A novel mechanism of action for salidroside to alleviate diabetic albuminuria: effects on albumin transcytosis across glomerular endothelial cells

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1Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology and Key Laboratory of Drug Target Research and Pharmacodynamic Evaluation of Hubei Province, Wuhan, Hubei, China; and 2Department of Endocrinology, Institute of Geriatric Medicine, Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China

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Wu D, Yang X, Zheng T, Xing S, Wang J, Chi J, Bian F, Li W, Xu G, Bai X, Wu G, Jin S. A novel mechanism of action for salidroside to alleviate diabetic albuminuria: effects on albumin transcytosis across glomerular endothelial cells. Am J Physiol Endocrinol Metab 310; E225–E237, 2016. First published December 8, 2015; doi:10.1152/ajpendo.00391.2015.—Salidroside (SAL) is a phenylethanoloid glycoside isolated from the medicinal plant Rhodiola rosea. R. rosea has been reported to have beneficial effects on diabetic nephropathy (DN) and high-glucose (HG)-induced mesangial cell proliferation. Given the importance of caveolin-1 (Cav-1) in transcytosis of albumin across the endothelial barrier, the present study was designed to elucidate whether SAL could inhibit Cav-1 phosphorylation and reduce the albumin transcytosis across glomerular endothelial cells (GECs) to alleviate diabetic albuminuria as well as to explore its upstream signaling pathway. To assess the therapeutic potential of SAL and the mechanisms involved in DN albuminuria, we orally administered SAL to db/db mice, and the effect of SAL on the albuminuria was measured. The albumin transcytosis across GECs was explored in a newly established in vitro cellular model. The ratio of albumin to creatinine was significantly reduced upon SAL treatment in db/db mice. SAL decreased the albumin transcytosis across GECs in both normoglycemic and hyperglycemic conditions. SAL reversed the HG-induced downregulation of AMP-activated protein kinase and upregulation of Src kinase and blocked the upregulation Cav-1 phosphorylation. Meanwhile, SAL decreased mitochondrial superoxide anion production and moderately depolarized mitochondrial membrane potential. We conclude that SAL exerts its proteinuria-alleviating effects by downregulation of Cav-1 phosphorylation and inhibition of albumin transcytosis across GECs. These studies provide the first evidence of interference with albumin transcytosis across GECs as a novel approach to the treatment of diabetic albuminuria.

albumin; transcytosis; salidroside; glomerular endothelial cell; diabetic nephropathy

DIABETIC NEPHROPATHY (DN) is a microangiopathic complication of diabetes mellitus (DM) and the leading cause of end-stage renal disease (ESRD) (25), of which albuminuria is a significant feature.

To retard the progression of DN, good glycemic control is emphasized. However, it is suggested that metformin (Met) be used with caution in patients with mild to moderate chronic kidney disease (16). A clinical research showed that, although

intensive life style control and Met treatment ameliorate DM, there was no improvement in urine albumin-to-creatinine ratios (9a). Thus, searching for novel approaches or drugs still appears especially crucial to improve the prognosis of DN.

Salidroside [SAL; 2-(4-hydroxyphenyl)-ethyl-ß-D-glucopyranoside], is the major ingredient in Rhodiola rosea, which grows at high altitudes and has been used as a roborant for a long time (9, 30). SAL has various pharmacological properties, including antidiabetic, hepatoprotective, and antioxidative effects, etc. (19, 34, 42). Ethanol extract of R. rosea has protective effects against early nephropathy in diabetic rats, and can inhibit high-glucose (HG)-induced mesangial cell proliferation (33, 37).

In a preliminary study to evaluate the effect of SAL on db/db mice, we found that, compared with Met, SAL not only had hypoglycemic effects on db/db mice (42) but also significantly alleviated the proteinuria. On the basis of this clue, we sought to further elucidate the molecular mechanism underlying its proteinuria alleviating action.

As the first line of the glomerular filtration barrier (GFB), glomerular endothelial cells (GECs) are essential for albumin filtration (11, 28). As a special phenotype of capillary endothelial cells, GECs are highly fenestrated with transcellular pores and are essential for the high hydraulic conductivity across the GFB (13). GECs are covered by a layer of the negatively charged glycocalyx. Only water and small solutes except albumin can gain high permeability through GECs.

Caveolin-1 (Cav-1), a plasma membrane intrinsic protein (20–22 kDa), is the primary component of caveolae and is the key signaling molecule in caveolae. Albumin traffic across the intact endothelium is predominantly through caveolae-mediated transcytosis (15, 22). Cav-1 is expressed on renal cortex both in mice and in humans, and albuminuria was not observed in the Cav-1−/− diabetic mice (12). Renal targeted interference with Cav-1/caveolae prevents DN (12, 23). Thus, converging lines of evidence have pointed to a possible link between albumin transcytosis across GECs and the pathogenesis of microalbuminuria in DN. Transcytosis has been reported to be initiated and regulated by Src family kinases (15). HG increased reactive oxygen species (ROS) production and inhibited AMPK, as well as activated Src kinase in various cell types (7, 32). ROS may increase endothelial permeability and activate Src kinase, which is able to phosphorylate Cav-1 at Tyr14 (15). Src kinase appears to represent a novel therapeutic target for DN (32).

The present study was thus designed to assess the effect of SAL on the development of DN in a type 2 diabetic model and

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to investigate the molecular mechanisms involved in Cav-1-mediated albumin transcytosis across GECs in a newly established in vitro cellular model.

MATERIALS AND METHODS

Animal experiments. C57BLKS/J db/m mice (WT) and C57BLKS/J db/db mice were used in the present study. All the experimental procedures were approved by the Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. Male WT C57BLKS/J db/m mice and C57BLKS/J db/db mice (8 wk old) were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Before the experiment, the mice were kept for 1 wk to acclimate. db/m mice (WT, n = 12) were orally treated with saline, and db/db mice (db/db, n = 15) were orally administered saline, SAL (25, 50, or 100 mg·kg⁻¹·day⁻¹, n = 15), or Met (200 mg·kg⁻¹·day⁻¹, n = 15), and methyl-β-cyclodextrin (MβCD, 100 mg·kg⁻¹·wk⁻¹, n = 15) or N-ethylmaleimide (NEM, 1.7 mg·kg⁻¹·wk⁻¹, n = 15) ip for 11 wk.

Postprandial blood glucose and body weight were measured. Blood samples and 24-h urine samples were collected. After mice were euthanized, kidneys were immediately excised and left kidney weight was measured. Serum and urine creatinine were measured using a Creatinine Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Urinary albumin was determined using an ELISA assay kit from Exocell (Philadelphia, PA). The level of albuminuria was presented as the ratio of urinary albumin (μg) to urinary creatinine (mg). Coded sections were read by two independent observers unaware of the experimental protocol. Thirty glomeruli, cut through the vascular pole, were randomly selected from each animal. The PAS-positive material in the mesangial matrix and glomerular tuft area was identified using the computer image analysis.

To determine the dose response of transcytosis inhibitor on proteinuria, 20-wk-old db/db mice were intraperitoneally administered saline, MβCD (50 or 100 mg/kg, n = 10), or NEM (0.85 or 1.7 mg/kg, n = 10) once. And a group of male db/m mice (WT, n = 10) were intraperitoneally administered saline once. The level of albuminuria was presented as the ratio of urinary albumin to urinary creatinine.

Antibodies and reagents. Rabbit monoclonal antibodies to p-AMPK (Thr172), Cav-1, and Src and rabbit polyclonal antibodies to AMPK, p-Cav-1 (Tyr14, p-CAV-1), and p-Src (Tyr416), were purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin monoclonal antibody or anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were from Abbkine (Redlands, CA). SAL was from the National Institute for Food and Drug Control (purity 98%, Beijing, China). Met and N-acetyl-L-cysteine (NAC) were from Beyotime (Jiangsu, China). Fluorescein isothiocyanate (FITC) was from Biosharp (Seoul, South Korea). AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside)

Fig. 1. Schematic diagram of the model of albumin transcytosis. Albumin were tagged with FITC and purified by dialysis. Glomerular endothelial cells (GECs) were cultivated in a monolayer on a transwell. In the noncompetitive group, medium in the upper chamber contained FITC-albumin; in the competitive group, 20-fold excess of unlabeled albumin was added to the upper chamber. To balance osmotic pressure, unlabeled albumin, whose concentration was the same as that in the upper chamber, was added to the lower chamber in both noncompetitive and competitive inserts. Samples were collected from the lower chamber and further dialysed. FITC fluorescence intensity was measured.
was from Selleckchem (Houston, TX). PP2 [1-tert-butyl-3-(4-chlorophenyl)-1H-pyrazo-[3,4-d]pyrimidin-4-amine] was from Biovision (Mountain View, CA). Bovine serum albumin (endotoxin free), mannitol, MβCD, NEM, polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), compound C, and other analytic reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and treatments. Mouse glomerular endothelial cells (GECs) (38) were purchased from Cell Biologics (Chicago, IL) and cultured following the supplier’s instructions. Before the experiment, the medium was replaced with medium containing 5.5 mmol/l [normal (NG)] or 30 mmol/l [high (HG)] glucose. Then, GECs were treated with vehicle, mannitol (5.5 mmol/l glucose + 24.5 mmol/l mannitol), SAL (1 OR 10 μmol/l), Met (10 μmol/l), MβCD (3 mmol/l), NEM (10 μmol/l), AICAR (1 mmol/l), compound C (10 μmol/l), PP2 (10 μmol/l), NAC (5 mmol/l, pretreated for 3 h), or PEG-SOD (1,000 U/mL) for 3 h.

Albumin labeling and establishment of albumin transcytosis model. As shown in Fig. 1, albumin was labeled with FITC and used to quantify the transcytosis of albumin, as described previously with minor modification (4, 39, 41). FITC and bovine serum albumin in a mole ratio of 3:1, both prepared using phosphate-buffered saline (PBS) at pH 7.4, were mixed and continuously shaken for 4 h. Unbound FITC was removed by dialysis against PBS for 96 h at 4°C. Protein concentration was measured with BCA reagent (Thermo Scientific, Rockford, IL). GECs were seeded on a polyester membrane (Costar transwell, 0.4 μm pore size, Cambridge, MA). The integrity of the cell monolayer was tested by a method described previously, which simply entailed filling the upper chamber to the top and then leaving the cells overnight, and the fall in the fluid level in the top chamber was measured to reflect the leak. Two inserts of cell monolayer with equal integrity were assigned to the same group: the noncompetitive insert and the competitive insert, respectively. The noncompetitive insert was incubated with 50 or 100 μg/ml FITC-albumin for 3 h to determine the total transport of transendothelial albumin; paracellular transport of albumin was determined by incubation with 50 or 100 μg/ml FITC-albumin and 20-fold excess of

Fig. 2. Physical and biochemical characteristics of mice. A: protocol of animal experiment. Blood glucose (B), body weight (C), left kidney weight (D), serum creatinine (E), and albumin-to-creatinine (μg/mg) ratio (F) were determined (n = 9–10). G: 20-wk-old db/db mice were administered saline, methyl-β-cyclodextrin (MβCD; 50 or 100 mg/kg ip), or N-ethylmaleimide (NEM; 0.85 or 1.7 mg/kg ip) once; a group of male db/db mice (WT) were administered ip saline once. Albumin/creatinine ratio was determined (n = 10). Data are means ± SE. **P < 0.01 vs. WT group; #P < 0.05 or ##P < 0.01 vs. db/db group treated with vehicle.
unlabeled albumin in competitive insert. To balance osmotic pressure, unlabeled albumin, the same concentration of which was that in the upper chamber of the transwell, was added to the lower chamber of the transwell in both the noncompetitive and competitive inserts. Samples were then collected from the lower chamber of the inserts and further dialysed against PBS to remove the free FITC. The FITC fluorescence intensity was measured. Meanwhile, background fluorescence determined by measuring the medium was subtracted from each measure. The amount of albumin transcytosis from the monolayer is the difference between the fluorescence intensity of the noncompetitive insert and that of the competitive insert.

In brief, the following equations are expected to describe the principles:

noncompetitive insert = paracellular transport + transcytosis

competitive insert = paracellular transport + competitively inhibited transcytosis

Fig. 3. Changes in glomerular pathomorphism in salidroside (SAL)-treated *db/db* mice. A: representative graph for kidney sections (4 μm) PAS stained. B and C: glomerular surface area and mesangial surface area evaluated by glomerulometry (*n* = 5). Data are means ± SE. **P < 0.01 vs. WT group; #P < 0.05 or ##P < 0.01 vs. *db/db* group treated with vehicle. Scale bar, 50 μm.

Fig. 4. Analysis of albumin transcytosis in an in vitro model. Receptor-mediated albumin transcytosis was calculated by subtracting the FITC intensity obtained in competitive insert (paracellular transport, ○ in A and B) from that obtained in noncompetitive insert (total transport, ● in A and B) and were summarized (C and D; *n* = 5). After incubation with 50 μg/ml (A) or 100 μg/ml (B) FITC-albumin for 1 or 3 h, total and paracellular transports were assessed. *P < 0.05 or **P < 0.01 vs. 1-h group. C: time-dependent transcytosis of 50 and 100 μg/ml FITC-albumin. D: concentration-dependent transcytosis of FITC-albumin at 1 and 3 h. Data are means ± SE. *P < 0.05 or **P < 0.01 vs. 1-h 50 μg/ml group.
noncompetitive incert – competitive incert = transcytosis – competitively inhibited transcytosis = transcytosis

Western blot analysis. Total protein from GECs was prepared as described previously (32). GECs were lysed with RIPA lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor (Roche, Mannheim, Germany). Lysates were centrifuged at 14,000 g for 15 min at 4°C, and insoluble debris was removed. The concentration of protein in supernatants was quantified with BCA reagent (Thermo Scientific, Rockford, IL). Equal amounts of protein were separated by 10% (vol/vol) or 12% (vol/vol) SDS-PAGE and electrotransfected to PVDF membranes (Millipore, Temecula, CA). After blocking with 5% fat-free milk, membranes were exposed to the desired primary antibodies overnight at 4°C and then to anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies. Western blot for the following primary antibodies: anti-AMPKα, p-AMPKα (Thr172), Src, p-Src (Tyr416), Cav-1, and p-Cav (Tyr14) were used at 1:1,000 dilutions. The anti-mouse or anti-rabbit secondary antibodies were used at 1:10,000 dilutions.

Knockdown of AMPK or Cav-1 by small interfering RNA in GECs. GECs were transfected with either scramble small interfering (si)RNA or siRNA for AMPKα1 and AMPKα2 or Cav-1 using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany). siRNAs were synthesized by RIBOBIO (Guangzhou, China) and annealed according to the manufacturer’s instructions. Sequences of AMPK siRNA were AMPKα1: sense 5′-CGGAUCCCUAGCCAUAGCCTT-3′, antisense 5′-UAGGUUGUAGGAUCCGAT-3′; AMPKα2: sense 5′-GGAUUGCCCCAGCUACCUTTTT-3′, antisense 5′-UAGGUAGCCGGGAAAUCCTTG-3′. Sequences of Cav-1 siRNA were sense 5′-AAGAUGUGAUUGCAGAACCA-3′, antisense 5′-UGGUCUGCAUAUCACU-3′.

Mitochondrial superoxide anion production. Mitochondrial superoxide anion was detected using MitoSOX Red (Invitrogen, Carlsbad, CA) (24). MitoSOX Red is live cell permeant and is rapidly and selectively targeted to the mitochondria. MitoSOX Red can be oxidized by superoxide but not by other ROS or reactive nitrogen species and exhibits red fluorescence in the mitochondria. GECs were incubated with MitoSOX Red (5 μmol/l) for 30 min at 37°C. Subsequently, cells were washed gently with warm PBS. The fluorescence intensity of MitoSOX Red was read at 535/610 nm (ex/em).

Measurement of mitochondrial membrane potential. JC-1 dye was used to measure mitochondrial membrane potential (ΔΨm) by using a method described previously (36). JC-1 (Beyotime, Jiangsu, China) is a dye with fluorescent emission that shifts from red to green with decreasing membrane potentials. The ratio of red (J-aggregate) to green (monomeric) emission is in proportion to the absolute value of ΔΨm. GECs were incubated in JC-1 staining solution at 37°C for 20 min. Then, cells were rinsed twice with JC-1 washing solution before fluorescence reading. J-aggregates and monomeric JC-1 were read at 535/610 nm (ex/em) and 485/535 nm (ex/em), respectively.

Imaging and analysis of albumin uptake. The analysis of FITC-labeled albumin uptake was carried out by a method described previously (3, 18). After treatment with different agents in normoglycemic or hyperglycemic conditions, GECs were incubated with FITC-albumin for 3 h. Images were obtained with a fluorescence microscope (Olympus). Using the Image-Pro Plus software, the integrated fluorescence intensities were analyzed and normalized to the number of cells.

Statistical analysis. The data are presented as means ± SE. One-way ANOVA with post hoc ranking was used for the multiple group statistical analysis. Comparisons between two groups were analyzed by Student’s t-test (two-tailed). Statistical significance was accepted at values of P < 0.05.

RESULTS

SAL ameliorates diabetic nephropathy in db/db mice. Figure 2A showed the protocol of the long-term animal experiment. Compared with db/m mice (WT), the levels of postprandial blood glucose, body weight, and left kidney weight were obviously higher in db/db mice (Fig. 2, B–D). SAL administration improved blood glucose (Fig. 2B), and the maximum effect of 100 mg/kg SAL was similar to that of 200 mg/kg Met. Both transcytosis inhibitors, MβCD and NEM, had no obvious effects on blood glucose. There were no detectable changes in the body weight of db/db mice treated with SAL, Met, MβCD, or NEM compared with their vehicle control counterparts (Fig. 2C). However, compared with db/db mice, 100 mg/kg SAL reduced the left kidney weights. The level of serum creatinine

Fig. 5. Effect of SAL on albumin transcytosis in GECs. A and B: GECs were incubated with vehicle, SAL, metformin (Met), MβCD, NEM, or mannitol. C: GECs were incubated with vehicle, AICAR, SAL, and compound C (Comp.C), or PP2 [1-tert-butyl-3-(4-chlorophenyl)-1H-pyrazo-[3,4-d]pyrimidine-4-amine]. Amounts of albumin transcytosis were determined (n = 4). Data are means ± SE. **P < 0.01 vs. vehicle in normoglycemic (NG) group; #P < 0.05 or ##P < 0.01 vs. vehicle in hyperglycemic (HG) group; &P < 0.05 vs. SAL in HG group.

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and the ratio urinary albumin/creatinine were markedly enhanced in db/db mice, which were reduced by SAL treatment in a dose-dependent manner (Fig. 2, E and F). However, Met had no effects on the level of serum creatinine and the urinary albumin/creatinine ratio. MβCD and NEM had no effects on serum creatinine but showed a reduced ratio of urinary albumin/creatinine (Fig. 2, E and F).

We also tested the dose response of MβCD and NEM on the urinary albumin/creatinine ratio in 20-wk-old db/db mice after a different dose of MβCD or NEM was intraperitoneally administrated once. As shown in Fig. 2G, only the higher dose of MβCD (100 mg/kg) or NEM (1.7 mg/kg) could alleviate the level of albuminuria.

As shown in Fig. 3, the mesangial matrix was more extensive in db/db mice. Glomerulometric determinations further showed reduced glomerular surface area in SAL-treated db/db mice as well as a decreased mesangial surface area. However, Met, MβCD, and NEM showed no significant effects on these parameters in db/db mice.

Establishment of the albumin transcytosis model and determination of albumin transcytosis across GECs. To quantify the albumin transcytosis, we developed a new model of albumin transcytosis (Fig. 1) to reveal the mechanism of SAL on alleviating albuminuria in DN mice.

The total transport and paracellular transport of 50 μg/ml (Fig. 4A) and 100 μg/ml (Fig. 4B) FITC-albumin across the monolayer of GECs were measured, respectively. The amount of albumin transcytosis was calculated by subtracting the paracellular transport from the total transport. The amounts of albumin transcytosis were displayed against time (Fig. 4C) and against the concentration of albumin (Fig. 4D). Albumin transcytosis was found increased with incubation time (3 h vs. 1 h). Furthermore, the amount of albumin transcytosis was apparently higher in 100 μg/ml albumin group than in the 50 μg/ml group. Thus, albumin transcytosis was higher in both time- and concentration-dependent manners.

SAL decreases albumin transcytosis in normoglycemic or hyperglycemic conditions. Using the established model, we tested whether SAL could decrease albumin transcytosis. SAL reduced the transcytosis of albumin across the GEC monolayer in a concentration-dependent manner in normoglycemia (Fig. 5A). HG increased albumin transcytosis, which was also inhibited by SAL, whereas mannitol showed no effect, excluding the potential effect of osmotic pressure (Fig. 5B). MβCD and NEM decreased albumin transcytosis in both NG and HG conditions (Fig. 5, A and B). Met slightly increased albumin transcytosis in the normoglycemic condition but had no sig-
significant effect on albumin transcytosis in the hyperglycemic condition (Fig. 5, A and B).

We tested whether AMPK or Src kinase signaling was involved in albumin transcytosis. Incubation with AICAR or PP2 inhibited the albumin transcytosis upregulated by HG but had no significant effect in the NG condition (Fig. 5C). Also, the inhibition of SAL on albumin transcytosis stimulated by hyperglycemia was partly blocked by compound C.

SAL activates AMPK but inhibits Src kinase or Cav-1 in normoglycemic condition. In the normoglycemic condition, SAL induced AMPK phosphorylation but inhibited the phosphorylation of Src and Cav-1 in a concentration-dependent manner (Fig. 6, A–F). However, mannitol had no effect on these proteins’ phosphorylation (Fig. 7).

Meanwhile, knockdown of Cav-1 by specific siRNA resulted in significant reduction of albumin transcytosis (Fig. 6, G–I).

SAL recovers inhibition of AMPK and prevents upregulation of Src kinase and Cav-1 in hyperglycemic condition. HG reduced the phosphorylation of AMPK and increased the phosphorylation of Src and Cav-1 (Fig. 8, A–D). SAL treatment dose-dependently reversed the alterations of AMPK, Src, and Cav-1 caused by HG. Met upregulated the phosphorylation of AMPK as well as the phosphorylation of Src and Cav-1 in normoglycemic condition, the latter of which was not similar to SAL. In HG-treated GECs, Met reversed the decreased phosphorylation only of AMPK, having no effects on HG-stimulated phosphorylation of Src and Cav-1 (Fig. 8, E–H).

AMPK activation and Src kinase inhibition are responsible for the effect of SAL on HG-upregulated Cav-1 phosphorylation. AICAR and PP2 significantly reversed the AMPK phosphorylation in HG-treated GECs, and blocked the HG-activated Cav-1 (Fig. 9, A, B, and D). Both SAL and PP2 prevented the HG-induced phosphorylation of Src. However, AICAR had no effect on phosphorylation of Src (Fig. 9, A and C). Meanwhile, compound C partly blocked the effect of SAL on phosphorylation of Cav-1 in hyperglycemic condition (Fig. 9, A and D).

SAL inhibits albumin transcytosis through AMPK/Cav-1 pathway. To further identify the role of AMPK in caveolae-mediated albumin transcytosis, GECs were transfected with AMPKα siRNA to specifically knock down AMPKα expression (Fig. 10, A and B), which caused an increase in both the phosphorylation of Cav-1 (Fig. 10, A and C) and albumin transcytosis (Fig. 10D). Concomitant treatment with AMPKα siRNA suppressed the inhibitory effects of SAL on phosphorylation of Cav-1 (Fig. 10, E and G). Figure 10, E–G, further shows that AMPKα siRNA partly inhibited SAL’s blocking effects on HG-upregulated Cav-1 phosphorylation but did not affect SAL’s blocking effects on Src phosphorylation.

SAL reduces the level of mitochondrial ROS. We also found that NAC, the ROS scavenger, abolished the HG-induced Src kinase activation, as well as blocking the Cav-1 phosphorylation (Fig. 11, A–C). As shown in Fig. 11D, SAL reduced mitochondrial ROS (mtROS) in NG and reversed the rise of mtROS production stimulated by HG. In contrast to SAL, Met slightly increased the level of mtROS in NG but had no effect on mtROS stimulated by HG. However, NAC decreased the level of mtROS stimulated by HG (Fig. 11D). ROS scavenger PEG-SOD and NAC decreased albumin transcytosis in GECs (Fig. 11E).

DISCUSSION

In db/db mice, we found that SAL reduced blood glucose and significantly retarded the development of DN. Specifically, the albuminuria was obviously reduced, which was not observed in Met treatment. The albuminuria in db/db mice was also reduced by MβCD or NEM, two frequently used, structurally different tool drugs for inhibiting transcytosis (3), confirming the role of transcytosis in the development of albuminuria. These interesting observations prompted us to...
further explore the mechanism of SAL to alleviate the proteinuria. We established an in vitro model of albumin transcytosis in GECs and quantified the albumin transcytosis in both normoglycemic and hyperglycemic conditions. We found that SAL reduced albumin transcytosis through activation of AMPK and inhibition of the Src kinase pathway, which both lead to downregulation of phosphorylated Cav-1, ultimately reducing the albumin transcytosis across GECs and alleviating proteinuria.

GECs are the first layers of GFB and the key regulators of glomerular microvascular permeability even though podocytes are absent (28). Since the intercellular space is smaller than the diameter of an albumin molecule, the albumin molecules actually traffic across the endothelial barrier mainly through the caveolae-mediated transcytosis pathway.

Transcytosis is a highly regulated, receptor-mediated, selective process. To quantitatively measure the transcytosis of albumin, we first established an in vitro model in GECs. In this model, we set a pair of transwell inserts for each group of treatments. One insert of fluorescence-labeled albumin was measured to reflect the total transport of albumin, which includes both intracellular transcytosis and intercellular leakage. The other insert was competitively saturated with 20-fold unlabeled albumin, which was the paracellular transport of albumin, since the non-receptor-mediated paracellular transport could not be competitively inhibited by excessive unlabeled albumin, whereas receptor-mediated selective transcytosis could. The difference between the total and paracellular measures was calculated as the transcytosis of albumin.

Using this model, we successfully identified that the transcytosis of albumin across GECs was a concentration-dependent and time-dependent process. We also found that HG significantly upregulated albumin transcytosis, whereas the same osmotic of mannitol did not affect this process. This maybe partly explains why diabetic patients with hyperglycemia develop significant proteinuria. SAL treatment not only decreased the basal transcytosis of albumin but also downregulated the elevated transcytosis of albumin stimulated by HG. However, Met failed to inhibit the elevated albumin transcytosis as SAL did. These results are in accord with the observations in db/db mice that SAL alleviates the proteinuria while Met could not, although both SAL and Met lower the blood glucose.

In hyperglycemia, AMPK activity is downregulated and Src kinase is activated (7, 32). Hence, we sought to elucidate whether these two molecules also affect albumin transcytosis. We found that both activation of AMPK by AICAR and inhibition of Src kinase by PP2 substantially reduced the enhanced albumin transcytosis brought by HG, which confirmed that Src kinase and AMPK were involved in HG-stimulated albumin transcytosis.

Fig. 8. Effects of SAL or Met on phosphorylation of AMPK, Src, and Cav-1 in HG. A–D: GECs were incubated with vehicle or SAL. E–H: GECs were incubated with vehicle or Met. Phosphorylation of AMPK, Src, or Cav-1 was assessed (n = 4). Data are means ± SE. *P < 0.05 or **P < 0.01 vs. vehicle in NG group; ##P < 0.01 vs. vehicle in HG group.
Both Src kinase activation and AMPK inhibition have been reported to activate Cav-1, which plays a central role in mediating albumin transcytosis. (15, 31) We also found that HG activated both Src kinase and Cav-1, whereas it inhibited AMPK in GECs, which could be largely reversed by SAL treatment. SAL reversed the phosphorylation of Cav-1 in HG; however, this effect was partly blocked by compound C. These data imply that AMPK activation is partly responsible for the effect of SAL on Cav-1 activation. These results are consistent with previous findings that indicate AMPK is a negative regulator of Cav-1 (31, 40).

To further confirm the role of AMPK in SAL’s inhibitory action on Cav-1 phosphorylation elevated by HG, we used siRNA to specifically knock down AMPK expression. Similarly, AMPK siRNA also partly blocked the reversing effect of SAL on elevated Cav-1 stimulated by HG; however, this effect was partly blocked by compound C. These data imply that AMPK activation is partly responsible for the effect of SAL on Cav-1 activation. These results are consistent with previous findings that indicate AMPK is a negative regulator of Cav-1 (31, 40).

To further confirm the role of AMPK in SAL’s inhibitory action on Cav-1 phosphorylation elevated by HG, we used siRNA to specifically knock down AMPK expression. Similarly, AMPK siRNA also partly blocked the reversing effect of SAL on elevated Cav-1 stimulated by HG. These data imply that SAL is able to downregulate the enhanced activation of Cav-1 through both AMPK-dependent and -independent pathways. Although AMPK activation indeed inhibits Cav-1, its absence is not sufficient to completely prevent the ameliorating effects of SAL on Cav-1 activation. The inhibition of Src kinase by SAL appears to play a more important role in reversing the HG-activated Cav-1, since PP2 almost completely prevented the phosphorylation of Cav-1 stimulated by HG. This may be due to the fact that Src kinase not only phosphorylates Cav-1 directly but also indirectly induces Cav-1 phosphorylation through an inhibitory action on AMPK, which was supported by the observation that PP2 increased AMPK phosphorylation. These data are also in accord with previous reports in other cell types (1), which also demonstrate that the reduction of Src kinase activity is able to enhance AMPK activity (2).

As a known activator of AMPK, Met failed to inhibit albumin transcytosis. This may be explained by the fact that plasma concentrations of Met (0 – 0.1 mmol/l) could also activate Src kinase (17, 43), which may overcome the inhibitory action on Cav-1 by AMPK. However, Met (30 mmol/l) inhibits phosphorylation of Src (20), but this concentration of Met is not clinically relevant (21).

Using NAC, the potent ROS scavenger, we found that the activation of Src kinase or Cav-1 by HG was dependent on ROS signaling. mtROS promotes DN (5, 10), and reduction of mtROS could protect mice from DN (29). Since mitochondria are the major source of intracellular ROS, Src kinase has been reported to reside in mitochondria (27) and Src kinase could be activated by mtROS (14, 35), we mainly focused on mtROS signaling. SAL blocked the overproduction of mtROS by HG. By contrast, Met further increased the mtROS in NG and failed to reverse HG-stimulated mtROS production. Previous reports also showed that Met increased mtROS in cancer cells (6, 8). The distinct effects of SAL and Met on mtROS production may partly explain the opposite effects of SAL and Met on Src and Cav-1 phosphorylation and ultimately lead to distinct effects on albumin transcytosis as well as on albuminuria in db/db mice.
Since the H9004/H9274 m has a critical role in mitochondrial superoxide production, we further studied SAL’s effects on the H9004/H9274 m. HG treatment for 3 h hyperpolarized the H9004/H9274 m. It is in accord with previous findings that HG induced initial hyperpolarization, followed by depolarization of the H9004/H9274 m in neurons (26). SAL reduced H9004/H9274 m in both normoglycemic and hyperglycemic conditions. Met per se depolarized the H9004/H9274 m and depolarized the elevated H9004/H9274 m by HG. Therefore, in the matter of H9004/H9274 m, SAL and Met appear to have similar effects. A recent report demonstrated that Met could target mitochondrial glycerol phosphate dehydrogenase and prevent the shuttle of cytosolic nicotinamide adenine dinucleotide (NADH) into mitochondria and result in the accumulation of NADH in the cytosol, which would reduce the cytosol concentration of H2O2 (21). This maybe contributes to the depolarization of Δψm by Met, since Δψm partly depends on the H2O2 gradient across the mitochondrial inner membrane. Hyperglycemia-induced ROS is produced from the mitochondrial electron transport chain. Since the ratio of electrons to oxygen is reduced, increased production of superoxide in mitochondria could be expected. As for the issue of SAL, its antioxidant effect may be due to its mild depolarizing effect on mitochondria. However, unlike Met, SAL does not alter the electron-to-oxygen ratio, whereby it maybe only affects the rate of oxidation phosphorylation in mitochondria, therefore slows down the rate of superoxide production.

The intermediate state of albumin transcytosis is that the albumin particles have already been taken up by the GECs at the luminal side but have not yet been exocytosed at the basolateral side. This intermediate state could also reflect the degree of active transcytosis. Consistent with the aforementioned quantification of transcytosis in transwell studies, our morphological observations further confirmed the effects of SAL or Met on the transport of albumin, thus providing further insights into the pathophysiology of proteinuria and pharmacology of relevant drugs.

In conclusion, hyperglycemia exacerbated albumin transcytosis by upregulation of the ROS/Src/Cav-1 pathway and downregulation of the AMPK/Cav-1 pathway. SAL prevents hyperglycemia-induced albumin transcytosis by antioxidant activity and activation of AMPK, whereas Met fails to inhibit Src kinase, although it activates AMPK as well. Our findings suggest that inhibiting albumin transcytosis across GECs may be a novel therapeutic target for diabetic albuminuria.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
Fig. 11. Effects of SAL on mitochondrial reactive oxygen species (mtROS) and membrane potential ($\Delta\psi_m$) in GECs. A–C: GECs were incubated with N-acetyl-L-cysteine (NAC). Phosphorylation of Src or Cav-1 was detected ($^{\ast}$P < 0.05 or $^{**}$P < 0.01 vs. vehicle in NG group; ##P < 0.01 vs. vehicle in HG group). Data are means ± SE. 

**AUTHOR CONTRIBUTIONS**


**REFERENCES**

Fig. 12. Analysis of FITC-albumin uptake in GECs. GECs were incubated with albumin, FITC-albumin, mannitol, SAL, Met, AICAR, SAL + Comp.C, NAC, or PP2 (n = 3). Fluorescence microscope images (A) and quantitative analysis (B) of FITC-albumin uptake. Data are means ± SE. *P < 0.05 or **P < 0.01 vs. vehicle group in NG group; ##P < 0.01 vs. vehicle in HG group; &P < 0.05 vs. SAL in HG group. Scale bar, 50 μm.


