

Rutin Prevents High Glucose-Induced Renal Glomerular Endothelial Hyperpermeability by Inhibiting the ROS/Rhoa/ROCK Signaling Pathway

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Key words

- rutin
- high glucose
- renal glomerular endothelial permeability
- RhoA/ROCK
- Nrf2/ROS

Abstract

▼ Diabetic nephropathy is a progressive kidney disease caused by damage to the capillaries in the glomeruli. Endothelial dysfunction is an early sign of diabetic cardiovascular disease and may contribute to progressive diabetic nephropathy. Hyperglycemia-induced endothelial hyperpermeability is crucial to diabetic nephropathy. Rutin has beneficial effects on diabetic nephropathy, but the exact mechanisms of its protective effect remain elusive. The aim of this study was to assess the role of pretreatment with rutin in an *in vitro* model of hyperglycemia-induced barrier dysfunction in human renal glomerular endothelial cells. Human renal glomerular endothelial cells were exposed to rutin and/or hyperglycemia for 24 h. Hyperglycemia increased permeability and decreased the junction protein occludin in the cell-cell junction area and the total expression in human renal glomerular endothelial cells, whereas

rutin treatment significantly corrected these abnormalities. Furthermore, hyperglycemia-induced activation of RhoA/ROCK was reversed by treatment with rutin or the knockdown of ROCK2. Interestingly, rutin prevented hyperglycemia-induced hyperpermeability, and dysfunction of the tight junction, a high level of reactive oxygen species, and activation of RhoA/ROCK were significantly abolished with the knockdown of Nrf2. In conclusion, rutin significantly prevented hyperglycemia-disrupted renal endothelial barrier function by inhibiting the RhoA/ROCK signaling pathway through decreasing reactive oxygen species, which was mediated by the activation of Nrf2. Our results may explain, at least in part, some beneficial effects of rutin that may be applicable to the treatment of vascular disorders in diabetic nephropathy.

Supporting information available online at <http://www.thieme-connect.de/products>

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Introduction

▼ The increase in the permeability of the glomerular barrier filtration to albumin is a well-known feature of diabetic microvasculature and a negative prognostic factor for vascular complications [1]. In patients with diabetes mellitus, microalbuminuria reflects a state of systemic endothelial dysfunction, also involving glomerular capillaries [2]. Hyperglycemia (HG) can rapidly increase systemic endothelial and glomerular barrier permeabilities *in vivo*. Such changes have also been amply demonstrated in the absence of insulin in endothelial monolayers *in vitro* [3–5]. Therefore, the endothelium plays an important role in the glomerular barrier upon HG [1,6]. By increasing the glomerular permeability, HG induces reactive oxygen species (ROS) production in endothelial cells and podocytes, as well as in mesangial cells.

HG-induced vascular dysfunction can be attributed to increased oxidative stress from ROS. The redox-sensitive transcription factor, nuclear factor-E2-related factor 2 (Nrf2), is a master regulator of antioxidant genes, such as heme oxygenase-1, involved in cellular defenses against oxidative stress. Activation of Nrf2 protects endothelial dysfunction under high glucose conditions [7]. Except for endothelial cells in glomeruli, the intracellular superoxide anion and hydrogen peroxide production as well as the permeability of podocytes, which are increased after being incubated upon HG, can be reduced by elevating the activity of the Nrf2 antioxidant pathway and nuclear Nrf2 expression [8]. These findings suggest protection against the activation of the Nrf2-ARE pathway in glomerular barrier permeability exposed to HG.

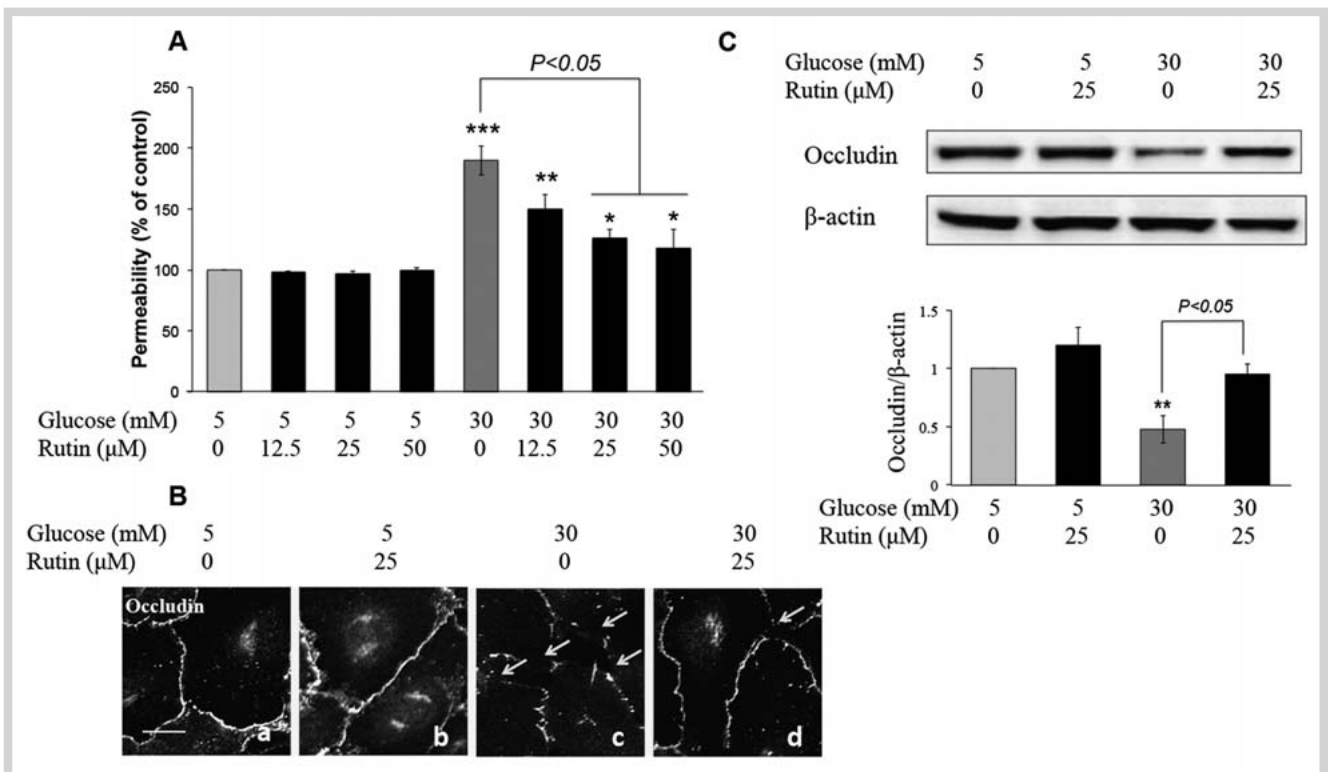


Fig. 1 Rutin prevented hyperpermeability in HG-cultured HRGECs. **A** HRGECs were cultured for 72 h to reach full confluence before the monolayer cells were treated with 5 mM glucose (NG) or 30 mM glucose (HG) and/or rutin for 24 h. Permeability assay was then performed to quantitatively determine the intercellular passage of 40 kDa weight FITC-conjugated dextran. **B** Monolayer HRGECs cells were treated with 25 µM rutin in NG or HG for 24 h. Occludin expression in intercellular junction was examined by immunofluorescence assay. Arrows indicate points where the intercellular junction was disrupted and occludin loss. Scale bar 25 µm. **C** Western blot analysis for occludin was performed. β -Actin served as the loading control. **D** Quantitative analysis was performed by measuring protein expression relative to the control. Each point represents the mean (\pm S. D.) of three independent experiments, each performed in triplicate. * $P < 0.05$ and ** $p < 0.01$ vs. NG control.

no fluorescence assay. Arrows indicate points where the intercellular junction was disrupted and occludin loss. Scale bar 25 µm. **C** Western blot analysis for occludin was performed. β -Actin served as the loading control. **D** Quantitative analysis was performed by measuring protein expression relative to the control. Each point represents the mean (\pm S. D.) of three independent experiments, each performed in triplicate. * $P < 0.05$ and ** $p < 0.01$ vs. NG control.

RhoA, a small GTPases of the Rho protein family, is a key regulator of the actin cytoskeleton [9]. Rho kinase (ROCK) is one of the first downstream targets of RhoA. RhoA/ROCK signaling regulates cell functions, including contraction [10], migration [11], adhesion [12], and cell cycle progression [13]. RhoA/ROCK signaling has recently attracted extensive attention as an important contributor to diabetic renal disease [14]. Activity of the RhoA/ROCK pathway is enhanced in vascular and renal cells in various types of kidney disorders. The factors involved in diabetes mellitus, such as high glucose, ROS, and the hexosamine pathway in vascular and renal cells, can activate the RhoA/ROCK signaling pathway [15, 16]. As a polyphenolic flavonoid, rutin has beneficial effects on renal damage by reducing oxidative stress [17, 18] with anticancer and anti-inflammatory activities as well. It has also been used to treat cardiovascular diseases and neurodegenerative disorders because of free radical-scavenging and antioxidant capacities [19]. Therefore, we hypothesized that rutin suppressed glomerular responses to HG in human renal glomerular endothelial cells (HRGECs). The aim of this study was to assess the role of rutin in an *in vitro* model of HG-induced barrier dysfunction in HRGECs.

Results

The endothelial tight junction (TJ) regulates the permeability of endothelial cells, and exposure of monolayers of cultured renal endothelial cells to HG can significantly increase the permeability

[20, 21]. In this study, HRGECs were treated with 12.5, 25, or 50 µM rutin and/or HG for 24 h. We detected HG-induced endothelial hyperpermeability by measuring the passage of FITC-dextran across the endothelium. As shown in **Fig. 1**, 24 h after incubation with HG, the endothelial permeability is obviously enhanced compared with that of cells treated with normal glucose (NG). However, treatment with rutin inhibited HG-induced endothelial barrier dysfunction (**Fig. 1A**). Immunofluorescence staining and Western blotting (**Fig. 1B, C**) showed that 24 h of HG treatment significantly downregulated occludin expression in HRGECs. Furthermore, less occludin was distributed in the cell-cell junction area. Interestingly, the abnormal distribution and decreased expression of occludin were significantly reversed by treatment with rutin (**Fig. 1B, C**). Taken together, HG disrupted renal endothelial barrier function and rutin blocked this response in cultured HRGECs.

Small GTPases, especially RhoA and its downstream effector ROCK, regulated TJ formation, and HG can induce TJ dysfunction of glomerular endothelial cells through RhoA/ROCK signaling [5, 22]. **Fig. 2** indicates that HG-treated HRGECs have significantly increased RhoA binding to GTP compared with that of NG-treated cells. ROCK was also activated because the phosphorylation rate of MYPT1 (a downstream effector of ROCK) was significantly increased by HG treatment (**Fig. 2B**). We then used siRNA to knockdown ROCK1 or ROCK2 or the inhibition of ROCK by using fasudil (ROCK inhibitor) in HRGECs. The results indicated that ROCK inhibition did not influence the expression of occludin

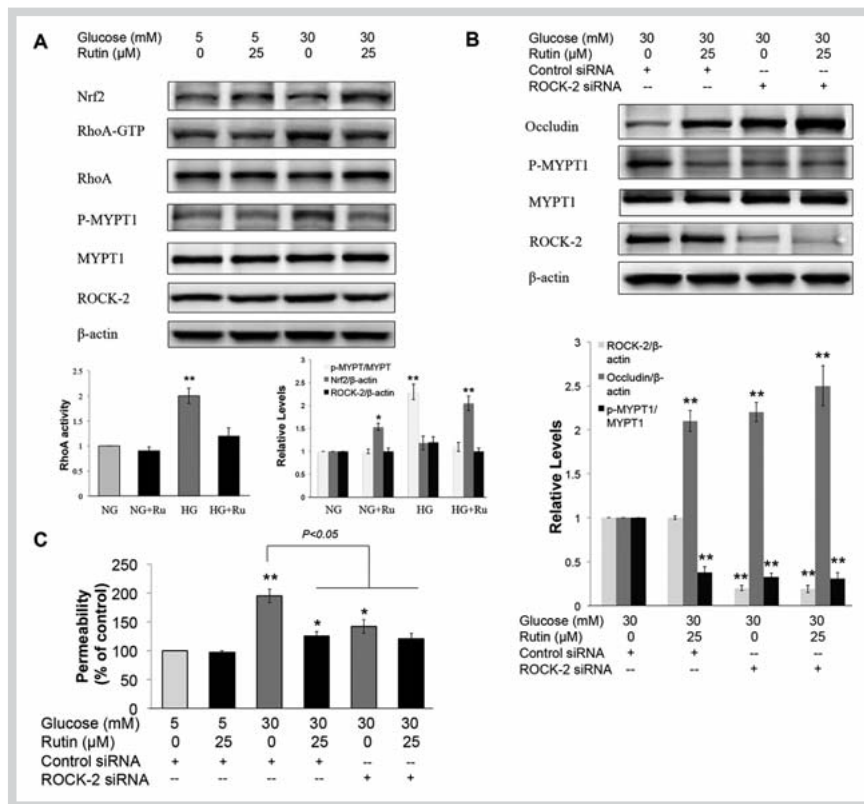


Fig. 2 Rutin prevented HG-induced hyperpermeability by inhibiting RhoA activation. Normal HRGECs (A) or Nrf2 knockdown HRGECs (B) were incubated with 5 mM glucose (NG) or 30 mM glucose (HG) and/or rutin for 24 h. RhoA activity was assessed by the pull-down assay, as described in Materials and Methods. ROCK activity was assessed by measuring the p-MYPT1 (Thr853)/MYPT1 ratio in HRGECs. The protein levels of Nrf2, ROCK-2, and occludin were analyzed by Western blot and (C) permeability assays were performed. * $P < 0.05$ and ** $p < 0.01$ vs. NG control.

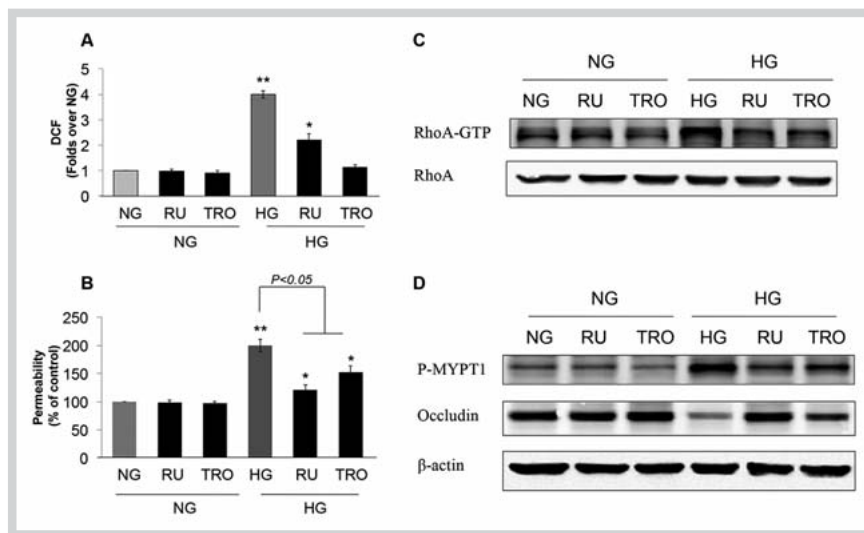


Fig. 3 Rutin inhibited RhoA/ROCK signaling by reducing ROS. A HRGECs were treated for 24 h with rutin, high glucose, or Trolox and loaded with DCF for 30 min. Then ROS signals were detected by flow cytometry. B HRGECs were cultured for 72 h to reach full confluence before the monolayer cells were treated with rutin, high glucose, or Trolox for 24 h. A permeability assay was then performed to quantitatively determine the intercellular passage of 40 kDa FITC-conjugated dextran. C Active GTP-RhoA was assessed by a pull-down assay after treatment with rutin or Trolox in high glucose-treated HRGECs. D Activation of ROCK (phospho-MYPT) and TJ protein (occludin) was assessed by Western blot. * $P < 0.05$ and ** $p < 0.01$ vs. NG control.

under NG conditions, but in the presence of HG, knockdown of ROCK or inhibition of ROCK activity, especially knockdown ROCK2, significantly increased occludin expression and decreased permeability (Fig. 2C and Fig. S1, Supporting Information). Moreover, in the presence of HG, treatment with rutin inhibited the activation of RhoA/ROCK, increased occludin expression, and decreased permeability. Therefore, rutin prevented HG-induced hyperpermeability through the inhibition of RhoA/ROCK signaling.

Accumulating evidence has indicated that rutin was able to prevent vascular damage by exerting a direct antioxidant effect [23–25]. In this study, we evaluated the inhibitory effect of rutin on HG-induced ROS generation in HRGECs. Fig. 3A shows that HG

treatment led to significant ROS generation in HRGECs and rutin effectively abrogates HG-induced ROS generation. Trolox, a ROS scavenger, was used as a positive control (Fig. 3A). Thus, we assessed the effects of Trolox (1 mM) on RhoA activation. As shown in Fig. 3C, Trolox blocks HG-induced RhoA activation. However, Trolox did not affect the basal activity of RhoA or ROCK in HRGECs cultured in NG ($p < 0.05$; Fig. 3C). Furthermore, Trolox also blocked HG-induced hyperpermeability in HRGECs (Fig. 3B, D). Thus, rutin inhibited RhoA/ROCK signaling by resisting oxidative stress.

Activation of the Nrf2 pathway attenuates HG-mediated high glucose-induced hyperpermeability by attenuating ROS production [8,26]. To clarify whether rutin prevented HG-disrupted renal

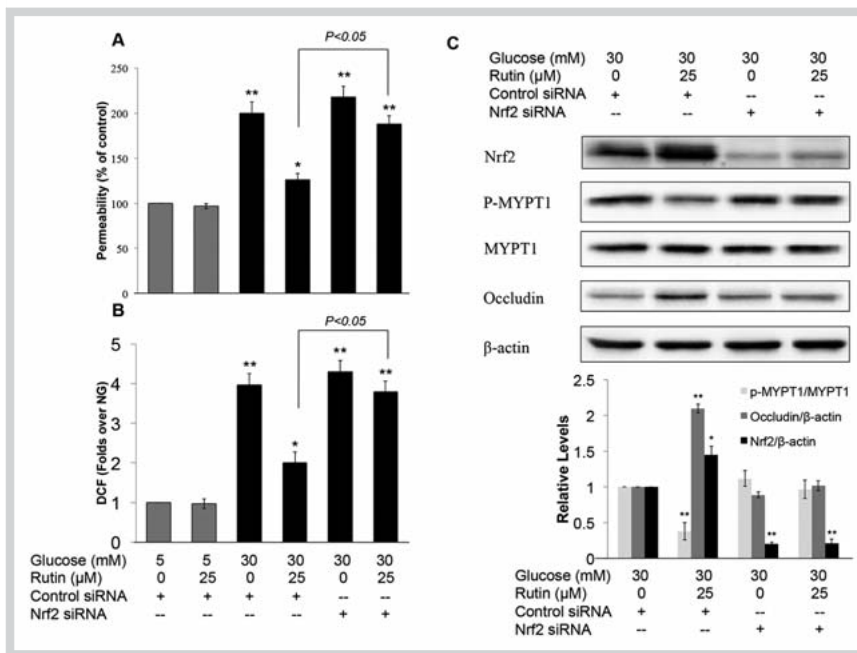


Fig. 4 Knockdown Nrf2 inhibited the preventive effects of rutin on HG-induced endothelial permeability. **A** Monolayer HRGECs cells were transformed with Nrf2 siRNA for 48 h, then treated with rutin and exposed to 5 mM glucose (NG) or 30 mM glucose (HG) for 24 h. A permeability assay was then performed to quantitatively determine the intercellular passage of 40 kDa FITC-conjugated dextran. **B** ROS levels of HRGECs were measured using flow cytometry. **C** Western blot analyses for Nrf2, occludin, and p-MYPT1/MYPT were performed. β -Actin served as the loading control. Each point represents the mean (\pm S. D.) of three independent experiments, each performed in triplicate. * $P < 0.05$ and ** $p < 0.01$ vs. control.

endothelial barrier function via the Nrf2 signaling pathway, HRGECs were transformed with Nrf2 siRNA for 48 h. Rutin significantly increase nrf2 expression in HG and NG conditions (● Fig. 2A). ● Fig. 4 indicates that knockdown of Nrf2 significantly inhibits the preventive effects of rutin on HG-induced hyperpermeability (● Fig. 4A), ROS increase (● Fig. 4B), activation of RhoA/ROCK, and occludin expression deficiency (● Fig. 4C). Thus, rutin prevented HG-disrupted renal endothelial barrier function by activating Nrf2, which was mediated by decreasing ROS and then deactivating RhoA/ROCK.

Discussion

The glomerular filtration barrier is a composite multilayered structure, and injury in one layer may spread to others and affect the whole function of the barrier [27]. It is well documented that endothelial glycocalyx plays a significant role in the glomerular filtration barrier [27,28]. Dysfunction of the endothelial TJ is a crucial step in the development of endothelial hyperpermeability that contributes to progressive DN [5]. HG is the main cause of all types of diabetic microvascular diseases. In addition, endothelial cell permeability is impaired by high concentrations of extracellular glucose upon diabetes mellitus [29,30]. It has previously been reported that rutin relieved renal damage by reducing oxidative stress [17,18]. However, the underlying mechanisms are still largely unexplored. In this study, HRGECs were exposed to rutin and/or HG for 24 h. HG significantly increased the permeability of HRGECs. Moreover, the distribution of the junction protein occludin in the cell-cell junction area and the total expression in HRGECs were significantly decreased by HG treatment. However, treatment with rutin significantly reversed HG-induced endothelial dysfunctions, demonstrating that it prevented HG-induced endothelial permeability.

Activation of RhoA/ROCK signaling by HG disrupted the expression and translocation of occludin/ZO-1 and resulted in hyperpermeability [5]. In the presence of HG, treatment with rutin or knockdown of ROCK2 inhibited such activation, increased occlu-

din expression, and decreased permeability. Therefore, rutin prevented HG-induced hyperpermeability through the inhibition of RhoA/ROCK signaling.

Oxidative stress is the most common disturbance resulting from HG. ROS generation is of particular importance in the alteration of endothelial permeability [2,26], and Nrf2 predominantly resists oxidative stress by decreasing ROS levels. Mounting evidence has indicated the potential role of Nrf2 in the protection and adaptation against HG-induced oxidative stress and inflammation [8,26]. Activation of the Nrf2 pathway attenuates HG-mediated high glucose-induced hyperpermeability by attenuating ROS production [8,26]. High glucose, ROS, and the hexosamine pathway in vascular and renal cells have been demonstrated to activate the RhoA/ROCK signaling pathway [15,16]. Rutin herein prevented HG-induced hyperpermeability, and junction protein defects, activation of the RhoA/ROCK signaling pathway, and ROS were significantly abolished with the knockdown of Nrf2. Collectively, rutin managed to prevent HG-disrupted renal endothelial barrier function by inhibiting the RhoA/ROCK signaling pathway, which was mediated by decreasing ROS.

In conclusion, the ROS/RhoA/ROCK/occludin signaling pathway was involved in HG-induced renal endothelial hyperpermeability. Since rutin inhibited the ROS/RhoA/ROCK signaling pathway by activating Nrf2, this strategy may be applicable to the treatment of vascular disorders in DN.

Material and Methods

Chemicals

Rutin ($\geq 94\%$ purity), Trolox, and DMSO were acquired from Sigma. Rutin was dissolved in DMSO to form a 100-mM solution. Nrf2, Keap1, occludin, phospho-MYPT1/MYPT1, β -actin, anti-rabbit IgG (H + L) 488, and goat anti-rabbit IgG HRP-conjugated antibodies were all purchased from Cell Signaling Technology. Control siRNA, ROCK-2 siRNA, and Nrf2 siRNA were all purchased from Santa Cruz Biotechnology.

Cell culture and transfection

HRGECs and CS-C complete medium (including 10% serum) or serum-free CS-C medium were purchased from Cell Systems. HRGECs were cultured in CS-C complete medium and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin). The cells were cultured in humidified air at 37 °C with 5% CO₂, and then transfected with the indicated siRNA using Lipofectamine LTX (Life Technologies, Cat No. 15338100) according to the manufacturer's protocol.

Fluorescein isothiocyanate-dextran transwell assay

Permeability across the HRGECs monolayer was studied in an *in vitro* vascular permeability system (Cat. No. ECM642), which has a 0.4-µm pore size. HRGEC monolayers were plated on the insert of the transwell and cultured until confluence. After treatment, the same amount of FITC-dextran tracer (40 kDa, final concentration 20 µg/mL) was added into the upper chamber and incubated for another 20 min at room temperature. The amount of tracer that penetrated the cell monolayer into the lower chamber was measured with the use of a fluorometer (Molecular Devices; excitation wavelength, 485 nm; emission wavelength, 530 nm) as the index of monolayer permeability of endothelial cells. The results are presented as fold change after correction with a blank control of NG. The data represent the means of three experiments.

Immunofluorescence staining

HRGECs were allowed to grow to confluence on fibronectin-coated glass chamber slides. After treatment, the medium was aspirated and the monolayers were washed with PBS, fixed with 4% paraformaldehyde, and washed three times with PBS for 10 min. Immunofluorescence staining was performed with a primary antibody against human occludin at a dilution of 1 : 500 overnight at 4 °C. The slides were photographed using an Olympus LCX100 Imaging System with an excitation wavelength of 546 nm.

Reactive oxygen species determination

The levels of cellular superoxide were assessed by using DCFDA (Invitrogen) according to the manufacturer's instructions. Briefly, HRGECs were washed with phosphate-buffered saline and labeled at 37 °C for 30 min in Hank's balanced salt solution (Gibco) containing 10 mM DCFDA. Then the cells were trypsinized and resuspended in Hank's balanced salt solution. Fluorescence was measured by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Western blot analysis

After incubation with HG and/or rutin, 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) and electrophoresed, and the proteins were transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in 5% nonfat milk at room temperature for 1 h, followed by overnight incubation at 4 °C with primary antibodies with constant rotation. After incubation with secondary antibodies for 1 h, proteins were detected using an enhanced ECL system (GE Healthcare). Semi-quantifications were performed according to densitometric analysis in conjunction with the Quantity One software package. The signal of each band was normalized to that of actin in the same sample.

Small GTPase activity (small GTPase GTP-loading) assay

Small GTPase GTP-loading assays were performed using the RhoA Activation Assay Biochem Kit (bead pull-down format) (Cytoskeleton). Briefly, the cell lysates were collected after treating HRGECs with HG and/or rutin. The GTP-bound small GTPases were captured by immobilized GST-tagged Rhotekin-RBD domain (Rho). After the beads were washed three times, the GTP-bound small GTPases were released by boiling with Laemmli sample buffer at 95 °C for 3–5 min. Following standard Western blotting protocols as mentioned above, the GTP content in small GTPases was determined as the amount of Rho pulled down. GTP loading was expressed as the amount of GTP-bound small GTPase relative to that of total GTPase.

Statistical analysis

All experiments were repeated at least three times. All data are presented as means and standard deviations. Differences between the hyperpermeability of the control and treatment groups were determined with an independent samples t-test. The differences between groups were tested with a one-way ANOVA and Fisher's post hoc least significant difference test. All statistical tests were two-sided and a p value < 0.05 was considered significant. All statistical analyses were performed with SPSS version 15.0 (SPSS).

Supporting information

Knockdown or inhibition of ROCK prevented HG-induced hyperpermeability.

Conflict of Interest

▼
We declare no conflict of interest.

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