Advanced glycation end products increase endothelial permeability through the RAGE/Rho signaling pathway

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ABSTRACT

Although increased vascular permeability is known to be a major characteristic of diabetic vasculopathy, the precise mechanisms and relevance of advanced glycation end products (AGE) to hyperpermeability of vessels remains unclear. Here we studied changes in cytoskeletal configuration and the signaling mechanism induced by AGE in human endothelial cells. AGE-BSA stimulation induced Rho activation, intercellular gap formation, prominent actin stress fiber and cell contraction without changing VE-cadherin, and subsequently transendothelial diffusion of FITC-labeled dextran. These processes induced by AGE-BSA were inhibited by either Rho-kinase inhibitor Y27632 or anti-RAGE antibody. We also showed that RhoA and RAGE spontaneously formed a complex. These findings suggest that activation of RAGE/Rho is involved in AGE-BSA-induced hyperpermeability through gap formation and actin reorganization in diabetes.

1. Introduction

Diabetic mellitus is a chronic progressive disease associated with serious complication including various vasculopathies. Increased capillary permeability occurs in the early stages of diabetes before the onset of structural angiopathy and is known to be a major characteristic of diabetic vasculopathy [1–4]. However, little is known about the intracellular signals that propagate these endothelial structural changes and lead to hyperpermeability during pathological processes of diabetes.

Endothelial structural changes, weak or loss of adherence junction, cell contraction, and focal adhesion redistribution are considered involved in hyperpermeability induced by various insults such as inflammatory stimuli [5]. Previous studies also reported the disorganization of tight junction structures in diabetic retina and cerebral microvessels [6], suggesting that these abnormalities could explain diabetic hyperpermeability. Another studies indicated that high glucose induces actin stress fiber formation in endothelial cells [7] and that microvascular leakage in the retinal microvasculature is enhanced in STZ-induced diabetic rats with high levels of F-actin stress fibers [8], suggesting a relation between hyperglycemia and cytoskeletal contraction, which leads to hyperpermeability.

Accumulated evidence indicates that advanced glycation end products (AGEs) increase at an accelerated rate in diabetes and in parallel with the severity of diabetic complications [9]. AGEs activate RAGEs, a major receptor for AGEs, modulate various cell functions and play a role in vasculopathies through multiple intracellular signal transduction pathways, including p21ras, MAP kinases, P3 kinase and many [4,10]. However, the relationship AGE with the receptor and the following signaling pathways was unclear. In this study to clarify the signaling mechanism that lead to papillary hyperpermeability in diabetes, we investigated changes in cytoskeletal configuration induced by AGE and the role of the RAGE/Rho signaling pathway in AGE-mediated microvascular hyperpermeability using human vascular endothelial cells in vitro.

2. Materials and methods

2.1. Antibodies and materials

Mouse monoclonal antibodies (mAb) to human VE-cadherin and human RAGE were purchased from R&D Systems (Minneapolis, MN).
Mouse mAb to human RhoA was from Santa Cruz Biotechnology (Santa Cruz, CA), mouse mAb to human β-actin was from Sigma (St. Louis, MO), and horseradish peroxidase-conjugated horse anti-mouse secondary antibody was from Amersham Biosciences (Arlington Heights, IL). Rhodamine–phalloidin and Alexa Fluor 488-conjugated goat anti-mouse secondary antibody were purchased from Invitrogen (Carlsbad, CA). AGE-bovine serum albumin (BSA) was purchased from BioVision (Mountain View, CA); BSA was from Sigma. Rho-kinase inhibitor Y27632 was obtained from Biomol (Plymouth Meeting, PA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex (Walkersville, MD) and cultured in EGM-2 (Lonza) complete medium containing 2% (v/v) fetal bovine serum (FBS), multiple recombinant human growth factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin growth factor-1 (IGF1), and epidermal growth factor, as well as hydrocortisone, heparin, and ascorbic acid. Experiments were performed using cells up to passage 3. Where appropriate, cells were starved in serum-free basal EBM-2 (Lonza) medium prior to treatment.

2.3. Endothelial monolayer permeability assay

HUVECs were plated in 24-well Transwell units (with polycarbonate membrane, 8-μm pores; Nunc) pre-coated with fibronectin (2.5 μg/well) and cultured to confluence in EGM-2. At the start of the experiment, the culture medium in the lower and upper compartments was replaced with EBM-2 containing AGE-BSA or control BSA. After incubation, FITC-labeled dextran (initial concentration 400 μg/ml) was added to the upper compartment. After 1 h of additional incubation at 37°C, the medium in the lower compartments was collected and analyzed in a fluorescence detector using 485 nm and 538 nm as the excitation and emission wavelengths, respectively.

2.4. Fluorescent staining of F-actin and VE-cadherin

HUVECs were plated in glass-bottom 35-mm dishes pre-coated with fibronectin (2.5 μg/well) and cultured to confluence in EGM-2. At the start of the experiment, the culture medium was replaced with EBM-2 containing AGE-BSA or control BSA. After incubation, the cells were fixed in 3.7% formaldehyde solution for 3 min and permeabilized with 0.1% Triton X-100 for 3 min. They were then incubated with rhodamine–phalloidin for 20 min in room temperature to stain F-actin. For staining VE-cadherin, cells were fixed in 3.7% formaldehyde solution for 10 min then incubated with mAbs against VE-cadherin at room temperature for 1 h, followed by Alexa Fluor 488-conjugated secondary antibody at room temperature in the dark for 1 h. Fluorescence was detected with a Zeiss Axiopt microscope.

2.5. Western blotting

HUVECs were cultured in cell culture dishes to confluence. Cells were starved in serum-free medium for 6 h and then exposed to AGE-BSA (50 μg/ml) or control BSA (50 μg/ml) for different times as indicated. Protein concentration was determined with Bradford reaction.

2.6. Rho activation assay

Rho activation was determined by a pull-down assay using GST-Rhotekin-Rho-binding domain (GST-RBD). HUVEC were cultured in cell culture dishes to confluence. Cells were starved in serum-free medium for 6 h and then exposed to AGE-BSA (50 μg/ml) or BSA (50 μg/ml) for different times as indicated, quickly washed with ice-cold Tris-buffered saline, and lysed in 500 μl of lysis buffer. Cell lysates were immediately centrifuged at 8000 rpm at 4°C for 5 min and equal volumes of lysates were incubated with 30 μg GST-RBD beads for 1 h at 4°C. The beads were washed with wash buffer, and bound Rho was eluted by boiling each sample in Laemmli sample buffer. Eluted samples from the beads and total cell lysate were then electrophoresed on 15% SDS–polyacrylamide gel electrophoresis gels, transferred to nitrocellulose, blocked with 5% non-fat milk, and analyzed by Western blotting using a polyclonal anti-Rho antibody.

2.7. Immunoprecipitation

Immunoprecipitations were carried out utilizing RhoA or RAGE antibodies. HUVEC were cultured in cell dishes to confluence. Cells were starved in serum-free medium for 6 h and then exposed to AGE-BSA (50 μg/ml) or serum-free medium only for 10 min, quickly washed with ice-cold Tris-buffered saline, and lysed in 500 μl of lysis buffer. Cell lysates were immediately centrifuged at 8000 rpm at 4°C for 5 min. The supernatants were incubated end-over-end for 60 min at 4°C with the mixture with Protein G sepharose beads, which were incubated end-over-end for 30 min at 4°C with anti-RAGE antibody (5 μg/ml) or anti-Rho antibody (5 μg/ml). The beads were washed three times with PBS-T, then treated with elution buffer (2.5% acetic acid), and the eluted immunoprecipitated samples were resuspended in Laemmli buffer and heated at 100°C for 3 min, and analyzed by SDS–PAGE, transferred to nitrocellulose, blocked with 5% non-fat milk, and analyzed by Western blotting using a monoclonal anti-Rho antibody or anti-RAGE antibody.

2.8. Statistical analysis

All data were obtained from at least five independent experiments performed in triplicate. Results were expressed as means ± S.D. Analyses of differences were carried out by ANOVA followed by posthoc Student Newman Keuls test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. AGE-BSA increases endothelial permeability

First, we investigated the effect of AGEs on permeability of human endothelial cells by observing diffusion of dextran in HUVEC. AGE-BSA increased transendothelial diffusion of FITC-labeled dextran in a time-dependent and a dose-dependent manner, whereas unmodified BSA had no effect on the permeability (Fig. 1A and B). These findings suggest AGE-BSA have a potential to induce endothelial permeability and disturb the physiological barrier of capillary endothelium.

3.2. AGE-BSA causes intercellular gap formation and reorganization of actin cytoskeleton

To test whether AGEs-induced changes in permeability are due to morphological changes in the endothelial cells, we stained actin and VE-cadherin in endothelial cells treated with or without AGE-BSA. While control cells and cells treated with BSA showed cobblestone morphology without intercellular gap, endothelial cells treated with AGE-BSA showed intercellular gap formation (Fig. 2A-a). Next, we focused on the changes in the cytoskeleton. Although
control cells and cells treated with BSA showed peripheral actin ring, but lacked with stress fibers, the cells treated with AGE-BSA showed prominent actin stress fiber and cell contraction (Fig. 2A-b). We also examined the protein level of VE-cadherin in HUVECs by Western blotting, but treatment with AGE-BSA for the indicated time periods did not modify the level of VE-cadherin (Fig. 2B).

3.3. AGE-BSA activates Rho and RhoA forms a complex with RAGE on HUVECs

To investigate whether these actin rearrangements induced by AGE-BSA are due to activation of Rho/Rho kinase, we pretreated the cells with the Rho kinase inhibitor Y27632 (1 h, 10 μM) and then stimulated the cells with AGE-BSA. As shown in Fig. 4A, Y27632 completely blocked the AGE-BSA-induced gap formation with actin reorganization. To investigate whether these changes induced by AGE-BSA are mediated through AGE–RAGE interaction, we pretreated cells with anti-RAGE antibody for 1 h and then stimulated the cells with AGE-BSA. Anti-RAGE antibody blocked AGE-BSA induced gap formation with actin reorganization. Y27632 or anti-RAGE antibody also blocked the AGE-BSA induced increase in endothelial cell permeability. These findings suggest that activation of RAGE/Rho is involved in AGE-BSA induced hyperpermeability through gap formation and actin reorganization.

4. Discussion

Previous studies proposed various mechanisms for the increased microvascular permeability in diabetic mellitus. Endothelial structural changes, weak or loss of adherence junction, cell contraction, and focal adhesion redistribution are considered involved in hyperpermeability induced by various insults such as inflammatory stimuli [5]. However, apart from these endothelial structural changes, little is known about the intracellular signals that propagate these endothelial structural changes and lead to hyperpermeability. Our study focused on the effect of AGES on vascular hyperpermeability. AGESs are thought to modulate various cell functions and play a role in vasculopathies and found the following a sequence of results; RhoA and RAGE spontaneously formed a complex, AGE-BSA induced Rho activation, intercellular gap formation, reorganization of actin stress fiber and cell contraction, AGE-BSA induced transendothelial diffusion of FITC-labeled dextran implying its hyperpermeability. However, our results showed no changes in VE-cadherin.

It is well known that activation of RAGEs by AGES transduces multiple intracellular signal transduction pathways, including p21ras, MAP kinases, PI3 kinase, NAD(P)H oxidase, and results in activation of nuclear factor-kappaB (NF-κB) [10]. Another study also reported that AGESs induced hyperpermeability through actin rearrangement via ERK and p38 MAPK pathways [4]. However the relationship with the receptor of AGE and these signaling pathways was unclear. On the other hand, little is known about AGESs and small G protein. Although it was reported that activation of RAGEs by AGES induced neurite outgrowth through the cdc42/Rac pathway [12], the relation between actin polymerization induced by AGESs and Rho signaling is not clear. In this study, we investigated the role of the Rho signaling pathway in AGE-mediated microvascular hyperpermeability. Rho is a major small GTP-binding protein and acts a molecular switch that controls a large variety of signal transduction pathways, many of which regulate actin cytoskeleton remodeling in various types of cells. Rho relays extracellular signals to a large number of downstream effectors. Therefore, Rho is a very important signaling element that cooperates with other small GTP-proteins to regulate various cell...
Fig. 2. AGE-BSA induced intercellular gap formation with reorganization of actin cytoskeleton, but no changes in total cellular level of VE-cadherin. (A) HUVECs grown on fibronectin-coated glass-bottom dishes were treated with 50 μg/ml AGE-BSA or BSA. After 6 h, cells were fixed and processed for immunofluorescence analysis of VE-cadherin (a) and actin (b). HUVEC treated with AGE-BSA showed gap formation and reorganization of the actin cytoskeleton. Untreated control cells or HUVEC treated with BSA showed no gap formation or stress fiber. (B) HUVECs were treated for the indicated time periods with 50 μg/ml AGE-BSA. After washing, cells were extracted with lysis buffer. Immunoprecipitated VE-cadherin and actin were detected using specific antibodies. Treatment with AGE-BSA for the indicated time periods did not modify the level of VE-cadherin protein. Representative results of five experiments performed with similar results.

Fig. 3. AGE-BSA activated Rho and RhoA forms a complex with RAGE on HUVECs. (A) HUVECs were treated with 50 μg/ml AGE-BSA (a) or BSA (b) as indicated time. After washing, cells were extracted with lysis buffer. Cell lysates were incubated with GST-RBD beads and RBD-bound Rho were eluted and immunoprecipitated with polyclonal anti-Rho antibody. AGE-BSA increased Rho-GTP level. (B) HUVECs were treated with or without 50 μg/ml AGE-BSA for 10 min. Lysates were immunoprecipitated with anti-RAGE monoclonal antibody (a), or anti-RhoA monoclonal antibody (b). Immunoprecipitates were subjected to Western blot analysis using anti-RAGE and anti-Rho monoclonal antibodies to confirm adequate immunoprecipitation. Representative results of five experiments performed with similar results.
functions [13]. Our study showed that AGEs activates RhoA in endothelial cells, leading to increased hyperpermeability through actin polymerization.

RAGE is a member of the immunoglobulin superfamily of receptors [14]. Receptor for IL-1β is also one of the immunoglobulin superfamily of receptors and the Rho family is a component of the IL-1 receptor complex [15]. In our study, RAGE and RhoA also formed a complex with or without stimulation with AGE. Because RhoA and RAGE spontaneously form a complex, it is likely that AGE directly activates RhoA. However, further studies including animal experiments are necessary to clarify the mechanism of RhoA activation by AGE-BSA.

In summary, our results demonstrated that AGE-BSA induces reorganization of the actin cytoskeleton through RAGE/Rho activation, leading to endothelial cell hyperpermeability.

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References


