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Caveolae May Enable Albumin to Enter Human Renal Glomerular Endothelial Cells

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ABSTRACT

Caveolae on human renal glomerular endothelial cells (HRGECs) are increased in glomerular disease and correlate with the degree of albuminuria. To assess the mechanism by which caveolae contribute to albuminuria, we investigated whether albumin enters into HRGECs through caveolae. HRGECs were incubated with Alexa Fluor 488 labeled BSA or transferrin, followed by immunofluorescence localization with antibody to caveolin-1 (Cav-1), the main structural protein of caveolae, or clathrin, the major structural protein of clathrin coated pits, to assess whether BSA colocalized with Cav-1. HRGECs were also incubated with albumin and caveolae disrupting agents, including methyl beta cyclodextrin (MBCD) and nystatin, to determine whether disrupting caveolae interfered with albumin endocytosis into HRGECs. HRGECs were also incubated with albumin after transfection with Cav-1 small interfering RNAs (siRNAs). Labeled BSA colocalized with Cav-1, but not with clathrin. In contrast, labeled transferrin colocalized with clathrin, but not with Cav-1. Incubation of HRGECs with MBCD or nystatin, or transfection with Cav-1 siRNA, significantly reduced the intracellular amounts of albumin and Cav-1, relative to normal HRGECs, as shown by western blotting and immunofluorescence. These findings indicate that albumin enters HRGECs through the caveolae, suggesting that caveolae play an important role in the pathogenesis of albuminuria by providing a pathway through which albumin can enter glomerular endothelial cells. J. Cell. Biochem. 116: 1060–1069, 2015.

KEY WORDS: CAVEOLAE; CAVEOLIN-1; ALBUMIN; ENDOCYTOSIS; GLOMERULAR ENDOTHELIAL CELLS

Although glomerular epithelial cells, basement membrane, and endothelial cells act as a glomerular filtration barrier, themechanism underlying urinary albumin excretion remains unknown. Glomerular endothelial cells are located on the inside of capillary walls and are thought to play an important role in albumin permeability. However albumin has been shown to pass through glomerular endothelial cells, via fenestrae without diaphragms, defined as 100 nm holes on the cell membranes, suggesting that glomerular endothelial cells have no effect on albumin permeability. If, however, glomerular endothelial cells do not act as a filtration barrier, allowing albumin to pass freely through them, the glomerular basement membrane or the next filtration barrier will soon clog up with proteins, resulting in immunoglobulin deposition on the basement membrane even in normal glomeruli. Moreover, if albumin can pass freely through the fenestrae, pre-eclampsia, a pathophysiologic condition that causes swelling of glomerular endothelial cells and reduces the sizes of fenestrae, would be unable to cause albuminuria, because albumin would be unable to pass through these small fenestrae [Ballermann, 2007; Obeidat et al., 2012]. As pre-eclampsia is accompanied by albuminuria, we hypothesized that another pathway in glomerular endothelial cells allows the passage of albumin.

Caveolae are 50- to 100-nm flask-shaped plasma membrane invaginations that play pivotal roles in the transcytosis of macromolecules, vascular permeability, regulation of endothelial nitric oxide (NO) synthesis, and transduction of various signaling molecules. These structures are abundant in vascular endothelial cells, smooth muscle cells, and cardiac myocytes. Their main components are caveolin-1, -2, and -3, which act as scaffolding proteins [Drab et al., 2001; Frank et al., 2003; Sowa, 2012]. Caveolin-1 (Cav-1) is the primary protein in the caveolae of vascular endothelial cells, as well as being present in glomerular endothelial cells. We previously reported that the numbers of caveolae on glomerular endothelial cells were significantly higher in patients with chronic glomerulonephritis than in normal controls, and that

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the numbers of caveolae correlated with the degree of albuminuria [Moriyama et al., 2011].

In this study, we investigated whether albumin enters human renal glomerular endothelial cells (HRGECs) through caveolae by using caveolae disrupting agents, such as methyl beta cyclodextrin (MBCD) and nystatin, both of which deplete cholesterol, and by transfection of these cells with Cav-1 small interfering RNA (siRNA). We also analyzed whether BSA colocalizes with Cav-1 in HRGEC. We found that albumin entered into HRGECs through caveolae, suggesting that caveolae act as gates to a previously unknown pathway that allows albumin to enter glomerular endothelial cells.

MATERIALS AND METHODS

CULTURE OF HRGECS

Fibronectin coated dishes and flasks (BD Biosciences, San Jose, CA) were incubated with fibronectin stock solution (Sigma Aldrich, St. Louis, MO), diluted 66.7-fold with Dulbecco's phosphate-buffered saline (DPBS), for 24 h. The fibronectin stock solution and DPBS were aspirated, and HRGECs (ScienCell Research Laboratories; Carlsbad, CA), in endothelial cell medium (ScienCell Research Laboratories) containing 10% fetal bovine serum (FBS), 5 mL endothelial cell growth supplement, and 5 mL penicillin/streptomycin solution, were seeded onto the fibronectin coated flasks and dishes.

ANTIBODIES AND REAGENTS

Mouse monoclonal antibody to human serum albumin was purchased from Abcam (Cambridge, MA), rabbit polyclonal antibody to Cav-1 and rabbit monoclonal antibody to clathrin heavy chain (HC) from Santa Cruz Biotechnology (Santa Cruz, CA), mouse monoclonal antibody to CD31 (platelet endothelial adhesion molecule-1 [PECAM-1]) from R&D Systems (Minneapolis, MN), and antibody to actin from Cytoskeleton Inc. (Denver, CO). Alexa Fluor 488 labeled BSA and transferrin, and Alexa Fluor 594 labeled donkey anti-rabbit IgG (H + L) antibody were purchased from Molecular Probes (Eugene, OR); and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was from Life Technologies (Grand Island, NY). Western blot analyses were performed using an Odyssey system (LI-COR, Inc., Lincoln, NE), IR Dye 680-conjugated affinity-purified anti-mouse IgG (H&L) and IR Dye 800-conjugated, affinity-purified anti-rabbit IgG (H&L) (Rockland Immunochemicals Inc., Gilbertsville, PA). MBCD and nystatin were purchased from Sigma-Aldrich and Millipore Corporation (Bedford, MA), respectively.

UPTAKE OF ALBUMIN OR TRANSFERRIN BY HRGECS

HRGECs passaged four to seven times were incubated with 20 μg/mL Alexa Fluor 488-labeled BSA or transferrin for up to 6 h. In some experiments, HRGECs were first incubated with MBCD or nystatin, at the indicated concentrations, for 1 h at 37°C. To each culture was added 100 μg/mL human serum albumin or 20 μg/mL Alexa 488 labeled BSA, followed by incubation for another 6 h at 37°C. HRGECs incubated in the absence of albumin and MBCD or nystatin constituted the negative controls, and HRGECs incubated with albumin alone were used as positive controls.

CYTOTOXICITY ASSAY

The cytotoxicities of MBCD and nystatin were assessed using CytoTox 96 nonradioactive cytotoxicity assays (Promega, Madison, WI), with the absorbance values representing the levels of lactate dehydrogenase (LDH) activity, according to the manufacturer's instructions and as described [Moriyama et al., 2007]. Briefly, HRGECs seeded onto 96-well plates were incubated with 100 μl normal medium with MBCD (0.625, 1.25, 2.5, 5, 10, or 25 mM) or nystatin (25, 50, 100, 200, 400, or 800 μg/mL) for 8 h. A 15 μl aliquot of lysis solution was added to each well, followed by incubation for 1 h at 37°C. Fifty microliters of each supernatant and cell lysate were transferred to wells of 96-well enzymatic assay plates, and 50 μl of LDH colorimetric determination solution was added to each. The plates were incubated at room temperature, protected from light, for 30 min, after which 50 μl of stop solution (1 M acetic acid) was added to each well. The absorbance of each well at 490 nm was measured using a 96-well plate reader.

EFFECT OF CAV-1 SIRNA

HRGECs were transfected with Cav-1 siRNA (Santa Cruz Biotechnology) as described by the manufacturer. Briefly, HRGECs were grown to 60 to 80% confluence on 35-mm fibronectin coated dishes in antibiotic free medium containing 10% FBS. The cells were washed with siRNA transfection medium, 1 mL of transfection reagent containing 100 nM Cav-1 or Control siRNA was added, and the cells were incubated at 37°C in a CO_2 incubator for 6 h. To each cell preparation was added 1 mL of normal medium containing twice the normal serum and antibiotic concentrations without removing the transfection reagent. After incubation for an additional 24 h, the medium with transfection reagent was aspirated and replaced by fresh normal medium. The cells were incubated for 72 h, and albumin internalization through the caveolae was analyzed.

WESTERN BLOT ANALYSIS

After incubation with albumin and/or inhibitors, cell monolayers were washed three times with PBS. The cells were scraped, harvested, and dissolved in gel buffer containing sodium dodecyl sulfate (SDS). Samples were loaded onto 5–20% Super Sep SDS polyacrylamide gels (Wako Pure Chemical Industries, Osaka, Japan) and electrophoresed, and the proteins were transferred to nitrocellulose membranes (Millipore Corporation). The membranes were blocked in Odyssey® blocking buffer (LI-COR Inc.) at room temperature for 1 h, followed by overnight incubation at 4°C with primary antibodies to albumin, Cav-1, and actin, with constant rotation. The membranes were washed three times with Tris-buffered saline containing 0.1% Tween-20 (TTBS) at room temperature for 5 min with agitation, incubated with secondary antibody conjugated to IRDye 680 or IRDye 800, and again washed three times with TTBS at room temperature for 5 min with agitation. The bands were visualized using an Odyssey® infrared imaging system, which measured the integrated intensity of each band. The signal of each band was normalized to that of actin in the same sample.

INDIRECT IMMUNOFLUORESCENCE ANALYSIS

HRGECs seeded on cover slips were washed three times with PBS, fixed in 100% methanol at −20°C for 20 min, and incubated in TTBS containing 3% FBS for 30 min. The cells were incubated with primary

antibody to Cav-1 or clathrin, both 1:200 in TTBS containing 1% FBS, for 1 h and washed three times with TTBS for 5 min each. The cells were subsequently incubated with secondary antibody conjugated to Alexa Fluor 594 (1:200) for 60 min and washed three times with TTBS for 5 min each. To stain nuclei, the cells were incubated with 300 nM DAPI for 5 min and washed three times with PBS. The samples were viewed by confocal microscopy (Carl Zeiss, LSM 710), with images captured by software (ZEN 2011, black version) from the manufacturer. The intensity of expression of BSA, Cav-1, and BSA or transferrin colocalizing with Cav-1 or clathrin was assessed using ZEN 2011 blue and black versions. To determine the background, we first analyzed the mean intensity of von Willebrand factor (vWF) in cells not treated with labeled albumin or transferrin, and not stained with Cav-1 or clathrin, because vWF is considered a cell identity marker. Next, we confirmed that the mean intensity of vWF was higher than that of nonspecific signal and noise in unlabeled and untreated cells, and defined the mean intensity of vWF as the background level. If the intensity of labeling was higher than the mean intensity of vWF after incubation with BSA, transferrin, Cav-1 or clathrin, we regarded this labeling as positive; if the intensity was lower than the mean intensity of vWF, we regarded it as background. After setting the threshold of intensity of labeled BSA (green), transferrin (green), Cav-1 (red), clathrin (red), and labeled BSA or transferrin with Cav-1 or clathrin (yellow), the ZEN 2011 blue version automatically, regularly and reproducibly recognized and measured the intensity of areas positive for BSA, transferrin, Cav-1, and clathrin and subtracted their background. ZEN 2011 blue version also automatically recognized and measured the intensity of labeled BSA, Cav-1, and labeled BSA or transferrin colocalized with Cav-1 or clathrin, after subtracting the background. We measured at least 20 cells per experiment, with each experiment performed at least three times and the results averaged.

STATISTICAL ANALYSIS

All experiments were repeated at least three times. Mean ± standard deviation (SD) was calculated from repeated data. Data were analyzed using JMP 10.0.1 software (SAS Institute, Cary, NC). Unpaired t-tests were used to compare the uptake of albumin and Cav-1 on western blot analysis. A P-value <0.05 was considered statistically significant.

RESULTS

EXPRESSION OF CD31 (PECAM-1) IN HRGECS

HRGECs show morphological changes during passage. Analysis of the expression of the endothelial cell marker CD31 (PECAM-1) during passage of these cells showed that this level remained relatively stable from passages 2 to 10. Morphologic assessments in bright fields showed that the morphology of these cells was unaltered from passages 3 to 9. We therefore performed all experiments using HRGECs between passages 4 and 7 (Fig. 1). To assess the morphological changes induced by Cav-1 disrupting agents and Cav-1 siRNA, we incubated these cells with MBCD, nystatin and Cav-1 siRNA, but found no alterations in cell growth or morphology on bright field images (Figs. 5c and 6b).

UPTAKE OF ALEXA FLUOR 488 LABELED BSA OR TRANSFERRIN AND COLOCALIZATION OF LABELED BSA OR TRANSFERRIN WITH CAVEOLIN-1 OR CLATHRIN ON HRGECS

To investigate albumin entry into HRGECs and determine whether albumin colocalizes with Cav-1, these cells were incubated with Alexa Fluor 488 labeled BSA and subsequently with antibody to Cav-1 or clathrin. Higher amounts of labeled BSA colocalized with

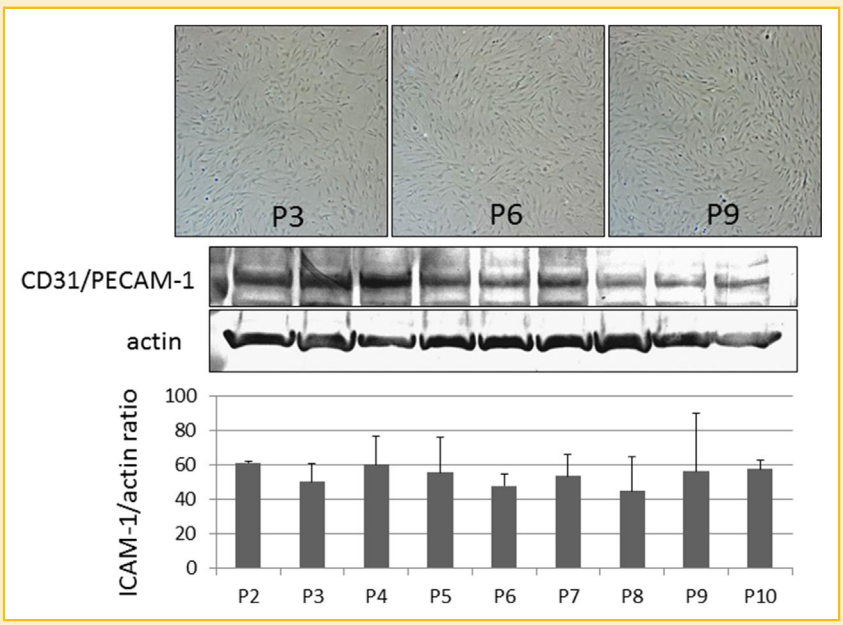

Fig. 1. Cell morphology in bright fields and expression of CD31 (PECAM-1) in HRGECs during passages 2–10. HRGECs passaged two to 10 times were grown to 80% confluence in 35 mm dishes and examined in bright fields; the cells were also subjected to western blot analysis to assess the level of expression of the endothelial cell marker CD31 (PECAM-1).

Cav-1 (Fig. 2a), but not with clathrin (Fig. 2b), at both lower and higher magnifications. Quantitative analysis showed that the relative intensity of labeled BSA colocalized with clathrin at 1 h was significantly lower than the relative intensity of labeled BSA colocalized with Cav-1 (31.6% vs. 100%, $P < 0.05$; Fig. 2c). To validate endocytosis through caveolae, we investigated the internalization of Alexa Fluor 488 labeled transferrin through caveolae or clathrin, because transferrin has been shown to enter cells through clathrin, but not through caveolae. Little labeled transferrin colocalized with Cav-1 (Fig. 3a), whereas larger amounts of transferrin colocalized with clathrin (Fig. 3b) at both lower and higher magnifications. The relative intensity of labeled transferrin colocalized with clathrin at 1 h was significantly higher than the relative intensity of labeled transferring colocalized with Cav-1 (340.1% vs. 100%, $P < 0.001$; Fig. 3c). Taken together, these results indicated that albumin enters HRGECs rapidly through caveolae, not through clathrin, whereas transferrin enters HRGECs through clathrin, not through caveolae.

CYTOTOXICITY OF MBCD AND NYSTATIN

The principal structural lipid present in caveolae is cholesterol. Caveolae can be disrupted using cholesterol-depleting agents such as MBCD and nystatin. The appropriate concentrations of MBCD and nystatin that disrupt caveolae in HRGECs without inducing cytotoxicity were assessed by measuring absorbance at 490 nm, representing LDH activity in these cells. The relative absorbances were similar at 0–2.5 mM MBCD and 0–200 μg/mL nystatin, but increased significantly at ≥5 mM MBCD and ≥400 μg/mL nystatin (Fig. 4a and b). Thus, to selectively disrupt caveolae, HRGECs should be incubated with ≤2.5 mM MBCD or ≤200 μg/mL nystatin.

MBCD AND NYSTATIN INHIBITION OF ALBUMIN ENDOCYTOSIS INTO HRGECS THROUGH CAVEOLAE

Western blot analysis showed that albumin was present in cells incubated with 100 μg/mL albumin, but not in untreated cells. The uptake of albumin by albumin-treated cells was significantly and dose-dependently reduced by MBCD, with significant reductions observed at MBCD concentrations of 0.5 mM ($P < 0.05$), 1 mM ($P < 0.0001$) and 2 mM ($P < 0.0001$) compared with positive controls (Fig. 5a). Western blotting also showed that the relative uptake of albumin was significantly lower in HRGECs co-incubated with albumin and 100 ($P < 0.05$) and 200 μg/mL ($P < 0.01$) nystatin than in HRGECs incubated with albumin alone (Fig. 5b). Immunofluorescence analysis clearly showed Cav-1 and labeled BSA colocalized in cells treated with 20 μg/mL Alexa Fluor 488 labeled BSA alone. Treatment with 0.5 mM MBCD or 200 μg/mL nystatin, however, dramatically reduced cellular uptake of labeled BSA and Cav-1 (Fig. 5c). When compared with untreated HRGECs, cells treated with MBCD showed significant reductions in the relative expression of Cav-1 ($P < 0.05$) and the uptake of labeled BSA ($P < 0.001$), as did cells treated with nystatin ($P < 0.001$ each).

EFFECT OF SIRNA-MEDIATED CAV-1 KNOCKOUT ON ALBUMIN INTERNALIZATION

To assess the appropriate effective dose and time for transfection, cells were incubated with 20, 50, and 100 nM Cav-1 siRNA for 24, 48, and 72 h. Compared with nontransfected cells, relative Cav-1 expression was significantly lower in cells transfected with 100 nM siRNA for 24 ($P < 0.005$), 48 ($P < 0.01$), and 72 h ($P < 0.001$), and in cells transfected with 50 nM siRNA for 48 and 72 h ($P < 0.05$ each) (Fig. 6a), indicating that transfection was optimal at 100 nM for 72 h. Following transfection for 72 h with 100 nM Cav-1 or Control siRNA, the cells were incubated with albumin for 6 h; as a control, cells were incubated with albumin alone. Cav-1 expression ($P < 0.05$) and albumin uptake ($P < 0.005$) were significantly lower in HRGECs transfected with Cav-1 siRNA, but not Control siRNA, than in control cells (Fig. 6b).

Taken together, these results indicate that albumin endocytosis into HRGECs through caveolae was reduced by the disruption of caveolae using cholesterol depleting agents or the transfection of Cav-1 siRNA.

DISCUSSION

Serum albumin can pass freely through the large fenestrae without diaphragms, which constitute 20–50% of the endothelial surface [Haraldsson and Jeansson, 2009]. However, if albumin, immunoglobulins, and macromolecules pass freely through the fenestrae, the glomerular basement membrane will soon become clogged with proteins that cannot pass the slit diaphragm of glomerular epithelial cells. The fenestrae in patients with thrombotic thrombocytopenic purpura and hemolytic uremic syndrome, which cause glomerular endothelial injury, are decreased owing to the thrombosis of intracapillary platelets and fibrin, leading to a reduction in glomerular filtration rate, but not albuminuria [Ballermann, 2007; Obeidat et al., 2012]. Early studies using transmission electron microscopy found that significant amounts of albumin could not pass freely through these fenestrae [Ryan and Karnovsky, 1976]. Electron micrographs of the glomerular capillary wall after oxygen-rich, fluorocarbon based perfusion in the presence of potassium ferricyanide during osmium tetroxide postfixation showed diaphragms between the glomerular endothelial cells [Hjalmarsson et al., 2004]. These results suggested that albumin may pass through glomerular endothelial cells via an as yet undetermined pathway, regardless of whether albumin could pass through the fenestrae.

Moreover, recent studies have described new mechanisms of glomerular albumin filtration and tubular reabsorption. Even in normal glomeruli, large amounts of albumin pass through the glomerular filtration barrier and leak into the primary urine, with almost all of this albumin absorbed by megalin located on the surface of tubular epithelial cells [Russo et al., 2007; Russo et al., 2009]. This leaky glomerular barrier theory suggests that the pathophysiology of albuminuria depends on a balance between the amount of glomerular filtered albumin and the ability of tubular epithelial cells to absorb albumin. If albumin can freely pass through the glomerular endothelial cells through the fenestrae, an increase in glomerular filtered albumin would require another pathway that allows albumin to pass through glomerular endothelial cells. The role of the leaky glomerular barrier in albuminuria has not yet been determined [Peti-Peterdi, 2009;

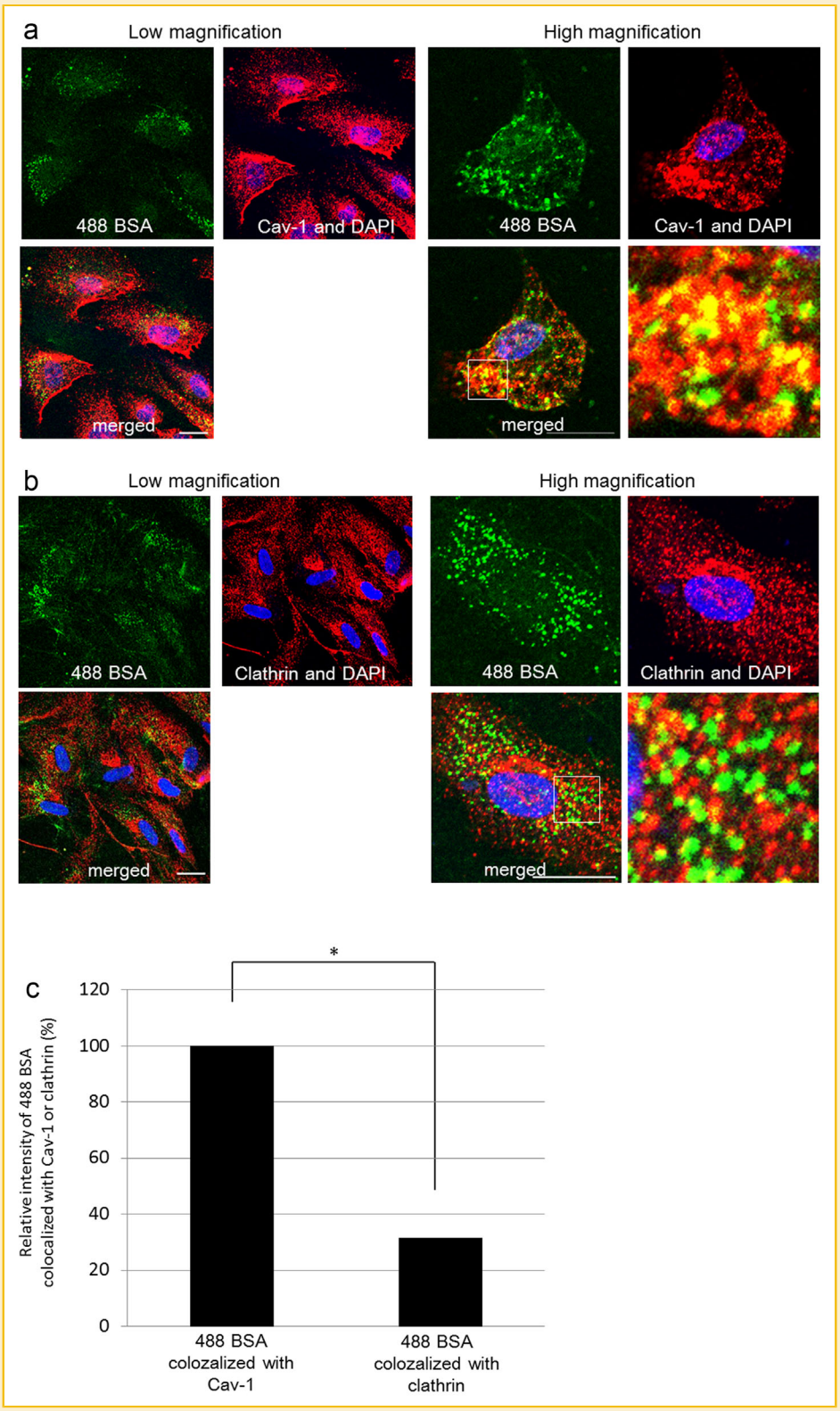

Fig. 2. Colocalization of Alexa Fluor 488 labeled BSA with caveolin-1, but not with clathrin. (a, b) HRGECs were incubated with Alexa Fluor 488 labeled BSA (green) for 0, 15, 30, or 60 min; or for 2, 4, or 6 h at 37°C. The cells were fixed and fluorochrome-conjugated with antibody to Cav-1 (red), or (b) clathrin (red) and DAPI (blue). Bars represent 20 μm (higher magnification). The pictures show cells after incubation for 1 h. (c) Bar graph showing that the relative intensity of labeled BSA colocalized with Cav-1 after 1 h was significantly greater than the relative intensity of labeled BSA colocalized with clathrin ($^{*}P < 0.05$).

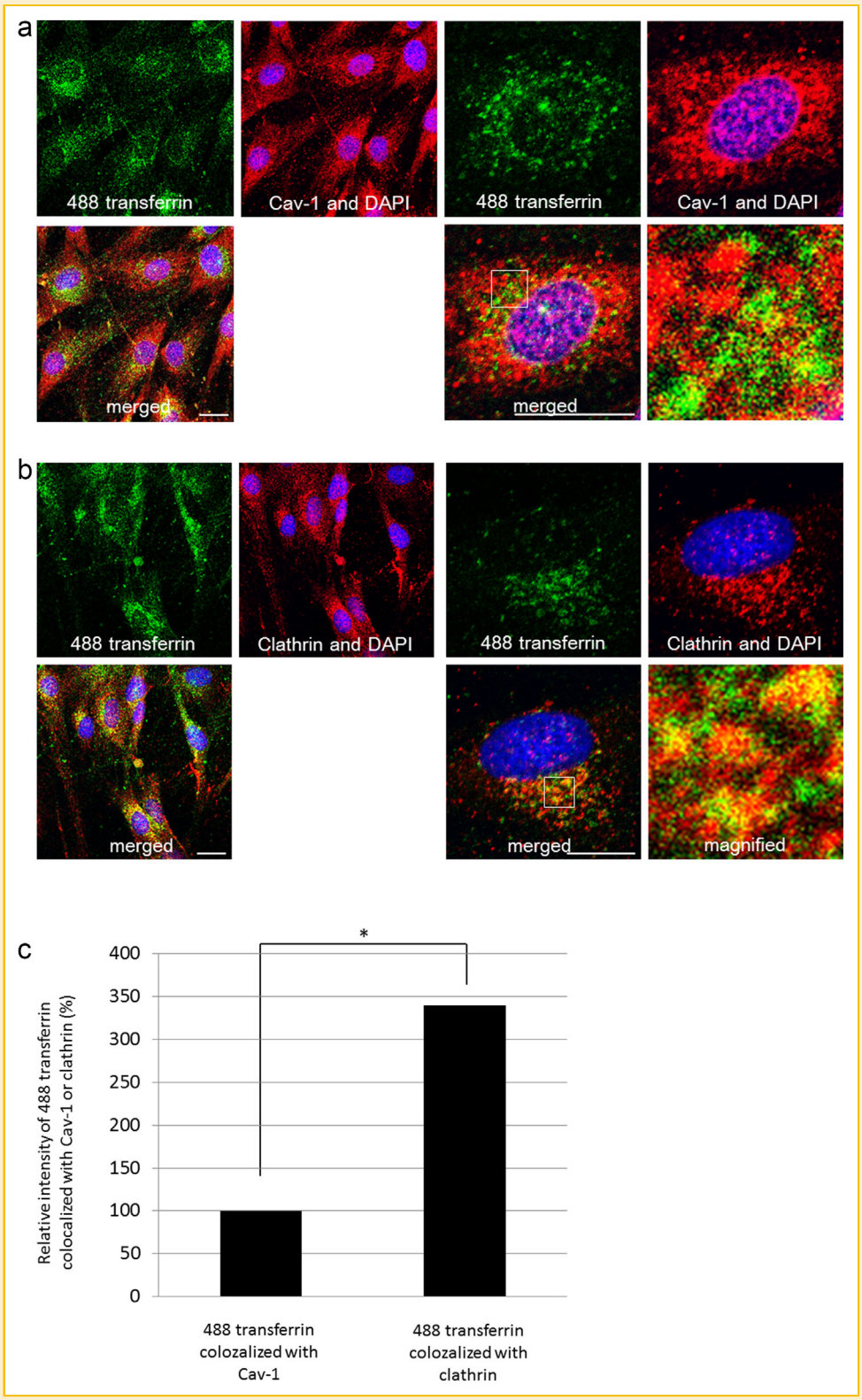

Fig. 3. Colocalization of Alexa Fluor 488 labeled transferrin with clathrin, but not with caveolin-1. (a, b) HRGECs were incubated with Alexa Fluor 488 labeled transferrin for 0, 15, 30, or 60 min, or for 2, 4, or 6 h at 37°C. The cells were fixed and incubated with fluorochrome-conjugated antibody to Cav-1 (red) , or clathrin (red) (b) and DAPI (blue). Bars represent 20 μm. The pictures show cells after incubation for 1 h. (c) Bar graph showing that the relative intensity of labeled transferrin colocalized with Cav-1 after 1 h was significantly lower than with the relative intensity of labeled transferring colocalized with clathrin ($^{*}P < 0.001$).

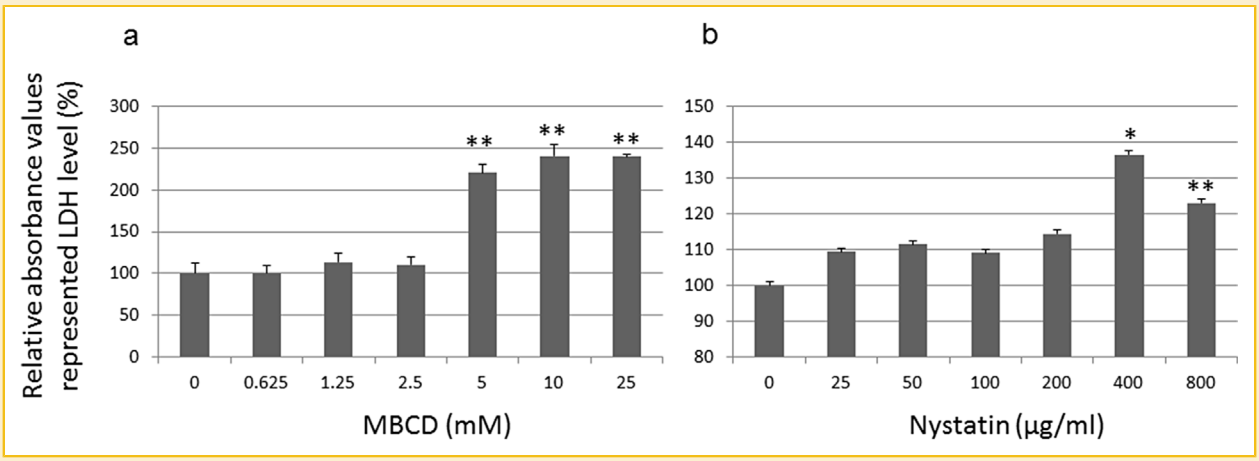

Fig. 4. Cytotoxic dosages of MBCD (a) and nystatin (b) toward HRGECs. HRGECs were incubated with MBCD (0.625, 1.25, 2.5, 5, 10, or 25 mM), nystatin (25, 50, 100, 200, 400, or 800 μg/mL), or vehicle for 8 h. The cells were lysed with lysis buffer, the supernatant and cell lysate were harvested, and their absorbance at 490 nm was measured. Each point represents the mean ± SD of eight different wells for each concentration of MBCD and nystatin ($P < 0.05$; $^{**}P < 0.0001$).

Tanner, 2009; Sandoval et al., 2012], because this hypothesis excludes the presence of a slit diaphragm on glomerular epithelial cells, the glycocalyx on glomerular endothelial cells, and the glomerular basement membrane [Haraldsson et al., 2008; Haraldsson and Jeansson, 2009; Jarad and Miner, 2009]. Regardless, the caveolae may constitute a new pathway by which albumin enters glomerular endothelial cells.

We found that, in glomerular endothelial cells, labeled BSA was highly colocalized with Cav-1, the main structural protein of caveolae, but not with clathrin, whereas labeled transferrin was highly colocalized with clathrin, but not with Cav-1, suggesting that albumin had been captured by these caveolae. Agents that disrupt caveolae by depleting membrane cholesterol, such as MBCD and nystatin, and transfection with Cav-1 siRNA, not only reduced the expression of Cav-1, but the amount of intracellular albumin, providing further evidence that albumin entered into these glomerular endothelial cells through the caveolae. These results have been confirmed, using the same method, in lung microvessel endothelial cells [John et al., 2003]. We previously found that the expression of Cav-1 was higher in the glomeruli of individuals with glomerular disease than in normal glomeruli, that steroid treatment for glomerular disease reduced Cav-1 expression to the level observed in normal glomeruli, and that the expression of Cav-1 correlated with the amount of urinary albumin excretion [Moriyama et al., 2011]. Moreover, the expression of Cav-1 was increased in the renal cortex of diabetic rats, as well as being suppressed by treatment with insulin or losartan [Demová and Komers, 2009]. In addition, Cav-1 levels were found to be higher in alveolar endothelial cells of diabetic than nondiabetic rats, as was the amount of albumin transported from capillary lumen through endothelial cells to the basement membrane and interstitium [Pascariu et al., 2004]. Taken together, these findings indicate that caveolae play pivotal roles in albumin uptake and urinary albumin excretion by glomerular endothelial cells.

Our results also suggest that deterioration of the caveolae may lead to a reduction in albuminuria. However, the amount of urinary albumin excretion was found to be similar in Cav-1 deficient and wild type mice [S□rrensson et al., 2002]. Moreover, the transcapillary escape rate of albumin from the glomeruli tended to be higher in Cav-1 knockout than in wild type mice because of the dilatation in the former of interendothelial junctions caused by increased microvascular pressure [Grände et al., 2009]. This unexpected result was confirmed in the lung and shown to result from an increase in endothelial NO [Schubert et al., 2002], although caveolar endocytosis and transcytosis of albumin in lung microvascular endothelial cells was previously confirmed [Schubert et al., 2001; Minshall et al., 2002; Schubert et al., 2002; John et al., 2003; Wang et al., 2009]. The concentration of NO was 5-fold higher in Cav-1 knockout than in wild-type mice [Minshall et al., 2003]. These findings suggested that the amount of albumin passing through the caveolae may be decreased in Cav-1 knockout mouse, but that albumin passage through another pathway in endothelial cells may be increased owing to increased microvascular pressure and NO. Thus, total albuminuria may be unaffected in Cav-1 knockout mice.

Caveolae are generally present in normal kidneys and other organs. Their functions include the transport of macromolecules, signal regulation, inhibition of endothelial NO synthase, and regulation of ion channels. Many receptors are located on the caveolae, including G-protein coupled receptors, transforming growth factor-beta type I and II receptors, vascular endothelial growth factor receptor, platelet-derived growth factor receptors, and insulin receptors [Drab et al., 2001; Frank et al., 2003; Sowa, 2012]. Therefore, knockout of Cav-1 would disrupt many caveolar functions, not only albumin entry into HRGECs. Preventing the proliferation of caveolae will likely decrease urinary albumin excretion. Indeed, overexpression of caveolin in diabetic mice increased the transcytosis of albumin [Pascariu et al., 2004] and may also be associated with microalbuminuria [Van Dokkum and Buikema, 2009].

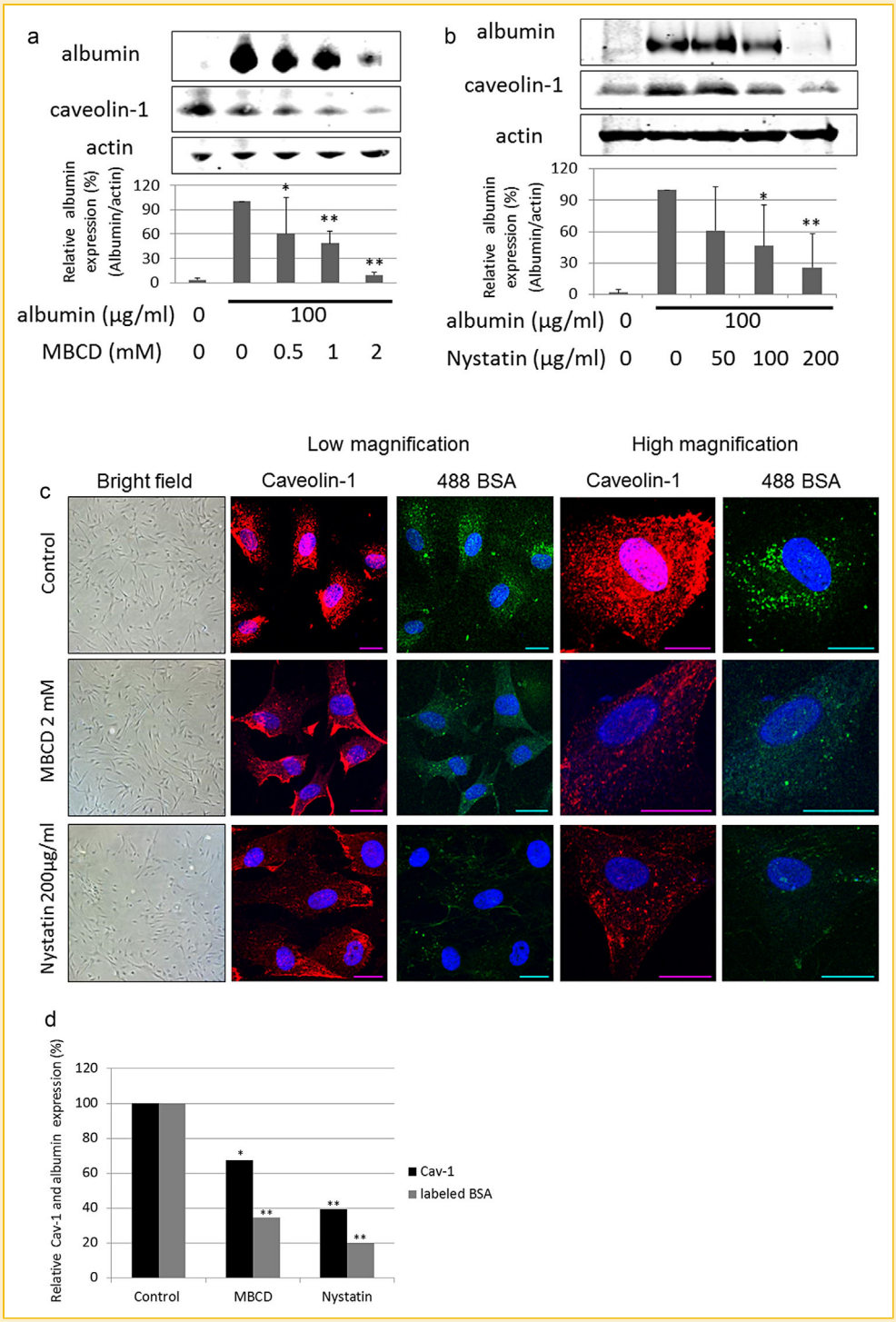

Fig. 5. MBCD or nystatin inhibits albumin internalization. (a, b) Western blot analysis of harvested cells. Serum albumin levels were measured using the Odyssey system and normalized relative to actin levels as the loading control. Each bar represents the mean ± SD of three independent experiments. HRGECs incubated with human serum albumin alone were used as the positive control. HRGECs were preincubated with MBCD (0.5, 1, or 2 mM) for 1 h prior to the addition of 100 μg/mL human serum albumin. The cells were subsequently incubated with MBCD and albumin for 6 h ($^{*}P < 0.05$; $^{**}P < 0.0001$ vs. positive control). HRGECs were preincubated with nystatin (50, 100, or 200 μg/mL) for 1 h prior to the addition of 100 μg/mL human serum albumin. The cells were subsequently incubated with nystatin and albumin for 6 h ($^{*}P < 0.05$; $^{**}P < 0.01$ vs. positive control). (c, d) Immunofluorescence analysis of fixed cells. (c) HRGECs were preincubated with 2 mM MBCD or 200 μg/mLnystatin for 1 h prior to the addition of 20 μg/mL Alexa Fluor 488 BSA. The cells were subsequently incubated with MBCD or nystatin and albumin for 6 h. The cells in the bright field (×20) were similar morphologically following incubation in normal medium and in medium containing MBCD or nystatin. Uptake of Alexa Fluor 488 labeled BSA or Cav-1 in each sample was assessed by confocal microscopy. In each experiment, cells were observed on three independent coverslips, with the experiment performed at least three times. HRGECs incubated with labeled BSA alone were used as the positive control. Bars represent 20 μm. (b) The relative expression of Cav-1 and uptake of labeled BSA in HRGECs treated with MBCD or nystatin was significantly lower than in untreated cells ($^{*}P < 0.05$; $^{**}P < 0.001$).

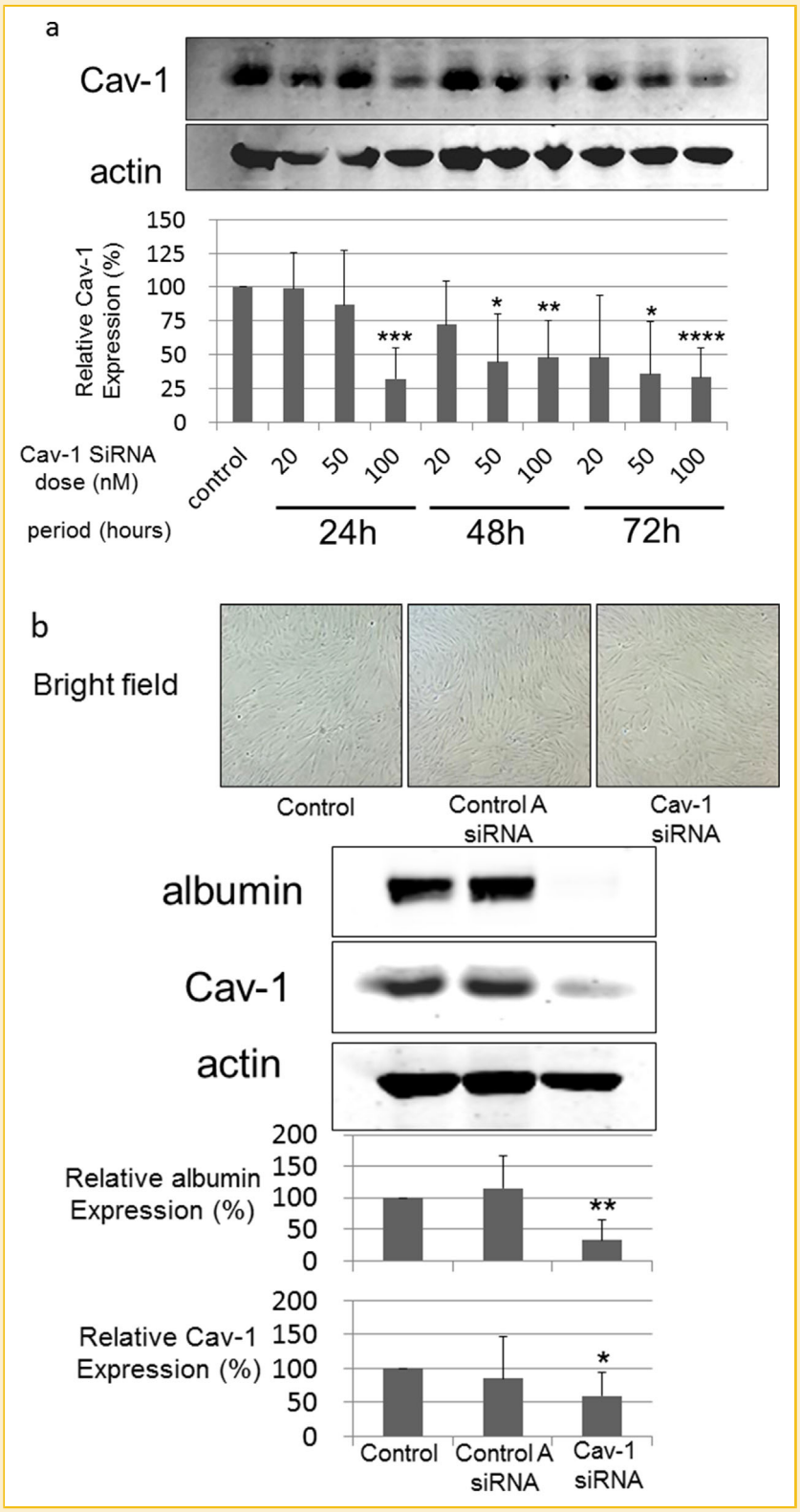

Fig. 6. Transfection with Cav-1 siRNA inhibits albumin internalization. (a) HRGECs were transfected with 20, 50, or 100 nM Cav-1 siRNA for 24, 48, or 72 h to determine the appropriate dosage and period of transfection. Each bar represents the mean ± SD of six independent experiments. HRGECs not transfected with Cav-1 siRNA were used as control ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.001$ vs. control). (b) HRGECs were transfected with 100 nM Cav-1 siRNA or control siRNA for 72 h, followed by incubation with albumin for another 6 h. Thereafter, cells were harvested and western blot analysis was performed. The cells transfected with Cav-1 siRNA in the bright field (×20) were similar to normal cells, without morphological changes. Each bar represents the mean ± SD of five independent experiments ($^{*}P < 0.05$; $^{**}P < 0.005$ vs. control).

CONCLUSION

In conclusion, we showed that albumin entered HRGECs through the caveolae, suggesting the importance of the caveolae for albumin entry into these cells. Caveolae may be the gate of a new pathway, allowing albumin to enter glomerular endothelial cells.

ACKNOWLEDGMENTS

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