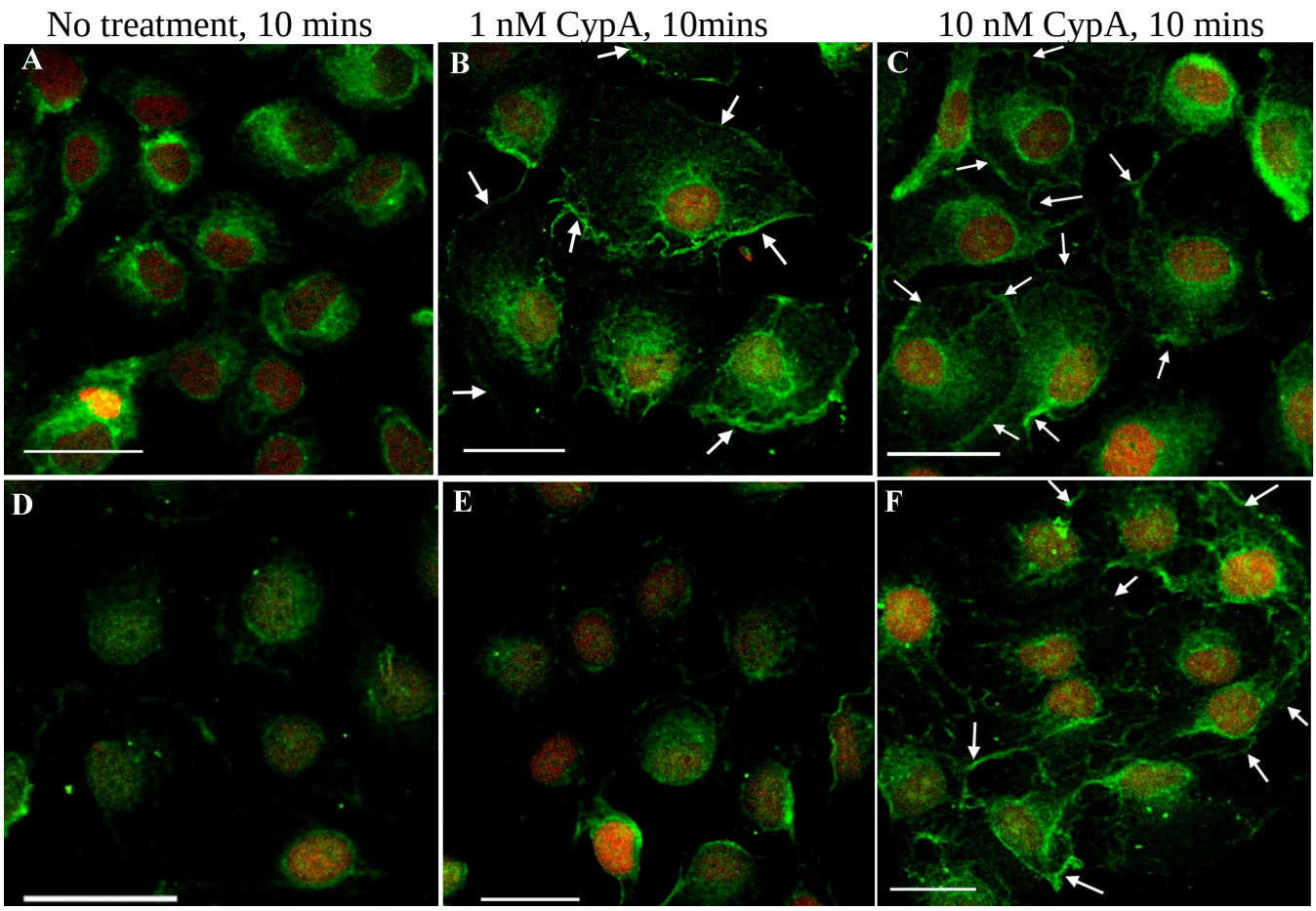



**Figure 17. Confocal microscopy for CD 147 in HK-2 cells treated with CypA and TGFβ1.**

(A) Without treatment of CypA, positive staining for CD147 (green staining) was mostly distributed in the cytoplasm near the nucleus (red staining by Propidium iodide).

(B) After treatment with 1 nM CypA for 10 minutes, the positive staining for CD147 moved closely to the cell membrane of HK-2 cells, and cell membranes can be seen very clearly (white arrow).

(C) After treatment with 10 nM CypA for 10 minutes, the positive

staining for CD147 moved further closely to the cell membrane of HK-2 cells and cell membranes can be seen much more clearly than the previous 1 nM CypA. Almost all contours of HK-2 cells can be seen clearly (white arrow).

(D) Without treatment of TGFβ1, positive staining for CD147 (green staining) was mostly distributed in the cytoplasm near the nucleus (red staining by Propidium iodide). The membrane of HK-2 cells cannot be detected.

(E)After treatment with 5 ng/ml TGFβ1 for 10 minutes, the positive staining for CD147 is mostly distributed in the cytoplasm near the nucleus. The membrane of HK-2 cells cannot be detected.

(F) After treatment with 5 ng/ml TGFβ1 for 24 hours, the positive staining for CD147 moved closely to the cell membrane of HK-2 cells, and cell membranes can be seen much more clearly (white arrow). Almost all contours of HK-2 cells can be seen clearly (white arrow).

scale bar, 50μm

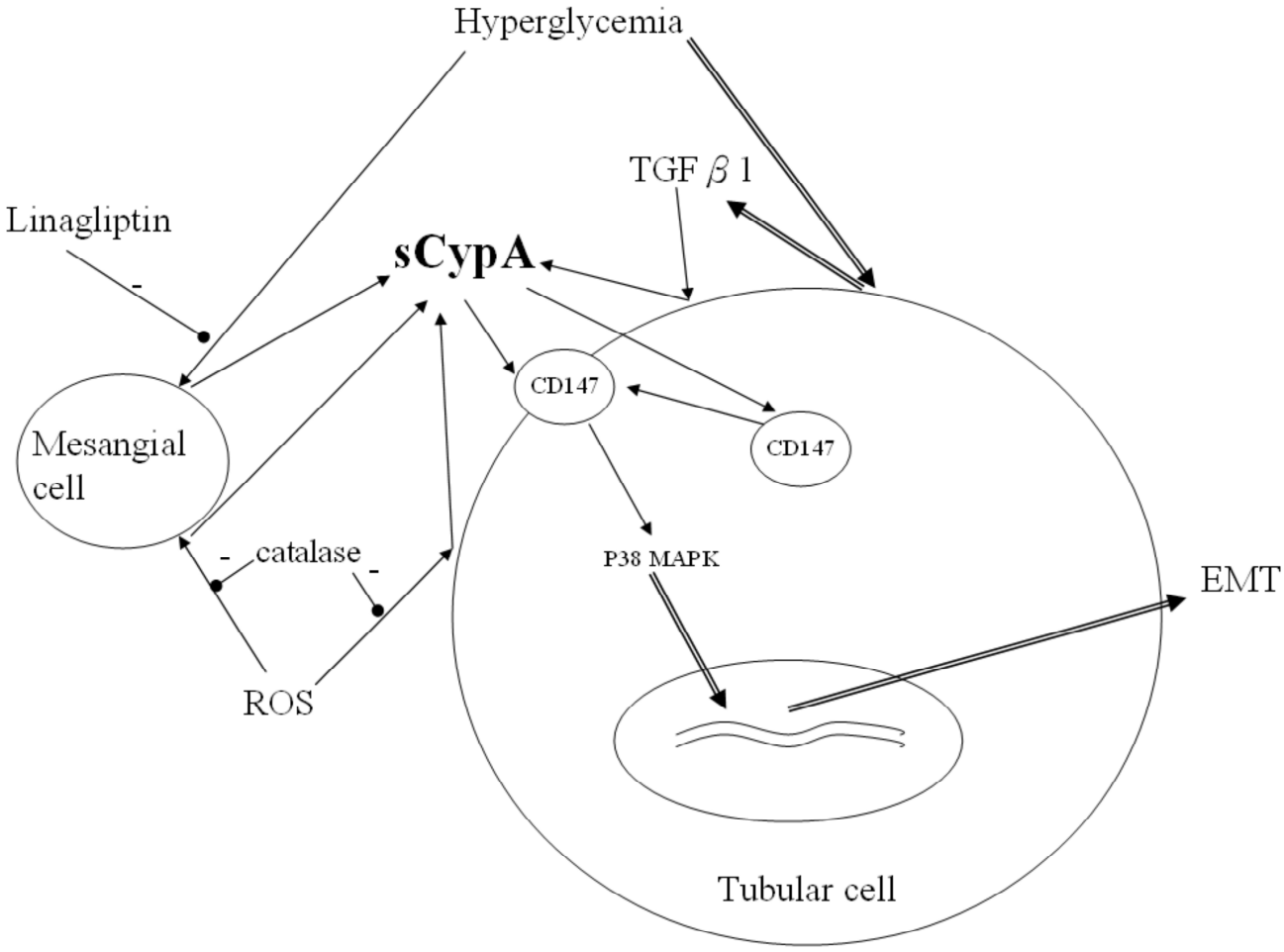



**Figure 18. Hypothesis of molecular pathway for the effects of sCypA on diabetic nephropathy and its association with Linagliptin.**

Both ROS (reactive oxidative stress) and hyperglycemia can stimulate MES-13 cells to secrete sCypA, which can be reversed by catalase and Linagliptin, respectively. ROS and hyperglycemia can also stimulate HK-2 cells to secrete sCypA. More precisely, hyperglycemia stimulates HK-2 cells to release TGFβ1, which induces HK-2 cells to secrete sCypA. The sCypA causes cytosolic CD147 to move to the cell membrane and serves as membrane receptors for sCypA. The binding of sCypA and CD

147 activates p38 as phosphorylated p38. Then the phosphorylated p38 may cause a downstream reaction, such as epithelial mesenchymal transition, which will cause diabetic nephropathy.

Two solid lines: cited from others published studies.

**Published papers**

1. **Tsai SF**, Su CW, Wu MJ, Chen CH, Fu CP, Liu CS, **Hsieh M**.

Urinary cyclophilin A as a new marker for diabetic nephropathy: a cross-sectional analysis of diabetes mellitus.

Medicine (Baltimore). 2015 Oct;94(42):e1802.

In 2014, IF=5.723, Rank=9.7% (15/154).

In 2015, IF= 2.133, rank=25.8% (40/155).

Categories: MEDICINE, GENERAL & INTERNA.

2. **Tsai SF**, Hsieh CC, Wu MJ, Chen CH, Lin TH, **Hsieh M**.

Novel findings of secreted cyclophilin A in diabetic nephropathy and its association with renal protection of dipeptidyl peptidase 4 inhibitor.

Clin Chim Acta. 2016 Dec 1;463:181-192

In 2014, IF=2.824, Rank=16.7% (5/30)

In 2015, IF=2.799, Rank=23.3% (7/30)

Categories: MEDICAL LABORATORY TECHNOLOGY

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OBSERVATIONAL STUDY

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# Urinary Cyclophilin A as a New Marker for Diabetic Nephropathy

## *A Cross-Sectional Analysis of Diabetes Mellitus*

*Shang-Feng Tsai, MD, Chien-Wei Su, Master, Ming-Ju Wu, MD, PhD, Cheng-Hsu Chen, PhD, Chia-Po Fu, MD, Chin-San Liu, MD, PhD, and Mingli Hsieh, PhD*

**Abstract:** Type 2 diabetes mellitus (DM) is the most common single cause of end-stage renal disease. Albuminuria is the most commonly used marker to predict onset of diabetic nephropathy (DN) without enough sensitivity and specificity to detect early DN. This is the first study to identify urinary cyclophilin A (CypA) as a new biomarker for early DN.

We recruited DM outpatients and healthy control subjects from January 2014 to December 2014. In this cross-sectional study, patients' urine samples were collected to determine the expression of urinary CypA. We also treated mesangial (MES-13) and tubular (HK-2) cells with glucose or free radicals to observe the expression of secreted CypA in Western blot analysis.

A total of 100 DN patients and 20 healthy control subjects were enrolled. All variables were matched. In univariate analysis, the concentration of urinary CypA correlated well with the progression of renal function. A significant increase in urinary CypA was noted in stage 2 DN and persisted in later stages. We could diagnose stage 2 DN using urinary CypA with a sensitivity of 90.0% and specificity of 72.7%. The area under curve was up to 0.85, indicating a good discriminatory power. In cellular models, MES-13 and HK-2 cells can both release CypA.

Urinary CypA is a good biomarker for early DN detection in humans and it can be released from either mesangial or tubular cells. The underlying molecular mechanisms still need further clarification in cellular and animal studies.

(*Medicine* 94(42):e1802)

**Abbreviations**: AUC = area under curve, CypA = cyclophilin A, CKD = chronic kidney disease, CAD = coronary arterial disease, CVD = cardiovascular disease, DM = diabetes mellitus, DN = diabetic nephropathy, ESRD = end-stage renal disease, GFR = glomerular filtration rate, L-FABP = liver-type fatty acid-binding protein, MES-13 = mesangial, PTEC = proximal tubule epithelial cell, RA = rheumatoid arthritis, sCypA = secreted cyclophilin A.

## INTRODUCTION

Type 2 diabetes mellitus (DM) is the most common single cause of end-stage renal disease (ESRD).[1] ESRD in almost half of patients is due to diabetic nephropathy (DN), and these cases have the worst outcome compared to patients with other causes of ESRD. Although there are many novel drugs for DM, there are no specific curative treatments yet for DN. Reasons for poor outcome include inadequate markers and the complicated mechanisms of DN.[2] Currently, the stage of severity is determined according to the levels of albuminuria. Albuminuria is the most commonly used marker to predict onset and progression of DN clinically. However, this traditional marker for DN lacks both sensitivity and specificity to detect early stage of DN.[3] Furthermore, some DN patients with ESRD do not present with significant albuminuria.[4–6] The lack of a strong association between glomerular filtration rate (GFR) and albuminuria suggests that an alternative to this albuminuria-based staging system is needed. Some studies have noted the existence of pathological change before microalbuminuria.[4] Therefore, even if microalbuminuria can be regarded as the earliest manifestation of DN, it is possible that a new biomarker for DN exists. Recently, different markers of DN were reviewed[4,7,8] including fibroblast growth factor 23,[9] tubular markers[10] (kidney injury molecule 1, neutrophil gelatinase-associated lipocalin, and liver-type fatty acid-binding protein [L-FABP]),[11] inflammatory markers (interleukin 6 [IL-6], IL-8, monocyte chemoattractant protein 1, and interferonγ–inducible protein),[12] urinary 8-hydroxy-20-deoxyguanosine,[13] serum cystatin C,[14] and so on. Among these, genetic susceptibility almost always leads to irreversible DN, and detection of the clinical markers mostly occurs too late to diagnose and monitor the progression of DN. As such, it is crucial to find an earlier and

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Urinary cyclophilin A is a new and earlier biomarker for diabetic nephropathy.
S-F T and C-W S carried out the molecular studies. S-F T, M-J W, C-H C, and MH drafted the manuscript. S-F T and C-W S carried out the immunoassays. S-F T, M-J W, C-H C, C-P F, C-S L, and MH participated in the design of the study. S-F T performed the statistical analysis. S-F T, M-J W, C-H C, C-S L, and MH conceived of the study. S-F T and MH participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.
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reliable marker for DN. Earlier diagnosis and intervention may provide an opportunity to stop the permanent damage caused by DN.

Cyclophilin A (CypA) is an 18-kDa protein with ubiquitous characteristics.[15] It is mostly distributed in the cytoplasm and facilitates protein folding and protein trafficking. It also acts as a cellular receptor for cyclosporine A (CsA). The expression of CypA is relatively high in the kidney,[16] where proximal tubular epithelial cells (PTECs) are reported to contain considerably more CypA than other kidney tissues.[17] With respect to kidney diseases, the majority of research has been on the cellular relationship between CypA and CsA, which is used as an immunosuppressant, and leaves behind its secreted form. This secreted CypA (sCypA) was reported to be correlated with cardiovascular disease (CVD), asthma, rheumatoid arthritis (RA), and lung and liver injury.[18] sCypA has been suggested to be a potential biomarker and mediator in CVD.[19]

In addition, sCypA is associated with inflammatory or infectious diseases such as RA, asthma, and periodontitis.[20] Interestingly, sCypA was also detected in diabetic patients' plasma[21] and was shown to be secreted by monocytes in response to hyperglycemia,[22] indicating that sCypA could be a potential secretory marker in type 2 DM.[22] Furthermore, a relatively high expression level of CypA in normal kidneys[16] has led to speculation that sCypA may be associated with solid organ damage. As a product directly produced by kidney, urine could be best measure for renal injury detection. Therefore, we postulated that CypA level in urine would be the most suitable indicator of DN.

## RESEARCH DESIGN AND METHODS

### Study Population

We recruited all the DM outpatients and healthy control groups with informed consent. In the group of DM patients, the different stages of DN were screened for the concentrations of urinary CypA. All subjects in this cross-sectional study were 20 years of age and older. Patients were free from infectious disease, inflammatory disease, liver disease, or malignancy, and all were nonsmokers. Only metabolic syndrome and/or CVD were noted. Patients who took drugs for hypertension, DM, hyperlipidemia, hyperuricemia, CVD, hyperuricemia, and gout were not excluded. Patients who took drugs for any other disease or condition were excluded. These data were collected in the outpatient department of metabolism and nephrology at Taichung Veterans General Hospital between January 2014 and December 2014. All of the study procedures were conducted in accordance with the ethical standards of Taichung Veterans General Hospital and were approved by the institutional review committee (CE14077, TCVGH).

### Data Collection

All DM patients were diagnosed according to the DM guidelines of the American Diabetes Association in 2013.[23] We collected the participants' clinical parameters including gender, age (years old), and duration after diagnosis of DM (years). The stages of DN were categorized according to the previous literature[24] where stage 1 is associated with hyperfiltration and a measured GFR exceeding the upper limit of the normal range (120 mL/min per 1.73 $m^2$) or beyond +2 standard deviation from mean GFR. Stage 2 DN develops silently over many years and is characterized by morphologic lesions without signs of clinical disease. Thus, it is usually called the silent stage. Stage 3 DN is characterized by ''microalbuminuria'' where urinary albumin excretion is between 30 and 300 mg/day or between 30 and 300 mg/g creatinine on a spot urine sample. Patients with normal GFR (no >2SD of GFR) and without microalbuminuria were defined as stage 2 DN. More importantly, some patients with normal GFR (no >2SD of GFR) and without microalbuminuria do not have DN. Patients included in our study should fit the above criteria and should have increased GFR (>2 SD of GFR) before timing of recruitment (progression of stage 1 DN) to make sure they really had DN and they were in the stage 2 of DN. Stage 4 DN is defined by severely increased albuminuria, also known as the ''macroalbuminuria'' (urinary albumin excretion above 300 mg/day or above 300 mg/g creatinine on a spot urine sample). The final stage, stage,[5] is known as ESRD. Blood samples were tested for fasting sugar (mg/dL), glycated hemoglobin (%), SCr (mg/dL), GFR (mL/min per 1.73 $m^2$),[25] total cholesterol (mg/dL), triglyceride (mg/dL), and low density lipoprotein cholesterol (mg/dL). Spot urine test was used to measure the concentration of CypA (ng/mL) and albumin creatinine ratio (ACR) (mg/g). The index estimated glomerular filtrate rate (eGFR) was calculated using the modification of diet in renal disease (MDRD) equation:[25] eGFR (mL/min per 1.73 $m^2$) = $186^{*}SCr^{-1.154}$ $^{*}Age^{-0.203*}0.742$ (if female). Patients were screened for CVD (hypertension, stroke, coronary artery disease, heart failure, and aortic aneurysm). Hypertension was defined as an average home systolic blood pressure greater than 140 mmHg and a diastolic blood pressure greater than 90 mmHg before medication according to the definition for stage I/II hypertension set forth in the JNC-7 guidelines.[26] Patients currently receiving antihypertensive agents were deemed to have hypertension. Stroke was confirmed by neurologists or brain images. Coronary artery disease (CAD) was defined according to arterial angiography. Some were diagnosed according to cardiologists, who made diagnosis of CAD according to if patients with typical angina pectoris, myocardial infarction, or silent myocardial ischemia. They used electrocardiogram, cardiac enzyme, coronary calcium score, and stress test to diagnose CAD. Heart failure was confirmed by cardiac sonography or the guidelines of the Framingham study.[27] Drugs such as angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers, dipeptidyl peptidase 4 inhibitor sulfonylurea, metformin, dipyridamole, pentoxifylline, and statin were also recorded to analyze possible correlations with urinary CypA concentration. We would also like to point out how we select patients. All data including medication, laboratory, and clinical parameters are without significant changes within 6 months or between 2-time outpatient department visits. We checked all parameters during the period of recruitment. If they remain similar to the previous data, we include them in the study. If there are significant changes, we will follow up this patient 3 months later and choose the data (the ones after 3 months) if it became similar to the previous data.

### Urine Collection and Analysis

Urine was collected in the morning from the outpatient subjects and stored in an ice package immediately. Within 4 hours, it was then restocked under −80 °C until analysis. The expression of urinary CypA was examined using an enzyme-linked immunosorbent assay kit (SEA979Hu, Uscn Life Science Inc., Texas, USA). All data of urinary CypA were double-checked at least twice.

## MATERIALS AND METHODS OF CELL STUDY

### Cell Culture

Mesangial cell (MES-13 cells, glomerular mesangial cells from an SV40 transgenic mouse) were obtained from the American Type Culture Collection (CRL-1927; Manassas, VA). MES-13 cells were cultured in a 3:1 mixture of M199 (Invitrogen, Carlsbad, CA) and Ham F-12 (Invitrogen), supplemented with 5% FBS, 1% penicillin–streptomycin, 1% L-glutamine, and 14 mM HEPES, and maintained at 37 °C in an incubator with 5% $CO_2$. All culture supplies were acquired from Life Technologies (Gaithersburg, MD). HK-2 cells (human PTEC) were obtained from American Type Culture Collection (CRL-2190; Manassas, VA). HK-2 cells were maintained in DMEM/F12 and supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/amphotericin B, and 1% glutamine (Invitrogen, Carlsbad, CA).

### Western Blotting

The cell lysates were collected from MES-13 and HK-2 cells, and Western blot analysis was performed as described previously.[28] Western blot reagents were obtained from Pierce (Rockford). Primary antibodies included polyclonal anti-CypA (1:10,000, Millipore, MA) and β-actin (Novus, Colorado) overnight at 4 °C followed by incubation with a horseradish peroxidase conjugated secondary antibody. Proteins were visualized using enhanced chemiluminescence (Amersham Biosciences, Amersham, UK). Protein bands of Western blot analysis were quantified using Quantity One software (BioRad). All other chemical supplies were acquired from Sigma (St Louis, MO).

### Glucose Treatment

MES-13 and HK-2 cells were seeded in a 6 cm cell culture plate with $4 \times 10^5$ and $3 \times 10^5$ cells/plate, respectively. They were incubated in M199:F12 (3:1) and DMEM:F12 (1:1) complete medium, respectively, for 1 day. Then culture media were replaced by 0.5% FBS (M199/low glucose:F12 = 3:1) for 2 days. The cells were incubated in the serum-free media supplemented with 0, 10, 25, and 50 mM of glucose. The procedure was conducted according to the methods described in a previous study (Su et al, unpublished data). After 24 hours of treatment, the secreted cellular proteins were collected for immunoblotting analysis.

## $H_2O_2$ TREATMENT

MES-13 and HK-2 cells were seeded in a 6 cm cell culture plate with $5 \times 10^5$ and $3 \times 10^5$ cells/plate, respectively. They were incubated in M199:F12 = 3:1 complete medium for 1 day and were then replaced by serum-free medium (M199:F12 = 3:1) and incubated for another 2 days. After 30 minutes of 0, 20 μM, or 40 μM of $H_2O_2$ stimulation, the secreted and cellular proteins were collected for immunoblotting analysis. To confirm the role of $H_2O_2$ in sCypA upregulation, mesangial cells and tubular cells were treated for 30 minutes with 20, and 40 μM of $H_2O_2$ in the presence or absence of 300 U/mL catalase (Sigma, St Louis, MO) according to a previously described method.[29]

### Statistical Analyses

Data were expressed as the mean ± SD in continuous variables. Mann–Whitney *U* test was used for continuous variables and the Chi-square test was used for categorical variables. A general linear model was used for categorical variables, and simple linear regression was used for continuous variables. The results from Western blot were expressed as mean ± SEM and were analyzed by Student's *t*-test. All statistical procedures were performed using the SPSS statistical software package, version 17.0 (Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

## RESULTS

### Baseline Characteristics of Cohorts

A total of 100 DN patients and 20 healthy control subjects were enrolled in this study (Table 1). The DN patients were categorized according to their stages of DN with matched basic variables. The control individuals were healthy subjects without any metabolic syndrome or medical drug treatment. Among all 100 DN patients, there were no significant differences in gender distribution ($P = 0.553$), age ($P = 0.469$), fasting sugar ($P = 0.403$), glycated hemoglobin ($P = 0.352$), total cholesterol ($P = 0.447$), triglyceride ($P = 0.324$), or low density lipoprotein cholesterol ($P = 0.199$). Prevalence rates of other metabolic syndromes and CVD were both similar, including hypertension ($P = 0.668$), stroke ($P = 0.480$), coronary artery disease ($P = 0.724$), heart failure ($P = 0.712$), aortic aneurysm ($P = 1.000$), and hyperlipidemia ($P = 0.075$). All included drugs were matched (except metformin) as well, such as angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers ($P = 0.144$), insulin ($P = 0.625$), dipeptidyl peptidase 4 inhibitor ($P = 0.710$), sulfonylurea ($P = 0.276$), dipyridamole ($P = 0.740$), pentoxifylline ($P = 0.121$), and statin ($P = 0.095$). It was not possible to match the usage of metformin because it is contraindicated in advanced DN. Among all the basic characteristics, it was difficult to match duration of DM ($P = 0.009$) because progression of DN is highly time-dependent. Patients with more severe DN had higher serum creatinine (SCr) ($P < 0.001$), lower GFR ($P < 0.001$), higher ACR ($P < 0.001$), and higher urinary CypA ($P < 0.001$). Taken together, all basic variables were matched (except metformin), and with later stage of DN, patients had worse renal function parameters, including SCr, GFR, and ACR. Importantly, the concentrations of urinary CypA were statistically different among the different stages of severity of DN.

### Correlation Between Urinary CypA and Other Clinical Variables

Because some variables are clinically associated with renal functions, we performed univariate analysis to verify the associations between these variables and urinary CypA (Table 2). Our analysis showed that if patients did not use metformin, the concentration of urinary CypA would increase by 3.281 ng/mL (Fig. 1A). The concentration of urinary CypA increased by 0.395 ng/mL for each 1 mg/dL increase of SCr. With each 1 mL/min decrease in GFR, the concentration of urinary CypA increased by 0.030 ng/mL. Without proteinuria, the concentration of urinary CypA decreased by 3.095 ng/mL (Fig. 1B). Even though there were no statistically significant differences among the different stages of chronic kidney disease (CKD) (Fig. 1D), there seemed to be a trend of increasing urinary CypA in the later stages of CKD. Also, there was a trend of higher urinary CypA in the group with GFR less than 60 mL/min per 1.73 $m^2$ as compared with the GFR group with more than 60 mL/min per 1.73 $m^2$ (Fig. 1C). For each 1 mg/g increase in ACR, the concentration of urinary CypA increased by 0.001 ng/mL (Fig. 2A and Table 2). All of the abovementioned

**TABLE 1.** Demography of Different Stages of Diabetic Nephropathy

| Diabetic Nephropathy Stage | Healthy Control Groups | 1 | 2 | 3 | 4 | 5 | $P^*$ Value | Total of DN Patients |
|---|---|---|---|---|---|---|---|---|
| Number | 20 | 20 | 20 | 20 | 20 | 20 | | 100 |
| Gender (male) (n, %) | 10, 50.0% | 10, 50.0% | 13, 65.0% | 12, 60.0% | 10, 50.0% | 10, 50.0% | 0.469 | 55, 55.0% |
| Age (y/o) | 45.8 ± 3.2 | 42.9 ± 13.1 | 59.2 ± 11.1 | 56.6 ± 11.2 | 59.3 ± 9.9 | 62.7 ± 8.1 | 0.469 | 57.3 ± 11.6 |
| Duration, years | – | 3.4 ± 2.7 | 5.7 ± 4.2 | 9.6 ± 8.7 | 10.6 ± 7.6 | 17.0 ± 9.3 | 0.009 | 7.9 ± 7.4 |
| Fasting sugar, mg/dL | 89.6 ± 5.4 | 147.8 ± 57.3 | 144.8 ± 57.3 | 151.0 ± 65.5 | 152.3 ± 47.4 | 121.4 ± 21.0 | 0.403 | 142.9 ± 50.5 |
| HbA1c, %[†] | 5.6 ± 1.3 | 7.2 ± 2.3 | 7.8 ± 2.8 | 7.6 ± 1.7 | 7.7 ± 2.6 | 6.4 ± 0.8 | 0.352 | 7.4 ± 2.3 |
| Creatinine, mg/dL | 0.70 ± 0.36 | 0.60 ± 0.10 | 1.09 ± 0.49 | 1.13 ± 0.49 | 3.28 ± 3.25 | 7.54 ± 4.12 | <0.001 | 2.41 ± 3.11 |
| GFR[‡], mL/min per 1.73 $m^2$ | 142.1 ± 10.0 | 133.6 ± 19.3 | 77.4 ± 28.2 | 78.2 ± 38.9 | 37.7 ± 30.1 | 7.4 ± 4.12 | <0.001 | 69.1 ± 48.2 |
| Total cholesterol, mg/dL | 190.2 ± 15.2 | 145.2 ± 50.0 | 149.2 ± 24 | 166.7 ± 43.6 | 180.9 ± 60.5 | 154.6 ± 42.1 | 0.447 | 162.7 ± 47.2 |
| Triglyceride, mg/dL | 100.8 ± 30.3 | 156.6 ± 55.1 | 133.6 ± 69.3 | 191.8 ± 103.5 | 162.7 ± 104.6 | 179.7 ± 134.7 | 0.324 | 161.0 ± 96.3 |
| Low density lipoprotein cholesterol, mg/dL | 77.3 ± 10.2 | 86 ± 29.8 | 88.0 ± 17.4 | 86.2 ± 31.6 | 119.5 ± 52.6 | 87.9 ± 34.8 | 0.199 | 96.4 ± 39.2 |
| ACR, mg/g[§] | 10.3 ± 5.6 | 11.7 ± 18.1 | 11.0 ± 27.3 | 142.6 ± 82.2 | 2426.0 ± 2409 | 4893 ± 3485 | <0.001 | 1238.4 ± 237.7 |
| CypA[‖], ng/mL | 0.7354 ± 0.4510 | 0.6088 ± 0.3795 | 1.8684 ± 2.0116 | 3.0157 ± 9.4954 | 4.11580 ± 3.6078 | 7.3818 ± 04.4710 | <0.001 | 3.1638 ± 5.3630 |
| Hypertension (n, %) | 0, 0% | 13, 65.0% | 12, 60% | 13, 65% | 12,60% | 13, 65% | 0.668 | 65, 65.0% |
| Stroke (n, %) | 0, 0% | 1, 5% | 2, 10% | 2, 10% | 2, 10% | 1, 5.0% | 0.480 | 8,8.0% |
| Coronary artery disease (n, %) | 0, 0% | 0, 0% | 2, 10% | 2, 10% | 2, 10% | 2, 10% | 0.724 | 8, 8% |
| Heart failure (n, %) | 0, 0% | 0, 0% | 2, 10% | 1, 5% | 3, 15% | 1,5.0% | 0.712 | 7, 7.0% |
| Aortic aneurysm (n, %) | 0, 0% | 0, 0% | 0, 0% | 0, 0% | 0, 0% | 0, 0% | 1.000 | 0, 0% |
| ACEi[¶] or ARB[#] (n, %) | 0, 0% | 12, 60.0% | 9, 45% | 9, 45% | 13, 65% | 18, 90.0% | **0.144** | 61, 61.0% |
| Insulin (n, %) | 0, 0% | 2, 10.0% | 6, 30% | 5, 25% | 7, 35% | 7, 35.0% | 0.625 | 27, 27.0% |
| DPP4i[$] (n, %) | 0, 0% | 10, 50.0% | 6, 30% | 9, 45% | 10, 50% | 10, 50.0% | 0.710 | 45, 45.0%. |
| Sulfonylurea (n, %) | 0, 0% | 4, 20.0% | 4, 20% | 8, 40% | 9, 45% | 10, 50.0% | 0.276 | 35, 35.0% |
| Metformin (n, %) | 0, 0% | 18, 90.0% | 17, 85% | 10, 50% | 8, 35% | 0, 0% | **<0.001** | 53, 53.0% |
| Dipyridamole (n, %) | 0, 0% | 0, 0% | 1, 5% | 0, 0% | 1,5% | 0, 0% | 0.740 | 2, 2.0% |
| Pentoxifylline (n, %) | 0, 0% | 0, 0% | 1,5% | 3, 15% | 6, 30% | 6, 30.0% | 0.121 | 16, 16.0% |
| Statin (n, %) | 0, 0% | 20, 100% | 14, 70% | 11, 55% | 17, 85% | 12, 60.0% | 0.095 | 74, 74.0% |
| Hyperlipidemia (n, %) | 0, 0% | 20, 100% | 16, 80% | 11, 55% | 19, 95% | 18, 90.0% | 0.075 | 84, 84.0% |
| Dialysis (n, %) | 0, 0% | 0, 0% | 0, 0% | 0, 0% | 0, 0% | 20, 100% | **<0.001** | 20, 20% |
| GFR[‡] >60 mL/min per 1.73 $m^2$ | 6, 100% | 20, 100% | 16, 80% | 12, 60% | 5, 25% | 0, 0% | **<0.001** | 53, 53.0% |
| CKD[-] stage | 0, 0% | 1.0 ± 0.0 | 1.9 ± 0.9 | 2.1 ± 0.9 | 3.5 ± 1.2 | 4.5 ± 0.9 | <0.001 | 2.5 ± 1.5 |

DN = diabetic nephropathy.
[*] Kruskal Wallis test between 5 stages of diabetic nephropathy.
[†] Glycated hemoglobin.
[‡] Glomerular filtration rate.
[§] Urine albumin creatinine ratio.
[‖] Cyclophilin A.
[¶] Angiotensin-converting-enzyme inhibitor.
[#] Angiotensin II receptor blockers.
[$] Inhibitors of dipeptidyl peptidase 4.

**TABLE 2.** Univariate Analysis and Multivariate Analysis

| **Univariate Analysis*** | ***P* Value** | $R^2$ |
|---|---|---|
| Age | 0.605 | |
| Gender | 0.305 | |
| Hypertension | 0.997 | |
| Stroke | 0.664 | |
| Coronary arterial disease | 0.470 | |
| Heart failure | 0.570 | |
| ACEi† or ARB‡ | 0.773 | |
| Sulfonylurea | 0.507 | |
| Insulin | 0.973 | |
| DPP4i§ | 0.952 | |
| Metformin | 0.004 | 0.095 |
| CypA$ = 1.581 + metformin (use)*0 or metformin (no use)*3.281 | | |
| Dipyridamole | 0.794 | |
| Pentoxifylline | 0.648 | |
| Statin | 0.177 | |
| DM | 0.425 | |
| Hyperlipidemia | 0.255 | |
| Proteinuria | 0.008 | 0.082 |
| CypA$ = 4.438 + proteinuria(yes)*0 or no proteinuria*(−3.095) | | |
| Dialysis | 0.389 | |
| GFR $\geq$ 60 mL/min per 1.73 $m^2$ | 0.060 | |
| Duration | 0.957 | |
| Fasting sugar | 0.898 | |
| HbA1c‖ | 0.686 | |
| Creatinine | 0.037 | 0.052 |
| CypA$ = 2.241 + Cr*0.395 | | |
| Glomerular filtration rate | 0.013 | 0.052 |
| CypA$ = 5.270 + GFR*(−0.030) | | |
| Total cholesterol | 0.133 | |
| Triglyceride | 0.567 | |
| Low density lipoprotein cholesterol | 0.796 | |
| ACR¶ | 0.034 | 0.054 |
| CypA$ = 2.461 + ACR*0.001 | | |
| Multivariate analysis | *P* value | $R^2$ |
| ACR | 0.414 | |
| GFR# | 0.547 | |
| Metformin | 0.081 | |

* General linear model for categorical variables and simple linear regression for continuous variables.
† Angiotensin-converting-enzyme inhibitor.
‡ Angiotensin II receptor blockers.
§ Inhibitors of dipeptidyl peptidase 4.
‖ Glycated hemoglobin.
¶ Urine albumin creatinine ratio.
# Glomerular filtration rate.
$ Cyclophilin A.

variables were renal function-related or renal function-dependent. In summary, the concentration of urinary CypA correlated well with the progression of renal function in DN patients, based on the albuminuria-based model.

## Urinary CypA Correlated With the Severity of DN Stages

The relationship between urinary CypA and ACR is summarized in Fig. 2A. The $R^2$ was 0.054 with a statistically significant correlation between urinary CypA and ACR. Moreover, we analyzed the correlation of urinary CypA among all 6 groups, including 5 stages of DN. The concentrations of urinary CypA were not different between the control group and stage 1 of DN ($P = 0.117$) (Fig. 2B). However, with progression of DN, urinary CypA significantly increased in stage 2 DN compared to that in stage 1 DN ($P = 0.012$). Most importantly, the concentration of urinary CypA increased as DN stages progressed ($P = 0.003$, $<0.0001$, and 0.005 between stage 2 and 3, stages 3 and 4, stages 4 and 5, respectively). Consistently, compared to patients with DN stage 1, the CypA concentrations in patients with DN stages 2 to 5 were significantly increased ($P = 0.006$) (Fig. 2C).

## Diagnosis of Silent Stage of DN via Urinary CypA

Since the concentration of urinary CypA significantly increased in stage 2 DN (silent stage) and it persistently increased significantly with the progression of DN, we performed an analysis of the receiver operating characteristic curve (Fig. 3). Our analysis demonstrated that when the concentration of urinary CypA was more than 0.7250 ng/mL, we could diagnose the silent stage of DN with a sensitivity of 90.0% and specificity of 72.7%. The area under curve (AUC) was up to 0.85, indicating that the use of urinary CypA for the diagnosis of silent stage of DN had a moderately good discriminatory power.

## Secreted CypA in Mesangial Cells Treated With High Concentration of Glucose or Free Radicals

At the microscopic level, there are 3 major histological changes in the glomeruli in DN: mesangial expansion, glomerular basement membrane thickening, and glomerular sclerosis.[30] A hyperglycemic state stimulates mesangial cell matrix production[31] and mesangial cell apoptosis.[32] Hence, we examined whether sCypA was secreted from mesangial cells following high glucose treatment. As shown in Figure 4A, glucose increased sCypA level in a dose-dependent manner (10, 25, and 50 mM). Statistical analysis showed that the increased expression of sCypA was found at 25 mM versus control ($P = 0.037$) and 50 mM versus control ($P = 0.037$). Expression of sCypA was much higher at 50 versus 10 mM ($P = 0.018$) (Fig. 4B). Meanwhile, it is known that reactive oxidative stress also plays an important role in DN. NADPH oxidase-mediated renal reactive oxidative stress promotes mesangial expansion and albuminuria.[33] We found that the expression of sCypA was significantly increased after 20 or 40 μM $H_2O_2$ treatment for 30 minutes (Fig. 4C). Quantitative assessment showed that either 20 or 40 μM $H_2O_2$ treatment significantly increased the expression of sCypA, which could be reversed by 300 U/mL of catalase (scavenger of free radicals) (Fig. 4D), which was used to counteract the effects of $H_2O_2$. It is worth noting that all the experiments were carefully performed with proper controls to eliminate CypA released from cell death. Taken together, free radicals or high concentrations of glucose stimulate the secretion of CypA from mesangial cells, suggesting that there is a link between sCypA and pathogenesis of DN.

## sCypA Released From HK-2 Cells Upon High Glucose Or Free Radical Treatment

Mesangial cell injury is the classical expression of DN, but recent studies suggested that DN is also a tubular disease. Early changes in tubular epithelial cells may be an essential factor in the development of progressive kidney diseases.[34] HK-2 cells, human PTEC, have been used as a cell model to study tubular diseases. Therefore, Western blotting was used to disclose the

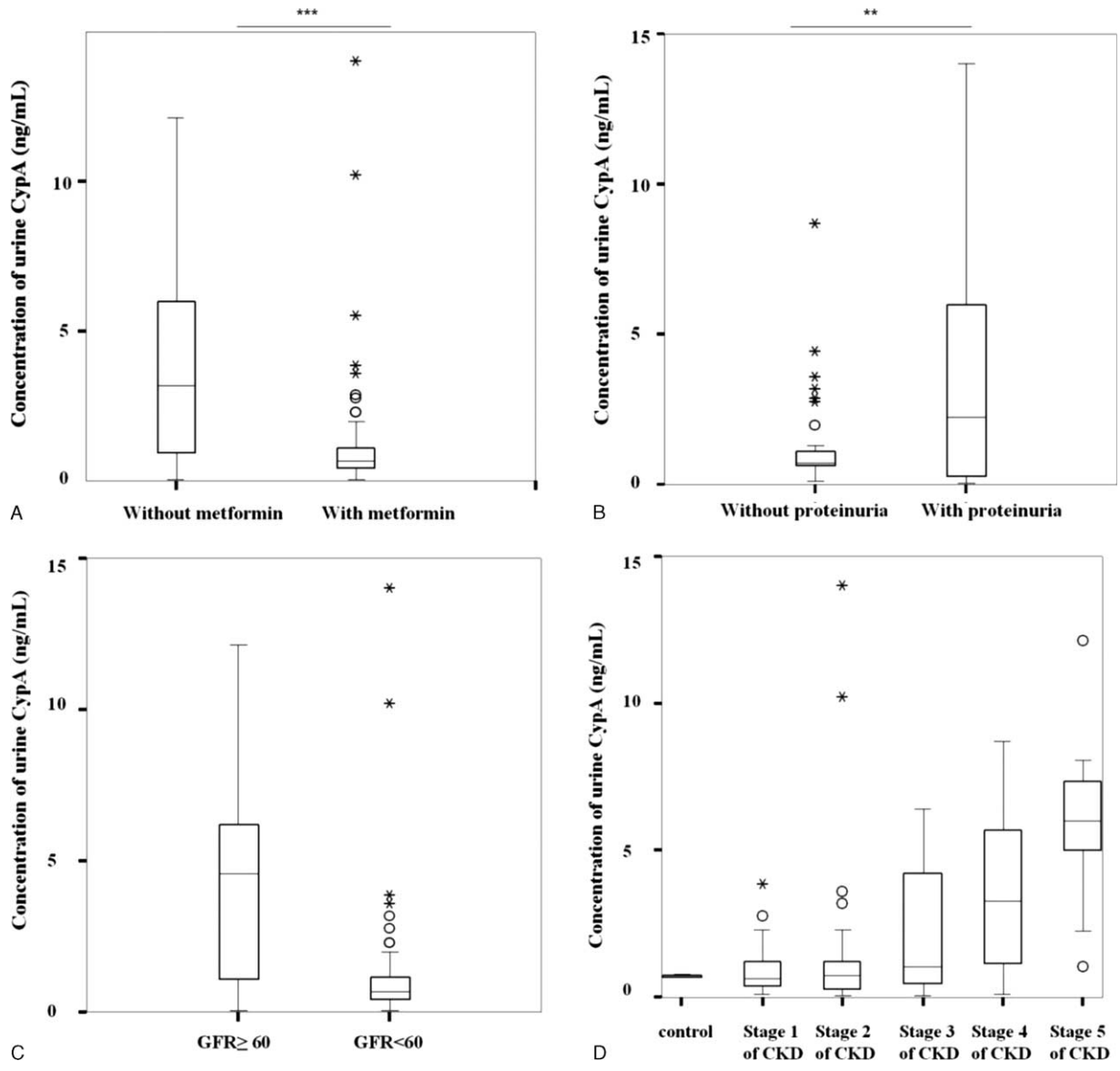


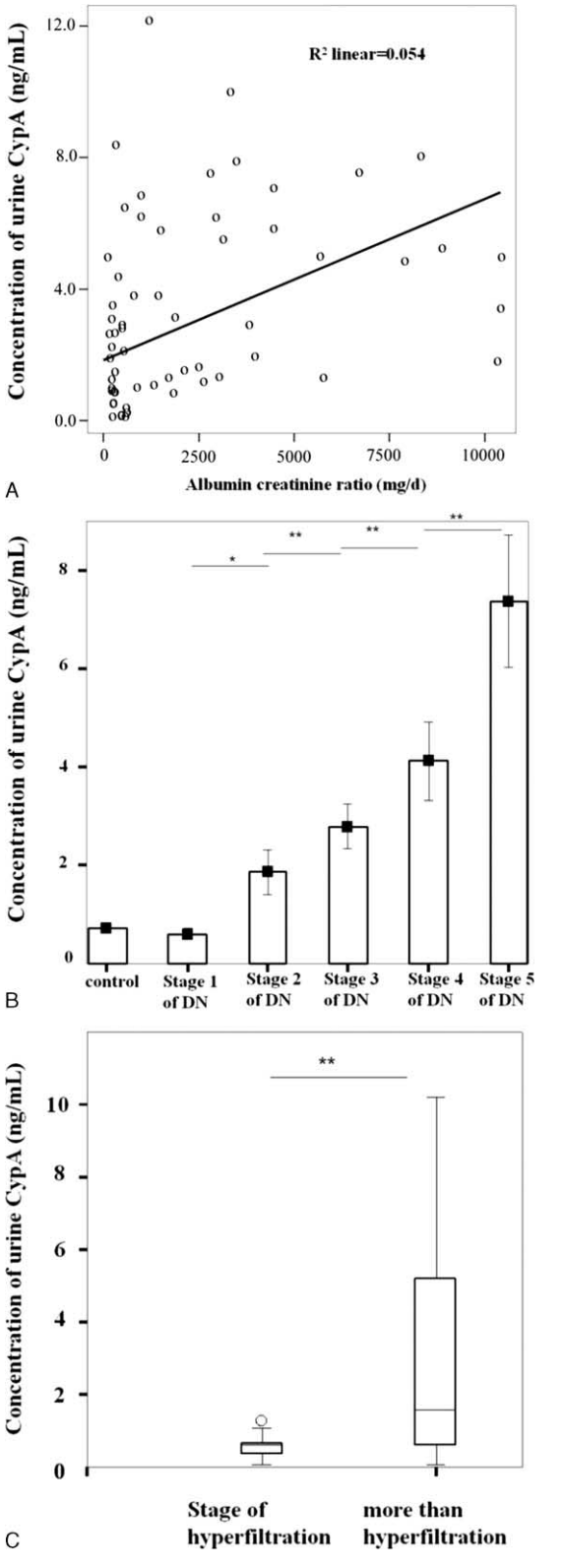

**FIGURE 1.** Univariate analysis between clinical parameters and urinary CypA. *P* value for A was <0.001, for B was 0.007, and for C was <0.060. *P* value between stages 1 and 2, 2 and 3, 3 and 4, 4 and 5 of CKD were 0.511, 0.633, 0.365, 0.203, and 0.061, respectively. CKD = chronic kidney disease, CypA = cyclophilin A.

expression of sCypA whereby various concentrations of glucose and $H_2O_2$ were applied to HK-2 cells. As expected, different concentrations of glucose (10, 25, and 50 mM) could effectively increase the expression of sCypA (Fig. 5A), indicating that hyperglycemia can also induce sCypA release from tubular cells. In addition, either 20 or 40 μM $H_2O_2$ treatment significantly increased the expression of sCypA, which could be reversed by 300 U/mL of catalase (Fig. 5B).

## DISCUSSION

The current clinical markers for DN are GFR and microalbuminuria. SCr is routinely measured for GFR, which can be used to stage CKD regardless of DM association. Since all renal diseases will progress to CKD and the cause and progression of CKD are heterogeneous, every cause related to CKD should theoretically have its own staging or detection criteria. Specific markers allow physicians to target and treat the definite cause, thereby potentially preventing further renal function deterioration. Albuminuria or proteinuria is the typical marker used for staging DN progression. However, it has become evident that there exists a subpopulation of patients with discrepant classifications of DN (albuminuria-based) and CKD (GFR-based).[4–6] Therefore, some committees are trying to develop a new classification of DN,[35] combining both GFR and albuminuria systems.

In this study, we measured renal function parameters and demonstrated that urinary CypA was significantly associated with SCr, GFR, proteinuria, ACR, stages of DN, and stages of CKD. In addition, either GFR-based or albuminuria-based

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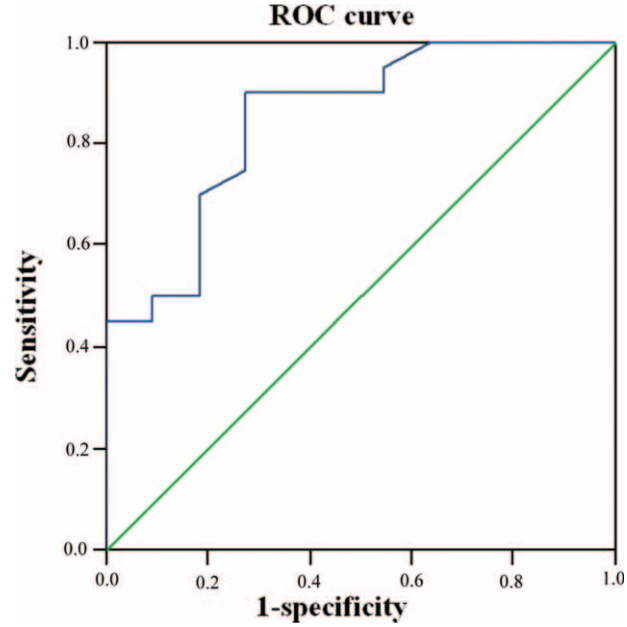


**FIGURE 3.** ROC curve for diagnosing silent stage of DN via urinary CypA. The concentration of urinary CypA to diagnose silent stage of DN was 0.7250 ng/mL with a sensitivity of 0.900 and a specificity of 0.727. The area under the ROC curve was 0.850. CypA = cyclophilin A, DN = diabetic nephropathy, ROC = receiver receiver operating characteristic.

classifications of DN correlated significantly with urinary CypA. When comparing different stages of DN or CKD, there was only a trend of higher CypA in higher CKD stages, but truly statistically significant difference existed among the different DN stages. This finding supports the notion that urinary CyA is better correlated using the albuminuria-based classification, which is the better and earlier detection method for monitoring DN compared with the GFR-based system in clinical practice. Although the albuminuria-based system is better than GFR, it is far from ideal for a number of reasons. First, increased albuminuria is actually a relatively late manifestation of early-stage DN, so it is not sensitive enough to detect early stages of DN. Second, only one-third of patients with microalbuminuria present with persistent macroalbuminuria according to 1 cohort study,[36] indicating a poor predictive power for outcome of DN. Third, some patients have renal pathological changes without

**FIGURE 2.** Concentrations of urinary CypA in different stages of DN. (A) Concentration of urinary CypA and ACR were plotted. When ACR increased by 1 mg/g, the concentration of urinary CypA increased 0.030 ng/mL (CypA = 2.461 + ACR*0.001). $R^2$ linear was 0.054. (B) No difference in concentration of urinary CypA was found between stage 1 DN and healthy control groups ($P = 0.117$). However, there were statistically significant differences between stages 1 and 2, stages 2 and 3, stages 3 and 4, and stages 4 and 5 DN ($P = 0.012$, 0.003, <0.001, and 0.005, respectively). (C) The differences between stage 1 DN and stages 2 to 5 DN were statistically significant ($P = 0.006$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ACR = albumin creatinine ratio, CypA = cyclophilin A, DN = diabetic nephropathy.

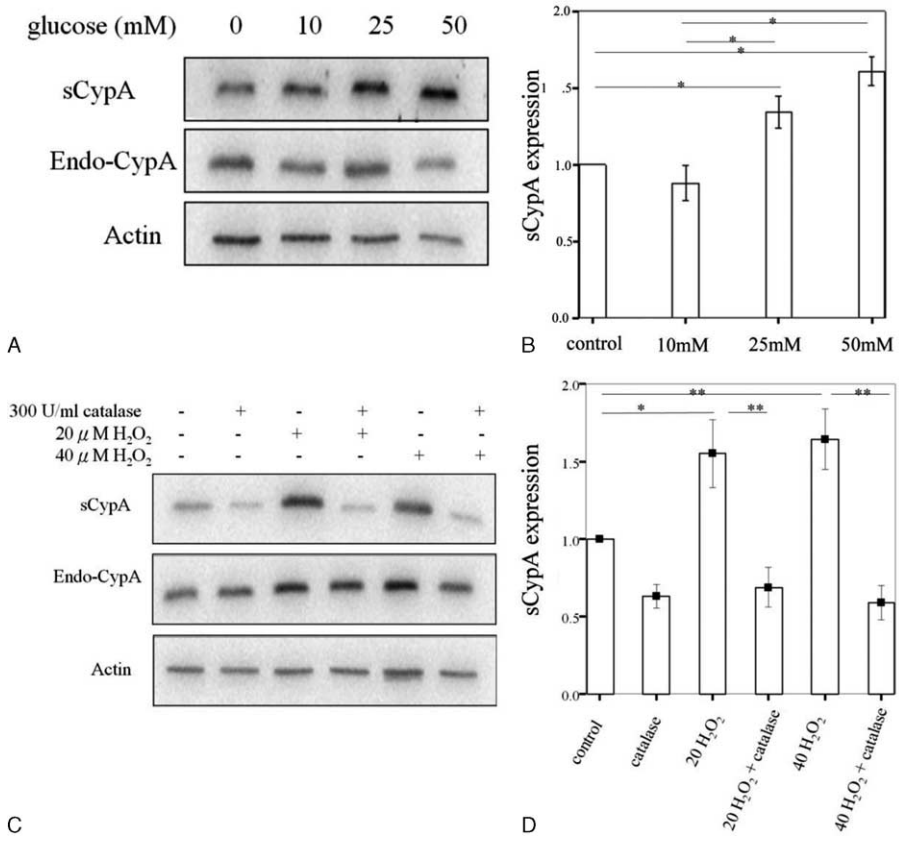


**FIGURE 4.** Western blotting of sCypA expression in MES-13 cells treated with different concentrations of glucose and $H_2O_2$. (A) Glucose increased the expression of sCypA, which was dose-dependent (10, 25, and 50 mM). (B) Statistical analysis showed that the increased expression of sCypA was found at 25 mM versus control ($P = 0.037$), and 50 mM versus control ($P = 0.037$). Increased expression of sCypA was observed at 50 versus 10 mM ($P = 0.018$). (C) The expression of sCypA was increased in cells with 20 or 40 μM $H_2O_2$ treatments. They could both be counteracted by 300 U/mL catalase. (D) Quantitative assessment showed that 20 μM $H_2O_2$ increased the expression of sCypA ($P < 0.05$) (n = 6), which could be reversed by 300 U/mL catalase ($P < 0.01$) (n = 6). The sCypA expression was also stimulated by 40 μM $H_2O_2$ ($P < 0.01$) (n = 6), which could be counteracted by 300 U/mL catalase ($P < 0.01$) (n = 6). (n = 4) $^{*}P < 0.05$, $^{**}P < 0.01$. Endo-CypA = endogenous cyclophilin A, MES-13 = mesangial, sCypA = secreted cyclophilin A.

microalbuminuria.[37] Finally, albuminuria is not specific enough for DN because it can be detected in other non-DM related nephropathy, such as retinopathy and congestive heart failure.[38] Therefore, urinary CypA could have enormous value as an earlier marker than albuminuria for identifying DN.

In this well-matched cohort of DN patients, urinary CypA correlated well to the different severity of DN according to the albuminuria-based classification. Compared with the control group, urinary CypA indeed increased significantly in stage 2 DN and this increase persisted throughout the later stages. The increment was more significant with worsening DN stage. In stage 1 DN, kidneys become dilated and glomerular capillary hydrostatic pressure increased in DN.[39] There was a hemodynamic change without any ultrastructure abnormality. Stage 2 DN is a silent stage but, to date, no useful markers for detection have been identified. No microalbuminuria can be measured in clinical practice. However, hyperglycemic effects are initiated in this stage. The glomerular basement membrane becomes thicker, followed by an increase in mesangial volume, and interstitial expansion.[24] The above structural changes do not become significant until stage 3 DN. If stage 2 DN could be detected early, intensive blood sugar monitoring, timely diet restriction, and exercise education would be useful to avoid further silent deterioration of DN. In this study, we propose that urinary CypA can be used as an early marker for identifying stage 2 DN with a high sensitivity (90%) and high diagnostic power (AUC = 0.885). Detection of urinary CypA is also very convenient because it is noninvasive. Now that urinary CypA appears to be capable of identifying DN in the silent stage, perhaps the term ''silent'' can be considered redundant. In an extensive review conducted by Lee et al,[40] urinary CypA was not mentioned as a potential biomarker for DN. This is the first study to use urinary CypA in early DN detection. CypA was mostly studied in CVD and lung or liver injury.[18] Asthma and RA are associated with this new marker.[16] According to an extensive review of CypA in human disease,[20] its association with DM was only mentioned once by Ramachandran et al.[22] They examined proteomic profiling of high glucose primed monocytes and found that CypA could be a potential secretory marker of inflammation in type 2 DM.[21] The present investigation is the 3rd study to identify a correlation between CypA and DM. Furthermore, this is the 1st study to verify the association between urinary CypA and DN with strong statistical significance in this well-designed human cohort.

It has been noted that urinary podocalyxin[41] and podocalyxin-positive element,[42] which increases after podocyte injury,

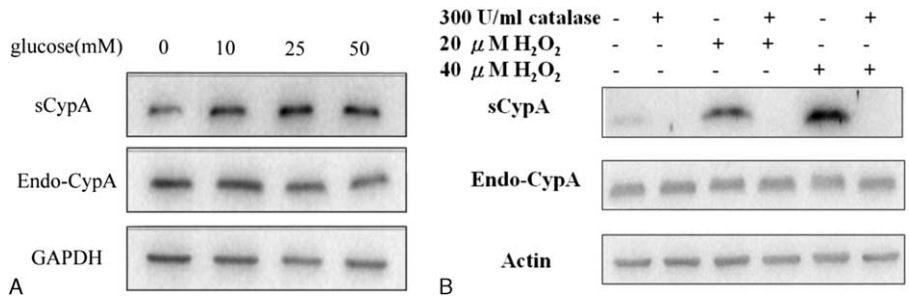


**FIGURE 5.** Western blotting of sCypA expression in HK-2 cells treated with different concentrations of glucose and $H_2O_2$. (A) HK-2 cells were treated with different concentrations (10, 25, and 50 mM) of glucose. All concentrations of glucose increased the expression of sCypA. (B) The expression of sCypA was increased in cells with 20 or 40 μM $H_2O_2$ treatments. They could both be counteracted by 300 U/mL catalase. Endo-CypA = endogenous cyclophilin A, sCypA = secreted cyclophilin A.

could be useful as new biomarkers for early DN. However, podocalyxin also increases in other diseases with podocyte injury,[41] including IgA nephropathy, focal segmental glomerulosclerosis, membranous nephropathy, and lupus nephritis, indicating that urinary podocalyxin is not specific to DN. In addition, urinary podocalyxin level or podocalyxin-positive element was not reported for early detection of stage 2 DN. Moreover, another biomarker, urinary L-FABP, expressed in the proximal tubules of the human kidney, was recently found to be associated with DN.[43] L-FABP increased in a stepwise manner with progression of DN.[7] In a study of type 1 DM,[44] urinary L-FABP was an independent predictor of progression of DN irrespective of disease stage. The AUC to predict the progression to stage 3 DN by measuring both urinary L-FABP and urinary albumin was up to 0.786. In another study of type 2 DM,[45] when the urinary L-FABP level was more than 8.4 μg/g creatinine, clinicians could predict the progression of DN to stage 3 DN with a sensitivity of 0.700 and specificity of 0.781. Compared to L-FABP as a marker for predicting stage 3 DN, urinary CypA is the first marker to be proposed for predicting progression to stage 2 DN with a much higher sensitivity (0.900 vs 0.700) and larger AUC (0.850 vs 0.786). In a recent extensive review of urinary biomarkers for early DN beyond albuminuria,[40] it was found that all of the studied biomarkers were limited to predicting microalbuminuria (stage 3 DN). Therefore, our data demonstrate that urinary CypA may have value as a novel biomarker for predicting DN as early as stage 2.

In kidneys, CypA is mostly distributed in tubules, followed by glomeruli.[17] Therefore, it is reasonable to hypothesize that urinary CypA could be secreted by tubular cells or mesangial cells. Because mesangial matrix expansion is a typical pathological finding of DN[30] and a high glucose state evokes an intrinsic proapoptotic signaling pathway in mesangial cells,[32] we first studied the expression of sCypA from mesangial cells. As shown in Figure 4, there was a significant release of CypA following glucose or free radical treatment. Even though DN has been traditionally considered as a glomerular disease, increasing evidence has shown that renal dysfunction correlates earlier and in association with the degree of tubular injury.[46] A novel mechanism for albuminuria from PTEC revealed that tubular epithelial cell injury occurs relatively earlier than glomerular injury. There are many chemokines released from PTEC which stimulate certain physiological signals and whose effects culminate in progressive tubular injury, interstitial inflammation, and fibrosis in DN.[47] Therefore, we next examined whether CypA can also be secreted by tubular epithelial cells as well. In Figure 5, after treatment of HK-2 cells with various concentrations of glucose or free radicals, sCypA was clearly increased in the conditioned medium. In the cell study using MES-13 and HK-2 cells, our results demonstrated that CypA was secreted after glucose or free radical stimulation, indicating that CypA could be secreted by either mesangial cell or PTEC into urine in early DN. Our results are consistent with those of previous studies that showed earlier renal dysfunction was associated with tubular change[46,47] and the later but typical change was related to mesangial cell dysfunction in the glomerulus.[31,32] Therefore, sCypA could be considered as both tubular and mesangial cell injury markers in DN.

In summary, we have demonstrated in this well-designed study of DN patients that urinary CypA is a good biomarker for early DN. Even though we cannot exclude the possibility that urinary CypA is released by renal cell lysis, our results from cellular models indicate it is very likely that CypA is secreted from either mesangial or PTEC in early DN. In addition to its role as a novel biomarker of early DN, sCypA may also play a pathological role in the development of DN and may be involved in the interplay between the tubulointerstitial and glomerular compartments. The underlying molecular mechanisms need to be elucidated in further cellular and animal studies.

## ACKNOWLEDGEMENTS

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# Novel findings of secreted cyclophilin A in diabetic nephropathy and its association with renal protection of dipeptidyl peptidase 4 inhibitor

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ABSTRACT

*Background:* Our previous clinical indicated that urinary cyclophilin A was a good marker for diabetic nephropathy.
*Methods:* We used animal and cell models of diabetic nephropathy to examine the role of cyclophilin A in disease progression.
*Results:* Significantly increased urinary cyclophilin A could be detected in *db*/*db* at the 8th week. Linagliptin (3 mg/kg/day and 15 mg/kg/day) could suppress urinary 8-hydroxy-2′-deoxyguanosine at the 8th and 16th week but only the high dose Linagliption could suppress cyclophilin A at the 8th week. Compared to 8-hydroxy-2′-deoxyguanosine, cyclophilin A was a stronger, earlier, and more sensitive marker. Immunohistochemical staining for cyclophilin A was also positive for *db*/*db*. In cell studies, oxidative stress and hyperglycemia could stimulate MES-13 and HK-2 cells to secrete cyclophilin A. Hyperglycemia stimulated HK-2 cells to secrete TGFβ1, which caused secretion of cyclophilin A. The secreted cyclophilin A further stimulated CD 147 to move outward from cytosol onto cell membrane in confocal microscopy, which was associated with the p38 MAPK pathway in the downstream.
*Conclusions:* Secreted cyclophilin A may play an important role in diabetic nephropathy in the mouse model and is associated with TGFβ1, CD 147, and the p38 MAPK pathway.



## 1. Introduction

Type 2 diabetes mellitus (DM) is the most common single cause of end-stage renal disease (ESRD) [1], and diabetic nephropathy (DN) is the cause of ESRD in almost half of all patients with ESRD. Despite the availability of many modern therapies for glycemic control, many diabetic patients still progressed to severe renal damage [2]. Therefore, it is important to identify new markers for DN and to further elucidate the molecular pathway that leads to DN. Cyclophilin A (CypA) is an 18-kD highly conserved protein [3] that mostly distributed in the cytoplasm, where it facilitates protein folding and trafficking. It can also act as a cellular receptor for cyclosporine A. Aside from the above described "cellular" form of CypA, the level of a secreted form of CypA (sCypA) correlates with cardiovascular disease [4], rheumatoid arthritis, and liver injury. Serum CypA level had been reported to be higher in diabetic patients and may be a new biomarker for DM [4,5]. The expression of CypA is at a relatively high level in the kidney [6], where proximal tubular epithelial cells (PTECs) contains a considerably greater level of CypA relative to other kidney cells [7]. The relationship between DN and sCypA has never been elucidated until our previous report [8]. Our study showed that the CypA was detectable in patients with stage 2 DN, with high sensitivity and specificity, and the level increased as DN progressed. Furthermore, high glucose treatment increased sCypA expression in cultured mesangial cells and PTECs [8]. Secreted CypA has been shown to be a good marker for DN according to our previous report [8]. Herein, we hypothesize that there might be an important pathological role of sCypA for DN as well.

Dipeptidyl peptidase 4 inhibitors (DPP-4is) benefit patients with renal protection independent from their glucose-lowering effects [9] and they also benefit patients by providing renal protection [10–13] without clear mechanism. Linagliptin (BI-1356, Trajenta) can lower albuminuria on top of the recommended standard treatment in patients with type 2 DM [13]. It can reduce renal events by 16% (composite of 6 renal outcomes) [14]. There are other preclinical studies describing possible mechanisms of the renal protection of DPP4i [10,15,16]. In this study, we examined the renal protective effect conferred by

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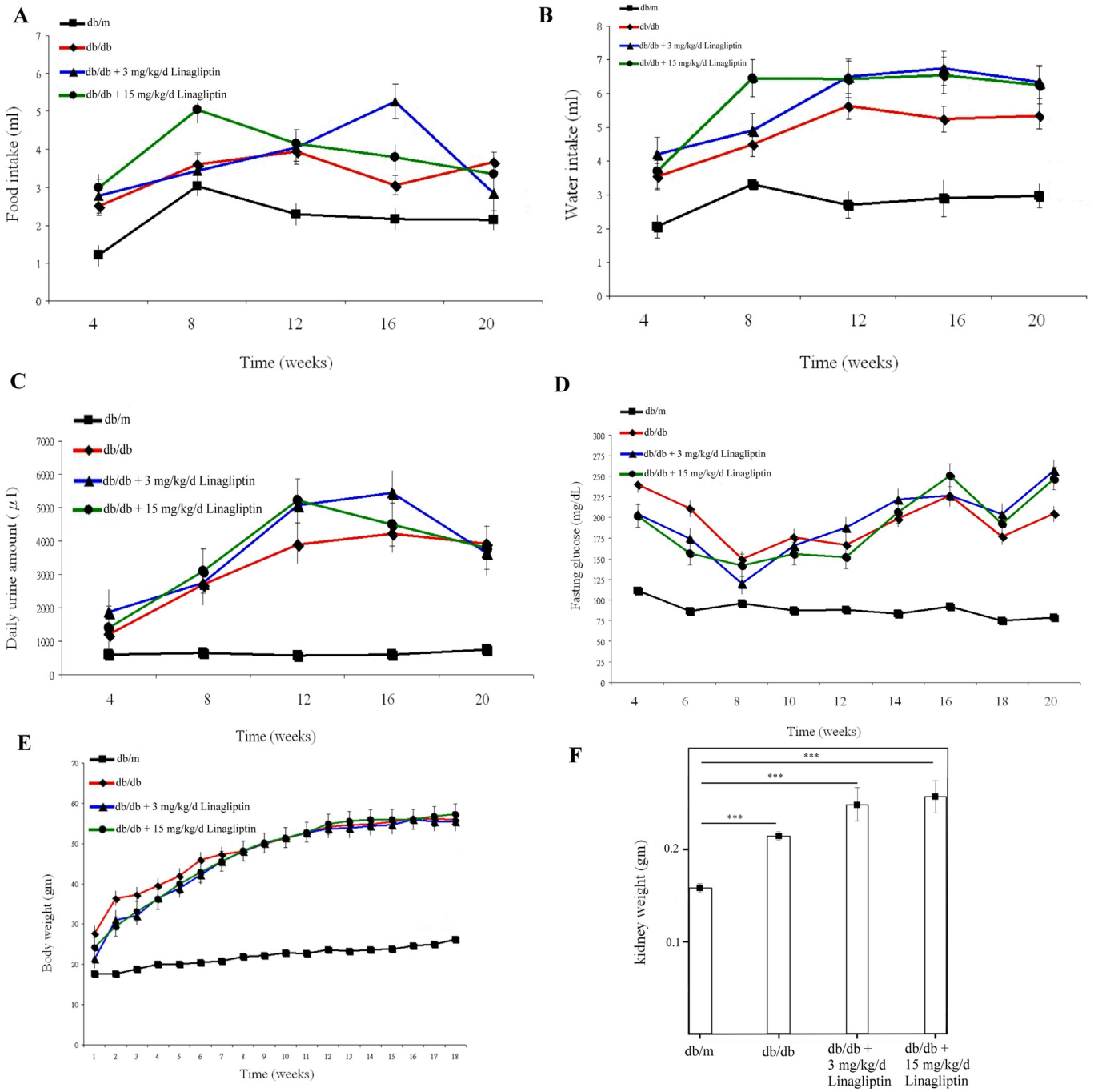



**Fig. 1.** Physical data of mice. (A) Three groups of mice with diabetic nephropathy (*db*/*db*) ate more food than mice without diabetic nephropathy (*db*/*m*). However, there was no difference among all 3 *db*/*db* groups. ($n = 10$). (B) Three groups of mice with diabetic nephropathy (*db*/*db*) drank more water than mice without diabetic nephropathy (*db*/*m*). However, there was no difference between all 3 *db*/*db* groups. ($n = 10$). (C) Three groups of mice with diabetic nephropathy (*db*/*db*) produced a greater urinary volume than mice without diabetic nephropathy (*db*/*m*). However, there was no difference between all 3 *db*/*db* groups. ($n = 10$). (D) Mice with diabetic nephropathy had significantly higher blood glucose than *db*/*m*. However, there was no difference between all 3 *db*/*db* groups. ($n = 10$). (E) Three groups of mice with diabetic nephropathy (*db*/*db*) had much higher body weight than mice without diabetic nephropathy (*db*/*m*). However, there was no difference between all 3 *db*/*db* groups. ($n = 10$). (F) Three groups of mice with diabetic nephropathy (*db*/*db*) had heavier kidneys than mice without diabetic nephropathy (*db*/*m*) did. However, there was no difference between all 3 *db*/*db* groups. ($n = 10$) $^{***}p < 0.001$.

Linagliptin in the *db*/*db* mouse model and the associations between Linagliptin and sCypA.

## 2. Material and methods

### *2.1. Type 2 DM mouse*

All experimental protocols were approved by a named Taichung Veterans general hospital and licensing committee (Affidavit of Approval of Animal Use Protocol in TVGH) and all experiments were performed in accordance with relevant guidelines and regulations. Four-week-old male C57BLKS/J *db*/*db* and *db*/*m* mice were purchased from National Applied Research Laboratories (Taiwan, R.O.C.); *db*/*m* mice were used as controls in all experiments. They were fed from the age of 4 weeks, and were sacrificed at the age of 20 weeks.

### *2.2. 24-h urinary sCypA and 8-hydroxy-2′-deoxyguanosine (8-OHdG)*

The amount of daily urine was collected from the metabolic cage and we used the ELISA kit (SEA979Mu, USCN Life Science Inc.) for determining

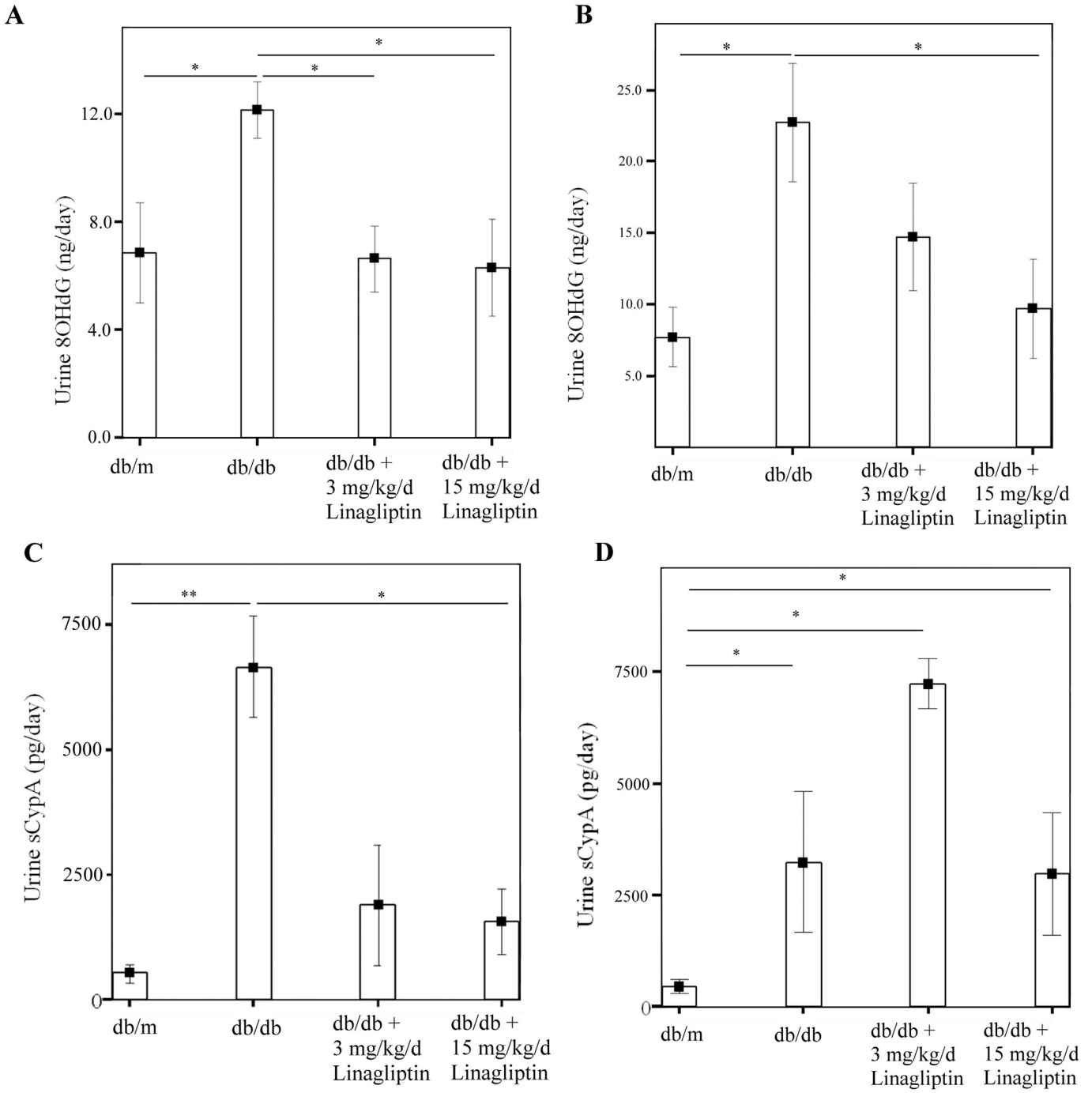



**Fig. 2.** Expressions of 8-OHdG and CypA from mice's urine at the 8th week and 20th week. (A) The expression of 8-OHdG in *db*/*db* from the urine at the 8th week was increased significantly compared to *db*/*m* ($p = 0.026$). This could be suppressed by administering both 3 and 15 mg/kg/day Linagliptin ($p = 0.018$ and $p = 0.028$ respectively). (B) The expression of 8-OHdG in *db*/*db* from urine at the 20th week was increased significantly compared to *db*/*m* ($p = 0.018$), but this could only be suppressed by higher dose (15 mg/kg/day) of Linagliptin ($p = 0.047$) instead of lower dose (3 mg/kg/day) ($p = 0.175$). (C) The expression of sCypA in *db*/*db* from urine at the 8th week was increased significantly compared to *db*/*m* ($p = 0.006$), and it could only be suppressed by higher dose (15 mg/kg/day) of Linagliptin ($p = 0.016$) instead of lower dose (3 mg/kg/day) ($p = 0.050$). (D) The expression of sCypA in *db*/*db* from urine at the 20th week was also increased significantly compared to *db*/*m* ($p = 0.019$), but could not be suppressed by either high or low dose of Linagliptin ($p = 0.773$ and $p = 0.149$ respectively). ($n = 6$ in *db*/*m*, $n = 5$ in *db*/*db*, $n = 6$ in *db*/*db* + 3 mg/kg/day Linagliptin, and $n = 5$ in *db*/*db* + 15 mg/kg/day Linagliptin.) $^{*}p < 0.05$, $^{**}p < 0.01$.

sCypA. The amount of daily urine and concentration of sCypA in urine were calculated as the daily sCypA excretion amount. Urine was stored in an ice package immediately. Within 4 h, it was then restocked under −80 °C until analysis. All data of urinary CypA were double-checked at least twice. To determine the oxidative DNA damage in the kidney, we determined 24-h urinary 8-OH-dG concentrations using the ELISA kit (8-OH-dG Check; Institute for the Control of Aging).

### 2.3. Histological analysis: light microscopic study

We selected 10 glomeruli from each mouse and there were 100 glomeruli from 10 mice in each group. The right kidney of each mouse was obtained for histological analysis. Histology was assessed after hematoxylin and eosin (HE) staining as well as the periodic acid-Schiff staining (PAS).

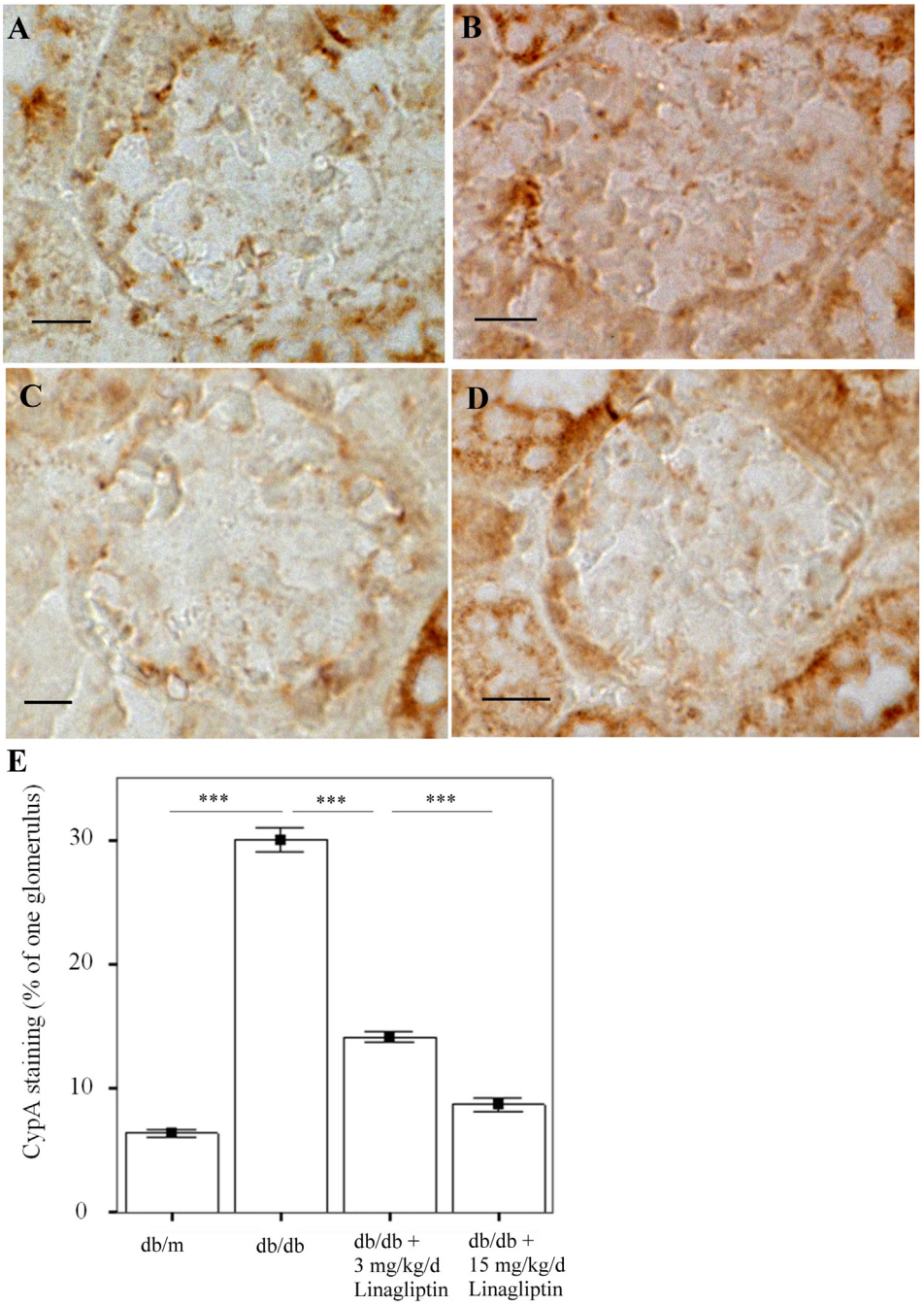



**Fig. 3.** IHC staining of glomeruli for CypA in *db/db* diabetic compared to *db/m* nondiabetic kidneys at 20th week. (A)(B)(C)(D) IHC staining for CypA (brown staining) was increased in the *db/db* group compared to *db/m* (B vs. A). The increased staining for CypA in *db/db* (B) could not be observed in high dose (C) or low dose (D) Linagliptin treatment. (E) Quantitative data ($n = 100$ in each group) for IHC staining for CypA. Increased staining for CypA in *db/db* could be reversed by both dose of Linagliptin treatment significantly. $^{***}p < 0.001$. Scale bar, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

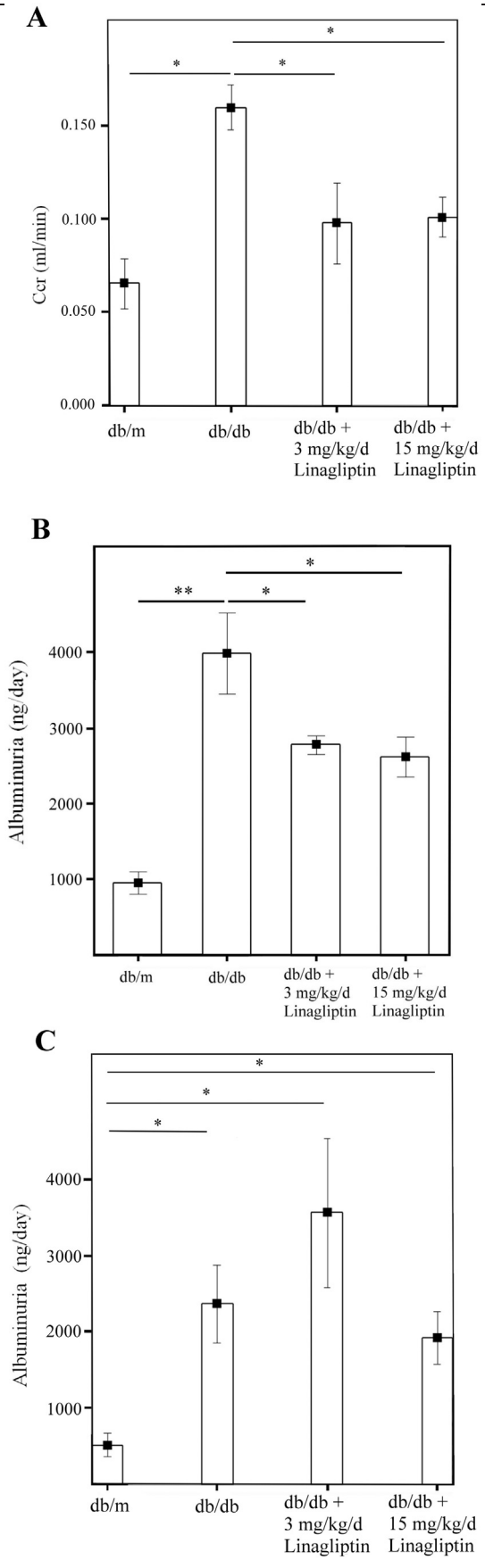
### 2.4. Cell culture

MES-13 cells (glomerular mesangial cells from an SV40 transgenic mouse) were obtained from American Type Culture Collection (CRL-1927; Manassas, VA, USA). MES-13 were cultured in a 3:1 mixture of M199 (Invitrogen) and Ham F-12 (Invitrogen), supplemented with 5% FBS, 1% penicillin-streptomycin, 1% L-glutamine, and 14 mmol/l HEPES and maintained in an incubator at 37 °C with 5% CO2. All culturing supplies were acquired from Life Technologies (Gaithersburg, MD, USA). Subsequently, the cell lysate and secreted cellular proteins were collected for Western blot analysis. HK-2 cells (human proximal tubular epithelial cells) were obtained from the laboratory of Taichung Veterans General Hospital. HK-2 cells were maintained in DMEM/F12 and supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin/amphotericin B, 1% glutamine (Invitrogen, Carlsbad, CA), and 1% Insulin-Transferrin-Selenium (Sigma, St. Louis, MO). Western blot analyses were used to determine the levels of endo-CypA, sCypA, p38, phosphorylated p38 and TGFβ1.

### 2.5. Chemicals, reagents and techniques

#### 2.5.1. Linagliptin effect on MES-13 cells

Linagliptin (5 mg/tab, BI-1356, film-coated tablet) and a pure powder form 1-[(4-methyl-quinazolin-2-yl)methyl]-3-methyl-7-(2-butyn-1-yl)-8-(3-(*R*)-amino-piperidin-1-yl)) xanthine) were sponsored by Eli Lilly Company. The film-coated tablets of Linagliptin were dissolved




in water for animal experiments and the pure powder form was dissolved in dimethyl sulfoxide (DMSO) for cell experiments.

*2.5.2. Glucose, TGFβ1 and CypA treatment on HK-2 cells*

HK-2 cells were seeded in a 6 cm cell culture plate with $3 \times 10^5$ cells/plate and were incubated in DMEM/F-12 1:11 medium (10% FBS) for 1 day. Then the medium was replaced with 0% FBS low glucose DMEM medium for 3-day glucose starvation. D-glucose (Sigma, Aldrich) TGFβ1 (PeproTech), CypA (Enzo Life Sciences, Inc.) were used to treat HK-2 cells.

*2.6. Statistical analysis*

The results from Western blot were expressed as mean ± SEM. The suitable cutoff value for the sCypA and 8-OHdG in urine at the 8th week were analyzed using ROC curve to determine the optimal sensitivity and specificity of the ROC curve. *Chi* square test was used to differentiate the two examinations. All statistical procedures were performed using the SPSS statistical software package, ver 12.0. A $p < 0.05$ was considered statistically significant.

## 3. Results

*3.1. Effects of Linagliptin on the physiology of mice with DM*

Since the *db/db* (*Lepr*$^{db}$) mouse model of leptin deficiency is currently the most reliable and widely used mouse for modeling type 2 DN [17], we treated both *db/db* and *db/m* mice with Linagliptin to observe the effects of Linagliptin on DN. All three groups of *db/db* mice exhibited the classical manifestations of DM: increased appetite (Fig. 1A), thirst (Fig. 1B), urinary frequency (1C), and weight (Fig. 1E). However, regardless of treatments (3 mg/kg/day or 15 mg/kg/day of Linagliptin), the blood sugar in all three groups remained the same (Fig. 1D). Therefore, we hypothesize all findings were independent from glucose-lowering.

*3.2. Secreted CypA as an earlier indicator than 8-OHdG for DN and their associations with Linagliptin*

Urinary 8-OHdG is a reliable and early marker of reactive oxidative stress (ROS) and DN because it can represent DNA damage in early DN [18]. The expression of 8-OHdG in the urine at the 8th week in the *db/db* was increased significantly compared to that in *db/m* ($p = 0.026$). This result could be suppressed by administering 3 and 15 mg/kg/day of Linagliptin ($p = 0.018$ and $p = 0.028$ respectively, Fig. 2A). The expression of 8-OHdG in the *db/db* at the 20th week increased significantly compared to that in *db/m* ($p = 0.018$), but it could only be suppressed by a high dose ($p = 0.047$) rather than low ($p = 0.175$) (Fig. 2B). In summary, we were able to detect the expression of 8-OHdG starting from the 8th week up to 20th. The receiver operating characteristic (ROC) curve is shown in supplementary data (Fig. S1, A). On the other hand, the sCypA in the urine at the 8th week in the *db/db* increased significantly compared to that of *db/m* ($p = 0.006$), and it could only be suppressed by high-dose Linagliptin ($p = 0.016$) rather than low-dose ($p = 0.050$) (Fig. 2C). The expression of sCypA in the urine from the 20th week in the *db/db* also increased significantly compared to that of *db/m* ($p = 0.019$), however, the sCypA expression could not be

**Fig. 4.** Renal function evaluations of mice, including creatinine clearance and daily albuminuria. (A) The Ccr was increased at the 20th week in the *db/db* group when compared to the *db/m* ($p = 0.034$). The increased hyperfiltration could be inhibited by high- or low-dose Linagliptin ($p = 0.021$ and $p = 0.014$ respectively). (B) Daily albuminuria at the 8th week was increased in *db/db* group ($p = 0.006$), which could be inhibited by 3 mg/kg/day-Linagliptin ($p = 0.045$) or 15 mg/kg/day-Linagliptin ($p = 0.046$). (C) Daily albuminuria at the 20th week was also increased in the *db/db* group ($p = 0.028$). The increased albuminuria could not be inhibited by either high- or low-dose Linagliptin ($p = 0.327$ and $p = 0.142$ respectively). ($n = 6$ in *db/m*, $n = 5$ in *db/db*, $n = 6$ in *db/db* + 3 mg/kg/day Linagliptin, and $n = 5$ in *db/db* + 15 mg/kg/day Linagliptin.) $^*p < 0.05$, $^{**}p < 0.01$.

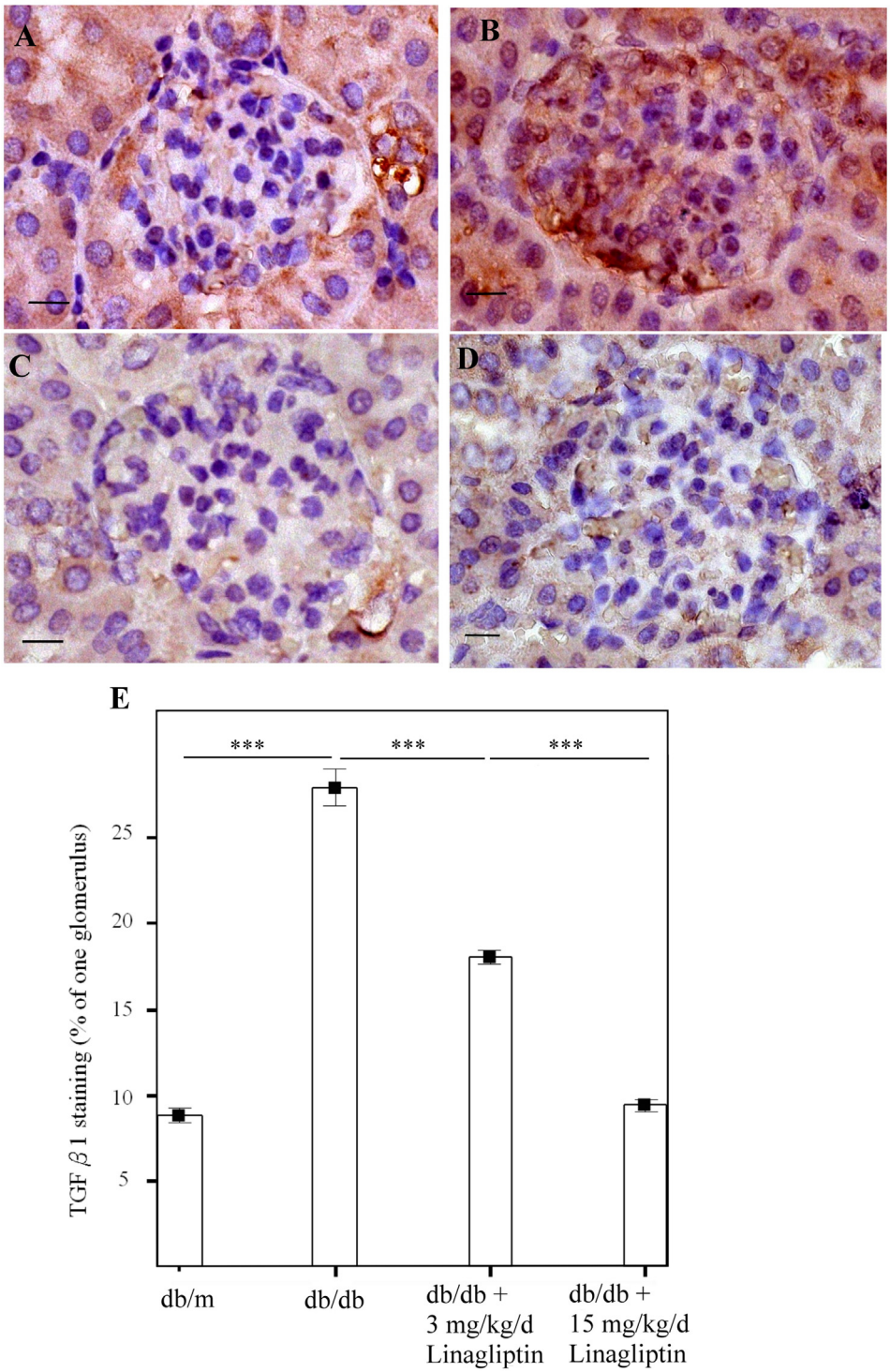



**Fig. 5.** IHC staining for TGFβ1 in *db/db* diabetic compared to *db/m* nondiabetic kidneys at 20th week. (A)(B)(C)(D) IHC staining for TGFβ1 (brown staining) in glomeruli was increased in the *db/db* group compared to *db/m* (B vs. A). The increased glomerular staining in *db/db* (B) could not be observed in either low-dose (C) or high-dose (D) Linagliptin treatment. (E) Quantitative data ($n = 100$ in each group) for IHC staining for TGFβ1 in glomeruli. Increased staining for TGFβ1 in *db/db* could be reversed by both doses of Linagliptin treatment significantly. ***$p < 0.001$. Scale bar, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suppressed regardless of high- or low-dose of Linagliptin ($p = 0.773$ and $p = 0.149$, respectively) (Fig. 2D). Similarly, we were able to detect the expression of sCypA starting from the 8th up to 20th. The ROC curve for sCypA is shown in supplementary data (Fig. S1, B). In contrast to 8-OHdG, however, a much higher dose of Linagliptin was needed to suppress the expression of sCypA at the 8th week.

### 3.3. Histological evidence of CypA in DN at the 20th week

The immunohistochemical (IHC) staining for CypA was significantly increased in the *db/db* (Fig. 3B) compared to *db/m* (Fig. 3A) in glomeruli, and the increased expression could be reversed by low-dose Linagliptin (Fig. 3C) and further reversed by high-dose Linagliptin (Fig. 3D). All

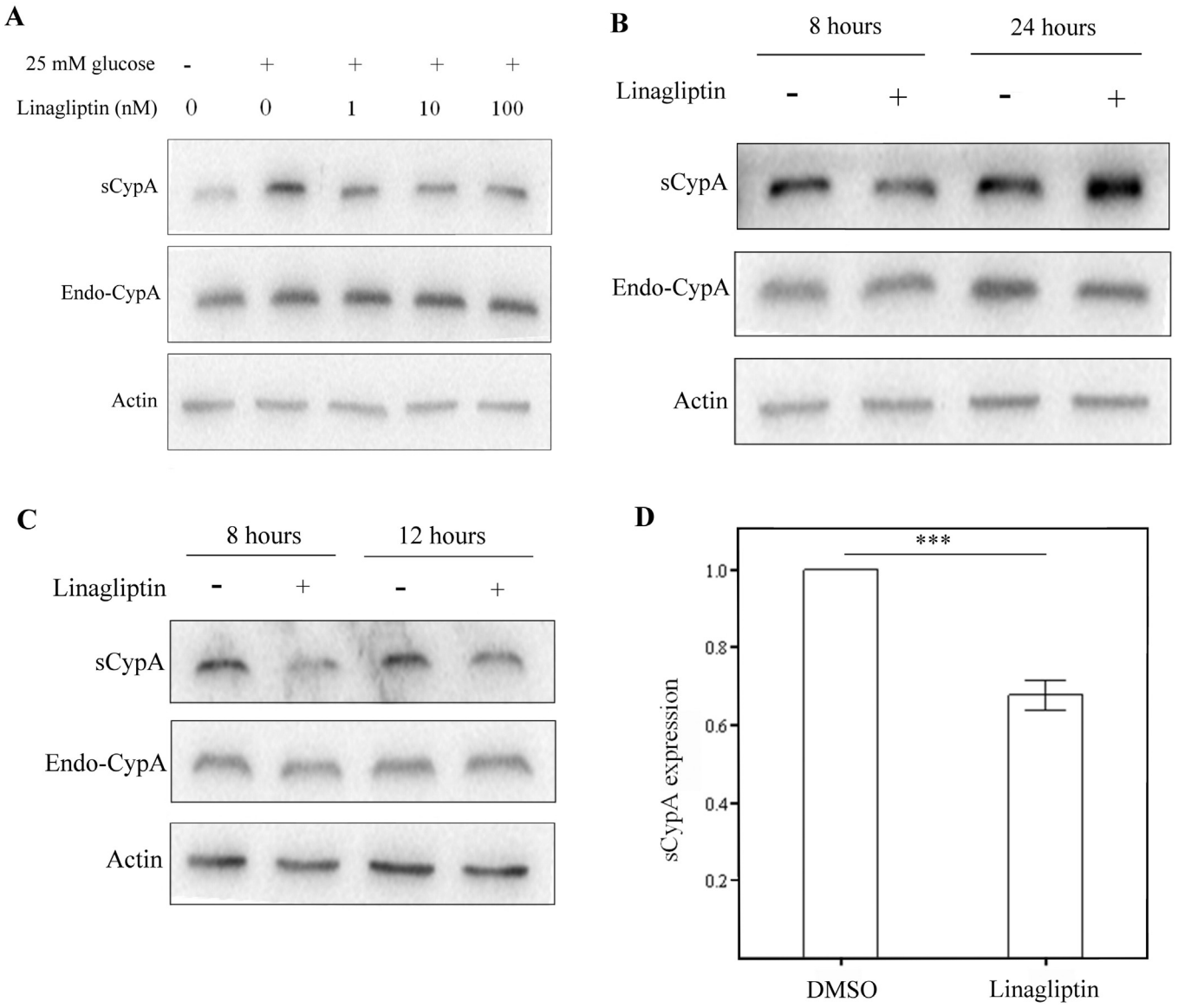



**Fig. 6.** Western blotting of sCypA expression in glucose treated MES-13 cells and rescued by Linagliptin. (A) The expression of sCypA treated by 25 mmol/l glucose was inhibited by Linagliptin in all 3 different concentrations (1, 10, and 100 nmol/l) for 8 h. (B) Under high glucose condition (25 mmol/l), the 10 nmol/l Linagliptin treatment was able to inhibit the expression of sCypA for 8 h. A longer treatment time for up to 24 h diminished the effect. (C) The effect of Linagliptin on the inhibition of sCypA could last for 12 h. (D) Statistical analysis showed that the inhibition could be sustained for at least 8 h with statistical significance ($p < 0.001$) ($n = 4$). ***$p < 0.001$; endo-CypA: endogenous cyclophilin A.

data were quantified in Fig. 3E. The data clearly indicate that a higher level of CypA exists in the mesangial area of glomeruli in DN compared to non-DN. In addition to IHC staining over glomeruli, there is increased IHC staining for CypA over peri-glomerrular tubules in the *db*/*db* (Supplementary data, Fig. S2, B) compared to *db*/*m* (Supplementary data, Fig. S2, A).

### 3.4. Linagliptin's effects on clinical markers of DN

The hyperfiltration and albuminuria are landmarks for DN [19]. At the 20th week, the Ccr (creatinine clearance) increased in the *db*/*db* group compared to *db*/*m* ($p = 0.034$) (Fig. 4A). The hyperfiltration could be inhibited by both doses of Linagliptin ($p = 0.021$ and $p = 0.014$ respectively) (Fig. 4A). On the other hand, albuminuria could be reduced at the 8th week at a low dose ($p = 0.045$) or high ($p = 0.046$) (Fig. 4B). Albuminuria was not reduced at the 20th week, even with a high dose of Linagliptin in the *db*/*db* ($p = 0.347$) (Fig. 4C).

### 3.5. Linagliptin's effects on pathological findings of DN at the 20th week

TGFβ1 is a pivotal mediator in the pathogenesis of renal fibrosis [20]. Microscopically, the IHC staining for TGFβ1 in glomeruli increased in the *db*/*db* group compared to *db*/*m* (Fig. 5B vs. A). The increased glomerular staining in the *db*/*db* (Fig. 5B) could be reversed by low- and high-dose Linagliptin (Fig. 5C and D). All data are quantified in Fig. 5E. These results suggest that Linagliptin can reduce the expression of TGFβ1 in glomeruli from DN. Increased TGFβ1 staining around peri-glomerular tubules can be detected in the *db*/*db* (Supplementary data, Fig. S3, B) compared to *db*/*m* (Supplementary data, Fig. S3, A). However, the expression of TGFβ1 cannot be relieved by low (supplementary data, Fig. S3, C) or high dose Linagliptin (Supplementary data, Fig. S3, D). These persistent increased stainings of TGFβ1 around tubules in all three *db*/*db* groups supported that very limited effect of Linagliptin on tubules because only 3–5% Linagliptin will enter tubular cells [21].

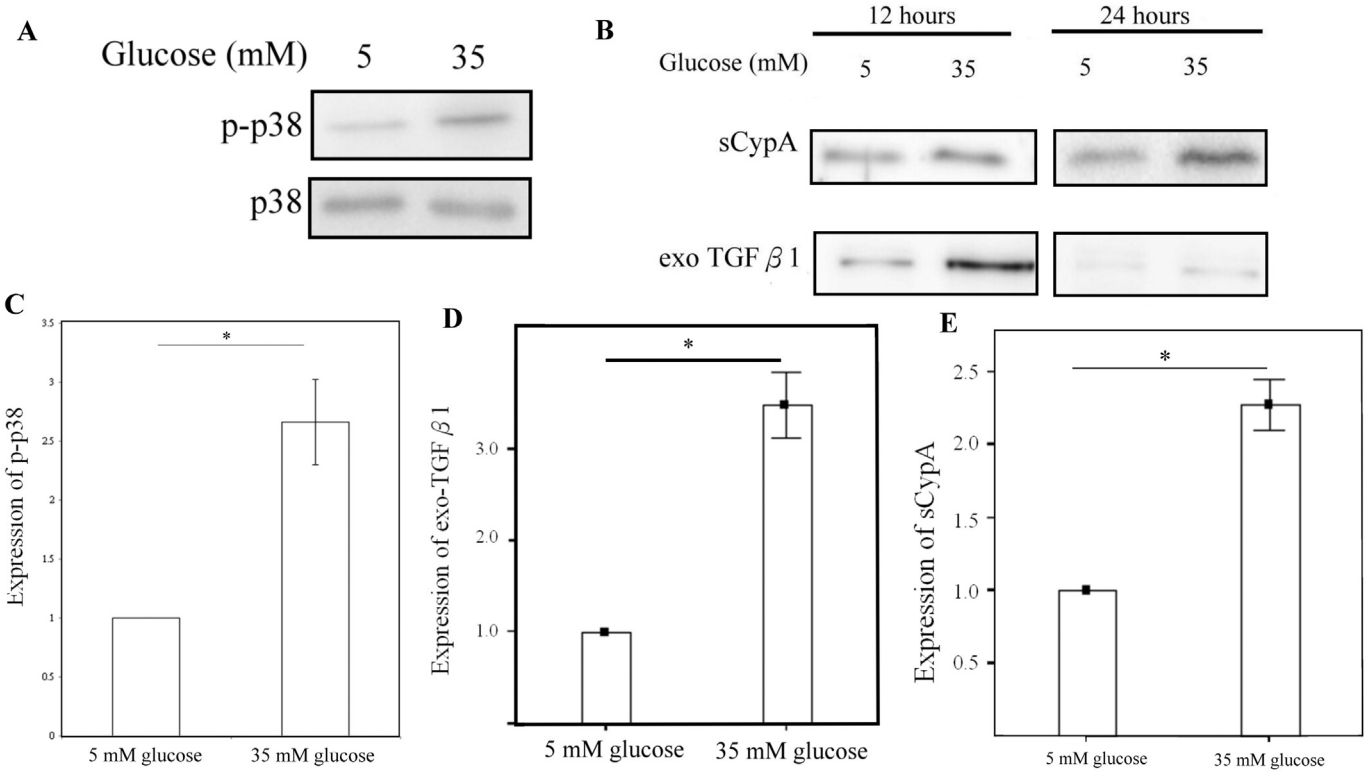



**Fig. 7.** Expression of phosphorylated p38, sCypA and TGFβ1 after high glucose treatment of HK-2 cells in Western blotting. (A) After high glucose (35 mmol/l) treatment for 30 min, the expression of p-p38 increased. (B) After high glucose (35 mmol/l) treatment for 12 h, the expression of TGFβ1 increased but not for sCypA. After high glucose (35 mmol/l) treatment for 24 h, the expression of sCypA increased but not for TGFβ1. (C) Quantitative data for the expression of p-p38 in (A). ($p < 0.05$) ($n = 3$). (D) Quantitative data for the expression of TGFβ1 after 12 h treatment of high glucose. ($p < 0.05$) ($n = 3$). (E) Quantitative data for the expression of sCypA after 24 h-treatment of high glucose. ($p < 0.05$) ($n = 3$)*$p < 0.05$; exo-TGFβ1: exogenous TGFβ1.

### *3.6. Effects of Linagliptin on expressions of sCypA on MES-13*

In our previous cell studies, oxidative stress and hyperglycemia could stimulate MES-13 and HK-2 cells to secrete cyclophilin A [8]. To understand whether Linagliptin affects the expression of sCypA in the cellular model, MES-13 cells were treated with high glucose to stimulate sCypA under different concentrations of Linagliptin. Our results showed that Linagliptin successfully inhibited the expression of sCypA in cells treated with 25 mM glucose in all three different concentrations (1, 10, and 100 nmol/l) (Fig. 6A). Under the same glucose concentration (25 mmol/l), the 10 nmol/l Linagliptin treatment was able to inhibit the expression of sCypA for 8 h with statistical significance ($p < 0.001$) (Fig. 6D). The effect could last for 12 h (Fig. 6C), but a longer treatment time of up to 24 h diminished the effect (Fig. 6B). These findings therefore indicate that Linagliptin certainly could act as a rescue reagent for MES-13 cells under hyperglycemia by reducing sCypA production. Since only 3–5% Linagliptin will enter tubular cells [21], we did not verify effects of Linagliptin on expressions of sCypA in HK-2 cells.

### *3.7. Molecular pathway of sCypA related DN*

Since sCypA can regulate p38-MAPK signaling [22], we hypothesize that p38-MAPK is also involved in sCypA-related DN. Instead of MES-13, we chose HK-2 cells because of the following reasons. Firstly, p38 MAPK signaling pathway was associated with DN in HK-2 cells [23]. Secondly, receptors of sCypA, CD 147, are mostly distributed over HK-2 cells [24]. After treating by high glucose on HK-2 cells, the phosphorylated-p38 (p-p38) increased in Western blotting (Fig. 7A). The increased expression of TGFβ1 could be detected earlier (12 h) compared to the increased CypA after a relatively longer duration (24 h) (Fig. 7B). All were quantified in Fig. 7C for p-p38, Fig. 7D for TGFβ1 (12 h) and Fig. 7E for sCypA (24 h). After treating by TGFβ1, the expression of p-p38 and sCypA both increased (Fig. 8A). Nevertheless, after treating by CypA, the expression of TGFβ1 did not increased (Fig. 8B), but the expression of p-p38 increased (Fig. 8C). Quantified data after the treatment of TGFβ1 are shown in Fig. 8D for p-p38 and Fig. 8E for sCypA. Quantified data of p-p38 after the treatment of CypA is shown in Fig. 8F. Taken together, our data indicate that hyperglycemia induced the secretion of TGFβ1 from HK-2 cells. Also, TGFβ1 stimulated the secretion of CypA, which may then result in the increment of p38-MAPK.

### *3.8. sCypA and its receptor (CD147) on HK-2 cells via confocal microscopy*

CD147 is a membrane receptor for sCypA and is mainly distributed in the cytoplasm [24]. CD147 is mostly concentrated in the PTEC [25]. Without any treatment, the CD147 is mostly distributed in the cytoplasm (Fig. 9A). After being treated with CypA (1 nmol/l) for 10 min, the cytosolic CD147 moved toward cell membranes and the contours of HK-2 cells could be identified (Fig. 9B). After further high dose of CypA treatment (10 nmol/l) for 10 min, almost all contours of HK-2 cells could be seen clearly (Fig. 9C). On the other hand, CD147 was mostly distributed in the cytoplasm (Fig. 9D) if there was no treatment of TGFβ1. After treatment of TGFβ1 for 10 min, the distribution of CD147 was mainly in the cytoplasm (Fig. 9E). However, after 24 h TGFβ1 treatment, the cytosolic CD147 moved toward cell membranes (Fig. 9F). In summary, CypA was capable of immediately stimulating cytosolic CD147 of HK-2 cells to move toward the cell membrane while it would take 24 h for TGFβ1 to do so.

## 4. Discussion

In the DN animal model, our results demonstrate that although both sCypA and 8-OHdG are early indicators for DN, sCypA is a better indicator than 8-OHdG. Firstly, we compared the extent for which the value of

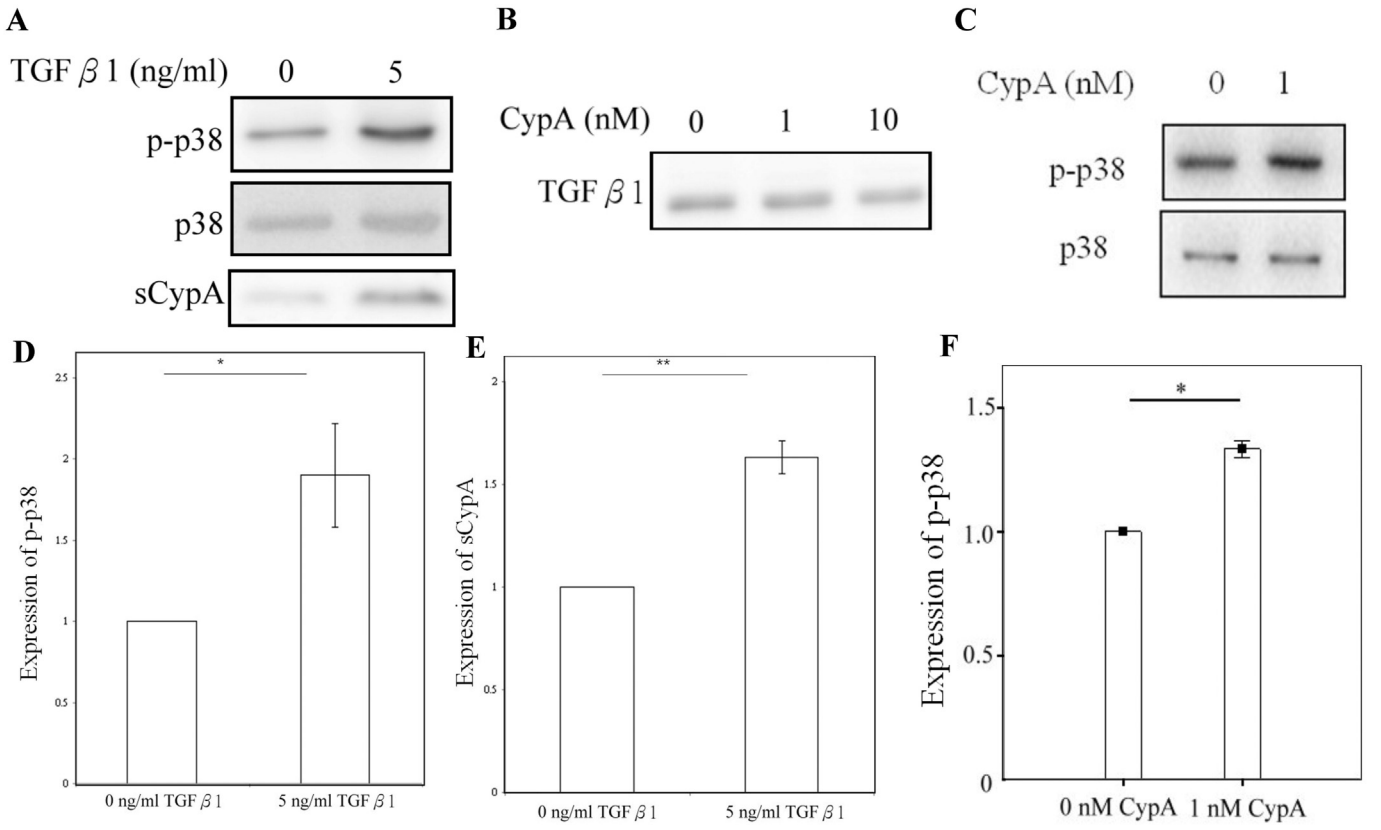



**Fig. 8.** Expression of phosphorylated p38 and sCypA by TGFβ1 treatment and expression of TGFβ1 by sCypA treatment in HK-2 cells. (A) After treatment of TGFβ1 (5 ng/ml) for 30 min and 24 h, the expression of p-p38 and sCypA both increased, respectively. (B) After treatment of CypA (1 and 10 nmol/l) for 24 h, the expression of TGFβ1 did not increase. (C) After treatment of CypA (1 nmol/l) for 30 min, the expression of p-p38 increased. (D) P-p38 increased significantly after treatment with TGFβ1 ($p < 0.05$) ($n = 3$). (E) SCypA increased significantly after treatment with TGFβ1 ($p < 0.01$) ($n = 3$). (F) P-p38 increased significantly after treatment with CypA ($p < 0.05$) ($n = 3$). *$p < 0.05$, **$p < 0.01$.

the indicator has increased at the same time. A 12.7 folds of increase [(6656.1 pg/day)/(523.1 pg/day)] of sCypA concentration at the 8th week in the *db*/*db* compared to 1.7 folds [(11.62 ng/day)/(6.83 ng/day)] of 8-OHdG concentration also suggested that sCypA is a more sensitive and specific indicator than 8-OHdG. We used *Chi* square to examine the ROC curve for sCypA (Supplementary Fig. 1, B) and 8-OHdG (Supplementary Fig. 1, A), and they differed statistically ($p < 0.0001$) for diagnostic power. Secondly, the sCypA detected at the 8th week is an early marker for DN since the blood sugar in the *db*/*db* began to rise slightly from the 4th week to the 8th (130 ± 4 mg/dl and 175 ± 29 mg/dl respectively) [26]. This period of time corresponds to the duration of early-stage DN when mesangial matrix expansion is still not detectable microscopically. The early-stage DN is characterized by hyperfiltration, resulting in a mere increase in 23% of glomerular surface (e.g. hypertrophy or hemodynamic hyperfiltration) [27]. Hence, urinary sCypA is an early DN marker because there is an obvious increase of sCypA at the 8th week when there are few pathological changes. On the other hand, the increased sCypA should not be considered as a general effect due to increased proteinuria in the *db*/*db* mice, because among the 4 groups of mice, there was no significant upsurge in albuminuria at the 16th week compared to that at the 8th ($p = 0.059$, 0.064, 0.400, and 0.203 in numerical order). Our data are consistent with previous reports that albuminuria or proteinuria was not the variable to represent the severity of DN in the *db*/*db* and *db*/*m* [27–30]. According to the above reasons, we believe that urinary sCypA is a much earlier and stronger maker than 8-OHdG for DN. Consistently, the increased IHC staining for CypA was also detected in mesangial cells (Fig. 3B vs. A; E) and tubular cells (Supplementary Fig. 2B vs. A) in DN. The above findings in animal models are all consistent with our previous human study that human urinary CypA can be detected since stage 2 DN [8]. Thus, we conclude that urinary sCypA could possibly be a much stronger and earlier factor involved in causing DN.

Significant protective role of Linagliptin on renal function are similar to the previous report that renal protection of Linagliptin is associated with TGFβ1 [31]: Linagliptin can interfere with the conversion of latent to active TGF-β1 and downstream fibrotic markers [31]. We also demonstrate that the increased staining for CypA in glomeruli of DN can be reduced by Linagliptin (Fig. 3), which suggests that renal protection of Linagliptin may be associated with CypA in glomeruli. Furthermore, it may be independent from tubular cells because <5% Linagliptin entered the tubules [21]. Besides, we postulate that sCypA may be a stronger pathological factor than that of 8-OHdG. At the 8th week, Linagliptin could suppress 8-OHdG at a low dose but suppression of sCypA required a high dose. Similarly, high dose of Linagliptin was able to suppress secretion of 8-OHdG but not sCypA at the 20th week. The more pathogenic marker, sCypA, could exist up to the 20th week and could not be suppressed by high dose Linagliptin. This is also an indirect evidence that sCypA has stronger pathogenic effects on DN than 8-OHdG. It is worth noting that renal protection of Linagliptin exists in this animal study independently from glucose lowering. Failure of glucose lowering by Linagliptin was similar to previous researches [11,32,33].

The control of blood sugar was sustained early with DPP4i in the animal model of db/db. Nonetheless, progression of insulin resistance (persisted increased body weight) appeared to block the improvement of glucose tolerance through DPP4i. Linagliptin is then effective in only the early stage of type 2 diabetes [32]. Other reason for the discrepancy of the blood sugar values obtained before and after 8-week Linagliptin treatments is that the *db*/*db* developed frank hyperglycemia (175 ± 29 mg/dl at the 8th week of age and 283 ± 77 mg/dl at the 10th) [26]. We highly suspect an unrestricted diet (Fig. 1A) leading to the increased weight gain (Fig. 1E) which also caused poor blood sugar control after the 8th week. We believe that one cannot rely merely on medication when treating diabetes. A restricted diet to prevent excessive weight

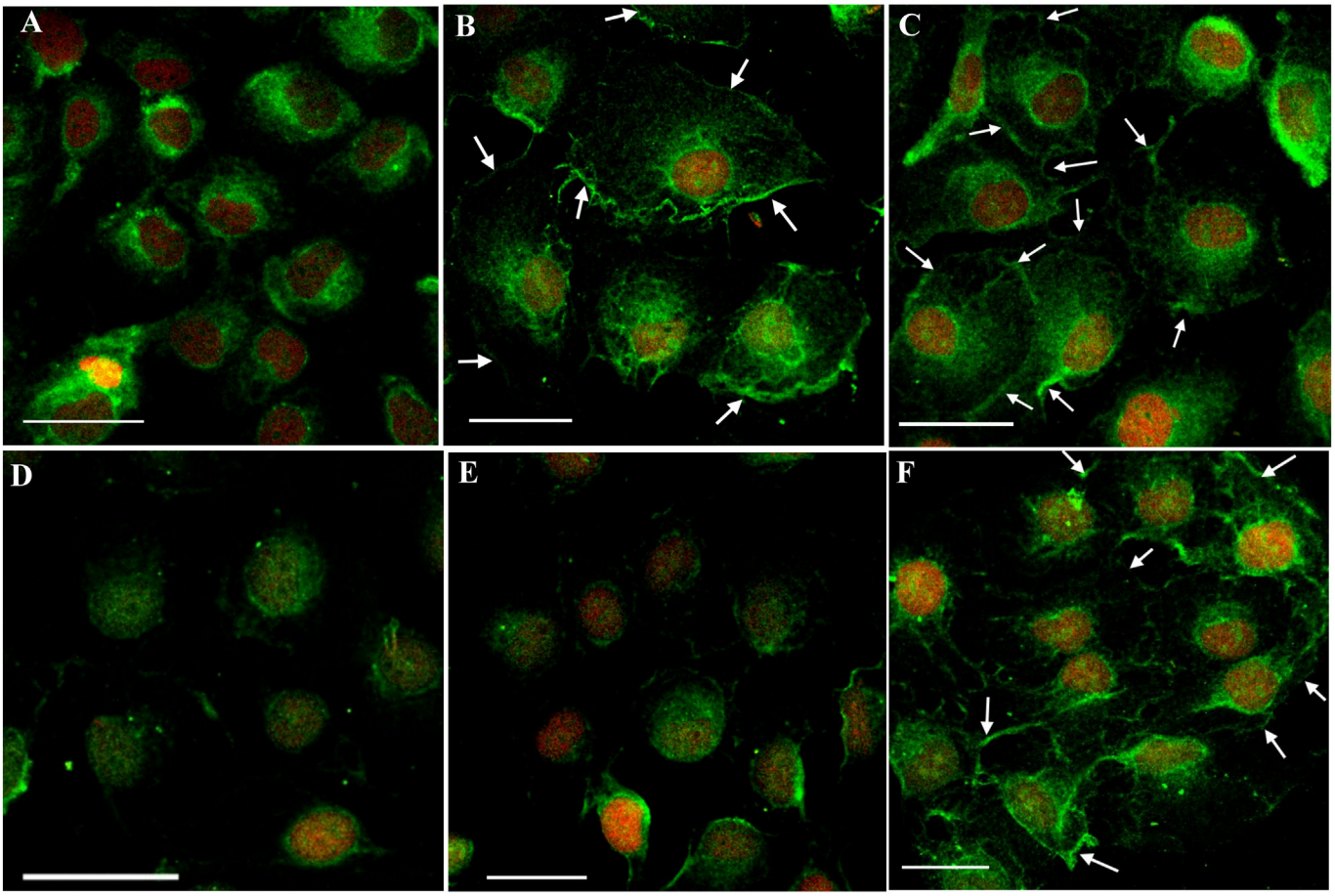



**Fig. 9.** Confocal microscopy for CD 147 in HK-2 cells treated with CypA and TGFβ1. (A) Without treatment of CypA, positive staining for CD147 (green staining) was mostly distributed in the cytoplasm near the nucleus (red staining by Propidium iodide). (B) After treatment with 1 nmol/l CypA for 10 min, the positive staining for CD147 moved closely to the cell membrane of HK-2 cells, and cell membranes can be seen very clearly (white arrow). (C) After treatment with 10 nmol/l CypA for 10 min, the positive staining for CD147 moved further closely to the cell membrane of HK-2 cells and cell membranes can be seen much more clearly than the previous 1 nmol/l CypA. Almost all contours of HK-2 cells can be seen clearly (white arrow). (D) Without treatment of TGFβ1, positive staining for CD147 (green staining) was mostly distributed in the cytoplasm near the nucleus (red staining by Propidium iodide). The membrane of HK-2 cells cannot be detected. (E) After treatment with 5 ng/ml TGFβ1 for 10 min, the positive staining for CD147 is mostly distributed in the cytoplasm near the nucleus. The membrane of HK-2 cells cannot be detected. (F) After treatment with 5 ng/ml TGFβ1 for 24 h, the positive staining for CD147 moved closely to the cell membrane of HK-2 cells, and cell membranes can be seen much more clearly (white arrow). Almost all contours of HK-2 cells can be seen clearly (white arrow). Scale bar, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gain is as important as prescription drugs, as reported in the study conducted by Ishibashi et al. [34] where the *db*/*db* mice were fed with two feeding methods: standard chow twice a day and ad libitum. In Ishibashi's study [34], they raised mice with two feeding methods which gave 3.2 g/day or 5 g/day of food at the 12th week. The resulting body weight was 29.8 ± 0.7 g vs. 42.6 ± 2.9 g respectively. DPP4i failed to control blood sugar in the *db*/*db* mice receiving chow ad libitum because of glucose toxicity and lipotoxicity [34]. In contrast, our study did not limit food intake for all three *db*/*db* groups. Compared to the Ishibashi's study, all our 3 groups of *db*/*db* at the 12th week weighted more (49.9 ± 0.64 g, 50.2 ± 0.47 g, and 50.2 ± 7.52 g). As it was also observed in Ishibashi's study, the body weight in our *db*/*db* mice remained high regardless of DPP4i treatment. However, DPP4i can achieve fair blood sugar control in human because unlimited weight gain is less likely. Interestingly, we could observe the renal protection effect of Linagliptin independently from its glucose lowering effect. Moreover, the similar weight gain among all 3 *db*/*db* groups was consistent with the clinical finding that DPP4i plays a neutral role in body weight in diabetic patients [35].

In addition, our results are consistent with a recent study regarding DPP4-deficinecy in an animal model [36]. Firstly, our findings suggest that the main effects of DPP4i were on glomeruli, with less effect on tubules, which are similar to the effects of DPP4 deficiency on expansion of glomerular area and albuminuria reported by Matsui T et al. [36]. Secondly, Matsui T et al. also found that increased 8-OHdG levels in the kidneys were suppressed significantly in DDP4-deficient rats. Our study echoed their finding. Thirdly, Matsui T et al. demonstrated that decreased Advanced Glycation End Product (AGE)-Receptor for AGEs (RAGE) axis in the genetically DPP4 deficiency rats provided renal protection even though the fasting blood glucose was similar in DN rats with or without DPP4 deficiency. In our study, Linagliptin reduced the increment of glucose-stimulated CypA without lowering fasting blood glucose. Both the internal (genetically DPP4 deficiency) and external (Linagliptin treatment) mechanisms resulted in less DN through less glucose toxicity (lower AGE-RAGE axis and lower glucose-stimulate CypA secretion, respectively), supporting the notion that the effects of renal protection from blocking DPP4 are the results from decreased glucose toxicity without lowering blood glucose,

We showed pathological evidence of strong positive staining for CypA over mesangial cells in glomeruli (Fig. 3B) and peri-glomerular tubules (Supplementary Fig. 2B vs. A). Typically, findings of DN are focused on mesangial cells in glomeruli. However, early changes in PTEC may be an essential factor in the development of progressive kidney diseases [37–39]. Based on our previous study [8], hyperglycemia stimulated both mesangial cells and PTEC to secret CypA. This finding is compatible with the distribution of CypA staining in the *db*/*db* mice. To this end, we propose that there is interplay between PTEC and mesangial cells, and sCypA is associated with this relationship. Secreted

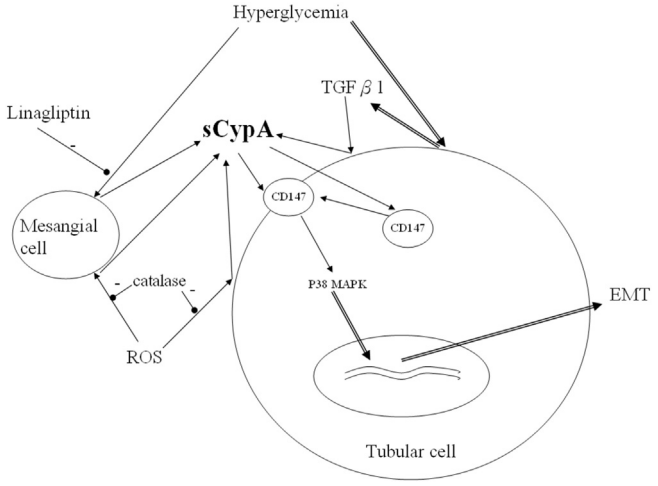



**Fig. 10.** Hypothesis of molecular pathway for the effects of sCypA on diabetic nephropathy and its association with Linagliptin. Both ROS (reactive oxidative stress) and hyperglycemia can stimulate MES-13 cells to secrete sCypA, which can be reversed by catalase and Linagliptin, respectively. ROS and hyperglycemia can also stimulate HK-2 cells to secrete sCypA. More precisely, hyperglycemia stimulates HK-2 cells to release TGFβ1, which induces HK-2 cells to secrete sCypA. The sCypA causes cytosolic CD147 to move to the cell membrane and serves as membrane receptors for sCypA. The binding of sCypA and CD 147 activates p38 as phosphorylated p38. Then the phosphorylated p38 may cause a downstream reaction, such as epithelial mesenchymal transition, which will cause diabetic nephropathy. Two solid lines: cited from others published studies.

CypA is associated with inflammatory or infectious diseases [5], especially in cardiovascular disease [4,40]. It is considered as a new promising target in cardiovascular therapy [4,40]. ROS inducers, including angiotensin II, stimulate CypA secretion from vascular smooth muscle cells. The sCypA activates ERK1/2 and promotes ROS production, thus augmenting the full response [40]. In rheumatoid arthritis, CypA-CD147 interaction might cause the destruction of cartilage and bone by upregulating MMP-9 expression [41]. CypA also induced CD147-dependent chemotaxis of activated CD4 + T cells in asthma [42]. CypA expression correlated with MMP-1, MMP-2, and MMP-9 expression in periodontitis [43]. In our previous report [8], we detected increased urinary CypA since the silent stage of DN. In this study, we further examined the mechanism that sCypA is involved in DN by using the cellular model. It is known that released sCypA will bind to its receptors, CD147, in many different types of cells. Given the fact that there are different ligands for CD147 binding, it is worth noting that the movement of cytosolic CD147 to cell membrane immediately after cells is treated with CypA (Fig. 9). The above finding indicates that sCypA is indeed involved in cell surface localization of CD147. All the above findings indicated that the interplay of sCypA may be a paracrine for MES-13 and an autocrine for HK-2 cells.

In this study, we showed that hyperglycemia stimulated PTEC to secret TGFβ1, which is consistent with the previous reports that the synergism of high glucose concentrations with cytokines can stimulate TGFβ1 synthesis by PTEC [44,45]. TGFβ1 is upstream to many fibrotic pathways and is a multifunctional regulator that modulates cell differentiation, proliferation, and migration and induces the production of extracellular matrix proteins [46]. All are pivotal processes that contribute to glomerulosclerosis [47]. In addition to the association of TGF-β1 with glomerular change, TGF-β1 has been shown to participate both directly and indirectly in tubule degeneration in DN [48]. The epithelial mesenchymal transition (EMT) is the mechanism in most studies [23,49,50]. TGF-β1 down-regulates the expression of epithelial cell adhesion molecules (E-cadherin and ZO-1), increases de novo α-SMA expression and actin reorganization, and finally enhances cell migration and invasion of the interstitium [49]. It is worth noting that TGF-β1 related EMT in PTEC had been recently studies by Zhi-Mei Lv et al. [23]. It is about the p38 MAPK signaling pathway in hyperglycemia induced EMT in PTEC. However, how the TGFβ1 stimulate increased expression of p38 MAPK is still unknown. Our study provides further evidence to confirm that TGFβ1 stimulates secretion of CypA which may cause CD147 to move outward to the cell membrane. CD147 may serve as the membranous receptor for sCypA. Secreted CypA induced cell surface localization of CD147 might cause increased expression of p38 MAPK, leading to a downstream reaction such as EMT [23].

The reasons that TGFβ1 is upstream to sCypA are as follows. Firstly, TGFβ1 can stimulate secretion of CypA (Fig. 8A) but not vice versa (Fig. 8B). Secondly, increased expressions of TGFβ1 can be detected at 12th h (Fig. 7B and D) from hyperglycemia-treated HK-2 cells, but expressions of sCypA was not detected until 24 h (Fig. 7B and E). Last but not the least, the surfacing of CD147 can be detected as soon as 10 min following treatment of CypA (Fig. 9B and C) but 24 h after TGFβ1 (Fig. 9F). Based on the above findings, in addition to functioning as a marker for DN, sCypA may also have a pathological role for DN. We propose that sCypA is involved in the cross-talk between mesangial cells and PTEC through TGFβ1, CD147, and p38 MAPK (Fig. 10).

## 5. Conclusion

Based on human, animal and cell studies, sCypA was shown to be not only a marker of DN but also appeared to play a pathological role for DN. The renal protective effect of Linagliptin may be associated with blockage of sCypA in glomeruli. The sCypA may have potential as a treatment target and thus further study is needed in the future.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2016.11.005.

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