Renal microvascular damaged in diabetes mellitus with hyperlipidemia through the regulation of MAMs by PACS2 in endothelial cell

Zhihao Shu

Central South University Third Xiangya Hospital Department of Cardiology

Shuhua Chen

Central South University School of Life Sciences

Hong Xiang

Central South University Third Xiangya Hospital

Ruoru Wu

Central South University Xiangya School of Medicine

Shaoli Zhao

Central South University Third Xiangya Hospital Department of Cardiology

Jie Xiao

Central South University Third Xiangya Hospital Department of Cardiology

Hengdao Liu

Zhengzhou University First Affiliated Hospital

Yulan Liu

Central South University Third Xiangya Hospital Department of Cardiology

Xuwen Wang

Central South University Third Xiangya Hospital Department of Cardiology

Wenfang Xiong

Central South University Third Xiangya Hospital Department of Cardiology

Jie Ouyang

Central South University Third Xiangya Hospital Department of Cardiology

Jing Zhang

Central South University Third Xiangya Hospital Department of Cardiology

Huiqin Liu

Central South University Third Xiangya Hospital Department of Cardiology

Alex F Chen

: University of Pittsburgh Department of Surgery

Hongwei Lu (✉ hongweilu@csu.edu.cn)

Central South University Third Xiangya Hospital Department of Cardiology

Original investigation

Keywords: Diabetic nephropathy, PACS2, fatty acid oxidation, MAMs

Posted Date: August 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-795542/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Renal microvascular damaged in diabetes mellitus with hyperlipidemia through the regulation of MAMs by PACS2 in endothelial cell

Zhihaoshu^{1,2}, Shuhua Chen³, Hong Xiang⁴, Ruoru Wu⁵, Shaoli Zhao², Jie Xiao², Hengdao Liu⁶, Yulan Liu², Xuewen Wang², Wenfang Xiong², Jie Ouyang², Jing Zhang², Huiqin Liu², Alex F Chen⁷, Hongwei Lu^{1,2}

¹ Health Management Center, the Third Xiangya Hospital of Central South University, Changsha, Hunan 410013, P.R. China;

² Department of Cardiology, the Third Xiangya Hospital of Central South University, Changsha, Hunan 410013, China;

³ Department of Biochemistry, School of Life Sciences, Central South University, Changsha, Hunan 410013, P.R. China;

⁴ Center for Experimental Medicine, the Third Xiangya Hospital of Central South University, Changsha, Hunan 410013, China.

⁵ XiangYa School of Medicine, Central South University, Changsha, Hunan 410013, China.

⁶ Department of Cardiology, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450000, China

⁷ Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Correspondence to: Dr. Hongwei Lu: hongweilu@csu.edu.cn;

Address: No. 138, Tongzi, Changsha, Hunan 410013, People's Republic of China;

Phone: +86-0731-88618428.

Keywords: Diabetic nephropathy, PACS2, fatty acid oxidation, MAMs,.

Running title: PACS2 in diabetes mellitus with hyperlipidemia induced endothelial dysfunction

Abstract:

Background: The prevalence of diabetic vascular complications is rapidly increasing, especially in the diabetes mellitus with hyperlipidemia. Consistent hyperglycemia and hyperlipidemia impairs microvascular, but lack of effective intervention target to prevention or reduced the risk of serious bad ending.

Methods: A mouse model of diabetes combined with hyperlipidemia were established by STZ injection and high fat diet to observe the possible damage of HGHF to renal blood vessels include vascular permeability, fibrosis and subcellular structure. Then, we replicated an in vitro endothelial cell injury model treated by 30mm Glucose and 0.1mm palmitic acid to verify its main functional changes. Proteomics and metabolomics were used to explore the molecular mechanisms behind diabetic microvascular damage. The mechanisms were further verified at siRNA interference and transgenic knockout mice.

Results: We found that renal vascular permeability impaired and fibrosis increased significantly in the stz+HFD mice. In human umbilical vein endothelial cells (HUVECs) treated with high glucose/high fat (HGHF), the number of mitochondrial-associated membranes (MAMs) and the expression of phosphofurin acidic cluster sorting protein 2 (PACS2) increased. In particular, gene manipulation of PACS2 altered endothelial cell MAMs. Knocking down PACS2 restored the barrier function of HUVECs. In vivo, knocking out PACS2 ameliorated the kidney injury in diabetic mice induced by streptozotocin and fed with high-fat diet for up to 20 weeks. PACS2^{-/-} mice leaked less vascular Evan's blue and improved glomerular fibrosis in the kidney tissue of hyperglycemia and hyperlipidemia mouse model. We further observed the reduction of fatty acid β -oxidation (FAO), CPT1 α expression, and NADPH production in endothelial cells induced by HGHF. These changes in fatty acid metabolism were rescued by silencing PACS2, but were blocked by the FAO inhibitor, etomoxir.

Conclusion: PACS2 impacts the metabolic response of endothelial cells to HGHF through MAMs. Loss of PACS2 expression reduces glomerular endothelial cells barrier injury, induced by VE-Cadherin internalized under HGHF. PACS2 play a

metabolism and MAMs regulators in the vascular endothelial cells of diabetes with hyperlipidemia.

Key words: Diabetic vascular disease, PACS2, fatty acid oxidation, MAMs, hyperlipidemia, diabetes mellitus

Background

Diabetes mellitus is a chronic metabolic disorder that can cause severe microvascular and macrovascular complications. Current diabetes treatment uses a variety of strategies to control blood glucose levels to reduce the occurrence of complications [1]. However, according to the results of large population studies, simple glucose control can effectively delay the onset of diabetic nephropathy, but even if glucose control is strengthened, the final incidence of vascular complications in diabetic patients has not been reduced [2, 3]. And prevalence of dyslipidemia associated with complications in diabetes are common and severe. The lipid-lowering treatment has proved can reduce cardiovascular events and mortality in severe cohort study [4, 5]. The combined effects of high glucose (HG) and free fatty acid levels [6] may be the main cause of vascular complications of diabetes. As a relatively common and serious high-glucose and high-fat (HGHF) condition, diabetes mellitus combined with hyperlipidemia also has a higher incidence of rapidly progressing diabetic vascular complications [7, 8]. Therefore, revealing the mechanism of HGHF on blood vessels may provide a new way to prevent and treat diabetic vascular damage.

In our research, the renal function and microvascular of kidney are main vascular research object. Recent studies have shown that the mitochondrial function of glomerular endothelium is crucial in diabetic nephropathy [9]. In addition, endothelial cell proliferation, apoptosis, angiogenesis, nitric oxide secretion, and permeability are also important in the progression of diabetic vascular complications [10]. According to reports, endothelial cells can transform into different morphologies or subtypes with their state or the presence of varying stimulating factors [11]. It was found that the expression of genes related to glycolysis, tricarboxylic acid cycle, nucleotide

purine and pyrimidine synthesis pathways in resting endothelial cells (QECs) was lower than that in proliferating endothelial cells (PECs), while the expression of genes related to the FAO pathway in the former cells is 3-4 times higher [12]. In addition, studies have reported that when QECs switch to an angiogenic phenotype, higher levels of glycolysis will occur to promote endothelial cell migration. As a high energy nutritional substrate, HGHF may mediate the damage and repair of endothelial function through the metabolic changes of endothelial cells. Therefore, we examined the mechanism of endothelial cell metabolism changes under HGHF.

In this study, we focused on diabetic microvascular dysfunction, especially the renal microvascular and glomerular function in hyperglycemia and hyperlipidemia condition. We first established a mouse model of diabetes combined with hyperlipidemia to observe the possible damage of HGHF to renal blood vessels. Then, we replicated an in vitro endothelial cell injury model of HGHF to verify its main functional changes. In addition, we used proteomics and metabolomics to explore the molecular mechanisms behind diabetic microvascular damage. Finally, the uncovered mechanisms were further verified at both the cellular and animal levels.

Materials and methods

Animal model

All animal manipulations were approved by the Institutional Animal Care and Use Committee of Central South University, Changsha, China (2019sydw0021). All efforts were made to minimize mouse suffering. Eight-week-old male mice C57BLKS/Nju (wildtype, WT) and phosphofurin acidic cluster sorting protein 2 (PACS2) knockout mice (B6/JGpt-PACS2 em2Cd771/Gpt, PACS2^{-/-}) were obtained from the Nanjing Biomedical Research Institute of Nanjing University and housed under standard pathogen-free conditions at the Xiangya Medical School of Central South University.

After one week of acclimatization, the mice were randomly assigned to the normal chow diet (ND) group and the streptozotocin (STZ) plus high-fat diet (HFD) (STZ + HFD) group. The latter was fed HFD (D12109C, Research Diets, USA) after grouping, and intraperitoneally injected with 10 mg/kg STZ (S0130, Sigma-Aldrich) once a week in the 9th and 10th week to simulate hyperglycemic and hyperlipidemic conditions [13]. STZ was dissolved in sodium citrate-hydrochloric acid buffer solution, pH 4.5 (SSC). The ND group was injected with the same volume of SSC at the 9th and 10th weeks. In the 11th week, blood glucose was measured, and mice with blood glucose > 11.1 mmol/L was considered to have diabetes. The flow chart of establishing HGHF-fed diabetic mouse model is showed in Fig. 1A.

Blood was drawn through the submandibular vein at 11, 14, 17, and 20 weeks to measure serum biochemistry parameters, including total cholesterol (CHO), triglyceride (TG), blood glucose, creatinine (CRE), and blood urea nitrogen (BUN). The 24-h urine microalbumin (u-mALB) was measured once a week from the 8th week, but the mouse urine was not collected next week after blood sampling to avoid the effect of transient blood loss on renal function. All mice were sacrificed at 20 weeks, and kidney tissues were fixed in 4% paraformaldehyde and stained with hematoxylin-eosin (HE) and Masson trichrome.

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC) and cultured in an Endothelial Cell Medium (ECM) (Carlsbad, CA, USA) containing 5% fetal bovine serum at 37°C in a 5% CO₂ incubator. Cells were treated with 30 mM glucose and 0.1 mM palmitic acid (PA) dissolved in 0.5% bovine serum albumin (BSA) in ECM for 48 h to induce HGHF.

siRNA transfection

To verify the function of PACS2 in HUVECs, PACS2 siRNA was delivered into cells according to the manufacturer's instructions. Briefly, cells were transfected with 50 nM PACS2 siRNA (RIBOBIO, Guangzhou, China) or negative control using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in Opti-MEM I Reduced Serum Media (Thermo Fisher Scientific, Inc.) for 12 h, and then stimulated with HGHF for another 48 h.

Vascular leakage assay

As an indicator of vascular permeability, albumin extravasation was evaluated in the Miles assay by measuring the extravasation of albumin-bound Evans blue. Briefly, mice were inhalational anesthetized with Isoflurane. Five min later, 150 µL of Evans Blue (30 mg/ml; Sigma-Aldrich, San Louis, MO, USA) was administered via the tail vein and allowed to circulate for 30 min, then extravasated Evans blue dye was evaluated [14]. Take pictures to show differences in Evans Blue extravasation of kidney. And dry one half of kidney at 150°C, on foil, for 48 h. Add 500 µl formamide to each tissue in 1.5ml tubes and incubate to 55 °C for 72h to extract Evans Blue from tissue. Measure OD620 of each sample and calculate plasma extravasation= $\frac{\text{OD620nm}}{\text{g dry weight}}$.

Fitc-dextran permeability assay

HUVECs were grown to confluence and treated with or without HGHF (30 mM glucose & 0.1 mM palmitic acid) for 48 h on type I collagen-coated culture inserts with 0.4- μ m pores (BD bioscience, USA). After that, 15 μ L of 5 mg/mL FITC-dextran 40 (Sigma-Aldrich) was added to the upper chamber and incubated at 37°C for 15min, 30min and 60min. The fluorescence intensity of FITC-dextran diffused into the lower chamber was measured at Ex/Em = 485/590 nm.

Endothelial cell permeability was detected by using an In Vitro Vascular Permeability Assay kit following the manufacturer's protocol (Millipore, Burlington, MA, USA). After forming a tighter monolayer and exposure to HGHF, the cells were stained with FITC-labeled bovine serum albumin (BSA) for 20 min, and the amount of FITC-BSA [15] diffused across the endothelial monolayer was observed under a fluorescent microscope with FITC filter and quantified on a fluorescence plate reader.

Leaked ratio = Transwell lower chamber fluorescence intensity
(Ex₄₈₀/Em₅₉₀)/upper chamber fluorescence intensity (Ex₄₈₀/Em₅₉₀)

Oxygen consumption assay

The kinetic changes of extracellular oxygen levels, an indicator of aerobic metabolism of living cells, are measured by using the MitoXpress Xtra oxygen consumption assay kit (Luxel Bioscience, USA) according to the manufacturer's manual [16]. Antimycin A, a potent electron transport chain complex III inhibitor, was used to shut down mitochondrial respiration (zero oxygen consumption control). The uncoupling agent carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) that disrupts the mitochondrial proton gradient was used to drive its maximal mitochondrial respiration rate. Basically, the phosphorescent signal of MitoXpress Xtra is quenched by oxygen and produces a signal that is inversely proportional to the amount of oxygen present. Time-resolved fluorescence (TR-F) was measured at Ex/Em = 380/650 nm and the recommended delay time. The parameters of oxygen consumption included PA-based maximal oxygen consumption rate (PA-OCR) obtained by adding 2.5 μ M FCCP plus PA-only-based substrate, and negative control

(NegOCR) obtained by adding 1 μ M antimycin A in the presence of PA-only-based substrate.

Cellular ATP assay

Cells were seeded in triplicate in 96-well plates and treated with different compounds for indicated time. Cellular ATP levels were evaluated by using an ATPlite assay (Perkin-Elmer, Waltham, MA, USA) [17].

NADPH/NADP measurement

Intracellular NADPH/NADP ratio was assayed using an NADP/ NADPH Quantification Colorimetric kit (K347-100; Biovision, Milpitas, CA, USA) according to the manufacturer's instructions.

Immunohistochemistry

The kidney tissues were fixed in 4% glutaraldehyde at 4°C for 48 h, and then post-fixed in 1% osmium tetroxide at room temperature for 2 h. After pre-staining with barbitone acetate for 10 min, histological samples were dehydrated with acetone, and embedded in paraffin wax. Immunohistochemical staining was performed to detect PACS2 and CD31 (ab28364, Abcam, Cambridge, UK) in mouse kidney endothelial cells as described previously, and visualized using fluorochrome-conjugated goat anti-mouse (ab150115, Abcam) or anti-rabbit IgG H&L (ab150077, Abcam). The numbers of PACS2-positive cells were counted and normalized to the total numbers of endothelial cells in the same field with the use of computer-assisted morphometric analysis.

Western blot analysis

Western blot analysis was performed as previously described.³⁹ The following antibodies were used: anti-CPT1A (12252S, Cell Signaling Technology, Beverly, MA, USA), anti-PACS2 (GTX17244, GeneTex, USA), VE-cadherin (ab33168, Abcam) and β -actin (A5441, Sigma-Aldrich), and HRP conjugated goat anti-rabbit IgG secondary antibody (AS09 602, Agrisera, Vannas, Sweden).

Electron microscope

Cells were isolated on nickel fitters, stained with 2% uranyl acetate for 10 min, and then stained with Reynold's lead citrate for 5 min. The endoplasmic reticulum (ER)-mitochondria contacts of endothelial cells were evaluated by a transmission electron microscopy (TEM; Hitachi-7650, Tokyo, Japan) at 60 kV. The ER mitochondrial contacts were quantified as described previously[18]. The images were analyzed using ImageJ (National Institutes of Health). The mitochondrial and ER membranes were delineated using the freehand tool. The selected areas were converted to masks and perimeters of ER were calculated. Two independent investigators quantified the images blindly. For the MAM quantification, we normalized the total ER connected to mitochondria to total ER perimeter.

Immunofluorescence microscopy

HUVECs were labeled with ER-Tracker Blue-White DPX dye (E12353, Thermo Fisher Scientific, USA) and Mito Tracker Deep Red FM (M22426, Thermo Fisher Scientific, USA) at 37°C for 30 min and observed under a confocal microscope (LSM800, Carl Zeiss Microscopy, Cambridge, MA, USA). The Pearson correlation coefficient mode, a well-defined and generally accepted means of describing the degree of overlap between image pairs, was applied to quantify the degree of co-localization between the fluorophores representing ER-Tracker Blue and Mito Tracker Deep Red. The Pearson correlation coefficient was analyzed using the built-in Carl Zeiss co-localization analysis module from the ZEN software and the threshold obtained from single-label control samples.

Label-free quantitative proteomics

Total proteins were extracted from HUVECs in HGHF or control. Peptide samples (2 µg) were separated using an EASY-nLC1200 system and then analyzed using a Q Exactive mass spectrometer (120 min/sample), which was performed at Kangchen Biotech (Shanghai, China) [19]. Identification of the differentially

expressed peptides was set at $P < 0.05$, fold-change > 2 , following gene ontology (GO) enrichment analysis using Blast2Go 4.0.7 software.

Untargeted Metabolomics of HUVECs

Every cell sample is slowly thawed at 4°C, mixed with 1mL of cold methanol/acetonitrile/H₂O (2:2:1,v/v/v) and adequately vortexed. The homogenate was sonicated at low temperature (30min/once, twice), then incubated for 60min at -20°C to precipitate the protein, and centrifuged (13000rpm, 4°C, 15 min). The supernatants were collected and dried under vacuum, and then stored at -80°C standby. Redissolve the sample in 100μL acetonitrile/water (1:1, v/v) and adequately vortexed, and then centrifuged (14000rpm, 4°C, 15 min). The supernatants were collected for LC-MS/MS analysis, which was performed at Applied Protein Technology Co., Ltd (Shanghai, China). The metabolites were blasted against the online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://geneontology.org/>) to retrieve their COs and were subsequently mapped to pathways in KEGG. The corresponding KEGG pathways were extracted.

Statistical analysis

The SPSS 22.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Data are expressed as mean $\pm$ standard deviation (SD). Statistical differences were assessed by Student t-test of the means between two groups or by one-way analysis of variance of the means between multiple groups. $P < 0.05$ was considered a significant difference.

Results

Renal vascular dysfunction in mice with diabetes and hyperlipidemia

In order to explore the effect of HGHF *in vivo*, we used low-dose STZ injection and a long-term high-fat diet to establish a mouse model of diabetes with hyperlipidemia (the STZ+HFD group) (Fig. 1A). Serum biochemical analysis and u-mALB quantitative results showed that after 20 weeks of continuous high fat feeding, the STZ+HFD group obtained a stable internal environment of hyperglycemia and hyperlipidemia compared with the ND group fed with normal diet (Fig. 1B). u-mALB increased significantly from the 16th week, indicating that the renal function had been impaired at this time. The blood CRE and BUN increased significantly at the 20th week, which also indicated that the renal function of the STZ+HFD group was abnormal.

Consistent with serum and urinary biochemical analysis, HE and Masson staining showed pathological changes in renal tissue, manifested as atrophy of glomerular vascular loops (Fig.1C) and renal microvascular fibrosis (Fig.1D and F). Furthermore, evaluation of the extravasation of Evan blue showed that the kidney tissue in the STZ+HFD group was significantly blue stained, and extravasation index was increased (Fig. 1E and G). These results support that HFD induced renal vascular dysfunction in diabetic mice.

HGHF induces endothelial hyperpermeability in HUVECs

Due to the definite damage of renal blood vessels, we hypothesized that endothelial cell function changes significantly in diabetic nephropathy. To test this, we further constructed a HGHF-treated HUVEC cell model. We observed that fluorescently labeled dextran permeated a single layer of endothelial cells in a 0.4- μ m pore size chamber and compared the upper and lower fluorescence values of the permeable chamber to reflect the endothelial barrier function. After measuring the leakage of FITC-labeled dextran through the endothelial cell monolayer, we found that the endothelial leakage in the HGHF group increased significantly at 15 and 30 min after adding FITC-dextran, and almost reached permeability equilibrium within

60 min (Fig. 2A). Furthermore, the immunofluorescence of VE-cadherin, a key regulator of endothelial adhesion junctions, was obviously internalized under HGHF treatment, and its distribution on the cell membrane was reduced (Fig. 2B). All these data indicate that long time (48 h) exposure to HGHF does significantly produce barrier dysfunction.

HGHF increases mitochondrial-associated membranes (MAMs) in endothelial cells

In order to study potential target proteins that can regulate endothelial cell dysfunction induced by HGHF, we evaluated proteome changes of endothelial cells treated with HGHF. By mining the mass spectrometry reference database, protein identification was performed on data-independent acquisition (DIA) data results (identification criteria: precursor threshold, 1.0% false discovery rate (FDR) and protein threshold, 1.0% FDR). In the two sets of samples, we identified 26,201 peptides and 3,678 protein molecules. The identified proteins were screened for differentially expressed proteins between the ND group and the HGHF group, and the relative fold change of expression > 1.2 and the Student t test q-value < 0.05 were used as filter criteria to process DIA quantitative data. On this basis, 320 differentially expressed proteins were selected out (Supplemental Fig. 1A). Among them, 138 were up-regulated and 182 were down-regulated after HGHF treatment. Gene Ontology (GO) annotation analysis showed that they are mainly related to the protein localization and unfolded protein response of the ER as well as the translational elongation and termination of mitochondria (Fig. 3A). Among all differential proteins, the localized mitochondria and ER were occupied respectively (Supplemental Fig. 1). Proteomics analysis showed that the mitochondrial and ER proteins of endothelial cells treated with HGHF changed significantly, the adhesion proteins changed significantly, and the active renal damage indicator lipocalin-2 (LCN2) was significantly increased (Supplemental Fig. 1).

The double staining of mitochondria and ER revealed higher co-localization in the presence of HGHF in HUVECs under a fluorescence microscopy (Fig. 3B and D).

The same cell samples were sent to TEM, which is observed enrichment of ER and MAMs (Fig. 3C and E). Next, we verified the changes in mitochondria-ER contact in vivo. TEM observation of renal endothelial cells showed a significant increase in MAMs of renal cortical glomerular endothelial cells, accompanied by mitochondrial swelling and mitochondrial cristae degeneration in the renal vascular endothelial cells (Fig. 3F). These results indicate that mitochondria are involved in endothelial cell dysfunction induced by HGHF.

PACS2 shows increased expression in response to HGHF at the MAMs

Based on the TEM and immunofluorescence observations of endothelial cells in HGHF-treated HUVECs and HFD-treated kidney tissues in vivo, we further explored the expression changes of three key proteins that regulate MAMs, acyl-CoA synthetase 4 (FACL4), glucose-regulated protein 75 (GRP75), and PACS2 in response to FFA treatment. Among these proteins, PACS2 was found to be significantly increased in HUVECs (Fig. 4A and B) and mouse renal vascular tissues (Fig. 4C). To further understand the role of PACS2, we silenced PACS2 in HUVECs using siRNA (Fig. 4D and E). Using confocal imaging technique, we found that knocking down PACS2 attenuated HGHF-enhanced co-localization of mitochondria and ER (Fig. 4F). In addition, we observed a decrease in the MAM interface of mitochondria and ER and an increase in the ER signal, indicating that the secretion and detoxification functions of the cell is enhanced. Besides, after knocking down PACS2 in HUVECs, the leaked FITC-dextran ratio was significantly reduced (Fig. 4H). These results indicate that PACS2 plays a role in the mitochondria-ER connectivity.

Loss of PACS2 expression improves kidney injury induced by HFD

To confirm the effect of PACS2 on vascular endothelium under HGHF in vivo, we constructed PACS2 knockout mice (PACS2^{-/-}) and used the same STZ injection and HFD treatment to produce the glucolipotoxic conditions depicted in Fig. 1A. The efficiency of gene knockout was verified by immunofluorescence microscopic

observations, showing that PACS2 was lost in CD31-labeled vascular endothelial cells (Fig. 5B). As showed in Fig. 1B, we observed that the STZ+HFD treatment altered the biochemical parameters of renal function in WT mice, however, PACS2 knockout eliminated the increase in serum parameters caused by STZ injection and high fat diet, including blood glucose, TG, CRE, and BUN, but had no effect on serum CHO at week 20 (Fig. 5A). Compared to the WT mice with the STZ+HFD treatment, PACS2-deficient mice showed a moderate reduction in glomerular fibrosis in Masson staining (Fig. 5B and C). In addition, we found that the glomerular cortex endothelial cells and vascular endothelial cell MAMs in the kidney tissue of PACS2^{-/-}+STZ+HFD mice were significantly reduced (Fig. 5D), as was the glomerular Evans blue leakage index (Fig. 5E and F), suggesting that PACS2 protects kidney tissue against HGHF by regulating MAMs

Silencing PACS2 improves free fatty and redox metabolism in HGHF-treated HUVECs

To further understand the downstream changes after the increase of MAMs, we performed metabolomics testing. KEGG pathway analysis results found that most of the differential metabolites were enriched in the metabolism pathways (Fig. 6A). There were significant changes in adenosine monophosphate, adenosine 5'-diphosphate, glycerol 3-phosphate (phosphoglycerol 3), N-acetylglucosamine-1phosphate, and phosphorylcholine (Supplemental Fig. 2), suggesting that these changes related to fatty acid metabolism may be crucial in HUVEC damage induced by HGHF.

To test the changes in fatty acid metabolism of endothelial cells under long-term HGHF treatment, we measured HUVECs' mitochondrial respiration using PA as an energy substrate. By adding FCCP or ETO into cultured cells, we evaluated FAO-dependent OCR (PA-OCR). The results showed that both FAO-dependent OCR were significantly reduced after HGHF exposure. However, silencing PACS2 reversed the PA-OCR inhibited by HGHF (Fig. 6B). Quantitative NADPH/NADP⁺ (Fig. 6C) and ATP (Fig. 6D) analysis showed that the NADPH/NADP⁺ ratio and ATP production in

HUVECs were significantly reduced after HGHF treatment. However, PACS2 knockdown prevented the effect of HGHF on the NADPH/NADP⁺ ratio, but did not affect ATP production.

Western blot analysis found that carnitine palmitoyl-transferase 1 α (CPT1 α), a key enzyme for FAO, decreased under HGHF stress, but was rescued by PACS2 knockdown (Fig. 6E-G). We also examined the CPT1 α in kidney tissues of diabetic mice fed with HGHF (Fig. 1) and found that it was obviously reduced (Fig. 6H) in Oil red-positive tissues (Fig. 6I).

These results suggest that PACS2 can regulate FAO, which may disturb the balance of endothelial redox homeostasis through the production of NADPH, thereby affecting the endothelial barrier function.

ETO block FAO to damage the barrier function of endothelial cells.

To further understand the role of PACS2 and FAO in HGHF treatment processing, we observed the synergistic effect of PACS2 silencing and FAO inhibition on the barrier function of endothelial cells. We found that regardless of PACS2 knockdown and HGHF treatment, ETO alone increased the internalization of VE-cadherin and expanded the distance between endothelial cells (Fig. 7A), and that had the most destructive effect on the barrier function of HGHF-treated cells. It was found that PACS2 siRNA inhibited the effect of HGHF on endothelial barrier function, but had no effect on ETO activity. This finding was consistent with the FITC-dextran leakage ratio, which was significantly higher in all ETO treatment groups (Fig. 7B). These results suggest that the changes in FAO may be downstream of MAMs, which will affect the response of PACS2 to energy conditions.

Discussion:

The purpose of this study was to determine the effect of hyperglycemia and hyperlipidemia condition on endothelial cell function and its mechanism in the occurrence and development of diabetic vascular complications. We observed mitochondria and ER at the cellular and animal levels through confocal and electron

microscope imaging, and found that MAMs increased significantly under the pressure of HGHF (Fig. 3B and C, Fig. 4F, and Fig. 5C), accompanied by an increase in PACS2 expression (Fig. 4A and C).

Previous studies have shown that MAMs can regulate Ca^{2+} [20], lipid synthesis [21], and mitochondrial fusion [22], but not in endothelial cells. Nevertheless, it has been reported that MAMs are associated with hepatocyte insulin resistance [23, 24] and diabetic smooth muscle cell phenotypic transformation [25]. In this study, we first discovered the relationship between HGHF and MAMs in endothelial cells, and explored the physiological significance of MAMs. We found that MAMs may affect the initialization of VE-cadherin, which sustains intercellular adherent junctions of endothelial cells [26]. Moreover, PACS2 downregulation can improve the barrier function of endothelial cells under HGHF (Fig. 7A). These findings may indicate the MAMs more important in the diabetes with hyperlipidemia patients, which already have definite dyslipidemia.

As previous studies have showed, HGHF can trigger unfolded protein response and cellular inflammation, leading to decreased insulin biosynthesis, impaired insulin secretion, or cell death [27, 28]. And lymphatic vessel integrity dysfunction in mice models of obesity and type 2 diabetes [29]. In our study, we found that mice with STZ+HDF treatment have high renal microvascular permeability (Fig. 1), HGHF induces endothelial barrier dysfunction in HUVECs (Fig. 2) and the VE-cadherin decreased in outer cell membrane were direct cause of high permeability of endothelial cells.

It was reported that HUVECs will increase FAO and decrease glycolysis when assembled into a formed network [30]. And when glucose and lipids both overload, the increased glucose will lead to the accumulation of malonyl-CoA, which affects the activation of CPT1 α and limits the oxidation of fatty acids in the mitochondria [27, 31]. Therefore, we examined the metabolomics of HUVECs under HGHF and found that the metabolic pathways changed the most in the KEGG analysis (Fig. 6A and Supplemental Fig. 2). In particular, long chain fatty acids, such as glycerol 3-phosphate, myristate, palmitate, and oleate, were increased in HGHF-treated cells,

which may cause fatty acid coenzyme A to accumulate in the cytoplasm in the form of complex lipids or ceramides, resulting in cell damage. This was supported by Oil Red staining, which showed significantly positive kidney tissues in the STZ+HDF group (Fig. 6I), indicating a decrease in fatty acid utilization. Therefore, in the OCR assay, we further verified that the FAO activity of endothelial cells treated with PA (Fig. 7A).

According to recent reports, CPT1 α is present in MAMs [32] and may have an important regulatory role in MAMs. We examined the expression of CPT1 α in HUVECs and mouse kidneys, both of which fully support that CPT1 α mediates the reduction of FAO under HGHF. Recent study also showed FAO is essential for endothelial function [33] [34]. It has been reported that endothelial cells rely on FAO to increase the regeneration of NADPH to maintain redox homeostasis [12]. Consistent with this, we found that blocking FAO by ETO reduced the endothelial cell barrier function under HGHF, which may due to the redox imbalance (Fig. 7). Interestingly, this damage caused by FAO inhibition could not be offset by PACS2 knockdown, which means that FAO and NADPH reduction are downstream of PACS2. Thus, endothelial cells on hyperglycemia and hyperlipidemia condition forms a chain of evidence from PACS2, MAMs to FAO and VE-cadherin (Fig. 8).

Our study shows that knocking down PACS2 led to a decrease in MAMs in HUVECs and vascular endothelial cells of mouse kidney tissues, which is in agreement with previous results in cancer cells [35], smooth muscle cells [36]. PACS2 is one of the multi-functional protein family [37], which were reported as a metabolic switches and nuclear trafficking signals regulator in HFD-induced steatosis and insulin resistance [38]. The down-regulation of PACS2 basically prevents the increase of mitochondrial Ca²⁺-mediated apoptosis [39]. Moreover, abnormally increased MAMs can evoke mitochondrial calcium overload, mitophagosome formation, and mitophagy, all of which can be regulated by PACS2 [25, 40].

In addition, in our animal model, renal fibrosis, especially glomerulosclerosis, appeared after STZ injection and 20 weeks of HFD feeding, indicating that some potential pathological process may occur under long-time hyperglycemia and

hyperlipidemia condition, such as endothelial-interstitial transformation [41]. Moreover, the blood glucose and TG decreased in PACS2^{-/-} mice, which may have more potential mechanisms to explore the relationship between PACS2 and glucose and lipid metabolism. Our limitation is that PACS2^{-/-} mice cannot exclude the loss of PACS2 in other cells or tissues, although the use of PACS2 siRNA in vitro can partially explain its specific effect in endothelial cells. Additionally, studies on MAMs in the heart [42], smooth muscle cells [25], and liver [43] also found that knocking out PACS2 can prevent lipotoxicity, indicating the importance of systemic understanding of high incidence of vascular damage in diabetic with hyperlipidemia patients.

In conclusion, we demonstrated the barrier dysfunction of endothelial cells in HGHF, and increased MAMs in the vascular endothelium. And knocking down PACS2 can induce changes in downstream FAO and protect the endothelium. In the diabetes mellitus patients, hyperlipidemia is the result of free fatty acid metabolism disorder. These metabolic change reflected in MAMs change on some degree, and PACS2^{-/-} maintain the FAO of endothelial cells, which shows the special effect in diabetes mellitus with hyperlipidemia. Overall, our study revealed the role of PACS2 and MAMs in endothelial cell dysfunction induced by HGHF, thereby providing molecular targets for reducing diabetic vascular damage in diabetes mellitus with hyperlipidemia.

DECLARATIONS

Funding

This study was supported by a grant from the National Natural Science Foundation of China (grant no. 81870352, 81970252) and the Key Research and Development Project of Hunan Province (grant no. 2019SK2041, 2020SK2087).

Conflict of interest

The authors confirm that there are no conflicts of interest.

Ethics approval

The animal experimental processes were approved by the Ethnic Committee of Third Xiangya hospital and conducted in strict accordance to the standard of the Guide for the Care and Use of Laboratory Animals published by the Ministry of Science and Technology of the People's Republic of China in 2019(2019sydw0021).

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets generated/analyzed during the current study are available.

Author's Contributions

Zhihao Shu wrote the paper and conceived and designed the experiments. Professor Alex F Chen Help modify and optimize experimental design. Professor Shuhua Chen and HongXiang collected and provided the cell samples for this study. Ruoru Wu, Shaoli Zhao, Jie Xiao, Hengdao Liu, Xuwen Wang, Jie Ouyang, Yulan Liu and Wenfang Xiong contributed to drafting the article. All authors have read and approved the final submitted manuscript. Professor Hongwei Lu is the GUARANTOR for the article who accepts full responsibility for the work and/or the conduct of the study, had access to the data, and oversaw the decision to publish.

Acknowledgements

Authors acknowledge the Junpu Wang and Jing Li at Department of Pathology, Xiangya Hospital, Central South University for providing the help of TEM of MAMs.

And we also would like to thank the Applied Protein Technology Co., Ltd (Shanghai., China) and KangChen Bio-tech(Shanghai., China) for metabolomics and proteomics.

REFERENCE

1. Dall TM, Yang W, Gillespie K, Mocarski M, Byrne E, Cintina I, Beronja K, Semilla AP, Iacobucci W, Hogan PFJDC: **The economic burden of elevated blood glucose levels in 2017: diagnosed and undiagnosed diabetes, gestational diabetes mellitus, and prediabetes.** 2019, **42**(9):1661-1668.
2. Coca SG, Ismail-Beigi F, Haq N, Krumholz HM, Parikh CRJAoim: **Role of intensive glucose control in development of renal end points in type 2 diabetes mellitus: systematic review and meta-analysis.** 2012, **172**(10):761-769.
3. Peltier A, Goutman SA, Callaghan BC: **Painful diabetic neuropathy.** *BMJ* 2014, **348**:g1799.
4. Moosaie F, Firouzabadi FD, Abouhamzeh K, Esteghamati S, Meysamie A, Rabizadeh S, Nakhjavani M, Esteghamati AJN, Metabolism, Diseases C: **Lp (a) and Apo-lipoproteins as predictors for micro-and macrovascular complications of diabetes: A case-cohort study.** 2020, **30**(10):1723-1731.
5. Fung CSC, Wan EYF, Chan AKC, Lam CLKJBcd: **Statin use reduces cardiovascular events and all-cause mortality amongst Chinese patients with type 2 diabetes mellitus: a 5-year cohort study.** 2017, **17**(1):1-9.
6. Prentki M, Joly E, El-Assaad W, Roduit R: **Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes.** *Diabetes* 2002, **51** Suppl 3:S405-413.
7. Gebreegziabiher G, Belachew T, Mehari K, Tamiru DJPo: **Prevalence of dyslipidemia and associated risk factors among adult residents of Mekelle City, Northern Ethiopia.** 2021, **16**(2):e0243103.
8. Haile K, Timerga AJD, Metabolic Syndrome, Targets O, Therapy: **Dyslipidemia and its associated risk factors among adult type-2 diabetic patients at Jimma University Medical Center, Jimma, Southwest Ethiopia.** 2020, **13**:4589.
9. Qi H, Casalena G, Shi S, Yu L, Ebefors K, Sun Y, Zhang W, D'Agati V, Schlondorff D, Haraldsson B *et al*: **Glomerular Endothelial Mitochondrial Dysfunction Is Essential and Characteristic of Diabetic Kidney Disease Susceptibility.** *Diabetes* 2017, **66**(3):763-778.
10. Carrizzo A, Izzo C, Oliveti M, Alfano A, Virtuoso N, Capunzo M, Di Pietro P, Calabrese M, De Simone E, Sciarretta SJIjoms: **The main determinants of diabetes mellitus vascular complications: endothelial dysfunction and platelet hyperaggregation.** 2018, **19**(10):2968.

11. Romaldini A, Ulivi V, Nardini M, Mastrogiacomo M, Cancedda R, Descalzi F: **Platelet Lysate Inhibits NF-kappaB Activation and Induces Proliferation and an Alert State in Quiescent Human Umbilical Vein Endothelial Cells Retaining Their Differentiation Capability.** *Cells* 2019, **8**(4).
12. Kalucka J, Bierhansl L, Conchinha NV, Missiaen R, Elia I, Brüning U, Scheinok S, Treps L, Cantelmo AR, Dubois CJCm: **Quiescent endothelial cells upregulate fatty acid β -oxidation for vasculoprotection via redox homeostasis.** 2018, **28**(6):881-894. e813.
13. Furman BL: **Streptozotocin-Induced Diabetic Models in Mice and Rats.** *Curr Protoc Pharmacol* 2015, **70**:5 47 41-45 47 20.
14. Shan K, Liu C, Liu B-H, Chen X, Dong R, Liu X, Zhang Y-Y, Liu B, Zhang S-J, Wang J-JC: **Circular noncoding RNA HIPK3 mediates retinal vascular dysfunction in diabetes mellitus.** 2017, **136**(17):1629-1642.
15. Aman J, van Bezu J, Damanafshan A, Huveneers S, Eringa EC, Vogel SM, Groeneveld AJ, Vonk Noordegraaf A, van Hinsbergh VW, van Nieuw Amerongen GPJC: **Effective treatment of edema and endothelial barrier dysfunction with imatinib.** 2012, **126**(23):2728-2738.
16. Du Q, Tan Z, Shi F, Tang M, Xie L, Zhao L, Li Y, Hu J, Zhou M, Bode A *et al*: **PGC1alpha/CEBPB/CPT1A axis promotes radiation resistance of nasopharyngeal carcinoma through activating fatty acid oxidation.** *Cancer Sci* 2019, **110**(6):2050-2062.
17. Liu H, Xiang H, Zhao S, Sang H, Lv F, Chen R, Shu Z, Chen AF, Chen S, Lu HJJoc *et al*: **Vildagliptin improves high glucose - induced endothelial mitochondrial dysfunction via inhibiting mitochondrial fission.** 2019, **23**(2):798-810.
18. Wu S, Lu Q, Ding Y, Wu Y, Qiu Y, Wang P, Mao X, Huang K, Xie Z, Zou M-HJC: **Hyperglycemia-driven inhibition of AMP-activated protein kinase α 2 induces diabetic cardiomyopathy by promoting mitochondria-associated endoplasmic reticulum membranes in vivo.** 2019, **139**(16):1913-1936.
19. Zhu Y, Bo H, Chen Z, Li J, He D, Xiao M, Xiang L, Jin L, Zhou J, Gong L *et al*: **LINC00968 can inhibit the progression of lung adenocarcinoma through the miR-21-5p/SMAD7 signal axis.** *Aging (Albany NY)* 2020, **12**(21):21904-21922.
20. Boyman L, Karbowski M, Lederer WJ: **Regulation of Mitochondrial ATP Production: Ca(2+) Signaling and Quality Control.** *Trends Mol Med* 2020, **26**(1):21-39.
21. Balla T, Sengupta N, Kim YJJBeBA-M, Lipids CBo: **Lipid synthesis and transport are coupled to regulate membrane lipid dynamics in the endoplasmic reticulum.** 2020, **1865**(1):158461.
22. Pinton P: **Mitochondria-associated membranes (MAMs) and pathologies.** In.: Nature Publishing Group; 2018.
23. Wang C-H, Wei Y-HJJobs: **Role of mitochondrial dysfunction and dysregulation of Ca 2+ homeostasis in the pathophysiology of insulin resistance and type 2 diabetes.** 2017, **24**(1):1-11.

24. Bassot A, Chauvin M-A, Bendridi N, Ji-Cao J, Vial G, Monnier L, Bartosch B, Alves A, Cottet-Rousselle C, Gouriou YJC: **Regulation of mitochondria-associated membranes (MAMs) by NO/sGC/PKG participates in the control of hepatic insulin response.** 2019, **8**(11):1319.
25. Moulis M, Grousset E, Faccini J, Richetin K, Thomas G, Vindis CJC: **The multifunctional sorting protein PACS-2 controls mitophagosome formation in human vascular smooth muscle cells through mitochondria-ER contact sites.** 2019, **8**(6):638.
26. De Rossi G, Vahatupa M, Cristante E, Arokiasamy S, Liyanage SE, May U, Pellinen L, Uusitalo-Jarvinen H, Bainbridge JW, Jarvinen TAH *et al*: **Pathological Angiogenesis Requires Syndecan-4 for Efficient VEGFA-Induced VE-Cadherin Internalization.** *Arterioscler Thromb Vasc Biol* 2021, **41**(4):1374-1389.
27. Bellini L, Campana M, Rouch C, Chacinska M, Bugliani M, Meneyrol K, Hainault I, Lenoir V, Denom J, Véret JJD: **Protective role of the ELOVL2/docosahexaenoic acid axis in glucolipotoxicity-induced apoptosis in rodent beta cells and human islets.** 2018, **61**(8):1780-1793.
28. Li J, Wu N, Chen X, Chen H, Yang X, Liu CJI: **Curcumin protects islet cells from glucolipotoxicity by inhibiting oxidative stress and NADPH oxidase activity both in vitro and in vivo.** 2019, **11**(6):152-164.
29. Sawane M, Kajiya K, Kidoya H, Takagi M, Muramatsu F, Takakura N: **Apelin inhibits diet-induced obesity by enhancing lymphatic and blood vessel integrity.** *Diabetes* 2013, **62**(6):1970-1980.
30. Patella F, Schug ZT, Persi E, Neilson LJ, Erami Z, Avanzato D, Maione F, Hernandez-Fernaund JR, Mackay G, Zheng L *et al*: **Proteomics-based metabolic modeling reveals that fatty acid oxidation (FAO) controls endothelial cell (EC) permeability.** *Mol Cell Proteomics* 2015, **14**(3):621-634.
31. Fucho R, Casals N, Serra D, Herrero LJTFJ: **Ceramides and mitochondrial fatty acid oxidation in obesity.** 2017, **31**(4):1263-1272.
32. Hung V, Lam SS, Udeshi ND, Svinkina T, Guzman G, Mootha VK, Carr SA, Ting AYJe: **Proteomic mapping of cytosol-facing outer mitochondrial and ER membranes in living human cells by proximity biotinylation.** 2017, **6**:e24463.
33. Ghosh A, Gao L, Thakur A, Siu PM, Lai CWJJobs: **Role of free fatty acids in endothelial dysfunction.** 2017, **24**(1):1-15.
34. Gray SP, Di Marco E, Okabe J, Szyndralewicz C, Heitz F, Montezano AC, de Haan JB, Koulis C, El-Osta A, Andrews KL *et al*: **NADPH oxidase 1 plays a key role in diabetes mellitus-accelerated atherosclerosis.** *Circulation* 2013, **127**(18):1888-1902.
35. Simmen T, Aslan JE, Blagoveshchenskaya AD, Thomas L, Wan L, Xiang Y, Feliciangeli SF, Hung CH, Crump CM, Thomas G: **PACS-2 controls endoplasmic reticulum-mitochondria communication and Bid-mediated apoptosis.** *EMBO J* 2005, **24**(4):717-729.
36. Moulis M, Grousset E, Faccini J, Richetin K, Thomas G, Vindis C: **The**

- Multifunctional Sorting Protein PACS-2 Controls Mitophagosome Formation in Human Vascular Smooth Muscle Cells through Mitochondria-ER Contact Sites.** *Cells* 2019, **8**(6).
37. Thomas G, Aslan JE, Thomas L, Shinde P, Shinde U, Simmen T: **Caught in the act - protein adaptation and the expanding roles of the PACS proteins in tissue homeostasis and disease.** *J Cell Sci* 2017, **130**(11):1865-1876.
 38. Krzysiak TC, Thomas L, Choi YJ, Auclair S, Qian Y, Luan S, Krasnow SM, Thomas LL, Koharudin LMI, Benos PV *et al*: **An Insulin-Responsive Sensor in the SIRT1 Disordered Region Binds DBC1 and PACS-2 to Control Enzyme Activity.** *Mol Cell* 2018, **72**(6):985-998 e987.
 39. Zhou F, Fu WD, Chen L: **MiRNA-182 regulates the cardiomyocyte apoptosis in heart failure.** *Eur Rev Med Pharmacol Sci* 2019, **23**(11):4917-4923.
 40. Gbel J, Engelhardt E, Pelzer P, Sakthivelu V, Jahn HM, Jevtic M, Folz-Donahue K, Kukat C, Schauss A, Frese CK *et al*: **Mitochondria-Endoplasmic Reticulum Contacts in Reactive Astrocytes Promote Vascular Remodeling.** *Cell Metab* 2020, **31**(4):791-808 e798.
 41. Cruz-Solbes AS, Youker KJKD, Disease: **Epithelial to mesenchymal transition (EMT) and endothelial to mesenchymal transition (EndMT): role and implications in kidney fibrosis.** 2017:345-372.
 42. Wu S, Lu Q, Ding Y, Wu Y, Qiu Y, Wang P, Mao X, Huang K, Xie Z, Zou MH: **Hyperglycemia-Driven Inhibition of AMP-Activated Protein Kinase alpha2 Induces Diabetic Cardiomyopathy by Promoting Mitochondria-Associated Endoplasmic Reticulum Membranes In Vivo.** *Circulation* 2019, **139**(16):1913-1936.
 43. Krzysiak TC, Thomas L, Choi Y-J, Auclair S, Qian Y, Luan S, Krasnow SM, Thomas LL, Koharudin LM, Benos PVJMc: **An insulin-responsive sensor in the SIRT1 disordered region binds DBC1 and PACS-2 to control enzyme activity.** 2018, **72**(6):985-998. e987.

Figure Legends

Fig. 1. Renal vascular function in diabetes mellitus with hyperlipidemia mice.

A. Schematic diagram of diabetic mice model induced by streptozotocin (stz) and fed with high-fat diet (HFD) for up to 20 weeks. **B.** Biochemical analysis of blood glucose, total cholesterol (CHO), triglyceride (TG), blood urea nitrogen (BUN), creatinine (CRE), and 24-h urine microalbumin (u-mALB) in mice fed with normal chow diet (ND) or HFD. **C.** Hematoxylin-eosin (HE) and **D.** Masson's trichrome staining of mouse kidney tissues. Magnification, 100× and 400×. Scale bar, 200 μm. **E.** Photographs of Evans blue (EB)-stained mouse kidneys. **F.** Histogram of changes in CVF. **G.** EB extravasation index. Representative images are shown or data are represented as the mean ± SD, $n \geq 6$.

Fig. 2. Effect of HGHF on endothelial hyperpermeability in primary human umbilical vein endothelial cells (HUVECs).

A. Cells were treated with 30 mM glucose and 0.1 mM palmitic acid (HGHF) for 48 h, then FITC-dextran leakage ratio was quantified at 15, 30, and 60 min after addition of the dye. Normal, 0.5% bovine serum albumin (BSA); Man, 30 mM mannitol in 0.5% BSA. Data are represented as the mean ± SD, $n = 3$. * $p < 0.05$ for HGHF vs Normal, # $p < 0.05$ for HGHF vs Man. **B.** Representative images of immunofluorescence staining of VE-cadherin (VE-cadh, green) and DAPI (blue). Scale bar, 20 μm.

Fig. 3. Effect of HGHF on mitochondrial-associated membranes (MAMs) in endothelial cells.

A. GO ontology analysis of changed cellular components, biological processes, and molecular functions in HUVECs treated with HGHF. **B.** Confocal imaging & Transmission electron microscope imaging of HUVECs double-labeled with ER-Tracker Green and Mito Tracker Deep Red. Bar, 5 μm. **C.** Transmission electron microscope imaging of HUVECs Blue arrow: MAMs. M: mitochondria. **D.** Quantitative analysis of the co-localization fluorescence intensity of mitochondria and ER in B. **E.** Quantitative analysis of mitochondrial MAM coverage in C. **F.** vascular

endothelial cells in kidney tissues depicted in Fig. 1. Representative images are shown or data are represented as the mean $\pm$ SD, n =3. ** p < 0.01.

Fig. 4. PACS2 is involved in mitochondrial-endoplasmic reticulum interaction in HUVECs treated with HGHF.

A. Western blot analysis of MAM-related proteins. ACTIN used as loading control. **B.** Quantitative analysis of the expression of FACL4, GRP75, and PACS2 in A. The band densities were quantitated and normalized to the corresponding ACTIN. **C.** Immunofluorescence imaging of cells stained for CD31 (red), PACS2 (green), and nucleus (DAPI, blue) in mouse kidney tissues. **D.** Transfection efficiency of PACS2 siRNA in HUVECs. **E.** Quantitative analysis of PACS2 knockdown in D. **F.** Confocal imaging of cells double-labeled with ER-Tracker Blue and Mito Tracker Deep Red in HUVECs. Single optical sections are shown and the purple ones indicate the co-localization of mitochondria and ER. Bar, 10 μ m. **G.** Quantitative analysis of the co-localization ratio in F. **H.** Quantification of FITC-dextran leakage of HUVECs within 15 min. **I.** Immunofluorescence staining of VE-cadherin (green) in HUVECs. Representative images are shown or data are represented as the mean $\pm$ SD, n =3. *p < 0.05.

Fig. 5. Loss of PACS2 expression improves kidney injury induced by HGHF

A. Biochemical analysis of serum parameters in wild type (WT) and PACS2^{-/-} mice fed with or without HFD. **B.** Immunofluorescence and Masson staining of kidney tissues. Magnification, 100 $\times$. Bar, 50 μ m **C.** Histogram of CVF in B. **D.** Transmission electron microscope imaging of mouse kidney ultrastructure. Magnification, 15,000 $\times$. Red M, mitochondria of renal endothelial cells; blue arrow, MAMs. **E.** Images of Evans blue staining of mouse kidneys. **F.** Histogram of EB extravasation index. Representative images are shown or data are represented as the mean $\pm$ SD, n $\geq$ 5. *p < 0.05.

Fig. 6. Silencing PACS2 improves free fatty and redox metabolism in HUVECs treated with HGHF.

A. KEGG analysis of metabolomics in HUVECs under HGHF. **B.** Palmitic acid (PA)-based oxygen consumption rate (OCR) was measured in cells transfected with

negative siRNA (NeSiRNA) or PACS2 siRNA (siPACS2) and treated with or without HGHF. **C.** Determination of cellular NADPH/NADP ratio. **D.** Determination of cellular ATP levels. **E.** Western blot analysis of PACS2 and carnitine palmitoyl-transferase 1 α (CPT1 α) expression. **F.** Quantitation and normalization of PACS2 expression in E. **G.** Quantitation and normalization of CPT1 α expression in E. **H.** Immunohistochemical staining of CPT1 α in mouse kidney. Magnification, 200 $\times$. **I.** Oil Red staining of mouse kidney. Magnification, 200 $\times$. Representative images are shown or data are represented as the mean $\pm$ SD, n = 3. *P < 0.05.

Fig. 7. Inhibition of fatty acid β -oxidation disturbs the barrier function of endothelial cells and eliminates the protective effect of PACS2.

A. Immunofluorescence staining of VE-cadherin (green) in cells transfected with NeSiRNA or siPACS2 and treated with the fatty acid β -oxidation inhibitor etomoxir (ETO) or phosphate buffered saline (PBS) as vehicle control. Blue, DAPI. Bar, 20 μ m. **B.** Quantification of FITC-dextran leakage ratio of HUVECs within 15 min. Representative images are shown or data are represented as the mean $\pm$ SD, n = 3. *P < 0.05.

Fig. 8. A flow chart model proposal to explain the mechanism of renal vascular endothelial cell injury in diabetes mellitus with hyperlipidemia.

PACS2 is a key component of the mechanism behind diabetic microvascular dysfunction caused by HGHF. Under high glucose/high fat conditions, PACS2 is upregulated and increases the number of MAMs, followed by a decrease in CPT1 α expression, free fatty (FA) β -oxidation, and NADPH production in renal vascular endothelial cells. All these metabolic changes will promote the internalization of VE-cadherin, disturb the barrier function of endothelial cells, and ultimately lead to renal vascular damage.

Fig. 1. Renal vascular function in diabetes mellitus with hyperlipidemia mice.

A. Schematic diagram of diabetic mice model induced by streptozotocin (stz) and fed with high-fat diet (HFD) for up to 20 weeks. **B.** Biochemical analysis of blood glucose, total cholesterol (CHO), triglyceride (TG), blood urea nitrogen (BUN), creatinine (CRE), and 24-h urine microalbumin (u-mALB) in mice fed with normal chow diet (ND) or HFD. **C.** Hematoxylin-eosin (HE) and **D.** Masson's trichrome staining of mouse kidney tissues. Magnification, 100 $\times$ and 400 $\times$. Scale bar, 200 μ m. **E.** Photographs of Evans blue (EB)-stained mouse kidneys. **F.** Histogram of changes in CVP. **G.** EB extravasation index. Representative images are shown or data are represented as the mean $\pm$ SD, $n \geq 6$.

A

B

Fig. 2. Effect of HGHF on endothelial hyperpermeability in primary human umbilical vein endothelial cells (HUVECs)

A. Cells were treated with 30 mM glucose and 0.1 mM palmitic acid (HGHF) for 48 h, then FITC-dextran leakage ratio was quantified at 15, 30, and 60 min after addition of the dye. Normal, 0.5% bovine serum albumin (BSA);

Man, 30 mM mannitol in 0.5% BSA. Data are represented as the mean $\pm$ SD, $n = 3$. * $p < 0.05$ for HGHF vs Normal, # $p < 0.05$ for HGHF vs Man. **B.** Representative images of immunofluorescence staining of VE-cadherin (VE-cadh, green) and DAPI (blue). Scale bar, 20 μ m.

Fig. 3. Effect of HGHF on mitochondrial-associated membranes (MAMs) in endothelial cells.

A. GO ontology analysis of changed cellular components, biological processes, and molecular functions in HUVECs treated with HGHF. **B.** Confocal imaging & Transmission electron microscope imaging of HUVECs double-labeled with ER-Tracker Green and Mito Tracker Deep Red. Bar, 5 µm. **C.** Transmission electron microscope imaging of HUVECs Blue arrow: MAMs. M: mitochondria. **D.** Quantitative analysis of the co-localization fluorescence intensity of mitochondria and ER in B. **E.** Quantitative analysis of mitochondrial MAM coverage in C. **F.** vascular endothelial cells in kidney tissues depicted in Fig. 1. Representative images are shown or data are represented as the mean ± SD, n = 3. ** p < 0.01.

Fig. 4. PACS2 is involved in mitochondrial-endoplasmic reticulum interaction in HUVECs treated with HGHF.

A. Western blot analysis of MAM-related proteins. ACTIN used as loading control. **B.** Quantitative analysis of the expression of FACIL4, GRP75, and PACS2 in A. The band densities were quantitated and normalized to the corresponding ACTIN. **C.** Immunofluorescence imaging of cells stained for CD31 (red), PACS2 (green), and nucleus (DAPI, blue) in mouse kidney tissues. **D.** Transfection efficiency of PACS2 siRNA in HUVECs. **E.** Quantitative analysis of PACS2 knockdown in D. **F.** Confocal imaging of cells double-labeled with ER-Tracker Blue and Mito Tracker Deep Red in HUVECs. Single optical sections are shown and the purple ones indicate the co-localization of mitochondria and ER. Bar, 10 μm. **G.** Quantitative analysis of the co-localization ratio in F. **H.** Quantification of FITC-dextran leakage of HUVECs within 15 min. **I.** Immunofluorescence staining of VE-cadherin (green) in HUVECs. Representative images are shown or data are represented as the mean ± SD, n = 3. *p < 0.05.

Fig. 5. Loss of PACS2 expression improves kidney injury induced by HGHF

A. Biochemical analysis of serum parameters in wild type (WT) and PACS2^{-/-} mice fed with or without HFD. **B.** Immunofluorescence and Masson staining of kidney tissues. Magnification, 100×. Bar, 50 μm **C.** Histogram of CVF in B. **D.** Transmission electron microscope imaging of mouse kidney ultrastructure. Magnification, 15,000×. Red M, mitochondria of renal endothelial cells; blue arrow, MAMs. **E.** Images of Evans blue staining of mouse kidneys. **F.** Histogram of EB extravasation index. Representative images are shown or data are represented as the mean ± SD, n ≥ 5. *p < 0.05.

Fig. 6. Silencing PACS2 improves free fatty and redox metabolism in HUVECs treated with HGHF.

A. KEGG analysis of metabolomics in HUVECs under HGHF. **B.** Palmitic acid (PA)-based oxygen consumption rate (OCR) was measured in cells transfected with negative siRNA (NeSiRNA) or PACS2 siRNA (siPACS2) and treated with or without HGHF. **C.** Determination of cellular NADPH/NADP ratio. **D.** Determination of cellular ATP levels. **E.** Western blot analysis of PACS2 and carnitine palmitoyl-transferase 1α (CPT1α) expression. **F.** Quantitation and normalization of PACS2 expression in E. **G.** Quantitation and normalization of CPT1α expression in E. **H.** Immunohistochemical staining of CPT1α in mouse kidney. Magnification, 200×. **I.** Oil Red staining of mouse kidney. Magnification, 200×. Representative images are shown or data are represented as the mean ± SD, n = 3. *P < 0.05.

Fig. 7. Inhibition of fatty acid β -oxidation disturbs the barrier function of endothelial cells and eliminates the protective effect of PACS2.

A. Immunofluorescence staining of VE-cadherin (green) in cells transfected with NeSiRNA or siPACS2 and treated with the fatty acid β -oxidation inhibitor etomoxir (ETO) or phosphate buffered saline (PBS) as vehicle control. Blue, DAPI. Bar, 20 μ m. **B.** Quantification of FITC-dextran leakage ratio of HUVECs within 15 min.

Representative images are shown or data are represented as the mean $\pm$ SD, n = 3. *P < 0.05

Fig. 8. A flow chart model proposal to explain the mechanism of renal vascular endothelial cell injury in diabetes mellitus with hyperlipidemia.

PACS2 is a key component of the mechanism behind diabetic microvascular dysfunction caused by HGHF. Under high glucose/high fat conditions, PACS2 is upregulated and increases the number of MAMs, followed by a decrease in CPT1 α expression, free fatty (FA) β -oxidation, and NADPH production in renal vascular endothelial cells. All these metabolic changes will promote the internalization of VE-cadherin, disturb the barrier function of endothelial cells, and ultimately lead to renal vascular damage.

Supplement fig1 Proteomics of endothelial cells under HGHF

E

Supplement fig2 Metabolomics of endothelial cell under HGHF

A

B

C

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SUPLLEDATA.xlsx](#)
- [WBGELS.tif](#)