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The inhibition of advanced glycation end-products-induced retinal vascular permeability by silver nanoparticles

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ABSTRACT

The increased permeability of the blood–retinal barrier is known to occur in patients with diabetes, and this defect contributes to retinal edema. This study aimed to determine the effects of silver nanoparticles (Ag-NPs) on advanced glycation end-products (AGEs)-induced endothelial cell permeability. Cultured porcine retinal endothelial cells (PRECs) were exposed to AGE-modified bovine serum albumin (AGE-BSA) and the endothelial cell permeability was detected by measuring the flux of RITC-dextran across the PREC monolayers. We found that AGE-BSA increased the dextran flux across a PREC monolayer and Ag-NPs blocked the solute flux induced by AGE-BSA. In order to understand the underlying signaling mechanism of Ag-NPs on the inhibitory effect of AGE-BSA-induced permeability, we demonstrated that Ag-NPs could inhibit the AGE-BSA-induced permeability via Src kinase pathway. AGE-BSA also increased the PREC permeability by stimulating the expression of intracellular adhesion molecule-1 (ICAM-1) and decreased the expression of occludin and ZO-1. Further, Ag-NPs inhibited the AGE-BSA-induced permeability by increased expression of tight junction proteins occludin and ZO-1, co-incident with an increase in barrier properties of endothelial monolayer. Together, our results indicate that Ag-NPs could possibly act as potent anti-permeability molecule by targeting the Src signaling pathway and tight junction proteins and it offers potential targets to inhibit the ocular related diseases.

1. Introduction

Diabetic retinopathy is one of the significant microvascular complications in diabetes and is a leading cause of acquired blindness among people of occupational age [1]. Chronic hyperglycemia is a major initiator of diabetic retinopathy. Advanced glycation end-products (AGEs), the senescent macroprotein derivatives, whose formation and accumulation occur at an accelerated rate in diabetes [2] have been strongly implicated in the pathogenesis of diabetic vascular complications [3]. Recent studies revealed that AGEs and their receptor RAGE interaction play a central role in the pathogenesis of diabetic vascular complications [4]. AGEs are known to modify proteins, such as bovine serum albumin (BSA) and collagen at lysine and/or arginine residues and stimulate the growth of microvascular endothelial cells by an autocrine induction of vascular endothelial growth factor (VEGF) leading to angiogenesis [5]. Clinical trials have demonstrated that VEGF plays a central role in several retinal vascular diseases [6].

AGEs can induce a range of pathogenic effects in retinal microvascular endothelium *in vitro*, many of which are mediated through AGE-receptors [7]. However in *in vivo* systems, the role of AGEs in diabetic retinopathy continues to remain equivocal. AGEs are known to accumulate in the neural retina and vascular cells of diabetic animals [8] where they appear to initiate pathophysiological changes in retinal microvascular function [9]. In fact, VEGF antagonists have been successfully used to reduce retinal/macular edema in neovascular eye diseases such as age-related macular degeneration with stabilization or even improvement of visual acuity in a subset of affected patients [10]. A critical early event in the pathogenesis of diabetic retinopathy is leukocyte adhesion to the diabetic retinal vasculature. The process is mediated in part by upregulation of intracellular adhesion molecule-1(ICAM-1) in the retinal microvascular endothelium [11] and contributes to blood–retinal barrier

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breakdown and capillary non-perfusion [12]. In one of the previous reports, the role of leukocyte adhesion in the production of retinal disease was illustrated, where diabetic retinopathy is now a recognized inflammatory disease. Leukocyte adhesion results in change in the functional state of the endothelium, affects surface protein expression, secretory function, permeability to macromolecules and leukocyte transmigration. These responses are also associated with the intracellular signaling, including cytoskeletal modifications, protein phosphorylation and calcium influx [13]. Both intracellular adhesion molecule-1(ICAM-1) and vascular cell adhesion molecule-1(VCAM-1) are barely detectable in normal brain cells, but their expressions are elevated on endothelial and glial cells during inflammatory conditions such as stroke. ICAM-1 cross-linking on rat brain endothelium cell lines (RBE4 and GP8 cells) induces activation of the tyrosine kinase $p60^{SRC}$ [13] and an associated phosphorylation of the cytoskeletal-associated proteins focal adhesion kinase, paxillin and $p130_{cas}$ [14]. More recently it was also reported that ICAM-1 cross-linking induces calcium signaling, which via PKCs, mediate the phosphorylation of actin-associated proteins and cytoskeleton rearrangement in brain endothelial cell lines. These calcium-mediated intracellular events are necessary for lymphocyte migration through the blood–brain barrier [15].

Endothelium is an important target for drug and gene therapy. The vascular endothelial monolayer forms a semi-selective permeability barrier between blood and the interstitial space to control the movement of blood fluid, proteins, and macromolecules across the vessel wall. Alteration of permeability barrier integrity plays a major role in drug-based therapies, as well as the pathogenesis of cardiovascular diseases, inflammation, acute lung injury syndromes, and carcinogenesis [16]. The tight junction (TJ) is essential to establish the tight barrier between endothelial cells in the retinal and brain blood vessels in order to maintain the proper environment for neuronal function [17]. Breakdown of this blood–retinal barrier (BRB) is a major causative factor in diabetic retinopathy (DR) and correlates directly with the macular edema leading to vision loss [18]. Therefore, understanding this TJ complex and how it is regulated is essential to develop new therapies for DR. Understanding the mechanism by which silver nanoparticles induces endothelial barrier properties may lead to potential therapies to reintroduce the BRB with reduced side effects.

In the present study, we have investigated the molecular mechanism of silver nanoparticles on AGE-BSA-induced endothelial cell permeability. The effect of silver nanoparticles on AGE-BSA-induced permeability in porcine retinal endothelial cells via Src dependent pathway was also studied. In addition to this, the role of Ag-NPs on the modulation of adhesion molecules and tight junction proteins was also investigated. The results obtained from this study may provide novel insights for understanding the molecular mechanism of silver nanoparticle in AGE-BSA-induced vascular permeability in general and this elucidation might lead to the emergence of silver nanoparticle as a potential therapeutic molecule to inhibit the ocular diseases such as diabetic retinopathy.

2. Materials and methods

2.1. Biosynthesis of Ag-NPs

In a typical experiment, 2 g of wet *Bacillus licheniformis* biomass was taken in an Erlenmeyer's flask. 1 mM $AgNO_3$ solution was prepared using deionized water and 100 ml of the solution mixture was added to the biomass. Then the conical flask was kept in a shaker at 37 °C (200 rpm) for 24 h for the synthesis of nanoparticles [19].

2.2. Purification of nanoparticles

The purification of Ag-NPs was performed as described earlier [20]. The biomass was removed by sonication and centrifugation (4, 000*g* for 10 min) leaving the particles suspended in the supernatant. The particles were washed five times by centrifugation and re-dispersed in water to remove excess of silver. They were then transferred to a dialysis tube with a 12,000 Da molecular weight cutoff. Nanoparticles were resuspended in 1 ml of HEPES buffer (20 mM, pH 7.4) supplemented with sucrose to reach a density of 2.5 g/ml and a gradient was made according to method described earlier [21]. The solution was placed at the bottom of a centrifuge tube (13 ml). Twelve milliliters of a linear gradient of sucrose (0.25–1 M) density was layered on the nanoparticle suspension and subjected to ultracentrifugation (200,000*g* at 4 °C for 16 h) by using an SW41 rotor (Beckman Instruments, Fullerton, CA, USA). Fractions (1 ml) were collected and purified sample was further characterized by UV–Vis spectra and TEM. Finally, the size distribution of the nanoparticles was evaluated using DLS measurements which were conducted with a Malvern Zetasizer ZS compact scattering spectrometer (Malvern Instruments Ltd., Malvern, UK).

2.3. Isolation and cell culture

Porcine retinal endothelial cells (PRECs) were isolated and cultured as described previously [22]. Briefly, the retinas were isolated from porcine eyes and washed with minimum essential medium (MEM; Sigma St Louis, MO) with 100 U/ml penicillin (Sigma St Louis, MO) and 100 μg/ml streptomycin (Sigma St Louis, MO) and cut into 3 mm segments and transferred into a 15 ml centrifuge tube (BD Falcon, USA) containing an enzyme cocktail which consisted of 500 μg/ml of collagenase of type IV (Sigma St Louis, MO), 250 μg/ml of Pronase (Biochemika, Sigma, Buchs) and 250 μg/ml of DNAse (Sigma St Louis, MO) in 1× phosphate buffered saline (PBS, pH 7.4). The enzyme mixture was vortexed for 10–15 s to disaggregate the tissue and kept at 37 °C for 30 min. The resultant enzyme digest was passed through 53 μm steel mesh (W.S Tyler, UK). The trapped vessels were washed with MEM medium by centrifugation at 400*g* for 5 min. The pellet containing microvessel fragments were finally suspended in Iscove's Modified Dulbecco's Medium (IMDM; Sigma St Louis, MO) with 10% fetal bovine serum(FBS) and seeded in a 35 × 10 mm tissue-culture dish (BD Falcon, USA) coated with 1.5% porcine gelatin type A (Sigma St Louis, MO), and incubated at 37 °C in 5% CO_2. The PRECs were characterized by using endothelial cells specific markers such as vWF, CD31, CD146 (Chemicon International, CA). Cells from fourth and fifth passages were used for all experiments.

2.4. Cell viability assay

The cell viability assay was performed as described earlier [22]. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide dye reduction assay using 96-well micro titer plates was performed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The assay depends on the reduction of MTT by the mitochondrial dehydrogenases of viable cells to a blue formazan product, which can be measured using a scanning multiwell spectrophotometer (Biorad, Model 680, and Japan). PRECs were seeded at a density of 2×10^3 cells per well into 96-well culture plates and starved in IMDM with 0.5% FBS for 5 h. To examine the effect of Ag-NPs on cell viability, PRECs were treated with Ag-NPs (from 100 to 1000 nM) and incubated for 24 h. After 24 h of incubation (37 °C, 5% CO_2 in a humid atmosphere), 10 μl of MTT (5 mg/ml in PBS) was added to each well, and the plate was incubated for a further 4 h (at 37 °C). The resulting formazan was dissolved in 100 μl of dissolving buffer (provided as part of the kit) and absorbance of the solution was read at 595 nm. All determinations were carried out in triplicate.

2.5. Pharmacological inhibitor assay

To assess the Src activity, the pharmacological inhibitor PP2 (Calbiochem, Germany) was used. Briefly, PRECs were seeded at a density of 2×10^3 cells per well into 96-well culture plates and starved in IMDM with 0.5% FBS for 5 h. Cells were incubated with 10 μM of PP2 for 30 min before treatment with AGE-BSA. The assays were conducted over a 24 h incubation period at 37 °C in a 5% CO_2 incubator, and endothelial cell proliferation was assessed.

2.6. Plasmid constructs and transient transfection assay

The mutants at Lys295 (Kinase-deficient HA-Src KD K295M) and Tyr527 (constitutive active HA-Src-CA Y527F) were used accordingly as described earlier [23]. PRECs were transiently transfected using nucleofection technique (Amaxa Biosystems, Koeln, Germany) and grown to 80% confluence in IMDM medium. Briefly, cells were harvested by trypsinization and centrifuged at 3000*g* for 10 min. The pellet was resuspended in the nucleofector solution (Basic nucleofector kit, Amaxa Inc, Germany) to a final concentration of 4–5 × 10^5 cells/100 μl. At the time of transfection, 1–3 μg of DNA encoding green fluorescent protein (pmaxGFP), constitutively active Src or dominant negative Src was added along with nucleofector solution and then subjected to electroporation using a nucleofector device-II (Amaxa Biosystems, Koeln, Germany: Program M-003) according to manufacturer's instructions. After electroporation, transfected cells were resuspended in 35 × 15 mm gelatin-coated dishes containing 1 ml of prewarmed IMDM media and incubated in 5% CO_2 at 37 °C. The transfection efficiency was about 80–90% determined using pmaxGFP plasmid (Amaxa Biosystems) and cell viability determined by trypan blue exclusion was about 90%.