Exendin-4 improving ABCA1-mediated cholesterol efflux

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ABSTRACT

The ATP-binding cassette transporter A1 (ABCA1), which promotes cholesterol efflux from cells and inhibits inflammatory responses, is highly expressed in the kidney. Research has shown that exendin-4, a glucagon-like peptide-1 receptor (GLP-1R) agonist, promotes ABCA1 expression in multiple tissues and organs; however, the mechanisms underlying exendin-4 induction of ABCA1 expression in glomerular endothelial cells are not fully understood. In this study we investigated the effect of exendin-4 on ABCA1 in glomerular endothelial cells of diabetic kidney disease (DKD) and the possible mechanism. We observed a marked increase in glomerular lipid deposits in tissues of patients with DKD and diabetic apolipoprotein E knockout (apoE−/−) mice by Oil Red O staining and biochemical analysis of cholesterol. We found significantly decreased ABCA1 expression in glomerular endothelial cells of diabetic apoE−/− mice and increased renal lipid, cholesterol, and inflammatory cytokine levels. Exendin-4 decreased renal cholesterol accumulation and inflammation and increased cholesterol efflux by upregulating ABCA1. In human glomerular endothelial cells (HRGECs), GLP-1R-mediated signaling pathways (e.g., Ca2+/calmodulin-dependent protein kinase, cAMP/protein kinase A (PKA), phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT), and extracellular signal-regulated kinase (ERK1/2) were involved in cholesterol efflux and inflammatory responses by regulating ABCA1 expression. We propose that exendin-4 increases ABCA1 expression in glomerular endothelial cells,
which plays an important role in alleviating renal lipid accumulation, inflammation, and proteinuria in mice with type 2 diabetes.

**INTRODUCTION**

Diabetes mellitus is a chronic disease that has become a global epidemic. One of the most common microvascular complications of diabetes mellitus is diabetic kidney disease (DKD), a progressive fibrotic kidney disease that is the leading cause of end-stage renal disease (1), beginning with normoalbuminuria, microalbuminuria, and macroalbuminuria before progressing to end-stage renal disease (2). Although several factors involved in the occurrence, progression, and outcome of DKD have been described, the exact pathogenic mechanism remains unclear. Renal lipotoxicity (i.e., dyslipidemia and renal lipid accumulation) is known to contribute to kidney damage in chronic kidney disease (3). The accumulation of intrarenal lipid droplets (LDs) appears to accelerate podocyte dysfunction, glomerulosclerosis, and interstitial fibrosis through lipid infiltration, induction of oxidative stress, and upregulation of proinflammatory and profibrotic cytokines and growth factors (3). Increasing evidence has shown that renal lipotoxicity also plays an important role in the pathogenesis of DKD (4-6).

Intracellular cholesterol accumulation in the kidney is a characteristic of high-fat induced kidney damage (7), which may be caused by alterations in cholesterol intake, intracellular synthesis, esterification, and efflux. The ATP-binding cassette transporter A1 (ABCA1), which is responsible for intracellular cholesterol efflux, along with ABCG1 and scavenger receptor class B member 1 (SR-B1), mediates intracellular cholesterol export from endothelial cells, β-cells, adipocytes, and podocytes. Excessive intracellular cholesterol is transferred to lipid-poor apolipoproteins such as apoA-I to generate HDL particles (8-11). ABCA1 also inhibits the expression of inflammatory cytokines IL-1β, IL-6, and TNFα in macrophages (12-15), suggesting a potential role in suppressing inflammation.

Glucagon-like peptide-1 receptor (GLP-1R), which is also expressed in kidney tissue (16), and its agonists (17) exert potential renoprotective effects in DKD by decreasing oxidative stress, lipid accumulation, macrophage infiltration, and proinflammatory cytokine release while improving metabolic abnormalities. GLP-1R-mediated signaling pathways such as Ca²⁺/calmodulin-dependent protein kinase (CaM kinase), cAMP/protein kinase A (PKA), phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT), and extracellular signal-regulated kinase (ERK)1/2 are involved in upregulating ABCA1 expression and inhibiting inflammatory responses. Therefore, in this study we investigated the effects of the GLP-1R agonist exendin-4 on renal lipotoxicity-induced glomerular endothelial cell inflammatory injury and ABCA1-mediated cholesterol efflux in diabetic apoE⁻/⁻ mice and in human renal glomerular endothelial cells (HRGECs).

**RESULTS**

*Lipid Deposits Increased in Glomerular Endothelial Cells in Patients with Early and Advanced DKD and Diabetic ApoE⁻/⁻ Mice*—We
evaluated renal lipid accumulation in glomerular endothelial cells from patients with early and advanced DKD and diabetic apoE<sup>−/−</sup> mice. Electron microscopy examination of kidney tissues from DKD patients showed widening of the glomerular basement membrane, mesangial expansion, podocyte foot process effacement, and extensive accumulation of LDs in fenestrated endothelial cells (Fig. 1A). Oil Red O staining also showed LDs in glomerular endothelial cells of human renal biopsies (Fig. 1B) and apoE<sup>−/−</sup> diabetic mice (Fig. 1C). We found evidence of cholesterol accumulation by filipin staining in diabetic apoE<sup>−/−</sup> mice (Fig. 1D). Oil Red O and CD31 staining confirmed lipid accumulation in the glomerular endothelial cells of diabetic apoE<sup>−/−</sup> mice (Fig. 1E). Biochemical analysis of lipid composition in the renal cortex of diabetic apoE<sup>−/−</sup> mice indicates that accumulation of neutral lipid deposits corresponds to significantly increased cholesterol content (Fig. 2A).

**Exendin-4 Decreased Blood Lipid Levels and Alleviated Kidney Damage in apoE<sup>−/−</sup> Mice**—Histological analysis of the kidneys of apoE<sup>−/−</sup> diabetic mice show that exendin-4 improves glomerular hypertrophy, basement membrane thickening, mesangial expansion (Fig. 2B and C), as well as HbA1c (glycated hemoglobin) level, creatinine clearance, albuminuria, urinary albumin excretion, ratio of kidney weight to body weight, total cholesterol, and LDL level. In addition, exendin-4–treated diabetic apoE<sup>−/−</sup> mice had a slightly higher plasma HDL level compared to untreated diabetic apoE<sup>−/−</sup> mice (Table 2), whereas serum triglycerides did not differ significantly between these two groups.

**Exendin-4 Attenuated LDs Accumulation and Inflammatory Injury in Glomerular Endothelial Cells of Diabetic ApoE<sup>−/−</sup> Mice by Increasing ABCA1 Expression**—Oil Red O staining, filipin staining, and biochemical analysis of lipid composition revealed greater accumulation LDs in the glomeruli of diabetic apoE<sup>−/−</sup> mice than in glomeruli of nondiabetic apoE<sup>−/−</sup> mice, which was improved by exendin-4 treatment (Fig. 1C and D and Fig. 2A). Results of double immunofluorescence staining showed that GLP-1R and ABCA1 were expressed in glomerular endothelial cells of nondiabetic apoE<sup>−/−</sup> mice (Fig. 3A and B). ABCA1 expression in the kidneys of diabetic apoE<sup>−/−</sup> mice was lower than that of nondiabetic apoE<sup>−/−</sup> mice (Fig. 3C), but was increased by exendin-4 treatment. Real-time PCR and Western blotting results also showed that ABCA1 expression in glomeruli from diabetic apoE<sup>−/−</sup> mice was lower than that of nondiabetic apoE<sup>−/−</sup> mice (Fig. 3E), but was significantly increased by exendin-4 treatment (Fig. 3D and E). We next assessed the effect of exendin-4 on inflammation in diabetes. Immunohistochemistry results showed that TNF-α and IL-6 protein levels were higher in the glomeruli of diabetic apoE<sup>−/−</sup> compared with that of nondiabetic apoE<sup>−/−</sup> mice, and these cytokines were downregulated by exendin-4 (Fig. 4A). Results of real-time PCR and Western blotting confirmed the downregulation of TNF-α and IL-6 (Fig. 4B and C) in exendin-4–treated diabetic apoE<sup>−/−</sup> mice.

**Exendin-4 Ameliorated Inflammation and Upregulated ABCA1-mediated Cholesterol Efflux in HRGECs by Activating the CaMKK/CaM Kinase Type IV (CaMKIV), cAMP/PKA, and**
PI3K/AKT pathways and Inhibiting the ERK1/2 Pathway—We first examined the effect of exendin-4 on the expression of ABCA1, which is critical for regulating cellular cholesterol homeostasis. Results of Western blot and real-time PCR analysis (Fig. 5A and B) showed decreased ABCA1 expression in HRGECs cultured under high glucose and high cholesterol conditions, but exendin-4 treatment increased ABCA1 expression in these cells. We next examined the effect of exendin-4 on apoA-I-specific cholesterol efflux in HRGECs. Our results showed that treatment with exendin-4 decreased cholesterol content (Tables 3 and 4) and increased the cholesterol/apoA-I ratio (Fig. 5C). These results indicate that exendin-4 upregulates apoA-I-specific cholesterol efflux and ABCA1 expression in HRGECs.

Double immunostaining showed that CaM-kinase kinase (CaMKK) was expressed in glomerular endothelial cells of nondiabetic apoE^{-/-} mice (Fig. 5D). The CaMKK/CaMKIV signaling pathway was investigated as a possible mechanism underlying the effect of exendin-4 on ABCA1 expression. Results of real-time PCR and western blot analysis (Fig. 5A and B) showed that exendin-4 increased ABCA1 expression in HRGECs under high glucose and high cholesterol conditions, but this effect was suppressed by the CaMKK inhibitor STO-609. We also examined the effect of exendin-4 on CaMKIV activity in HRGECs and found increased CaMKIV phosphorylation in exendin-4–treated cells, this effect was also inhibited by STO-609. Similarly, siRNA-mediated knockdown of CaMKIV suppressed the exendin-4–induced increase in ABCA1 mRNA and protein levels (Fig. 5E and F), whereas ABCA1 expression increased in cells transfected with scrambled siRNA. These findings demonstrate the involvement of CaMKK/CaMKIV signaling in exendin-4–induced ABCA1 expression.

To further investigate the mechanism underlying the effect of exendin-4 on ABCA1-mediated cholesterol efflux in HRGECs, we analyzed GLP-1R-mediated signaling pathways, such as the cAMP/PKA pathway, which plays a role in cholesterol efflux (18, 19). Intracellular cAMP activity was assessed by ELISA, and PKA activity was assessed by the Kemptide phosphorylation assay. We found that the cAMP and PKA activity were markedly decreased under high glucose and high cholesterol conditions compared with normal glucose conditions, and exendin-4 increased both cAMP and PKA activity (Fig. 6A and B). Results of real-time PCR and western blot analysis showed that PKA siRNA blocked the effect of exendin-4 on ABCA1 expression (Fig. 6C and D). Taken together, these findings suggested the involvement of cAMP/PKA signaling in the upregulation of ABCA1 by exendin-4.

Previous studies suggested that PI3K/AKT signaling contributes to the regulation of ABCA1 expression (20). Therefore, we examined PI3K/AKT activation in exendin-4–treated HRGECs. We found that exendin-4 increased PI3K and AKT phosphorylation (Fig. 7A), and this effect was attenuated by the PI3K inhibitor LY294002 (Fig. 7A). In addition, LY294002 inhibited exendin-4–induced upregulation of
ABCA1 mRNA and protein levels (Fig. 7A and B). These results suggest that exendin-4 regulates ABCA1 expression by activating the PI3K/AKT pathway.

We also investigated the effect of exendin-4 on ERK1/2 activation in HRGECs by real-time PCR and western blot analysis. Our results showed that ERK1/2 phosphorylation was increased under high glucose and high cholesterol conditions, but phosphorylated ERK1/2 levels were significantly decreased by exendin-4 and by the selective ERK1/2 inhibitor PD98059 (Fig. 8A). We then examined the effect of PD98059 on ABCA1 expression and found that, similar to exendin-4, PD98059 upregulated ABCA1 expression (Fig. 8A and B). These results suggest that exendin-4 regulates ABCA1 expression by inhibiting the ERK1/2 pathway.

Real-time PCR and western blot analysis also showed the TNF-α and IL-6 levels were significantly increased in HRGECs under high glucose and high cholesterol conditions, but this effect was inhibited by exendin-4. However, exendin-4 did not decrease TNF-α and IL-6 mRNA and protein levels in cells transfected with ABCA1 siRNA (Fig. 8C and D). Furthermore, TNF-α and IL-6 expression in exendin-4–treated cells was increased by pretreatment with STO-609, CaMKIV siRNA, PKA siRNA, or LY294002 and decreased by PD98059.

DISCUSSION

Excess lipid accumulation in the liver, heart, and pancreas is associated with lipotoxicity, inflammation, and fibrosis. In addition, renal accumulation of lipids is thought to play a role in the pathogenesis of DKD (21,22). DKD is associated with excessive generation of reactive oxygen species and endoplasmic reticulum stress, damage to podocytes and interstitial tubular cells, mesangial expansion, and inflammatory cell infiltration, ultimately leading to alterations in the glomerular filtration barrier and renal failure (23-25). In our study we observed lipid deposition and cholesterol accumulation in glomerular endothelial cells of patients with early and advanced DKD and apoE–/– diabetic mice. We also showed that decreased ABCA1 expression in the glomerular endothelial cells of diabetic mice is the key factor linking lipid accumulation to the development of nephropathy. Treatment with the GLP-1R agonist exendin-4 improved ABCA1 expression, ABCA1-mediated cholesterol efflux, and inflammation in glomerular endothelial cells.

A novel finding of our study is that the exendin-4–mediated upregulation of ABCA1 in glomerular endothelial cells alleviates diabetic kidney damage. This effect of exendin-4 on ABCA1 levels was previously shown to play an important role in cholesterol efflux in adipocytes (26), hepatocytes (27), and pancreatic cells (28). Increasing evidence also suggests that glomerular endothelial cells, which are an integral part of the glomerular filtration barrier, and crosstalk between glomerular endothelial cells and podocytes are important in the development and progression of DKD (29-31). Endothelial cells express several genes involved in reverse cholesterol transport such as ABCA1, ABCG1, and SR-B1 (32-34), which encode transporters and receptors that promote the cellular efflux or uptake of cholesterol. Previous studies reported that endothelial
expression of ABCA1 protects against endothelial dysfunction (35,36).

Defects in ABCA1, an ABC transporter superfamily member, are the cause of Tangier disease (37), which is characterized by decreased blood HDL levels. Efflux of free cholesterol from cells, an early step in reverse cholesterol transport, is mediated by ABCA1 (11), which directly interacts with apolipoproteins and promotes the solubilization of lipids and their release from cells (38). In the mouse model of type I diabetes mellitus, ABCA1 expression is downregulated in both kidneys and circulating macrophages (39,40). In humans, ABCA1 genetic variants are strongly associated with the risk of coronary artery disease (41), which is inversely correlated with the cholesterol efflux capacity of macrophages (42). Renal lipid accumulation is due to increased cholesterol and fatty acid synthesis, as well as reduced ABCA1-mediated cholesterol efflux (43) in glomerular mesangial cells and tubular cells (44). However, the level of ABCA1 expression in glomerular endothelial cells and its role in glomerular endothelial cell injury was unclear. Inducing diabetes with a high-fat diet and streptozotocin significantly decreased renal expression of ABCA1, and this alteration appears to precede the development of diabetic nephropathy. We also showed that ABCA1 is expressed in glomerular endothelial cells, and this expression is downregulated in apoE−/− diabetic mice and under high glucose conditions. Taken together, these findings suggest that defects in ABCA1 could promote cholesterol accumulation in renal cells. Cholesterol accumulation is closely associated with chronic metabolic inflammation, including atherosclerosis, obesity, and other metabolic diseases (45). ABCA1 is reported to have anti-inflammatory effects, suggesting a putative mechanism and potential target for protection against atherosclerotic cardiovascular disease (46-48). Accordingly, humans with dysfunctional ABCA1 and familial HDL deficiency tend to have chronic low-grade inflammation (49,50). The decreased ABCA1 expression and lipid accumulation in glomerular endothelial cells in DKD appears to promote inflammatory injury. Thus, exendin-4 treatment may prevent glomerular endothelial cell damage by increasing ABCA1 expression.

Exendin-4 acts directly on GLP-1R to decrease body weight, peri-epididymal fat, 24-h urine volume, and creatinine clearance in diabetic (db/db) mice (51), and renoprotective effects of exendin-4 in patients with DKD have been described (52,53). In animal models of diabetes, exendin-4 activates GLP-1R in THP-1 macrophages, thereby increasing ABCA1 expression, enhancing reverse cholesterol transport, and preventing the maturation of oxidized LDL and atherogenic foam cell formation (54,55). In our study, we observed that ABCA1 and GLP-1R were co-expressed in glomerular endothelial cells. Similar to previous studies, we found that exendin-4 has renoprotective and anti-inflammatory effects in diabetic mice with nephropathy. Exendin-4 also decreased LD accumulation in vivo and increased glomerular endothelial cell cholesterol efflux in vitro. Additionally, exendin-4 inhibited the expression of the proinflammatory cytokines IL-6 and TNF-α. The siRNA-mediated knockdown of ABCA1
increased inflammatory cytokine production in exendin-4–treated HRGECs, indicating that ABCA1 plays a crucial role in the anti-inflammatory activity of exendin-4.

Our results suggest that exendin-4 regulates ABCA1 through GLP-1R-mediated signaling (CaMKK/CaMKIV, cAMP/PKA, PI3K/AKT, and ERK1/2 pathways). The CaMKK/CaMKIV pathway functions in various cell types (56-58). For example, a recent study reported that exendin-4–stimulated ABCA1 transcription plays an important role in insulin secretion and cholesterol ester content in pancreatic β-cells by interfering with CaMKK/CaMKIV signaling (57). We hypothesize that exendin-4 promotes ABCA1 expression via the CaM kinase pathway in glomerular endothelial cells. In our study CaMKK expression was observed in the glomerular endothelial cells of mice in vivo, and CaMKIV phosphorylation did not differ significantly under normal glucose, high glucose, or high cholesterol conditions in vitro. However, exendin-4 treatment increased CaMKIV phosphorylation, and this effect was inhibited by the CaMKK inhibitor STO-609. In addition, STO-609 or CaMKIV siRNA suppressed exendin-4–induced ABCA1 expression, ABCA1–mediated cholesterol efflux, and anti-inflammatory effects. Taken together, our findings indicate that exendin-4 increases phosphorylation of CaMKIV by CaMKK, which upregulates ABCA1 expression, thereby enhancing cholesterol efflux and ameliorating inflammation.

Upstream mechanisms involved in GLP-1–mediated renal protection in diabetes are unclear but may involve activation of cAMP/PKA (52), which is a positive regulator of ABCA1 (18). Our results suggest that cAMP/PKA signaling contributes to the exendin-4–mediated restoration of ABCA1 expression under high glucose and high cholesterol conditions in HRGECs. This conclusion is supported by the fact that exendin-4–induced upregulation of ABCA1 was abrogated by inhibiting the cAMP/PKA pathway. PI3K/AKT and ERK1/2 pathways also play key roles in GLP-1R action. For example, ABCA1 expression is regulated by the PI3K/AKT pathway (32,59). In addition, the GLP-1R agonist (liraglutide), suppresses damage in microvascular endothelial cells through the PI3K/AKT pathway (33). In this study, our results showed that exendin-4 upregulates ABCA1 expression through phosphorylation of PI3K and AKT to increase cholesterol efflux and decrease inflammation in HRGECs. In animal studies, ERK1/2 signaling is activated under hyperglycemic conditions in aortic vascular smooth muscle cells in vivo (34,60) and under high glucose conditions in cultured vascular smooth muscle cells. Similar to our findings, previous studies have shown that exendin-4 can inhibit ERK1/2 signaling (53,61). Moreover, the ERK1/2 inhibitor PD98059 increased ABCA1 expression in HRGECs similar to exendin-4.

In summary, we have provided new evidence of LD accumulation and lipotoxicity in glomerular endothelial cells in a mouse model of DKD and in the kidneys of patients with DKD. We found that diabetes and hyperglycemia significantly decrease ABCA1 expression in glomerular endothelial cells both in vitro and in vivo. Exendin-4 significantly decreases LD
Exendin-4 improving ABCA1-mediated cholesterol efflux accumulation, promotes cholesterol efflux, and exerts anti-inflammatory effects in glomerular endothelial cells by upregulating ABCA1 expression via activation of the CaMKK/CaMKIV, cAMP/PKA, and PI3K/AKT pathways and inhibition of the ERK1/2 pathway. Our findings demonstrate that lipid accumulation in glomerular endothelial cells plays a crucial role in the development and progression of DKD. Thus therapeutic agents that enhance GLP-1R action may prevent glomerular endothelial dysfunction and slow the progression of diabetic nephropathy.

EXPERIMENTAL PROCEDURES

Human Kidney Sample Preparation—This study was approved by the Institutional Ethics Committee of the West China Hospital of Sichuan University. Three patients with early and advanced DKD were included in this study, and kidney tissues were obtained from renal biopsies. Three other patients with renal carcinoma and eGFR > 60 ml/min/1.73 m² but without hypertension, diabetes, or other comorbidities served as controls. The clinical data and laboratory test results of these patients are shown in Table 1. Kidney specimens from the controls were obtained during surgical nephrectomy. LD deposition in the kidney biopsy specimens was detected by transmission electron microscopy (JEOL 1010, Japan) in the West China Hospital pathology department. Frozen sections of the kidney tissues were prepared for Oil Red O staining.

Animal Model—Animal experiments were performed with the approval of the Sichuan University Animal Ethics Committee. Male apoE-/- mice and C57BL/6J mice (6 weeks old, Huafukang Animal Centre, Beijing, China) were housed at the Laboratory Animal Centre of West China Hospital and given unrestricted access to water and food. The mice were divided into the following groups (each group, n = 6): (1) wild type C57BL/6J controls (Con), (2) nondiabetic apoE-/- mice (ApoE-/-), (3) diabetic apoE-/- diabetes mice (ApoE-/- DM), and (4) exendin-4–treated diabetic apoE-/- mice (ApoE-/- DM+Ex4). All groups of mice were fed a high-fat diet (regular diet plus 27.3% lard, 54.6% sucrose, 16.4% cholesterol, and 1.6% sodium cholate [w/w]) (Beijing Keao Xieli Feed Co., Ltd, Beijing, China). Diet-induced obesity causes peripheral insulin resistance in rodents (62), resulting in inflammation and lipid accumulation (63). In our study, high-fat diet-fed apoE-/- mice with high homeostatic model assessment of insulin resistance (HOMA-IR) values (64) (fasting plasma glucose [mmol/L] × fasting insulin [mIU/L]/22.5) were defined as insulin-resistant and injected with four doses of low-dose streptozotocin (55 mg/kg; Sigma-Aldrich, St. Louis, MO) in citrate buffer (pH 4.5) after overnight fasting. These mice served as a model of accelerated renal damage associated with diabetes due to dyslipidemia, which is also a feature of diabetic nephropathy in humans. The apoE-/- mice with fasting blood glucose > 16.7 mmol/L for consecutive 3 days, as determined by a glucometer (Accu-Chek, Roche, Basel, Switzerland), served as the diabetic group in this study. The wild type C57BL/6J mice were injected with citrate buffer (1 ml/kg). All mice were maintained on their respective diets until the end of the study. The group of exendin-4–treated diabetic apoE-/- mice received intraperitoneal injections (1.0
nmol/kg/day; Sigma-Aldrich) for 8 weeks after diabetes induction.

**Biochemical Measurements**—Prior to euthanasia, blood glucose was measured using a glucose analyzer after a 6-h daytime fast. Urine samples were collected after the mice were housed individually in metabolic cages for 24 h. Blood samples were obtained by cardiac puncture. Blood urea nitrogen, plasma creatinine, plasma total cholesterol, plasma triglycerides, and urinary protein excretion were measured on a biochemistry autoanalyzer (Cobas Integra 400 Plus, Roche) using commercial kits.

**Oil Red O Staining**—Sections (6 μm) of renal biopsies from mice and patients with early and advanced DKD were stained with Oil Red O according to the manufacturer’s protocol (Sigma-Aldrich). The frozen kidney sections were rinsed with distilled water, and then rinsed with 60% isopropanol, stained for 15 min in the Oil Red O working solution, and returned to distilled water. The sections were counterstained with hematoxylin for 1 min and mounted in glycerine jelly.

**Filipin Staining**—Sections (4 μm) of fixed frozen mouse kidneys were fixed with 4% paraformaldehyde for 30 min, washed with PBS three times, and then stained with freshly prepared filipin solution (125 g/ml, Sigma-Aldrich) for 30 min. Then, the slides were washed with PBS, and a drop of glycerol was added. The slides were mounted with coverslips and examined by confocal laser scanning microscopy. A semi-quantitative analysis of filipin-positive areas was performed using the software package Image-Pro Plus version 6.0.

**Isolation of Glomeruli**—Glomeruli were isolated from the renal cortex of mice by Dynabead perfusion using a previously reported method (65), with some modifications. The kidneys were removed, minced into 1-mm³ pieces, and digested with collagenase (1 mg/ml collagenase A, 100 U/ml deoxyribonuclease I in Hank’s balanced salt solution [HBSS]) at 37°C for 30 min with gentle agitation. The collagenase-digested tissue was gently pressed through a 100-μm cell strainer (Sangon Biotech, RA441) with a flattened pestle, and the cell strainer was washed with 5 ml HBSS. The filtered cells were passed through a new cell strainer without pressing, and the cell strainer was washed with 5 ml HBSS. The resulting cell suspension was then centrifuged at 2000 × g for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 2 ml HBSS. Finally, glomeruli containing Dynabeads were gathered by a magnetic particle concentrator and washed at least three times with HBSS. During the procedure, the kidney tissues were kept at 4°C, except for the collagenase digestion at 37°C, and acridine orange staining was used to identify the isolated glomeruli. The glomeruli were analyzed by real-time PCR and western blot.

**Immunohistochemistry**—Paraffin sections (4 μm) were stained with periodic acid-Schiff (PAS) stain and hematoxylin-eosin (HE) and incubated with the following primary monoclonal antibodies: mouse anti-ABCA1 (1:100, Abcam, Cambridge, MA), anti-IL-6 (1:200, Santa Cruz Biotechnology), and anti-TNF-α (1:50, Abcam). The sections were then incubated with ChemMate™EnVision + HRP (EnVision™
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Detection Kit, GK500705, Gene Tech, Shanghai, China) for 45 min at 37°C. The sections were stained with diaminobenzidine and counterstained with hematoxylin and eosin. Six mice per group were analyzed, and more than 20 cortical glomeruli from each mouse were assessed. Immunostained areas were quantified by Image-Pro Plus version 6.0 software.

**Immunofluorescence**—Frozen kidney tissue sections (3 μm) were stained with the following primary antibodies: mouse anti-GLP-1R (1:200, Abcam), mouse anti-CaMKK (1:100, Abcam), and rabbit anti-ABCA1 (1:200, Abcam) along with the endothelial cell markers mouse anti-CD31 (1:100, Abcam) or rabbit anti-CD31 (Bioworld Technology Inc., MN) for 2 h at 37°C. The sections were then stained with FITC-labeled rabbit anti-goat immunoglobulin (Dako, USA), TRITC-labeled rabbit anti-mouse immunoglobulin (Dako), FITC-stained swine anti-rabbit immunoglobulin (Dako), or goat anti-mouse IgG (heavy and light chains) (Millipore, USA). The tissue sections were analyzed with a Nikon A1Si confocal laser scanning microscope and NIS-Elements AR software version 4.0 (Nikon, Japan), and S Plan Fluor ELWD 40 ×DIC. The subsequent steps were carried out as described previously (64).

**Cell Culture**—Well-characterized HRGECs were purchased from ScienCell Research Laboratories (USA) and cultured in endothelial cell medium (ScienCell) containing 5% FBS and 1% endothelial cell growth supplement at 37°C in a 5% CO₂ atmosphere (64). Cells were harvested with 0.25% trypsin (Gibco, Life Technologies, Carlsbad, CA) at approximately 80% confluence, and the cells were used for experiments within six passages. The cells were transferred to serum-free medium 12 h prior to treatment, which was then replaced with endothelial cell medium containing normal glucose (NG, 5 mmol/L), high glucose (HG, 25 mmol/L), high glucose plus cholesterol (HC, 400 μg/ml, Sigma-Aldrich), high glucose plus cholesterol and exendin-4 (HCE, 100 nmol/L, Abcam) for 24 h. In the inhibition study, cells were pretreated for 24 h with the CaMKK-specific inhibitor STO-609 (HCES, 1 μg/ml, Sigma-Aldrich), CaMKIV siRNA (HCECa), control CaMKIV siRNA (HCEcoCa), PKA siRNA (HCEP), control PKA siRNA (HCEcoP), PI3K inhibitor LY294002 (HCEL, 50 μM, Cell Signaling Technology), selective ERK1/2 inhibitor PD98059 (HCEPd and HCPd, 10 μmol/L, Sigma-Aldrich), ABCA1 siRNA (HCEA) or control ABCA1 siRNA (HCEcoA). After the 24-h pretreatment, the cells were treated with 20 mg/ml apoA-I (Biodesign, Memphis, TN) for 6 h to induce cholesterol efflux. Cells were then harvested with 0.25% trypsin for real-time PCR and western blotting analysis.

**Transfection of siRNA**—HRGECs were seeded in 6-well plates and flasks and maintained in endothelial cell medium with 5% FBS. Silencer negative control siRNA and Silencer validated siRNA against CaMKIV and ABCA1 were purchased from Biology Engineering Corporation (Shanghai, China). Cells were transfected with siRNA diluted in Opti-MEM I (100 nmol/L) using HiPerFect transfection reagent (Invitrogen) according to the manufacturer’s protocol. The culture medium was changed 24 h after
transfection. After washing twice with PBS, cells were collected for RT-PCR and western blot analysis.

**cAMP Assay**—HRGECs (3 × 10^5) were seeded in a 24-well plate, and after being subjected to the experimental treatments, the cells were collected and lysed by freeze and thaw cycle 5 times. The samples were centrifuged at 1300 × g for 5 min at room temperature. Intracellular cAMP concentrations in these supernatants were determined by using an ELISA kit (Jiang Lai Biotechnology, Shanghai, China) according to the manufacturer's instructions. Absorbance at 595 nm was detected using a microplate reader (BioTek Instruments, San Jose, CA) (66).

**PKA Activity Assay**—After treatment, cells were collected and placed in PBS containing protease inhibitor cocktail (1:500, Sigma-Aldrich) and phosphatase inhibitor cocktail (1:200, Sigma-Aldrich). Cells were then subjected to sonication to disrupt the cell membrane, and cytosolic proteins were harvested by centrifugation. PKA activity was determined using the Kemptide phosphorylation assay, as previously described (66).

**Lipid Extraction and Measurement of Lipid Composition**—Lipids were extracted from the renal cortex as previously described (21,22). Triglyceride and cholesterol content was measured using kits (Sigma-Aldrich) according to manufacturer's instructions.

**Cholesterol Efflux Assay**—After incubating with various concentrations of glucose, HRGECs were labeled with 3H-cholesterol (1 mCi/well, Abcam) for 24 h at 37°C. The cells were then equilibrated with 0.5% BSA for 4 h and treated with apoA-I (20 mg/ml, Abcam), which was used to induce cholesterol efflux from the labeled cells for 6 h, as previously described (44). Both cells and cell culture medium were collected, and radioactivity was quantified by liquid scintillation counting using a Packard scintillation counter (Perkin Elmer, Waltham, MA). The percentage of cholesterol efflux from cells was calculated as the radioactivity in the medium divided by the total radioactivity (in both cells and medium).

**High-performance Liquid Chromatography Assay**—Cell sterol analyses were performed as previously described (19,67). Briefly, cells were washed three times with PBS, and then sonicated using an ultrasonic processor for 2 min. An appropriate volume of 0.5% NaCl (usually 1 ml) was added to 50–200 μg/ml cellular protein. A 0.1-ml aliquot of the cell solution (containing 5–20 μg protein) was used to measure free cholesterol, and a 0.1-ml aliquot was used to measure total cholesterol. Free cholesterol was dissolved in isopropanol (1 μg cholesterol/ml) and stored at -20°C as a stock solution. Cholesterol standard calibration solutions ranging from 0 to 40 μg cholesterol/ml were obtained by diluting the cholesterol stock solution in the same cell lysis buffer. Then 0.1 ml of each sample (cholesterol standard or cell suspension) was supplemented with 10 ml of the reaction mixture (500 mM MgCl₂, 500 mM Tris HCl [pH 7.4], 10 mM dithiothreitol, and 5% NaCl). To determine free cholesterol, 0.4 U cholesterol oxidase in 10 ml 0.5% NaCl was added to each tube. To determine total cholesterol, 0.4 U cholesterol oxidase and 0.4 U cholesterol esterase were added to each tube.
The tubes were incubated at 37°C for 30 min, and the reaction was stopped by adding 100 ml methanol: ethanol mixture (1:1). The reactions were kept cold for 30 min to allow protein precipitation and then centrifuged at 1000 × g for 10 min at 15°C. The supernatant (10 ml) was applied to the chromatograph system (PerkinElmer, Inc., Waltham, MA), which consisted of a PerkinElmer series 200 vacuum degasser, pump, PerkinElmer series 600 LINK, PerkinElmer series 200 UV/Vis detector, and Discovery C-18 HPLC column (Supelco, Inc., PA). The column was eluted using an isopropanol: n-heptane: acetonitrile mixture (35:13:52) at a flow rate of 1 ml/min for 8 min. Absorbance at 216 nm was monitored. Data were analyzed using TotalChrom software (PerkinElmer, Inc.).

**RNA Isolation and Real-time PCR Analysis**—Total RNA from glomeruli or cultured cells was extracted using TRIzol reagent (Thermo Fisher Scientific Inc., Waltham, MA), and its concentration was measured using a microspectrophotometer (Thermo Fisher Scientific Inc.). After confirming RNA quality by agar gel electrophoresis, cDNA was synthesized. Real-time PCR was performed with the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using SYBR Premix Ex Taq™ (Tli RNaseH Plus, Takara) as previously described (64). The gene encoding β-actin was used as an internal standard. Relative mRNA levels were calculated as the ratio of target mRNA to β-actin mRNA and expressed as mean ± standard error of the mean (SEM).

**Western Blot Analysis**—Cells and isolated glomeruli were harvested, and the proteins were extracted with RIPA lysis buffer, separated by SDS polyacrylamide gel electrophoresis, and transferred to a PVDF membrane for western blotting. Briefly, after blocking nonspecific binding with 5% BSA, the membranes were incubated with primary antibodies against ABCA1, phospho-CaMKIV Thr196, phospho-ERK1/2, phospho-PI3K, phospho-AKT, TNF-α (Abcam, Cambridge, MA), and IL-6 (Santa Cruz Biotechnology); β-actin was used as an internal reference. Membranes were then incubated with a secondary antibody, followed by an ECL reagent (Western Blotting Chemiluminescence Luminol reagent; Santa Cruz Biotechnology) and exposed to Canon EOS 60D film (USA). The signals were detected with an Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE) and quantified using the ImageJ program (NIH). Relative protein levels were calculated as the ratio of target protein to β-actin and expressed as mean ± SEM.

**Statistical Analysis**—Data are expressed as mean ± SEM. Multiple groups were compared by one-way analysis of variance. Two-way comparisons were performed using two-sample and paired t-tests. All analyses were performed with SPSS software (version 11.5, IBM Corp., NY, USA), and p < 0.05 was considered significant.
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**FIGURE LEGENDS**

**TABLES**

**TABLE 1.** Clinical data and laboratory test results of patients with diabetic kidney disease and controls.

**TABLE 2.** General and metabolic parameters after 8 weeks in nondiabetic and diabetic apoE<sup>−/−</sup> mice, with and without treatment (n = 6 per group).

Footnote: a. Nondiabetic apoE<sup>−/−</sup> mice vs. control mice (p < 0.05), b. diabetic apoE<sup>−/−</sup> mice vs. nondiabetic apoE<sup>−/−</sup> mice (p < 0.05), c. exendin-4–treated diabetic apoE<sup>−/−</sup> mice vs. untreated diabetic apoE<sup>−/−</sup> mice (p < 0.05).

**TABLE 3.** Effect of exendin-4 on total cholesterol (TC), free cholesterol (FC), and cholesterol ester (CE) in human glomerular endothelial cells.

Foot note: Results are expressed as mean ± SEM from three independent experiments; *p < 0.05 vs. control.

**TABLE 4.** Effect of exendin-4 on total cholesterol (TC), free cholesterol (FC), and cholesterol ester (CE) in human glomerular endothelial cells.

Footnote: Results are expressed as mean ± SEM from three independent experiments; *p < 0.05 vs. 0 h.

**FIGURE 1.** Increased lipid accumulation in patients with diabetic kidney disease (DKD). (A) Electron microscopy (EM) of glomeruli overloaded with lipid droplets (LDs). LDs in fenestrated glomerular endothelial cells (indicated by arrows) from patients with early and advanced DKD. (B) Oil Red O staining of frozen kidney sections (glomeruli; 40×magnification). Oil Red O-stained glomerular and
Exendin-4 improving ABCA1-mediated cholesterol efflux

tubulointerstitial regions of kidney biopsies of patients with early and advanced DKD. (C) Oil Red O staining in renal tissues from control mice (Con), apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup>), untreated diabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup> DM) and exendin-4–treated diabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup> DM + Ex-4). (D) Filipin cholesterol staining of renal tissues from Con, ApoE<sup>−/−</sup>, ApoE<sup>−/−</sup> DM, and ApoE<sup>−/−</sup> DM + Ex-4 mice. (E) Oil Red O and double immunofluorescence (IF) staining (with endothelial cell marker CD31). Representative images are shown. Scale bars = 50 μm.

FIGURE 2. Biochemical analysis of lipid composition and histological analysis of glomerular injury in mice. (A) The cholesterol content in renal cortex from controls (Con), nondiabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup>), untreated diabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup> DM), and exendin-4–treated diabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup> DM + Ex-4). Hematoxylin-eosin (HE) staining (B) and periodic acid-Schiff (PAS) staining (C) of kidney sections show glomerular or cortical interstitial morphological changes. The bar graph show quantitative analysis of the PAS-positive area, and the box area is enlarged. Scale bar = 50 μm. Images are representative of two separate experiments. Results are expressed as mean ± SEM (n = 6); *p < 0.05, **p < 0.01.

FIGURE 3. ABCA1 expression in the kidneys of mice. Double-immunofluorescence staining of (A) GLP-1R and (B) ABCA1 with CD31 in kidneys from apoE<sup>−/−</sup> mice. (C) Immunohistochemical (IHC) analysis of ABCA1 expression in kidneys from mice, and quantitative analysis of the ABCA1-positive area (scale bar = 50 μm). (D) Real-time PCR and (E) western blotting analyses of ABCA1 expression in isolated glomeruli from controls (Con), nondiabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup>), untreated diabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup> DM), and exendin-4–treated diabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup> DM + Ex-4). Results are expressed as mean ± SEM (n = 6); *p < 0.05, **p < 0.01.

FIGURE 4. IL-6 and TNF-α expression in the kidneys of mice. (A) IHC analysis of IL-6 and TNF-α expression in kidneys from controls (Con), nondiabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup>), untreated diabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup> DM), and exendin-4–treated diabetic apoE<sup>−/−</sup> mice (ApoE<sup>−/−</sup> DM + Ex-4) (scale bar = 50 μm). Real-time PCR (B) and (C) western blotting analyses of for IL-6 and TNF-α expression in isolated glomeruli from the Con, ApoE<sup>−/−</sup>, ApoE<sup>−/−</sup> DM, and ApoE<sup>−/−</sup> DM + Ex-4 groups. Results are expressed as mean ± SEM (n = 6); *p < 0.05, **p < 0.01.

FIGURE 5. Exendin-4 increased the effect of ABCA1 on GLP-1R/CaMKK/CaMKIV signaling. Western blot (A) and real-time PCR (B) analysis of ABCA1 expression and CaMKIV phosphorylation in HRGECs cultured under normal glucose (5 mM, NG), high glucose (25 mM, HG), or high glucose (25 mM) plus high cholesterol (400 μg/ml) (HC) conditions and treated exendin-4 (100 nmol/L) (HCE) or STO-609 (1 μg/ml) (HCES). (C) Cellular cholesterol efflux from HRGECs to apoA-I in cells exposed to NG, HG, HG
plus exendin-4 (100 nmol/L) (HE), HE plus ABCA1 siRNA (HEA), or CaMKIV siRNA (HCECa). (D) Double-immunofluorescence staining of CaMKK with CD31 in kidneys of apoE/− mice (scale bar = 50μm). Western blot (E) and real-time PCR (F) analysis of ABCA1 in HRGECs exposed to NG, HG, HC, HCE, HCE plus CaMKIV siRNA (HCECa), or co-CaMKIV siRNA (HCEcoCa). Results are expressed as mean ± SEM (n = 6); *p < 0.05, **p < 0.01.

FIGURE 6. Exendin-4 increased the effect of ABCA1 on cAMP/PKA signaling. Exendin-4 activates the cAMP/PKA pathway in HRGECs (A) Intracellular cAMP levels assessed by ELISA. (B) PKA activity assessed by the Kemptide phosphorylation assay. Western blot (C) and real-time PCR (D) analysis of ABCA1 and PKA expression in HRGECs exposed to normal glucose (5 mM, NG), high glucose (25 mM, HG), or high glucose (25 mM) plus cholesterol (400 μg/ml) (HC), exendin-4 (100 nmol/L) (HCE), HCE plus PKA siRNA (HCEP), or co-PKA siRNA (HCEcoP). Results are expressed as mean ± SEM (n = 6); *p < 0.05, **p < 0.01.

FIGURE 7. Exendin-4 increased the effect of ABCA1 on PI3K/AKT signaling. Western blot (A) and real-time PCR (B) analysis of ABCA1 expression, PI3K phosphorylation, and AKT phosphorylation in HRGECs exposed to normal glucose (5 mM, NG), high glucose (25 mM, HG), high glucose (25 mM) plus cholesterol (400 μg/ml) (HC), exendin-4 (100 nmol/L) (HCE), or HCE plus LY294002 (50 μM, HCEL). Results are expressed as mean ± SEM (n = 6); *p < 0.05, **p < 0.01.

FIGURE 8. Effect of exendin-4 on ERK1/2 signaling, inflammation and lipid accumulation in HRGECs. Western blot (A) and real-time PCR (B) analysis of ABCA1 expression and ERK1/2 phosphorylation in HRGECs exposed to normal glucose (5 mM, NG), high glucose (25 mM, HG), high glucose (25 mM) plus cholesterol (400 μg/ml) (HC), exendin-4 (100 nmol/L) (HCE), or HCE plus PD98059 (10 μmol/L, HCEPd). Western blot (C) and real-time PCR (D) analysis of TNF-α and IL-6 expression in HRGECs exposed to NG, HG, HC, HCE, HCEA, or HCE plus co-ABCA1 siRNA (HCEcoA). Results are expressed as mean ± SEM (n = 6); *p < 0.05, **p < 0.01.
**Table 1.** Clinical and biochemical characteristics of control and patients with DKD

| Variable                        | Control group | Patient 1 with early DKD | Patient 2 with early DKD | Patient with advanced DKD |
|---------------------------------|---------------|--------------------------|--------------------------|---------------------------|
| Gender (male/female)            | male          | male                     | female                   | female                    |
| Age (year)                      | 46            | 63                       | 51                       | 58                        |
| Type of diabetes                | -             | type 2                   | type 2                   | type 2                    |
| Duration of diabetes (year)     | -             | 2                        | 14                       | 10                        |
| Renal biopsy pathology          | -             | stage I DKD              | stage II DKD             | stage IV DKD              |
| Glycated hemoglobin (%)         | -             | 7.1                      | 6.2                      | 6.8                       |
| Urine albumin/creatinine ratio (mg/g) | 3.8          | 2295.2                   | 3675.9                   | 391.5                     |
| Serum creatine (μmol/L)         | 63.8          | 77                       | 58                       | 64                        |
| Total cholesterol (mmol/L)      | 3.01          | 6.91                     | 5.96                     | 6.23                      |
| Triglycerides (mmol/L)          | 0.98          | 1.62                     | 1.38                     | 1.56                      |

**Table 2.** General and metabolic parameters after 8 weeks of study in apoE<sup>−/−</sup> mice, in the presence and absence of diabetes, with and without treatment (n = 6 per group)

| Variable                        | Control      | ApoE<sup>−/−</sup> | ApoE<sup>−/−</sup> DM  | ApoE<sup>−/−</sup> DM+Exendin-4 |
|---------------------------------|--------------|---------------------|-------------------------|---------------------------------|
| Body weight (g)                 | 25.90±1.34   | 31.38±1.17<sup>a</sup> | 21.33±1.32<sup>b</sup> | 24.97±1.62<sup>c</sup> |
| Kidney weight/Body weight (%)   | 0.54±0.05    | 0.56±0.02           | 0.86±0.07<sup>b</sup>  | 0.71±0.02<sup>c</sup>  |
| Glycated hemoglobin (%)         | 3.58±0.41    | 4.00±0.39           | 13.8±0.59<sup>b</sup>  | 9.65±1.10<sup>c</sup>   |
| Plasma glucose (mmol/L)         | 7.35±0.43    | 8.32±0.59           | 29.42±2.29<sup>b</sup> | 17.55±4.12<sup>c</sup> |
| Fasting blood-glucose (mmol/L)  | 5.8±0.52     | 6.87±0.97           | 18.8±1.85<sup>b</sup>  | 13.60±2.43<sup>c</sup> |
| Blood urea nitrogen (mmol/L)    | 12.23±0.49   | 12.47±0.54          | 13.22±0.75             | 13.41±0.74<sup>c</sup> |
| Creatinine clearance (umol/L)   | 16±0.48      | 16.32±0.62          | 29.65±0.88<sup>b</sup> | 21.31±1.26<sup>c</sup> |
| Total cholesterol (mmol/L)      | 2.51±0.35    | 8.03±0.27<sup>a</sup> | 18.05±0.74<sup>b</sup> | 9.68±0.59<sup>c</sup>  |
| Triglycerides (mmol/L)          | 0.68±0.04    | 0.73±0.04<sup>b</sup> | 0.82±0.03              | 0.7±0.07                      |
| HDL (mmol/L)                    | 1.±0.16      | 1.55±0.28<sup>a</sup> | 0.87±0.19<sup>b</sup>  | 1.21±0.19<sup>c</sup>  |
| LDL (mmol/L)                    | 1.35±0.14    | 5.73±0.37<sup>a</sup> | 13.47±0.60<sup>b</sup> | 6.93±0.43<sup>c</sup>  |
| C-reactive protein (mg/L)       | 0.23±0.02    | 0.22±0.02           | 0.29±0.15<sup>b</sup>  | 0.23±0.02                    |
| Albumin excretion (ug/24 h)     | 22.67±1.15   | 22.93±1.07          | 151.58±6.22<sup>b</sup> | 106.9±2.93<sup>c</sup> |

Note: a. ApoE vs Control (P<0.05), b. ApoE DM vs ApoE (P<0.05), c. ApoE DM+Exendin-4 vs ApoE DM (P<0.05).
Table 3. Effect of extendin-4 on total cholesterol (TC), free cholesterol (FC) and cholesteryl ester (CE) at different concentration in HRGECs.

|           | control | 25nmol/L | 50nmol/L | 100nmol/L | 200nmol/L |
|-----------|---------|----------|----------|-----------|-----------|
| TC (mg/dL)| 504 ± 36| 497 ± 37 | 483 ± 38 | 301 ± 20* | 298 ± 38* |
| FC (mg/dL)| 193 ± 28| 187 ± 25 | 192 ± 29 | 127 ± 23* | 126 ± 32* |
| CE (mg/dL)| 311 ± 22| 303 ± 28 | 291 ± 27 | 174 ± 33* | 177 ± 21* |
| CE/TC (%) | 61.7    | 60.9     | 60.4     | 58.1      | 57.7      |

Values represent the mean ± SEM from three independent experiments. *p < 0.05 VS. control.

Table 4. Effect of extendin-4 on total cholesterol (TC), free cholesterol (FC) and cholesteryl ester (CE) at different time in HRGECs.

|           | BSA (24h) | 0h | 6h | 12h | 24h | 48h |
|-----------|-----------|----|----|-----|-----|-----|
| TC (mg/dL)| 495 ± 39  | 491 ± 37 | 478 ± 29 | 394 ± 32* | 297 ± 25* | 294 ± 20* |
| FC (mg/dL)| 192 ± 21  | 189 ± 22 | 188 ± 32 | 161 ± 27 | 124 ± 25* | 123 ± 21* |
| CE (mg/dL)| 303 ± 28  | 301 ± 30 | 290 ± 24 | 233 ± 25* | 173 ± 31* | 171 ± 26* |
| CE/TC (%) | 61.2      | 61.5 | 60.6 | 59.1 | 58.2 | 58.1 |

Values represent the mean ± SEM from three independent experiments. *p < 0.05 VS. 0h
Figure 1

A

B

C

D

E

Exendin-4 improving ABCA1-mediated cholesterol efflux
Figure 2

A

Cholesterol

mg/mg protein

Con  ApoE−/−  ApoE−/− DM  ApoE−/− DM+Ex-4

B

Con

ApoE−/−

ApoE−/− DM

ApoE−/− DM+Ex-4

C

Con

ApoE−/−

ApoE−/− DM

ApoE−/− DM+Ex-4

PAS

Area (μm²)

Con  ApoE−/−  ApoE−/− DM  ApoE−/− DM+Ex-4

** ** ** *
Figure 4

A

| Con | ApoE<sup>-/-</sup> | ApoE<sup>-/-</sup> DM | ApoE<sup>-/-</sup> DM+Ex-4 |
|-----|------------------|----------------------|--------------------------|
| IL-6 | ![IL-6 Image] | ![IL-6 Image] | ![IL-6 Image] |
| TNF-α | ![TNF-α Image] | ![TNF-α Image] | ![TNF-α Image] |

B

| Con | ApoE<sup>-/-</sup> | ApoE<sup>-/-</sup> DM | ApoE<sup>-/-</sup> DM+Ex-4 |
|-----|------------------|----------------------|--------------------------|
| IL-6 | ![IL-6 Image] | ![IL-6 Image] | ![IL-6 Image] |
| TNF-α | ![TNF-α Image] | ![TNF-α Image] | ![TNF-α Image] |

C

| Con | ApoE<sup>-/-</sup> | ApoE<sup>-/-</sup> DM | ApoE<sup>-/-</sup> DM+Ex-4 |
|-----|------------------|----------------------|--------------------------|
| IL-6 | ![IL-6 Image] | ![IL-6 Image] | ![IL-6 Image] |
| TNF-α | ![TNF-α Image] | ![TNF-α Image] | ![TNF-α Image] |

β-actin

**Statistical Significance**

- **P < 0.05**
- **P < 0.01**
- **P < 0.001**
- **P < 0.0001**
Exendin-4 improving ABCA1-mediated cholesterol efflux

Figure 5

A

| GLU (mM) | CHO | EX-4 | STO-609 | ABCA1 | p-CaMKIV |
|----------|-----|------|---------|-------|----------|
| 5        | -   | -    | +       | ++    | +        |
| 25       | -   | +    | ++      | +     | +        |
| 25       | -   | +    | ++      | +     | +        |
| 25       | -   | +    | ++      | +     | +        |
| 25       | -   | +    | ++      | +     | +        |

B

ABC1

C

p-CaMKIV

D

CaMKK

CD31

DAPI

Merged

E

| GLU (mM) | CHO | EX-4 | CaMKIV-siRNA | ABCA1 | p-CaMKIV |
|----------|-----|------|---------------|-------|----------|
| 5        | -   | -    | -             | ++    | +        |
| 25       | -   | +    | -             | ++    | +        |
| 25       | -   | +    | -             | ++    | +        |
| 25       | -   | +    | -             | ++    | +        |
| 25       | -   | +    | -             | ++    | +        |

F

ABC1

Fold of mRNA expression

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Exendin-4 improving ABCA1-mediated cholesterol efflux
Figure 7

A

| GLU(mM) | CHO | EX-4 | LY294002 | ABCA1 | p-PI3K | p-AKT | β-actin |
|---------|-----|------|-----------|-------|--------|--------|---------|
| 5       | -   | +    | -         | -     | -      | -      | +       |

B

Figure 8

A

| GLU(mM) | CHO | EX-4 | PD98059 | ABCA1 | p-ERK1/2 | β-actin |
|---------|-----|------|---------|-------|-----------|---------|
| 5       | -   | +    | -       | -     | -         | +       |

C

| GLU(mM) | CHO | EX-4 | ABCA1-siRNA | co-ABCA1-siRNA | ABCA1 | IL-6 | TNF-α | β-actin |
|---------|-----|------|--------------|-----------------|-------|------|-------|---------|
| 5       | -   | +    | -            | -               | -     | -    | -     | +       |

D

| IL-6 | TNF-α |
|------|-------|

Exendin-4 improving ABCA1-mediated cholesterol efflux
Exendin-4 Ameliorates Lipotoxicity-induced Glomerular Endothelial Cell Injury by Improving ABC Transporter A1-mediated Cholesterol Efflux in Diabetic apoE Knockout Mice
Qing-hua Yin, Rui Zhang, Li Li, Yi-ting Wang, Jing-ping Liu, Jie Zhang, Lin Bai, Jing-qiu Cheng, Ping Fu and Fang Liu

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