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Ascorbate reverses high glucose- and RAGE-induced leak of the endothelial permeability barrier

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ABSTRACT

High glucose concentrations due to diabetes increase leakage of plasma constituents across the endothelial permeability barrier. We sought to determine whether vitamin C, or ascorbic acid (ascorbate), could reverse such high glucose-induced increases in endothelial barrier permeability. Human umbilical vein endothelial cells and two brain endothelial cell lines cultured at 25 mM glucose showed increases in endothelial barrier permeability to radiolabeled inulin compared to cells cultured at 5 mM glucose. Acute loading of the cells for 30–60 min with ascorbate before the permeability assay prevented the high glucose-induced increase in permeability and decreased basal permeability at 5 mM glucose. High glucose-induced barrier leakage was mediated largely by activation of the receptor for advanced glycation end products (RAGE), since it was prevented by RAGE blockade and mimicked by RAGE ligands. Intracellular ascorbate completely prevented RAGE ligand-induced increases in barrier permeability. The high glucose-induced increase in endothelial barrier permeability was also acutely decreased by several cell-penetrant antioxidants, suggesting that at least part of the ascorbate effect could be due to its ability to act as an antioxidant.

1. Introduction

The endothelium presents a barrier to movement of cells and plasma constituents out of the blood vessel, the permeability of which varies greatly in different vascular beds and under different conditions of blood flow, hormonal activation, and disease state [1,2]. Among the conditions known to increase endothelial barrier permeability is the hyperglycemia of diabetes, where it is one of the earliest signs of endothelial dysfunction [3–5] and an established precursor to diabetes microvascular complications [6,7], including insulin resistance [8]. Several mechanisms may underlie high glucose-induced increases in endothelial barrier permeability. There is evidence that it is caused by increases in cellular oxidative stress and superoxide generation that are a consequence of excessive glucose metabolism [9] and protein kinase C activation [7,10,11]. High glucose also generates advanced glycation end-products (AGE) that along with other ligands produced by damaged cells (e.g., HMGB1, calgranulins) bind to the receptor for AGE (RAGE) and increase both its activation and expression [12,13]. RAGE activation in turn activates NADPH oxidase, which is associated with increased retinal endothelial permeability [14].

Agents known to reverse dysfunctional increases in endothelial permeability include increases in intracellular cyclic AMP [15], cyclic GMP [16,17], nitric oxide [1,18,19], prostaglandin E_2 [20], and sphingosine 1-phosphate [21]. We have also found that an increase in intracellular vitamin C, or ascorbic acid (ascorbate), tightens the endothelial barrier as measured by decreased paracellular transfer of the polysaccharide inulin and of ascorbate itself across endothelial monolayers [22].

Whether and how ascorbate might reverse high glucose-induced increases in endothelial barrier permeability is unknown. In this work we tested this hypothesis in three types of endothelial cells: two immortalized microvascular brain endothelial cell lines and human umbilical vein endothelial cells (HUVECS).

2. Materials and methods

2.1. Materials

Sigma/Aldrich Chemical Co. (St. Louis, MO) supplied the reagent chemicals, including ascorbate, *N*-2-hydroxyethylpiperazine-*N*′-2-

Abbreviations: AGE, advanced glycation end-products; FPS-ZM1, *N*-benzyl-4-chloro-*N*-cyclohexylbenzamide; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HMGB1, high mobility group box 1; KRH, Krebs-Ringer Hepes; NAC, *N*-acetylcysteine; RAGE, receptor for advanced glycation end-products; SVCT2, sodium-dependent vitamin C transporter-2.

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ethanesulfonic acid (Hepes), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (4-hydroxy-TEMPO, Tempol), and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox). *N*-benzyl-4-chloro-*N*-cyclohexylbenzamide (FPS-ZM1) was purchased from EMD Millipore (Billerca, MA, catalog #553030). FPS-ZM1 was initially dissolved in a small amount of dimethylsulfoxide, and then diluted with culture medium such that the final dimethylsulfoxide concentration was 0.06% or less. HMGB1 was purchased from ProSpec (Ness Ziona, Israel, catalog # pro-610). AGE-conjugated bovine serum albumin (AGE-BSA) was purchased from BioVision, Inc. (Milpitas, CA, catalog #2221-10). Perkin-Elmer Life and Analytical Sciences, Inc. (Boston, MA) supplied the [carboxyl-^{14}C]inulin (molecular weight range 5000–5500, 2 mCi/g).

2.2. Cell culture

Early passage HUVECS were obtained from Sciencell Research Laboratories (Carlsbad, CA, catalog #8000) and cultured in Endothelial Cell Medium from the same company (catalog #1001) that contained 10% (v/v) heat-inactivated fetal bovine serum and the indicated glucose concentration.

The human cerebral microvascular endothelial cell line hCMEC/D3 [23] was a kind gift from Dr. Ashwath Jayagopal. Cells were cultured in Medium 131 (Gibco/Invitrogen, Carlsbad, CA, catalog # M-131-500) containing microvascular growth supplement (Gibco/Invitrogen catalog #S-005-25) after coating of the plate or filter with 0.1% gelatin (Gibco/Invitrogen catalog #S-006-100) according to the manufacturer's instructions.

Murine brain endothelial cells (bEnd.3 cells) were obtained from ATTC (VA, USA) and cultured in a 1:1 ratio of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and astrocyte conditioned media, kindly provided by the laboratory of Michael Aschner. Astrocyte-conditioned media from primary rat brain astrocytes is known to tighten the endothelial cell barrier [24].

Cells were cultured at 37 °C in humidified air containing 5% CO_2.

2.3. Assay of intracellular ascorbate

To measure intracellular ascorbate, confluent cells cultured in 6-well plates were rinsed 3 times with Krebs-Ringer Hepes buffer (KRH) that consisted of 20 mM Hepes, 128 mM NaCl, 5.2 mM KCl, 1 mM NaH_2PO_4, 1.4 mM $MgSO_4$, and 1.4 mM $CaCl_2$, pH 7.4. After removal of the last rinse, the cells were triturated with 0.1 ml of 25% (w/v) metaphosphoric acid with mixing. The lysate was partially neutralized with 0.35 ml of 0.1 M Na_2HPO_4 and 0.05 mM EDTA, pH 8.0, centrifuged at 3 °C for 1 min at 13,000*g*, and the supernatant was taken for assay of ascorbate. Ascorbate assay was performed in duplicate by HPLC as previously described [25]. Intracellular ascorbate concentrations were calculated based on the measured intracellular distribution space of 3-*O*-methylglucose relative to protein: 3.0 μl/mg protein in hCMEC/D3 cells and 2.9 μl/mg protein in bEnd.3 cells [26]. The water space in HUVECs was taken as that previously measured in HUVEC-derived EA.hy926 endothelial cells (3.6 μl/mg protein) [26].

2.4. Assay of trans-endothelial inulin transfer

Endothelial cells were cultured to confluence in a 6-well format on polyethylene terephthalate cell culture inserts (0.4 micron pores at a density of $2 \pm 0.2 \times 10^6$ pores per cm^2, Falcon BD Biosciences, Franklin Lakes, NJ). After reaching confluence, cells were cultured for another 5–6 days with 1.7 ml of medium in the upper well and 2.8 ml of medium in the lower well. Agents were added above the cells/filter, followed by incubation at 37 °C for the times indicated. [Carboxyl-^{14}C]inulin was added during the last hour of the transfer experiment. Aliquots of medium above and below the cells/filter were sampled for liquid scintillation counting of the radiolabeled inulin.

The permeability of the endothelial cell layer to [carboxyl-^{14}C]inulin and was measured as previously described [27], with minor modification [22]. The permeability coefficient for [carboxyl-^{14}C]inulin was corrected for [carboxyl-^{14}C]inulin transfer across filters after removal of cells by treatment with ammonium hydroxide [28]. This adjusts for any changes in permeability due to deposition of matrix material by the cells during culture.

2.5. Measurement of trans-endothelial electrical resistance (TEER)

TEER, primarily reflecting the flux of ions through the endothelial barrier, was measured at room temperature across confluent cell monolayers using an EVOM resistance meter (EVOM2, World Precision Instruments, Sarasota, FL). Measurements were taken concomitantly with the inulin transfer experiments. Triplicate readings were taken for each well after the treatment time period. Once the cells were removed from the insert, blank measurements were recorded to adjust for changes due to deposition of matrix material by the cells during culture and to account for the insert itself. Values are reported in $\Omega \times cm^2$.

2.6. Data analysis

Results are shown as mean + standard error. Statistical comparisons were made using GraphPad Prism version 5.04 for Windows

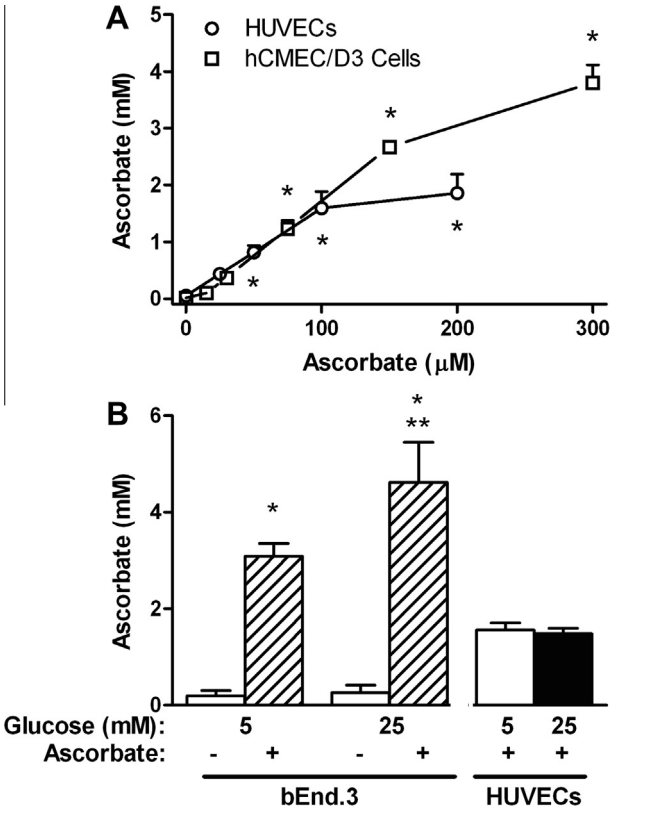

Fig. 1. Ascorbate loading of endothelial cells in 5 mM and 25 mM glucose conditions. Panel A: cells (HUVECs, circles; HCMEC/D3 cells, squares) in culture at 5 mM glucose, were treated with the ascorbate concentrations noted for 60 min at 37 °C. Panel B: bEnd.3 cells in culture at 5 mM or 25 mM glucose were treated where indicated for 60 min with 200 μM ascorbate. HUVECs cultured in 5 mM or 25 mM glucose were treated with 150 μM ascorbate. Results are shown from 3 to 5 experiments with $^{*}p < 0.05$ compared to no ascorbate control and $^{**}p < 0.05$ compared to 5 mM glucose at the same ascorbate loading concentration.

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