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

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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

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.
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, C). 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.

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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
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 occludin 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 (≥ 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.
**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.

**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.

**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).

**Conflict of Interest**

We declare no conflict of interest.

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