A Novel Mechanism of Action for Salidroside to Alleviate Diabetic Albuminuria: Effects on Albumin Transcytosis across Glomerular Endothelial Cells

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Abbreviated Title: Salidroside alleviates albuminuria

Key terms: albumin; transcytosis; salidroside; glomerular endothelial cell; diabetic nephropathy

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Disclosure Statement: The authors have nothing to disclose.
Salidroside (SAL) is a phenylethanoid glycoside isolated from the medicinal plant Rhodiola rosea. Rhodiola 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 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/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 (AMPK) and upregulation of Src kinase, as well as blocked the upregulation Cav-1 phosphorylation. Meanwhile, SAL decreased the 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 for the interference with albumin transcytosis across GECs as a novel approach to the treatment of diabetic albuminuria.

KEYWORDS: transcytosis, salidroside, glomerular endothelial cell, diabetic albuminuria
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 (1). Thus, searching for novel approaches or drugs still appears especially crucial to improve the prognosis of DN.

Salidroside (SAL) [2-(4-hydroxyphenyl) ethyl beta-D-glucopyranoside], is the major ingredient in Rhodiola rosea, which grows at high altitude zone 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 Rhodiola 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 to Met, SAL not only has hypoglycemic effects on db/db mice (42), but also significantly alleviated the proteinuria. Based on this clue, we seek to further elucidate the molecular mechanism underlying its proteinuria alleviating action.

As the first line of glomerular filtration barrier (GFB), glomerular endothelial cells (GECs) are very essential for albumin filtration (11, 28). As a special phenotype of capillary endothelial cells, GECs are highly fenestrated, which are 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 human, and albuminuria was not observed in the Cav-1 knockout 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 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 Ethical Committee of
Tongji Medical College, Huazhong University of Science and Technology. Male wide-type
C57BLKS/J db/m mice (WT) and C57BLKS/J db/db mice (8-week-old) were purchased from
Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Before the experiment,
the mice were kept for 1 week to acclimate. db/m mice (WT, n=12) were orally treated with saline,
and db/db mice (db/db, n=15) were orally administered with saline, SAL (25, 50 or 100 mg/kg/day,
n=15) or Met (200 mg/kg/day, n=15), and Methyl-β-cyclodextrin (MβCD, 100 mg/kg/week, n=15) or
N-ethylmaleimide (NEM, 1.7 mg/kg/week, n=15) ip for 11 weeks. Postprandial blood glucose and
body weight were measured. Blood samples and 24 h-urine samples were collected. After mice were
sacrificed, kidneys were immediately excised and left kidney weight was measured. Serum and urine
creatinine was measured using Creatinine Assay Kit (Nanjing Jiancheng Bioengineering Institute,
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-week-old db/db mice were intraperitoneally administrated with 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 administrated with saline once. The level of albuminuria was presented as the ratio of urinary albumin (µg) to urinary creatinine (mg).

**Antibodies and reagents.** Rabbit monoclonal antibodies to p-AMPKα (Thr 172), CAV-1, Src and rabbit polyclonal antibodies to AMPKα, p-caveolin-1 (Tyr 14, p-CAV-1), p-Src (Tyr 416) were purchased from Cell Signaling Technology (Danvers, MA). Anti-β-actin monoclonal antibody, anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were from Abbkine (Redlands, CA). SAL was from 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) were from Biosharp (Seoul, South Korea). AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) 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 supplier’s instructions. Before experiment, the medium were replaced with the medium containing 5.5 (NG) or 30 (HG) mmol/L glucose. Then GECs were treated with vehicle, mannitol (5.5 mmol/L glucose + 24.5 mmol/L mannitol), SAL (1, 10 μmol/L), Met (10 μmol/L), MβCD (3 mmol/L), NEM (10 μmol/L), AICAR (1 mmol/L), compound C (10 μmol/L) or PP2 (10 μmol/L), NAC(5 mmol/L, pretreated for 3 h) or PEG-SOD (1000 U/mL) for 3 h.
Albumin labeling and establishment of albumin transcytosis model. As shown in Fig. 31, albumin was labeled with FITC 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 cell monolayer was tested by a method described previously, which simply referred to fill the upper chamber to the top and then leave 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 into 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 unlabeled albumin in competitive insert. To balance osmotic pressure, unlabeled albumin, the same concentration of which as that in upper chamber of the transwell, was added into 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 dialyzed against PBS to remove the free FITC. The FITC fluorescent 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 fluorescent 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

Noncompetitive insert - Competitive insert = 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 in 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 electro-transfected 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 (Thr 172), Src, p-Src (Tyr 416), CAV-1 and p-Cav (Tyr 14) were used at 1:1000 dilutions. The anti-mouse or anti-rabbit secondary antibodies were used at 1:10000 dilutions.

Knockdown of AMPK or Cav-1 by small interfering RNA (siRNA) in GECs. GECs were transfected with either scramble siRNA or siRNA for AMPKα1 and AMPKα2 or Cav-1 using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany). Small interfering RNAs (siRNAs) were synthesized by RIBOBIO (Guangzhou, China) and annealed according to the manufacturer's instructions. Sequences of AMPK siRNA are as follows: AMPKα1, sense 5’ CGGGAUCCAUCAGCAACUATT 3’, antisense 5’ UAGUUGCUGAUGGAUCCGAT 3’; AMPKα2, sense 5’ GGAUUUGCCCAGCUACCUATT 3’, antisense 5’ UAGGUAGCUGGCAAUCCTG 3’. Sequences of CAV-1 siRNA are as follows: sense 5’ AGAUGUGAUUGCAGAACCA 3’, antisense 5’ UGGUUCUGCAUCACAUUCU 3’.

Mitochondrial superoxide anion production. Mitochondrial superoxide anion was detected using the MitoSOX Red (Invitrogen, Carlsbad, CA) (24). MitoSOX Red is live-cell permeant and is rapidly and selectively targeted to the mitochondrial. 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 ($\Delta \psi_m$). JC-1 dye was used to measure mitochondrial membrane potential ($\Delta \psi_m$) by using a method described previously (36). JC-1 (Beyotime, Jiangsu, China) is a dye with fluorescent emission which shifts from red to green with decreasing membrane potentials. The ratio of red (J-aggregate)/green (monomeric) emission is in proportion to the absolute value of $\Delta \psi_m$. 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 the albumin uptake. The analysis of FITC-labeled albumin uptake was carried out by a method described previously (4, 18). After treated with different agents in normoglycemic or hyperglycemic, 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 the mean ± SE. One way ANOVA with post-hoc testing 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 to 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 to their vehicle control counterparts (Fig. 2C). However, compared to db/db mice, 100 mg/kg SAL reduced the left kidney weights. The level of serum creatinine and the ratio of 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 ratio of urinary albumin/creatinine. MβCD and NEM had no effects on serum creatinine but showed a reduced ratio of urinary albumin/creatinine (Fig. 2E and F).

We also tested the dose-response of MβCD and NEM on the ratio of urinary albumin/creatinine in 20-week-old db/db mice after 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. Glomerulo-metric 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 (Fig. 4A) and 100 (Fig. 4B) µg/mL FITC-albumin across the monolayer of GECs was 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 compared to that in 50 µg/mL group. Thus, albumin transcytosis was 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 GECs monolayer in a concentration-dependent manner in normoglycemia (Fig. 5A). HG increased albumin transcytosis, which was also inhibited by SAL,
whilst mannitol showed no effect, excluding the potential effect of osmotic pressure (Fig. 5B). MβCD and NEM decreased albumin transcytosis in both normoglycemic and hyperglycemic conditions (Fig. 5, A and B). Met slightly increased albumin transcytosis in normoglycemic condition, but had no significant effect on albumin transcytosis in 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 normoglycemic 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 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 has 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 the upregulation of Src kinase and Cav-1 in hyperglycemic condition.** HG reduced the phosphorylation of AMPK and also, 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 causes by HG. Met up-regulated the phosphorylation of AMPK as well as the phosphorylation of Src and Cav-1 in normoglycemic condition, the latter of which was not similar as SAL. In HG-treated GECs, Met only reversed the decreased phosphorylation of AMPK, but had 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 knockdown AMPKα expression (Fig. 10, A and B), which caused an increase in both the phosphorylation of Cav-1 (Fig. 10, A and C) and in 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). Fig. 10, E-G further showed 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 blocked the Cav-1 phosphorylation (Fig. 11, A-C). As shown in Fig. 11D, SAL reduced mitochondrial ROS (mtROS) in normoglycemic condition, and reversed the rise of mtROS production stimulated by HG. In contrast to SAL, Met slightly increased the level of mtROS in normoglycemic condition, 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).

SAL moderately depolarizes the $\Delta \Psi_m$ and prevents HG-induced hyperpolarization in $\Delta \Psi_m$. Fig. 11F showed SAL and CCCP decreased the $\Delta \Psi_m$ in normoglycemic condition. HG which was slightly hyperpolarized $\Delta \Psi_m$, was prevented by SAL, NAC, or CCCP. Met also induced a decline in the $\Delta \Psi_m$, as well as prevented HG-stimulated increase in $\Delta \Psi_m$.

Albumin uptake in the cultured GECs. After incubation with FITC-albumin for 3 h, some small, individual, discrete vesicles were found in GECs. The fluorescent intensity in each individual cell reflected the extent of albumin uptake. Fig. 12 showed that HG significantly increased the intracellular albumin particles, which were prevented by AICAR, NAC or PP2. Similarly, SAL also inhibited elevated albumin uptake induced by HG, which was partly blocked by compound C. Met increased the albumin uptake in normoglycemic condition, but had no effect in hyperglycemic condition. Mannitol also had no effect on the albumin uptake.
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 (4), confirmed the role of transcytosis in the development of albuminuria. These interesting observations prompt 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 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 the glomerular microvascular permeability even though podocytes are absent (28). Since the intercellular space is smaller than the diameter of albumin molecule, the albumin molecules actually traffic across the endothelial barrier mainly through caveolae-mediated transcytosis pathway.

Transcytosis is a highly regulated, receptor-mediated selective process. To quantitatively measure the transcytosis of albumin, we firstly 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 by 20-folds of 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, while 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 a time-dependent process. We also found that HG significantly upregulated albumin transcytosis, while 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 accordance 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.

Both Src kinase activation and AMPK inhibition have been reported to activate Cav-1, which plays a central role in mediating albumin transcytosis. We also found HG activated both Src kinase and Cav-1, whilst inhibited AMPK in GECs, which could be largely reversed by SAL treatment. SAL reversed the phosphorylation of Cav-1 in hyperglycemic condition. However, this effect was partly blocked by compound C. These data imply AMPK activation is partly responsible for the effect of SAL on Cav-1 activation. These results are consistent with previous findings which 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 specific knockdown AMPK expression. Similarly, AMPK siRNA also partly blocked the reversing effect of SAL on elevated Cav-1 stimulated by HG. These data implicate 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 accordance with previous reports in other cell types (2), which also demonstrate that the reduction of Src kinase activity is able to enhance AMPK activity (3).

As a known activator of AMPK, Met failed to inhibit albumin transcytosis. This may be explained by the fact that plasma concentrations of Met (0-0.1mmol/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 promote 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 normoglycemic condition, 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 maybe partly explain the opposite effects of SAL and Met on Src and Cav-1 phosphorylation, ultimately led to distinct effects on albumin transcytosis, as well as on albuminuria in db/db mice.

Since the $\Delta\psi_m$ has critical role in the mitochondrial superoxide production, we further studied SAL’s effects on the $\Delta\psi_m$. HG treatment for 3 h hyperpolarized the $\Delta\psi_m$. This is in accordance with previous findings that HG induced initial hyperpolarization, followed by depolarization of the $\Delta\psi_m$ in neurons (26). SAL reduced $\Delta\psi_m$ in both normoglycemic and hyperglycemic conditions. Met per se depolarized the $\Delta\psi_m$, as well as depolarized the elevated $\Delta\psi_m$ by HG. Therefore, in the point of $\Delta\psi_m$, 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 H$^+$ (21). This maybe contributes to the
depolarization of $\Delta \psi_m$ by Met since $\Delta \psi_m$ partly depends on the $H^+$ gradient across mitochondrial inner membrane. Hyperglycemia-induced ROS is produced from mitochondrial electron transport chain.

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

**GRANTS**

This study was funded by the National Natural Science Foundation of China (81373413, 81072634, 81573432, 81470458 and 81070190) and grants from the Ministry of Education of China (NCET-10-0409, 2015ZHYX006).

**DISCLOSURES**

The authors have no conflicting financial interests.
AUTHOR CONTRIBUTIONS


W.D., Y.X.Y. and J.S. wrote the manuscript.

REFERENCES


Fig. 1. Schematic diagram of the model of albumin transcytosis. Albumin were tagged with FITC and purified by dialysis. GECs were cultivated in a monolayer on a transwell. In noncompetitive group, the medium in the upper chamber contained FITC-albumin. In competitive group, 20-fold excess of unlabeled albumin were added into the upper chamber. To balance osmotic pressure, unlabeled albumin, which concentration was as same as that in upper chamber, were added into the lower chamber in both the noncompetitive and competitive inserts. Samples were collected from the lower chamber and further dialyzed. The FITC fluorescent intensity was measured.

Fig. 2. Physical and biochemical characteristics of mice. (A) The protocol of animal experiment. Blood glucose (B), body weight (C), left kidney weight (D), serum creatinine (E) and albumin (µg)-to-creatinine (mg) ratio (F) were determined (n = 9-10). (G) 20-week-old db/db mice were intraperitoneally administrated with saline, MβCD (50 or 100 mg/kg) or NEM (0.85 or 1.7 mg/kg) once. And a group of male db/m mice (WT) were intraperitoneally administrated with saline once. Albumin (µg)-to-creatinine (mg) 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;

Fig. 3. Changes in glomerular pathomorphism in SAL-treated db/db mice. (A) Representative graph for kidney sections (4µm) in PAS-stained. (B-C) Glomerular surface area and mesangial surface area evaluated by glomerulo-metry (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-B) from that obtained in noncompetitive insert (total transport, ● in A-B) and were summarized (C-D) (n=5). After incubation with 50 (A) or 100 (B) µg/mL FITC-albumin for 1 or 3 h, the total transport and paracellular transport 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 h and 3 h. Data are means ± SE. *P < 0.05 or **P < 0.01 vs. 1 h 50 µg/mL group.

Fig. 5. Effect of SAL on albumin transcytosis in GECs. (A-B) GECs were incubated with vehicle, SAL, Met, MβCD, NEM or mannitol. (C) GECs were incubated with vehicle, AICAR, SAL, SAL + compound C (Comp.C) or PP2. The amount of albumin transcytosis were determined (n=4). Data are means ± SE. **P < 0.01 vs. vehicle in NG group; #P < 0.05 or ##P < 0.01 vs. vehicle in HG group; &P < 0.05 vs. SAL in HG group.

Fig. 6. Effects of SAL on phosphorylation of AMPK, Src, Cav-1 and effect of Cav-1 siRNA on albumin transcytosis in NG group. (A-F) GECs were incubated with SAL in NG group. The phosphorylation of AMPK, Src or Cav-1 was determined (n=4). Data are means ± SE. *P < 0.05 or **P < 0.01 vs. vehicle in NG group. (G-H) GECs were transfected with Cav-1 siRNA (20 nmol/L) for 48 h in NG. The expression of Cav-1 was assessed (n=4). **P < 0.01 vs. Scramble. (I) 3 h of albumin transcytosis were evaluated in transfected GECs (n=4). *P < 0.05 vs. Scramble.

Fig. 7. Effects of mannitol on phosphorylation of AMPK, Src or CAV-1 in GECs (n=3).

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 by vehicle or Met. The phosphorylation of AMPK, Src or Cav-1 were 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.
Fig. 9. Roles of AMPK and Src pathway in inhibitory effect of SAL on the phosphorylation of Cav-1 in hyperglycemia. GECs were incubated with vehicle, AICAR, SAL, SAL + compound C (Comp.C) or PP2. The phosphorylation of AMPK, Src or Cav-1 were assessed \((n = 4)\). Data are means ± SE. *\(P < 0.05\) or **\(P < 0.01\) vs. vehicle in NG group; #\(P < 0.05\) or ##\(P < 0.01\) vs. vehicle in HG group; &\(P < 0.05\) vs. 10 µM SAL in HG group.

Fig. 10. AMPK activation is partly responsible for the inhibitory effect of SAL on Cav-1 activation. GECs were transfected with AMPKα1 siRNA (50 nmol/L) and AMPKα2 siRNA (50 nmol/L) for 72 h in NG. (A-C) The protein expression of AMPK and phosphorylation of Cav-1 were determined \((n = 4)\). (D) 3 h of albumin transcytosis were evaluated \((n = 3)\). (E-G) GECs were transfected and then incubated with vehicle or SAL. The phosphorylation of Src or Cav-1 were assessed \((n = 4)\). Data are means ± SE. *\(P < 0.05\) or **\(P < 0.01\) vs. Scramble in NG group; #\(P < 0.05\) vs. Scramble in HG group; &\(P < 0.05\) vs. SAL + Scramble in HG group.

Fig. 11. Effects of SAL on mtROS and \(\Delta \psi_m\) in GECs. (A-C) GECs were incubated with NAC. The phosphorylation of Src or Cav-1 were detected \((n = 4)\). (D) GECs were incubated with SAL, Met or NAC; The level of mtROS was indicated by MitoSOX Red \((n = 4)\). (E) GECs were incubated with NAC or PEG-SOD for 3 h. The albumin transcytosis cross GECs was determined \((n = 4)\). (F) GECs were incubated with SAL, Met or NAC for 3 h, or incubated with 10 µmol/L carbonyl cyanide m-chlorophenylhydrazone (CCCP) for 20 min, \(\Delta \psi_m\) were indicated by JC-1 \((n = 4)\). Data are means ± SE. *\(P < 0.05\) or **\(P < 0.01\) vs. vehicle in NG group; ##\(P < 0.01\) vs. vehicle in HG group.

Fig. 12. Analysis of FITC-albumin uptake in GECs. GECs were incubated with albumin, FITC-albumin, mannitol, SAL, Met, AICAR, SAL + compound C (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.
(1) Albumin labeling
PBS
25°C, in the dark,
Shaking, 4h

(2) Purification
PBS
Dialysis bag
FITC, FITC-albumin

(3) Transcytosis in transwell
Noncompetitive group
Competitive group

(4) Purification
PBS
Dialysis bag
FITC-albumin, FITC

(5) Fluorescence measurement at Ex 490 nm, Em 520 nm
A C57BLKS/J db/m mice vehicle
Vehicle
Met 200 mg/kg/day
SAL 25 mg/kg/day
SAL 50 mg/kg/day
SAL 100 mg/kg/day
MβCD 100 mg/kg/week
NEM 1.7 mg/kg/week

Treatment start
Age (weeks)
8w 9w 20w

B Blood glucose (mM)

WT db/db
Vehicle Met 25 50 100 MβCD NEM

C Body weight (g)

WT db/db
Vehicle Met 25 50 100 MβCD NEM

D Left kidney weight (g)

WT db/db
Vehicle Met 25 50 100 MβCD NEM

E Serum Cr (mg/dl)

WT db/db
Vehicle Met 25 50 100 MβCD NEM

F Albumin/creatinine (µg/mg)

WT db/db
Vehicle Met 25 50 100 MβCD NEM

G Albumin/creatinine (µg/mg)

WT db/db
Vehicle 50 100 0.85 1.7 MβCD NEM