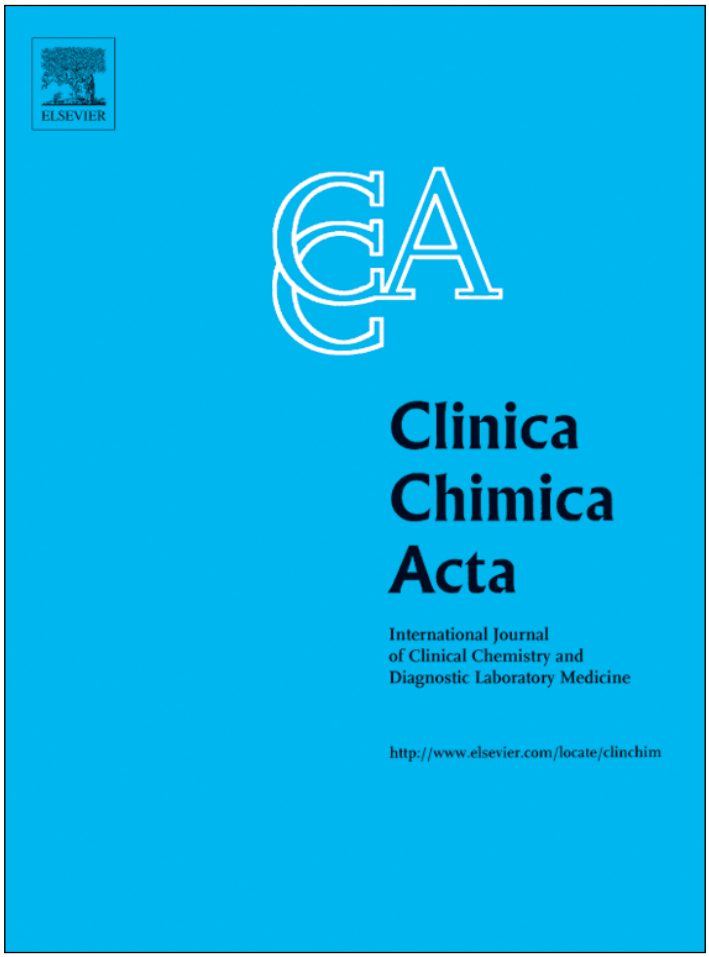

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Novel findings of secreted cyclophilin A in diabetic nephropathy and its association with renal protection of dipeptidyl peptidase 4 inhibitor

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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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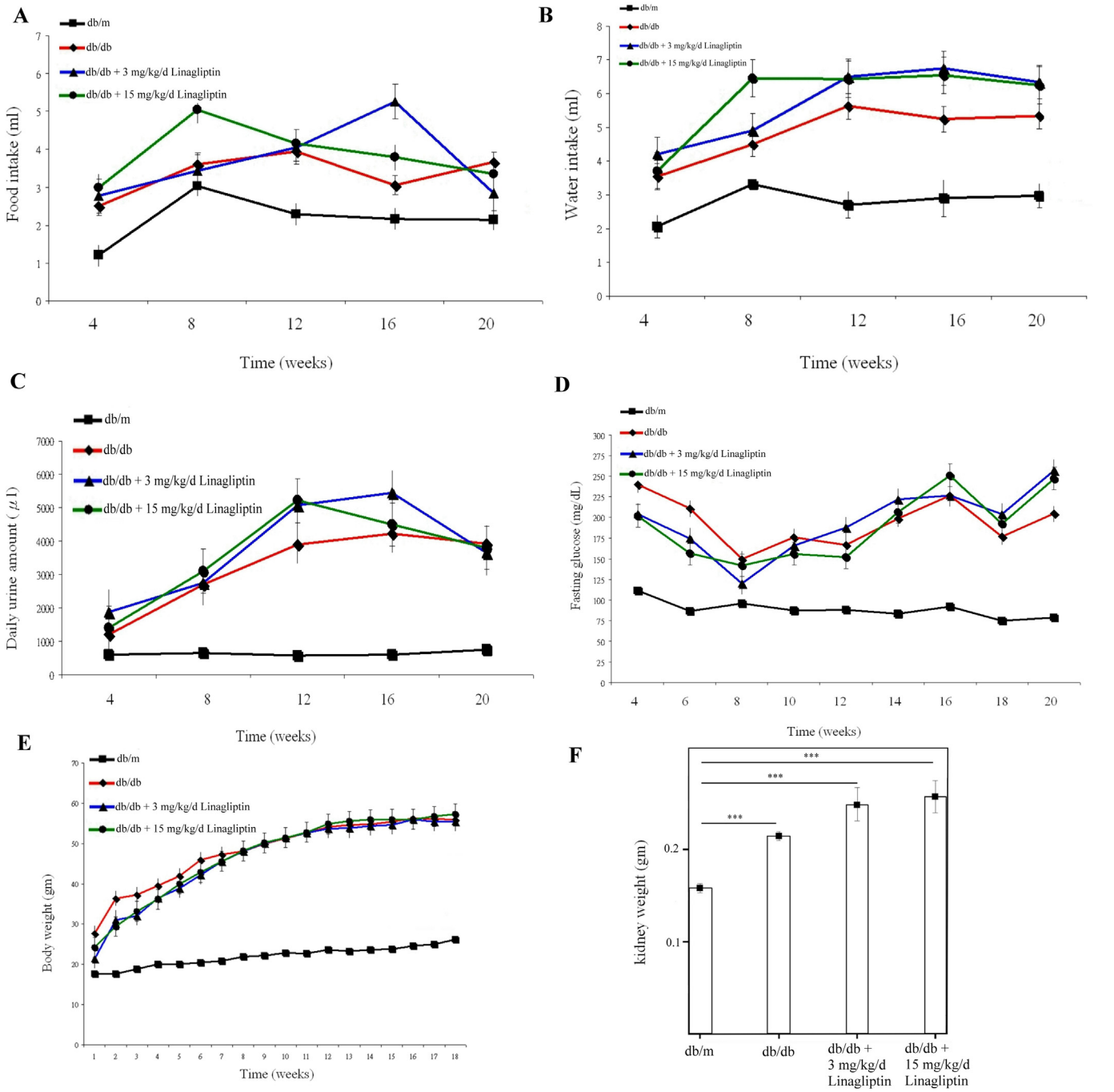


Fig. 1. Physical data of mice. (A) Three groups of mice with diabetic nephropathy (*db*/*db*) ate more food than mice without diabetic nephropathy (*db*/*m*). However, there was no difference among all 3 *db*/*db* groups. ($n = 10$). (B) Three groups of mice with diabetic nephropathy (*db*/*db*) drank 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

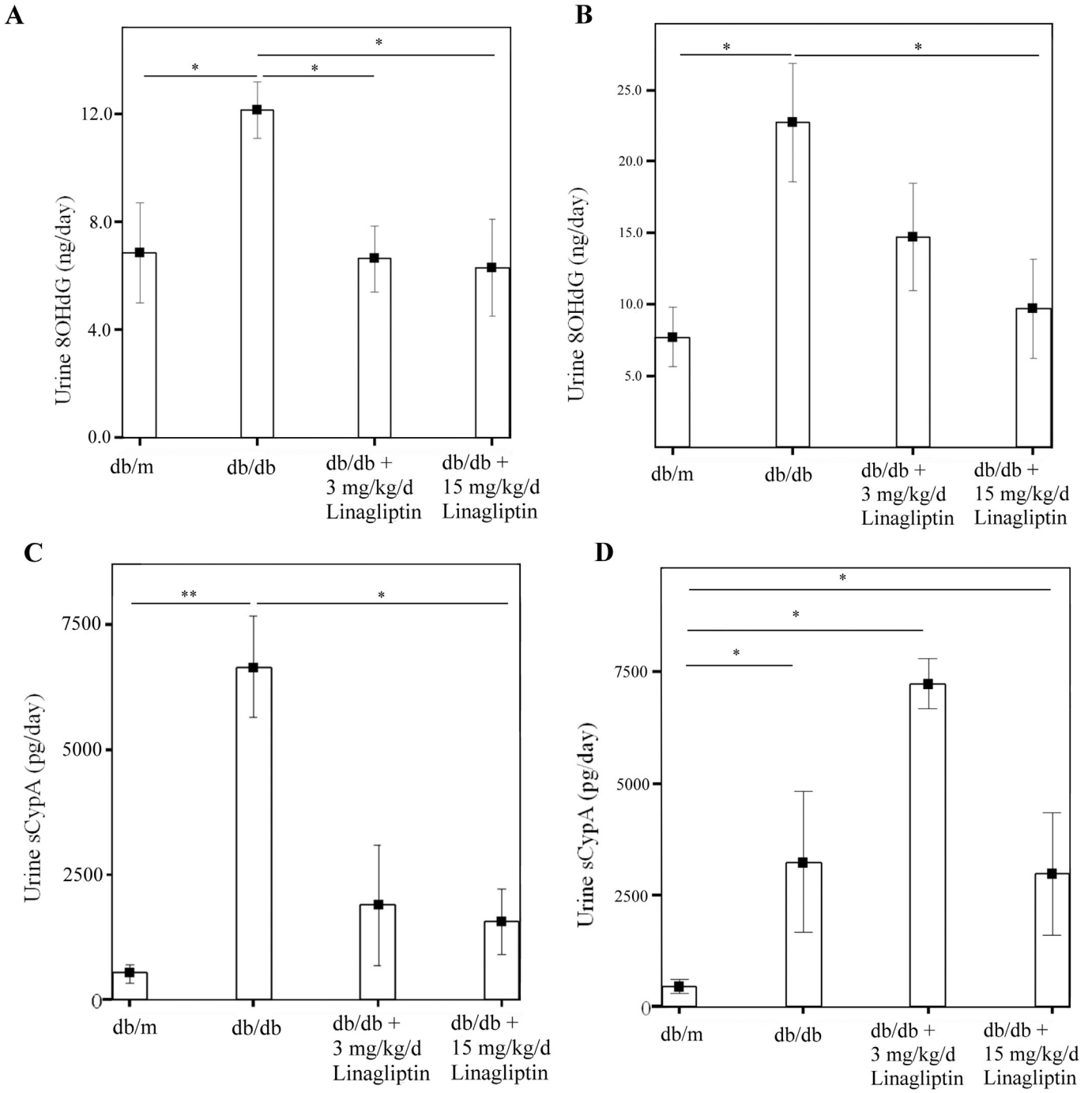


Fig. 2. Expressions of 8-OHdG and CypA from mice's urine at the 8th week and 20th week. (A) The expression of 8-OHdG in *db*/*db* from the urine at the 8th week was increased significantly compared to *db*/*m* ($p = 0.026$). This could be suppressed by administering both 3 and 15 mg/kg/day Linagliptin ($p = 0.018$ 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).

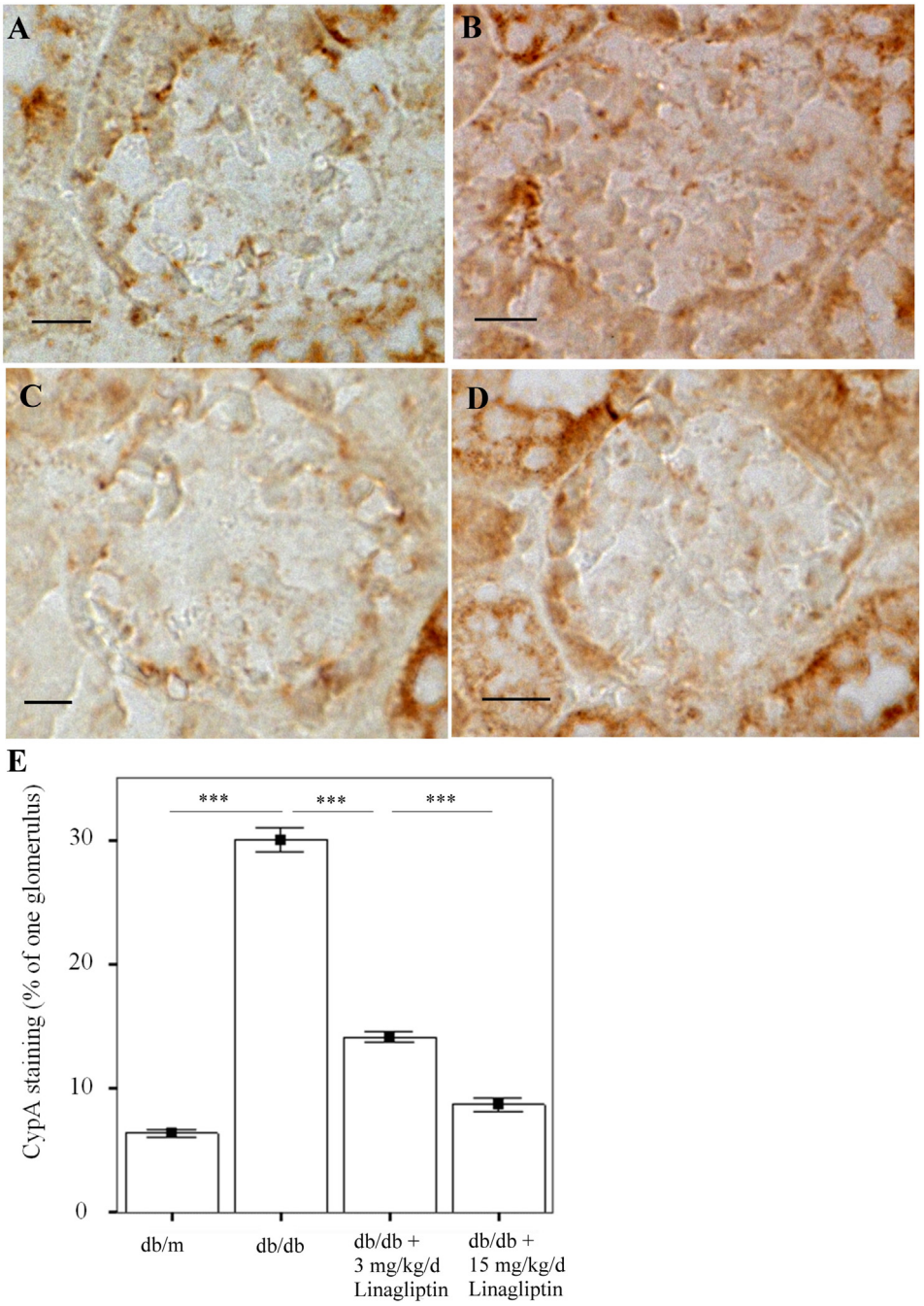


Fig. 3. IHC staining of glomeruli for CypA in *db/db* diabetic compared to *db/m* nondiabetic kidneys at 20th week. (A)(B)(C)(D) IHC staining for CypA (brown staining) was increased in the *db/db* group compared to *db/m* (B vs. A). The increased staining for CypA in *db/db* (B) could not be observed 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.)

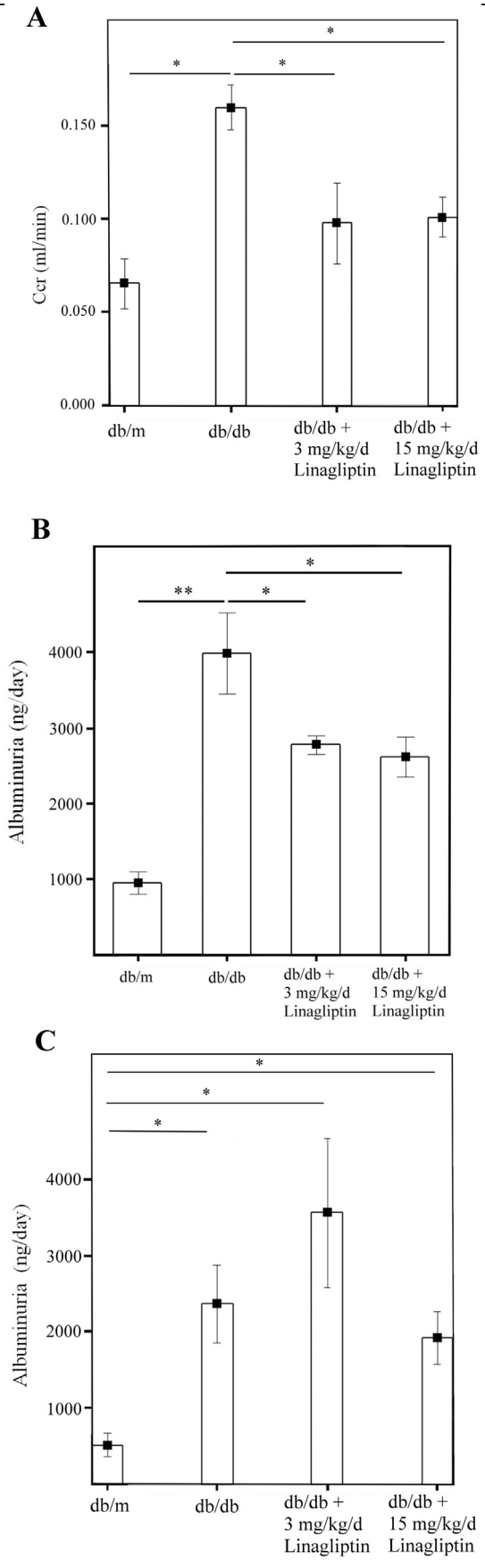
2.4. Cell culture

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 3×10^5 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.) $^*p < 0.05$, $^{**}p < 0.01$.

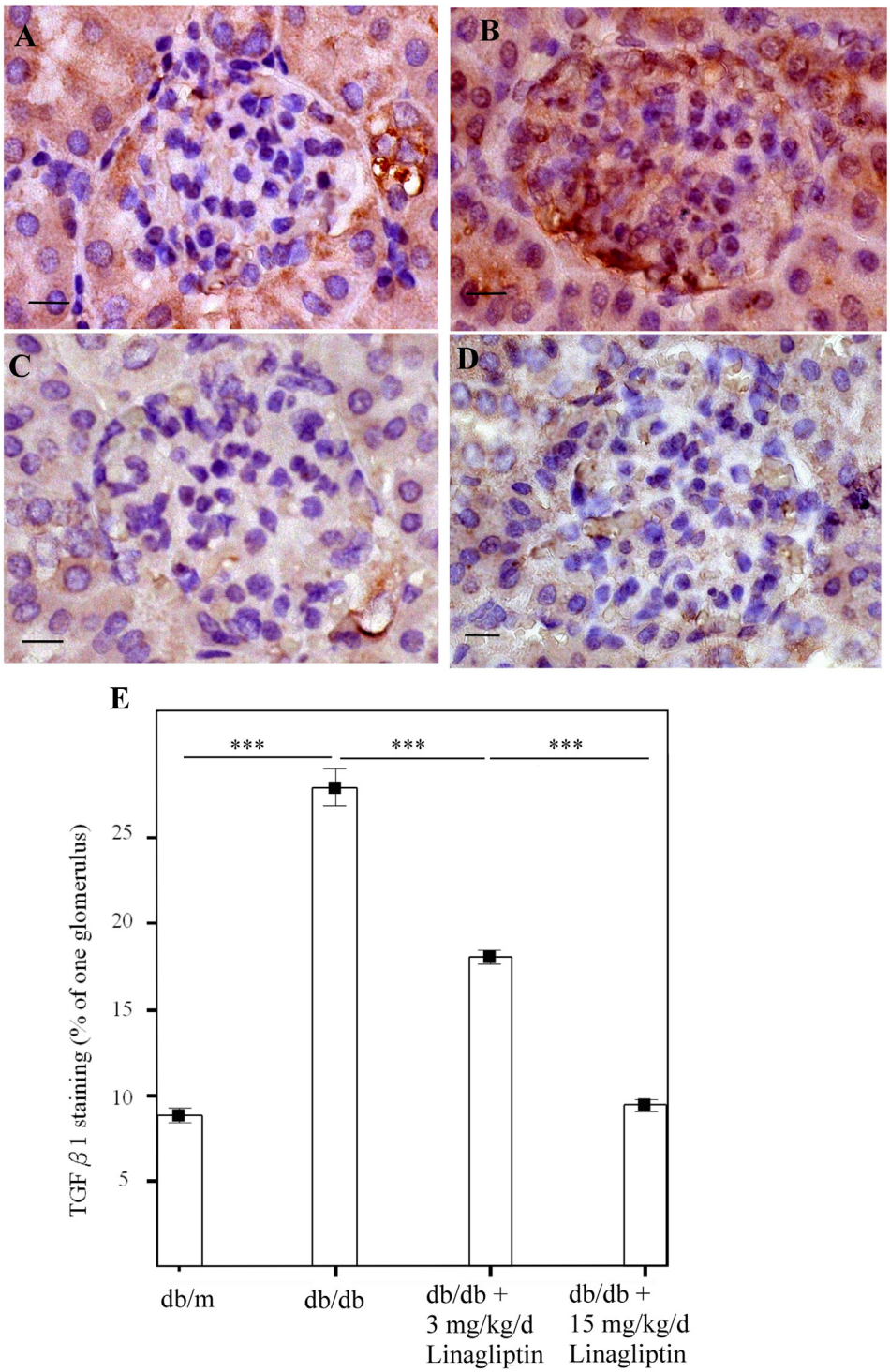


Fig. 5. IHC staining for TGFβ1 in *db/db* diabetic compared to *db/m* nondiabetic kidneys at 20th week. (A)(B)(C)(D) IHC staining for TGFβ1 (brown staining) in glomeruli was increased in the *db/db* group compared to *db/m* (B vs. A). The increased glomerular staining in *db/db* (B) could not be observed 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

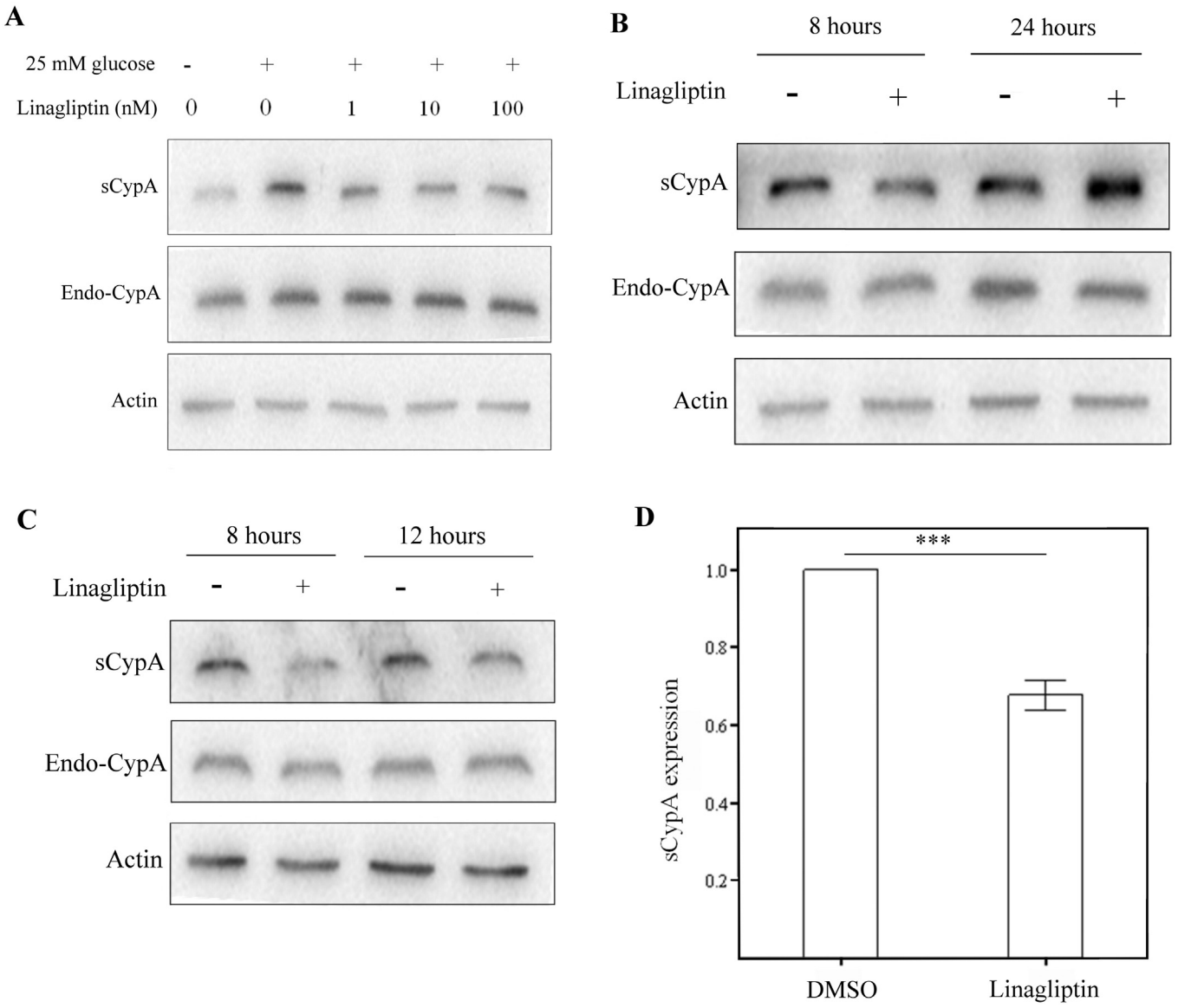


Fig. 6. Western blotting of sCypA expression in glucose treated MES-13 cells and rescued by Linagliptin. (A) The expression of sCypA treated by 25 mmol/l glucose was inhibited by Linagliptin in all 3 different concentrations (1, 10, and 100 nmol/l) for 8 h. (B) Under high glucose condition (25 mmol/l), 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].

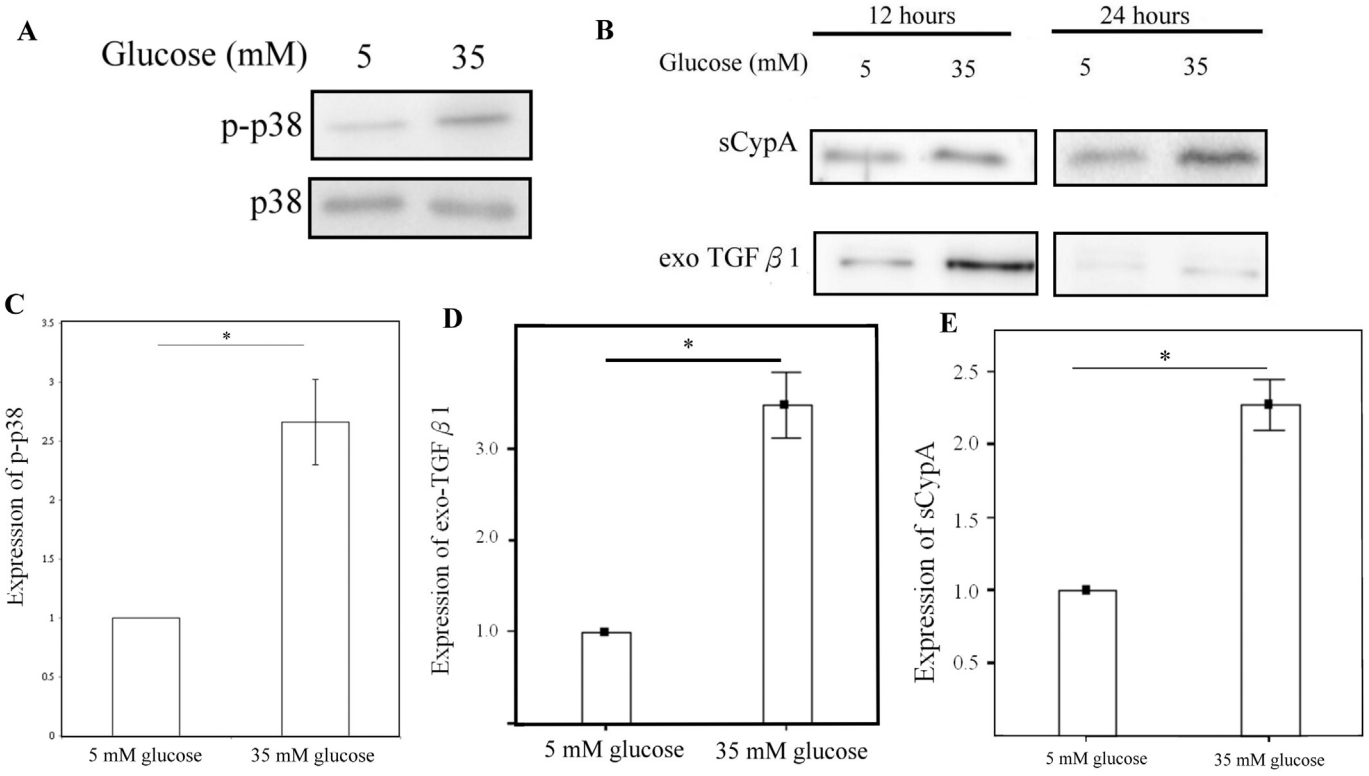


Fig. 7. Expression of phosphorylated p38, sCypA and TGFβ1 after high glucose treatment of HK-2 cells in Western blotting. (A) After high glucose (35 mmol/l) treatment for 30 min, the expression of p-p38 increased. (B) After high glucose (35 mmol/l) treatment for 12 h, the expression of TGFβ1 increased 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

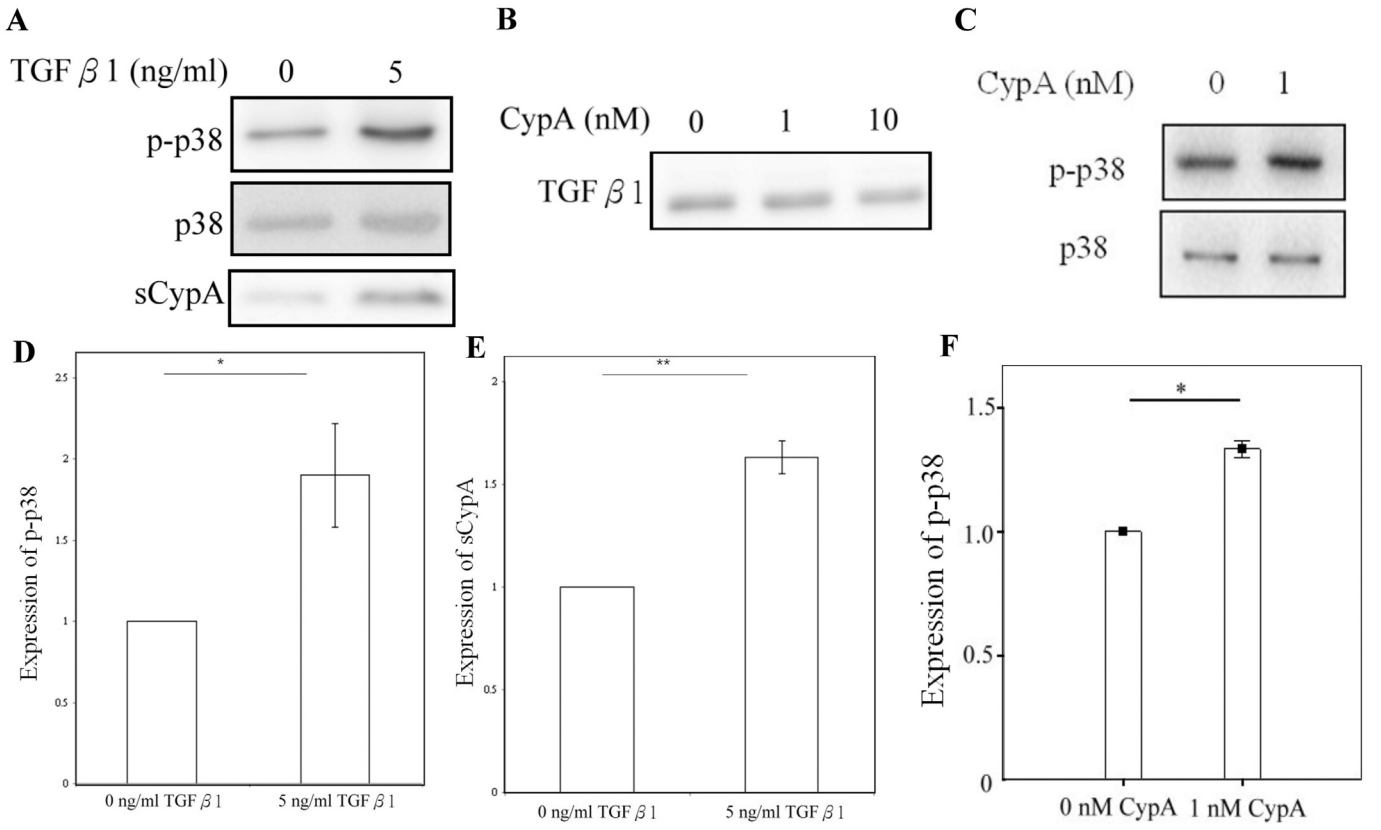


Fig. 8. Expression of phosphorylated p38 and sCypA by TGFβ1 treatment and expression of TGFβ1 by sCypA treatment in HK-2 cells. (A) After treatment of TGFβ1 (5 ng/ml) for 30 min and 24 h, the expression of p-p38 and sCypA both increased, respectively. (B) After treatment of CypA (1 and 10 nmol/l) for 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

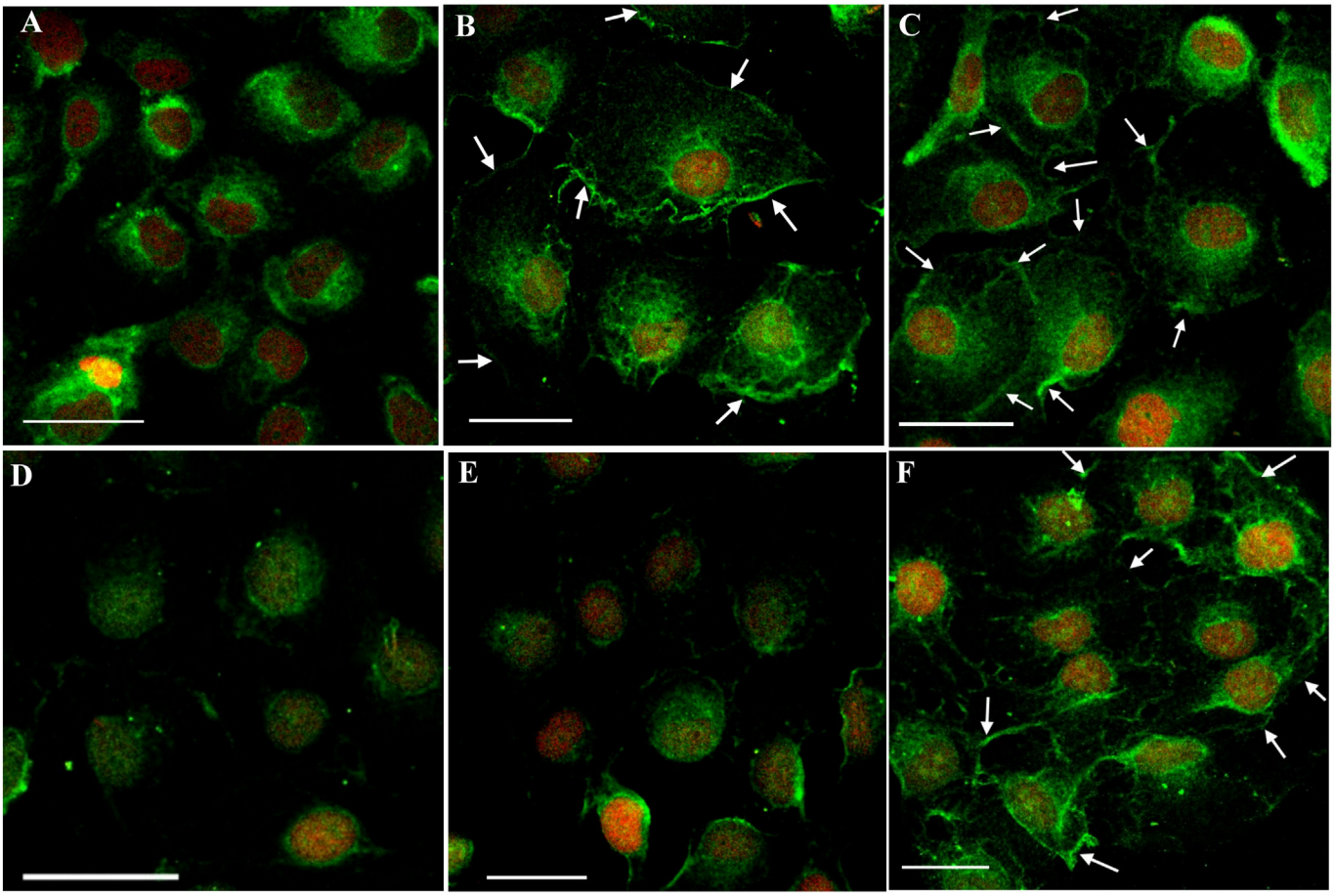


Fig. 9. Confocal microscopy for CD 147 in HK-2 cells treated with CypA and TGFβ1. (A) Without treatment of CypA, positive staining for CD147 (green staining) was mostly distributed in the cytoplasm near the nucleus (red staining by Propidium iodide). (B) After treatment with 1 nmol/l CypA for 10 min, 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

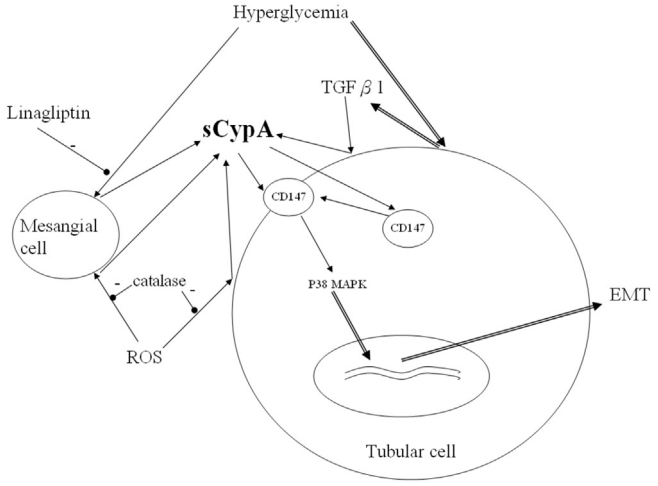


Fig. 10. Hypothesis of molecular pathway for the effects of sCypA on diabetic nephropathy and its association with Linagliptin. Both ROS (reactive oxidative stress) and hyperglycemia can stimulate MES-13 cells to secrete sCypA, which can be reversed by catalase and Linagliptin, respectively. ROS and 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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