

Advanced glycation end-products affect the cytoskeletal structure of rat glomerular endothelial cells via the Ras-related C3 botulinum toxin substrate 1 signaling pathway

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Abstract. The present study aimed to determine the molecular mechanisms leading to the production of advanced glycation end-products (AGEs) and their effect on the morphology and function of rat glomerular capillary endothelial cells (GECs). Primary rat GECs were treated with AGE-modified human serum albumin (AGE-HSA) and divided into groups according to AGE concentration and treatment time. The structure and distribution of cytoskeletal protein F-actin and the cortical actin binding protein, cortactin, were analyzed using immunofluorescence and confocal microscopy. As the Ras-related C3 botulinum toxin substrate 1 (Rac1) signaling pathway was previously identified to be involved in mediating the contraction of endothelial actin-myosin activity, Rac1 was examined subsequent to treatment of the cells with the Rac1 agonist 2'-O-methyladenosine-3',5'-cyclic monophosphate (O-Me-cAMP) for 1 h using a pull-down assay. Cell permeability was determined by the leakage rate of a fluorescein isothiocyanate fluorescent marker protein. AGE-HSA treatment resulted in alterations in the structure and distribution of F-actin and cortactin in a dose- and time-dependent manner, while no effect was observed with HSA alone. The effect of AGE on the cytoskeleton was inhibited by the addition of O-Me-cAMP. AGE-HSA significantly reduced the level of Rac1 activity ($P<0.05$); however, no effect was observed on total protein levels. Furthermore, AGE-HSA treatment led to a significant increase in the permeability of endothelial cells ($P<0.01$), which was inhibited by O-Me-cAMP ($P<0.01$). The Rac1 signaling

pathway is thus suggested to serve an important function in mediating AGE-induced alterations in GEC morphology and function.

Introduction

Previous studies have demonstrated that an increase in glomerular capillary permeability is a key step leading to proteinuria in patients with diabetes and nephropathy (1). Glomerular capillary endothelial cells (GECs) constitute the first barrier preventing blood macromolecules, such as proteins, from passing through the endothelial wall (2-4). Therefore, the structure and distribution of GECs is closely associated with the permeability of the capillary. Previous studies have suggested that the reorganization and redistribution of the cytoskeleton protein F-actin and the cortical actin binding protein cortactin in endothelial cells is crucial to the increase in capillary permeability observed (5-7). In addition, the Ras-related C3 botulinum toxin substrate 1 (Rac1) signaling pathway has been identified to be involved in mediating the contraction of endothelial actin-myosin. This induces an alteration in cell morphology, destroying the cell-cell connections and forming the gap between cells (8-12), suggesting an association between the Rac1 signaling pathway and capillary permeability. It has been reported that advanced glycation end-products (AGEs) are involved in inducing alterations in the distribution of cytoskeletal proteins and endothelial cell permeability in diabetic patients with microvascular complications (13-16). However, the function of the Rac1 signaling pathway in this process remains elusive. In the present study, immunofluorescence staining and confocal microscopic analysis were conducted in order to determine the effect of AGEs on the structure and distribution of F-actin and cortactin in GECs. In addition, the levels of Rac1 activity were investigated using a pull-down assay, endothelial permeability was analyzed using a Transwell assay and the potential involvement of the Rac1 signaling pathway in this process was examined. The present study aimed to improve the understanding of the pathogenesis of nephropathic proteinuria in diabetics and provide novel therapeutic targets for diabetes and nephropathy.

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Materials and methods

Materials. Dulbecco's modified Eagle's medium, MCDB131, trypsin and fetal bovine serum (FBS) were purchased from Gibco Life Technologies (Carlsbad, CA, USA). Vascular endothelial growth factor was purchased from BD Biosciences (San Jose, CA, USA) and fluorescein isothiocyanate (FITC)-phalloidin was obtained from Molecular Probes Life Technologies (Grand Island, NY, USA). FITC-labeled goat anti-rabbit antibody, human serum albumin (HSA) and FITC-bovine serum albumin were from Sigma-Aldrich (St. Louis, MO, USA). DAPI was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA) and 2'-*O*-methyladenosine-3',5'-cyclic monophosphate (O-Me-cAMP) was purchased from Aladdin Reagents Co., Ltd (Shanghai, China). Transwell was purchased from Corning Life Sciences (Tewksbury, MA, USA) and a Rac1 Activity Detection kit was purchased from EMD Millipore (Billerica, MA, USA). A Bradford kit was purchased from Shenneng Bocai Biotechnology Co., Ltd. (Shanghai, China).

Isolation and culture of GECs. The isolation and culture of primary GECs was conducted as previously described (17-19). All studies were approved by the Ethics Committee of Anhui Provincial Hospital (Hefei, China) for Animal Experiments and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. Primary GECs were isolated from the kidneys of two male Wistar rats (body weight, 80-120 g) (Animal Department of Anhui Medical University, China; certificate no. 003). Primary GECs were cultured in MCDB131 medium supplemented with 10% FBS in a humidified atmosphere with 5% CO₂. All operations were performed under 10% chloral hydrate (Hechang Chemical Company, Wuhan, China) and all efforts were made to minimize suffering.

Preparation of AGE-modified HSA. AGE-HSA was prepared by incubating HSA with glucose, as previously described (20-23). The reaction system contained 1.5 g HSA and 3.0 g D-glucose (Meilun Bio, Dalian, China), which were dissolved in 10 ml phosphate-buffered saline (PBS; 0.2 mol; pH 7.4; Gibco Life Technologies) and filtered with 0.22 µm microporous membranes (EMD Millipore). The solution was then maintained in a container filled with nitrogen, which was sealed, protected from light and incubated at 37°C for three months. The unbound materials were removed using a dialysis bag (molecular weight, 10,000; Corning, Inc., Corning, NY, USA). The same procedure was completed without the addition of D-glucose for the control. The AGE value of the samples (1 mg/ml) was detected by fluorescence scanning (BX43; Olympus Corp., Tokyo, Japan) and samples were stored at -20°C.

Immunofluorescence staining. Staining was conducted as previously described (24-27). The slides were washed in PBS twice and fixed in 4% paraformaldehyde at room temperature for 30 min. Subsequently, the slides were treated with 0.1% Triton X-100 (Sangon Biotech Co., Ltd, Shanghai, China) for 15 min (F-actin staining). Subsequent to blocking with 1% bovine serum albumin (BSA; Sigma-Aldrich) for

1 h at room temperature, the samples were incubated with FITC-phalloidin for 1 h at room temperature in the dark (F-actin staining), or with rabbit anti-mouse monoclonal cortactin antibody (SAB1305513; 1:100) overnight at 4°C. For cortactin staining, samples were then incubated with DyLight 605-labeled goat anti-rabbit antibody (SAB4600398; 1:500) for 1 h at room temperature in the dark following washing three times with PBS for 5 min. Antibodies were obtained from Santa Cruz Biotechnology, Inc. (Beijing, China) and samples were incubated with antibodies for 2 h at room temperature. Glycerol (50%; Xilong Chemical Company, Guangzhou, China) was used to mount the glass slides with cells, and the images were captured using a confocal microscope.

Rac1 activity analysis. Rac1 activity was analyzed using a pull-down assay, as previously described (9,28). The protein was extracted using a chemical cleavage method, and the protein concentration was detected using the Bradford assay. Samples (50 µg) underwent SDS-PAGE (10%) and were subsequently transferred to polyvinylidene difluoride membranes (EMD Millipore). The membranes were incubated with RAC1-GTP antibody and were then incubated with horseradish peroxidase-conjugated secondary antibodies (Zhongshan Jinqiao Company, Beijing, China). The chemiluminescent images were obtained using a Kodak Image Station 2000R system (Kodak, Rochester, NY, USA) and the results were analyzed using ImageJ software version 1.44e (National Institutes of Health, Bethesda, MD, USA).

Cell permeability analysis. Using a previously reported method (29), GECs were seeded into the top compartment of a Transwell chamber with FITC-albumin (100 µl; 1 mg/ml; Sigma-Aldrich). Subsequent to incubation, the fluorescence intensity of samples was analyzed using a HTS-7000 Bio Assay Reader (BioAssay Systems, Hayward, CA, USA) with 495 nm excitation and 520 nm emission filters. The apparent permeability coefficient [$P_a = (F/t)(1/A)(v/L)$] was used, where F indicates the fluorescence intensity in the bottom chamber, t indicates time (sec), A is the membrane area (cm²), v is the solution volume in the bottom chamber and L indicates the fluorescence intensity in the top chamber. The results are expressed as a percentage [$P_a\% = (\text{experimental } P_a \text{ value}/\text{control } P_a \text{ value}) \times 100\%$]. The experiments were repeated a minimum of five times.

Statistical analysis. Statistical analysis was performed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). All data are presented as the mean ± standard error and were analyzed by a one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of AGE-HSA on F-actin and cortactin morphologies in GECs. Under normal conditions, endothelial cells appear smooth and intact (30). F-actin is filamentous among the long axis and near cell junctions and is present in reticular, intact and continuous lines, predominantly in the edges of cells and the inner membranes. A small amount of reticular nuclear matrix is also present around the nucleus. Cortactin is predominantly

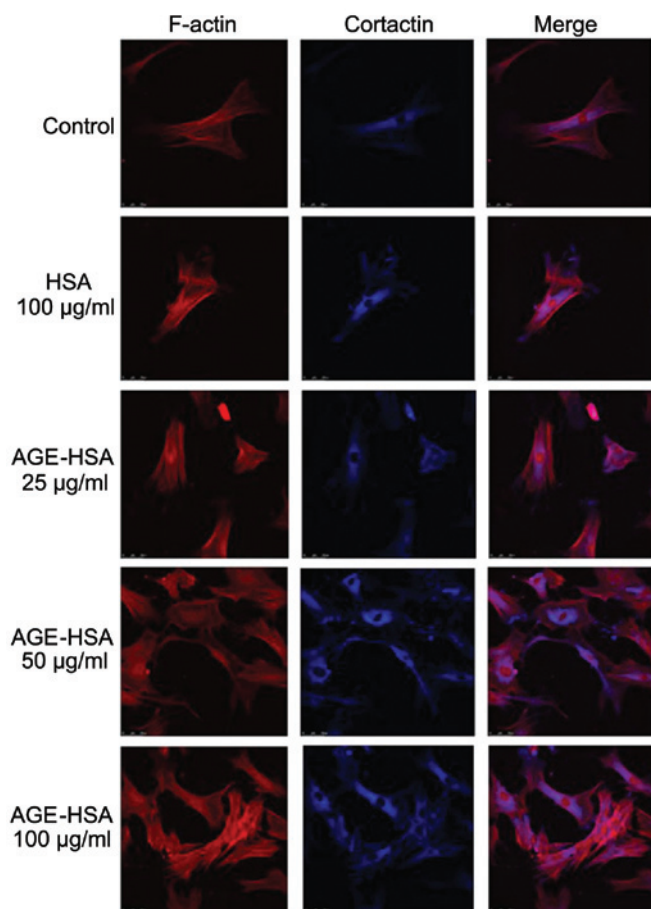


Figure 1. AGE alters the morphology and distribution of F-actin and cortactin in a dose-dependent manner. Rat GECs were treated with different concentrations of AGE-HSA (25, 50 or 100 $\mu\text{g/ml}$) for 8 h. Cells cultured in pure MCDB131 medium (control) or with HSA (100 $\mu\text{g/ml}$) alone were designated as the controls. With increasing AGE-HAS concentration, the edge of the F-actin peripheral dense band was observed to become rough and irregular with a jagged appearance, F-actin was diffusely distributed in cells and the number of stress fibers increased. Cytoplasmic cortactin was unclear in the cortex and membrane and its distribution became disorganized. AGE, advanced glycation end-products; GEC, glomerular capillary endothelial cells; HSA, human serum albumin.

distributed in the cytoplasm and is occasionally also present in the cell membrane. With an increase in AGE-HSA concentration or treatment time, the edge of the F-actin peripheral dense band was observed to become rough and irregular, with a jagged appearance. F-actin was observed to be diffusely distributed in cells and the number of stress fibers, composed of a single row of non-polar actin filaments, increased. The distribution of cytoplasmic cortactin became disorganized and it was unclear in the cortex and membrane. Cells became round and retracted when treated with AGE-HSA for 8 h at a concentration of 100 $\mu\text{g/ml}$; however, HSA alone did not produce these effects (Figs. 1 and 2).

Activation of Rac1 inhibits AGE-HSA-induced morphological alterations to F-actin and cortactin in GECs. The reconfiguration of the structure of F-actin, formation of central stress fibers and retraction in GECs induced by AGE-HSA was markedly inhibited by pre-treatment with O-Me-cAMP. In addition, similar inhibitory effects were observed in

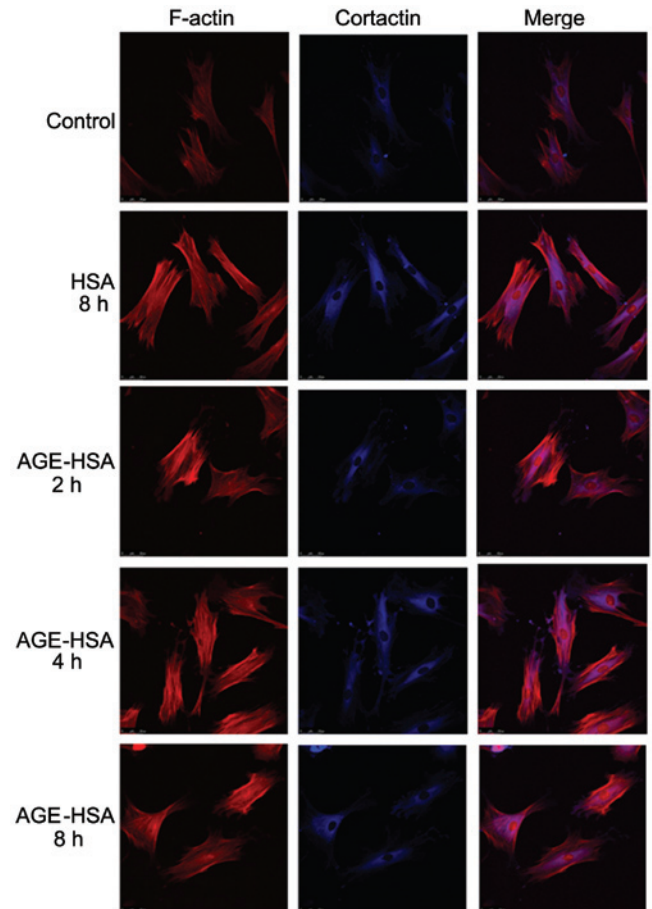


Figure 2. Time-dependent AGE-induced alterations to the morphology and distribution of F-actin and cortactin. GECs were treated with 100 $\mu\text{g/ml}$ AGE-HSA for 2, 4 or 8 h. Cells cultured in pure MCDB131 medium (control) or with HSA (100 $\mu\text{g/ml}$) alone were designated as the controls. With increasing AGE-HAS treatment time, the edge of the F-actin peripheral dense band was observed to become rough and irregular with a jagged appearance, F-actin was diffusely distributed in cells and the number of stress fibers increased. Cytoplasmic cortactin was unclear in the cortex and membrane and its distribution became disorganized. AGE, advanced glycation end-products; GEC, glomerular capillary endothelial cells; HSA, human serum albumin.

AGE-HSA-induced cortactin disorganization and cell retraction with O-Me-cAMP-pre-treatment. However, HSA alone did not produce this inhibitory effect (Fig. 3). These observations further suggested an involvement of the Rac1 signaling pathway in the development of AGE-induced morphological and structural alterations in GECs.

Effect of AGE-HSA on the levels of Rac1 activity in GECs. The levels of Rac1 activity (but not total Rac1 levels) in the AGE-HSA (100 $\mu\text{g/ml}$) treatment group were significantly reduced compared with those in the control group ($P=0.002$), whereas HSA alone did not produce this effect (Fig. 4). These results suggested that the Rac1 signaling pathway is important in the mediation of AGE-induced functional alterations in endothelial cells.

Rac1 agonist inhibits the AGE-HSA-induced increase in GEC permeability. The permeability of GECs to FITC-BSA was significantly increased with AGE-HSA-treatment ($P<0.05$),

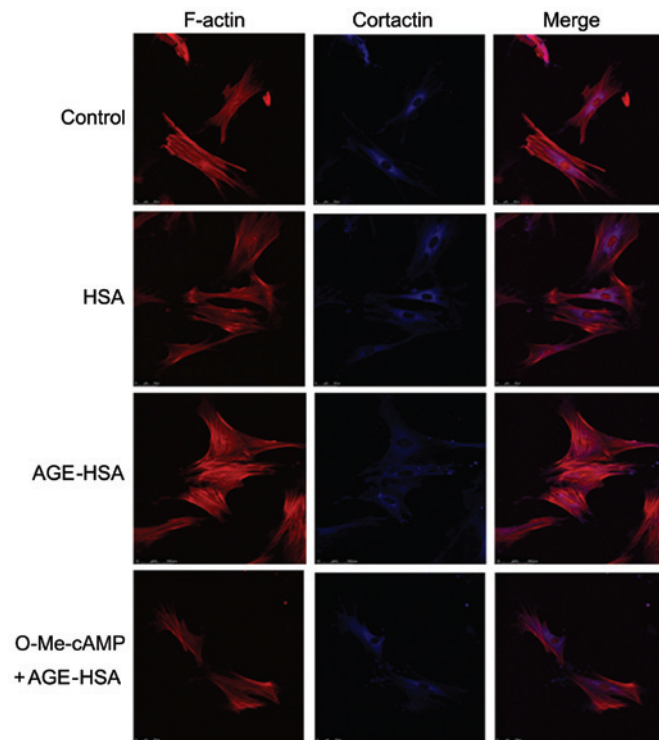


Figure 3. Effect of the Rac1 agonist O-Me-cAMP in regulating AGE-induced alterations in F-actin and cortactin morphology and distribution in endothelial cells. In the O-Me-cAMP group, cells were pre-treated with O-Me-cAMP (2 μ mol/ml) for 60 min, then treated with AGE-HSA (100 μ g/ml) for 8 h. The cells cultured in pure MCDB131 medium (control) or with HSA (100 μ g/ml) alone were designated as the controls. AGE-induced F-actin structure reconstruction, the disorganized distribution of cortactin, altered cell morphology and the formation of stress fibers in endothelial cells were all markedly inhibited by O-Me-cAMP-pre-treatment. O-Me-cAMP, 2'-O-methyladenosine-3',5'-cyclic monophosphate; AGE, advanced glycation end-products; GEC, glomerular capillary endothelial cells; HSA, human serum albumin.

but not with HSA alone ($P>0.05$). This effect was inhibited by O-Me-cAMP, as demonstrated by reduction in P_a from 189.32 ± 6.16 to $128.52\pm3.53\%$ subsequent to treatment with O-Me-cAMP (Fig. 5).

Discussion

The initial step in the development of diabetes and nephropathy is the structural and functional impairment of GECs, which induces glomerular capillary lesions and leads to disease progression (31,32). The increase in glomerular capillary permeability is considered to indicate the development of diabetes and nephropathy and leads to the development of pathological proteinuria (33). The proximal damage among cells is considered to be the basis for the increase in endothelial cell gap formation and vascular permeability (2,34-36). A number of studies have demonstrated that the alterations in cellular morphology and the distribution of cytoskeletal proteins is closely associated with the integrity of the endothelial cell-cell connections (37,38). The AGE content in diabetic patients has been observed to be significantly increased, which may induce damage to GEC structure and function (39-41). Additional studies have demonstrated that AGE increases the permeability of umbilical vein endothelial cells and induces

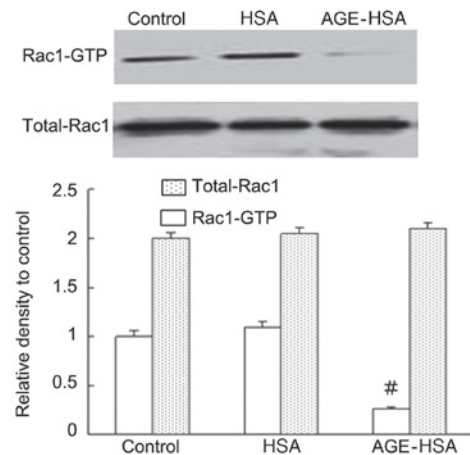


Figure 4. Effect of AGE on the levels of Rac1 activity in GECs. AGE-HSA (100 μ g/ml) treatment for 8 h significantly reduced the levels of Rac1 activity compared with controls, but did not affect the levels of total protein. [#] $P<0.05$ vs. control group. AGE, advanced glycation end-products; GEC, glomerular capillary endothelial cells; HSA, human serum albumin; GTP, guanosine triphosphate.

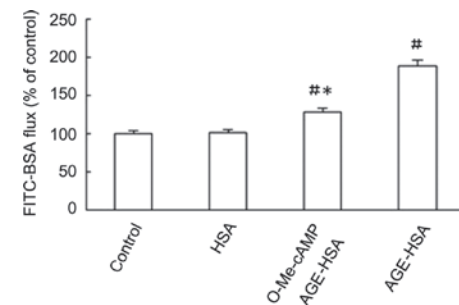


Figure 5. Effect of O-Me-cAMP on mediation of AGE-induced alterations in endothelial cell permeability. Compared with the control group, treatment with AGE-HSA (100 μ g/ml) for 8 h significantly increased permeability in endothelial cells ($P<0.05$), which was inhibited by O-Me-cAMP. [#] $P<0.05$ vs. control; ^{**} $P<0.05$ vs. AGE-HSA. O-Me-cAMP, 2'-O-methyladenosine-3',5'-cyclic monophosphate; AGE, advanced glycation end-products; GEC, glomerular capillary endothelial cells; HSA, human serum albumin; BSA, bovine serum albumin.

alterations in the distribution and morphology of cytoskeletal proteins (8,11,12,14). To mimic the pathological process of glomerular capillary endothelial damage in diabetes and nephropathic patients, primary GECs were used in the present study. The effects of AGE on the distribution and morphology of F-actin and cortactin in GECs were investigated and it was determined whether or not these processes were mediated by the Rac1 signaling pathway. Rac1 is a member of the Ras protein superfamily and belongs to the Rho family of guanosine triphosphatases (42). Rac1 has multiple functions, including controlling cellular morphology, actin movement, transcriptional activation and apoptotic signals (43-46). Rac1 has two active forms, including an active form bound to guanosine triphosphate (GTP) on the cell membrane and an inactive form bound to GTP in the cytoplasm. The two forms can change as a result of upstream stimuli, Rac 1 combining with GTP on the cell membrane is activated, whereas on the cytoplasm it is inactivated, which in turn regulate the functions of downstream effectors.

It has been demonstrated that Rac1 can be activated by specific extracellular signals, which in turn induce actin cytoskeleton-directed assembly, resulting in characteristic morphological alterations, including cell stretch (47), an increase in cortical actin polymerization (48) and an enhancement of connections between cells (10). Therefore, the Rac1 signaling pathway is suggested to be necessary for maintaining the stability of vascular endothelial cell connections. Previous studies have suggested that activation of the Rac1 signaling pathway can promote cortactin translocation to the plasma membrane and cortex, thus inhibiting cell collapse and gap-formation and strengthening cell-cell connections (49-51). The mechanism for strengthening these connections is frequently associated with the increase in cortical actin polymerization, cortical cortactin and the enhancement of binding to the cortical actin cytoskeleton. Thus, cortical actin assembly is suggested to be closely associated with cortactin.

In the present study, the observations suggested that the AGE-induced increase in GEC permeability was closely associated with inhibition of Rac1 activity. It was observed that the F-actin peripheral dense band became thicker and disorganized, the number of central stress fibers increased, the expression levels of cortactin in cell membranes were reduced, the boundaries of cells were unclear and cells retracted and deformed upon treatment with AGE in a time- and dose-dependent manner (Figs. 1 and 2). These alterations are associated with the damage to endothelial cell integrity and the increase in permeability. The pull-down assay was used to investigate Rac1 activity upon treatment with different concentrations of AGE-HSA, and it was observed that AGE-HSA was able to significantly reduce Rac1 activity ($P < 0.05$; Fig. 4). This suggested an important involvement for Rac1 in the mediation of functional alterations in GECs induced by AGE. In addition, the present study demonstrated that with O-Me-cAMP pre-treatment, AGE-induced alterations in cell morphology and stress fiber-formation (Fig. 3), in addition to increased GEC permeability, were significantly inhibited ($P < 0.05$; Fig. 5).

In conclusion, the results of the present study suggested that Rac1 signaling is important in mediating AGE-induced morphological and functional alterations in GECs. This may aid in the development of novel therapeutic targets for microvascular complications in patients with advanced diabetes. Furthermore, it was observed that O-Me-cAMP was not able to completely reverse AGE-induced alterations, including the disorganization of F-actin and cortactin distribution and morphology, and the increase in cell permeability. This suggested that in addition to the Rac1 signaling pathway, other pathways may be involved in the pathological processes induced by AGE, which require further investigation.

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