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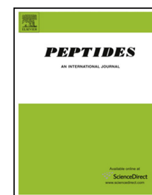

Adropin reduces paracellular permeability of rat brain endothelial cells exposed to ischemia-like conditions

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ABSTRACT

Adropin is a peptide encoded by the energy homeostasis associated gene (*Enho*) and plays a critical role in the regulation of lipid metabolism, insulin sensitivity, and endothelial function. Little is known of the effects of adropin in the brain and whether this peptide modulates ischemia-induced blood-brain barrier (BBB) injury. Here, we used an *in vitro* BBB model of rat brain microvascular endothelial cells (RBE4) and hypothesized that adropin would reduce endothelial permeability during ischemic conditions. To mimic ischemic conditions *in vitro*, RBE4 cell monolayers were subjected to 16 h hypoxia/low glucose (HLG). This resulted in a significant increase in paracellular permeability to FITC-labeled dextran (40 kDa), a dramatic upregulation of vascular endothelial growth factor (VEGF), and the loss of junction proteins occludin and VE-cadherin. Notably, HLG also significantly decreased *Enho* expression and adropin levels. Treatment of RBE4 cells with synthetic adropin (1, 10 and 100 ng/ml) concentration-dependently reduced endothelial permeability after HLG, but this was not mediated through protection to junction proteins or through reduced levels of VEGF. We found that HLG dramatically increased myosin light chain 2 (MLC2) phosphorylation in RBE4 cells, which was significantly reduced by adropin treatment. We also found that HLG significantly increased Rho-associated kinase (ROCK) activity, a critical upstream effector of MLC2 phosphorylation, and that adropin treatment attenuated that effect. These data indicate that treatment with adropin reduces endothelial cell permeability after HLG insult by inhibition of the ROCK-MLC2 signaling pathway. These promising findings suggest that adropin protects against endothelial barrier dysfunction during ischemic conditions.

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1. Introduction

Ischemic stroke is a leading cause of death and permanent disability worldwide. Opening of the blood-brain barrier (BBB) correlates with increased edema, hemorrhagic transformation, and ultimately worse outcome in ischemic stroke patients [12,25,41]. One of the main components of the BBB is the endothelial cell monolayer, held tightly together by tight junctions and adherens junctions. Reducing BBB breakdown by protecting the endothelium is a critical target for potential stroke therapeutics.

Adropin is a highly conserved polypeptide containing a secretory signal peptide encoded by N-terminal amino acid residues 1–33, and a putative secreted domain, adropin$^{34-76}$ [24]. This polypeptide is abundantly expressed in the liver and brain of many mammals including humans, and is encoded by the energy homeostasis associated gene (*Enho*) [24,42]. Previously, adropin has been shown to be an important regulator of adiposity, insulin resistance, and glucose tolerance [17,18,24,42]. Recent studies found that low plasma adropin levels were closely associated with endothelial dysfunction in patients with diabetes or cardiovascular diseases [10,20,39,43,46]. Also, an *in vitro* study found that adropin-treated human umbilical vein and coronary artery endothelial cells exhibited reduced endothelial monolayer permeability and greater cell proliferation and migration mediated through the vascular endothelial growth factor receptor-2

Abbreviations: BBB, blood-brain barrier; RBE4, rat brain microvascular endothelial cells; HLG, hypoxia/low glucose; FITC-dextran, fluorescein isothiocyanate–dextran; HIF-1α, hypoxia inducible factor-1alpha; VEGF, vascular endothelial growth factor; TJ, tight junction; AJ, adherens junction; ZO-1, zonula occludens-1; VE-cadherin, vascular endothelial cadherin; ELISA, enzyme-linked immunosorbent assay; MLC2, myosin light chain 2; ROCK, Rho-associated kinase; MLCP, myosin light chain phosphatase; MLCK, myosin light chain kinase; *Enho*, energy homeostasis associated gene; Akt, protein kinase B; ERK1/2, extracellular signal-regulated kinases 1 and 2; eNOS, endothelial nitric oxide synthase; Papp, apparent permeability coefficient; CNS, central nervous system; HBMECs, human brain microvascular endothelial cells; OGD, oxygen-glucose deprivation.

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(VEGFR2)/Akt- and VEGFR2/ERK1/2-mediated endothelial nitric oxide synthase (eNOS) activation signaling pathways [28]. Taken together, there is substantial evidence indicating that adropin plays an important role in the regulation of endothelial dysfunction. However, little is known of the effects of adropin on brain endothelial cells and whether this peptide can modulate brain endothelial cell permeability in ischemic conditions.

In this study, we hypothesized that adropin attenuates paracellular permeability of brain endothelial cell monolayers subjected to hypoxia/low glucose (HLG) conditions. We investigated the effects of synthetic adropin$^{34–76}$ on endothelial cell barrier permeability induced by HLG and explored molecular mechanisms that may contribute to its protective effects on endothelial cell permeability. We utilized an *in vitro* BBB model composed of rat brain microvascular endothelial cells (RBE4) grown in Transwell inserts in the presence of astrocyte-conditioned media. We found that adropin$^{34–76}$ significantly reduces endothelial permeability by decreasing Rho-associated kinase (ROCK) activation and myosin light chain 2 (MLC2) phosphorylation, which are key molecular events affecting cytoskeletal structure, cell contractility, and endothelial permeability.

2. Materials and methods

2.1. Cell culture

The rat astrocyte cell line (CTX-TNA2) was obtained from the American Type Culture Collection (Cat. No. CRL-2006; ATCC, Manassas, VA), which was originally established from primary cultures of type 1 astrocytes from brain frontal cortex tissue of one-day old rats. CTX-TNA2 cells (passage 5–20) were maintained in growth media composed of 44% alpha-MEM: 44% Ham's F-10 Nutrient (Cat. Nos. 12571-063 and 11550-043, respectively; Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Cat. No. F4135; Sigma-Aldrich, Saint Louis, MO), 1% (100 U/ml) penicillin/(100 μg/ml) streptomycin (Cat. No. 15140-122; Invitrogen, Grand Island, NY), and 1% geneticin (G418, Cat. No. ALX-380-013-G005; 300 μg/ml; Enzo Life Sciences, Farmingdale, NY) at 37 °C in a 5% CO_2 incubator. After reaching confluency, cells were subcultured and conditioned media was collected for use in endothelial cell culture as detailed below.

Rat brain endothelial (RBE4) cells, provided as a gift by Dr. Michael Aschner from the Albert Einstein College of Medicine at Yeshiva University, were grown until confluent on T75 flasks coated with rat tail collagen I at 50 μg/ml (Cat. No. C3867; Sigma-Aldrich, Saint Louis, MO). Cells were initially cultured at 2×10^4 cells/cm^2 for 5 days in astrocyte-conditioned media (ACM) plus freshly prepared 1 ng/ml basic fibroblast growth factor (Cat. No. PHG0264; Invitrogen, Grand Island, NY) and maintained in a humidified 37 °C, 5% CO_2 incubator. Media were replaced every 3 days. The ACM was produced by plating CTX-TNA2 cells on 150-mm dish at 2×10^4 cells/cm^2 and media were harvested every 3 days. The ACM consists of equal parts (1:1, V/V) of fresh astrocyte growth media (alpha-MEM/Ham's F-10 Nutrient containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 300 μg/ml G418) and conditioned astrocyte growth media harvested from the confluent CTX-TNA2 cells. When confluent, RBE4 cells (passages 5 and 15) were trypsinized and harvested.

2.2. In vitro *BBB model using RBE4 cell monolayers*

To establish a BBB model *in vitro*, confluent RBE4 cells grown in T75 flasks for 5 days were trypsinized and seeded at an initial density of 2×10^5 cells/cm^2 on the inner surface of polyethylene terephthalate (PET) cell culture inserts coated with 50 μg/ml of rat tail collagen I (ThinCert^{TM} inserts for 24-well plates, 0.4 μm membrane pore size, Cat. No. 662641; Greiner Bio-One, Monroe, NC). The luminal and abluminal compartments were filled with 200 μl and 800 μl fresh ACM plus EGM-2MV SingleQuot Kit Supplements and Growth Factors (Cat. No. CC-4147, Lonza Walkersville, Inc., Walkersville, MD), respectively. Media were replaced every 3 days. Cells were allowed to grow for 6 days at 37 °C in 5% CO_2 incubator to achieve confluent monolayer and confirmed under a phase contrast microscopy before they were subjected to hypoxia/low glucose (HLG) treatment.

2.3. Hypoxia/low glucose (HLG) conditions

To mimic acute ischemia-like conditions *in vitro*, RBE4 cells grown in the inserts were subjected to HLG treatment. In brief, when the monolayer of cells in 24-well cell culture inserts (for permeability experiments) or 35 mm dishes (for molecular biology experiments) reached confluency after 6 days incubation with ACM plus EGM-2MV SingleQuot Kit Supplements and Growth Factors in a humidified 37 °C, 5% CO_2 incubator, media were replaced with pre-warmed light serum Earle's balanced salt solution (EBSS) containing 1.1 mM D-Glucose plus 5% FBS, where EBSS solution consisted of 117 mM NaCl, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, 26 mM $NaHCO_3$ and 1 mM NaH_2PO_4. Cultures were then transferred and incubated in a hypoxic atmosphere of 93% N_2, 2% O_2 and 5% CO_2 at 37 °C for 16 h. Once in the chamber, the hypoxic conditions within the chamber were monitored using an electronic oxygen/carbon dioxide analyzer (ProOx Model C21, BioSpherix, Lacona, NY). Control cultures were incubated with normal EBSS containing 5.6 mM D-Glucose plus 5% FBS for 16 h at 37 °C in 95% air and 5% CO_2. After 16 h treatment, cell culture supernatant and cells in 35 mm dishes were collected separately for molecular mechanism analyses, while both luminal and abluminal sides of the inserts were replaced with glucose- and serum-free EBSS. For the paracellular permeability measurements, FITC-dextran was added to the upper chamber of the inserts as described below.

To study the effects of adropin on HLG-induced increase in endothelial permeability, cells were treated with different concentrations (1–100 ng/ml) of adropin$^{34–76}$ (Cat. No. 032-35, Phoenix Pharmaceuticals, Inc., Burlingame, CA) 1 h before and during HLG exposure. To rule out that changes in endothelial permeability in each treatment condition were not due to changes in cell viability, a cytotoxicity/viability assay was performed utilizing the calcein AM fluorescence kit following the manufacturer's protocol (Cat. No. C3099, Molecular Probes, Grand Island, NY).

2.4. Measurement of endothelial monolayer permeability

After exposure to HLG conditions for 16 h, media in both luminal and abluminal sides of the inserts were replaced with EBSS solution. Paracellular permeability was measured by adding 1 mg/ml of fluorescein isothiocyanate (FITC)-conjugated dextran (MW = 40,000 Da; Cat. No. FD40S; Sigma-Aldrich, Saint Louis, MO) to the upper chamber of the insert. After 15 min incubation, FITC-dextran in the lower compartment was detected at excitation/emission wavelengths of 485 and 528 nm, respectively, using a fluorescent multi-mode microplate reader (Biotek, Winooski, VT). A standard curve of different concentrations of the tracer (10–250 ng/ml) was utilized to determine the concentration of FITC-dextran in the lower compartment. The paracellular permeability was then assessed by calculating the apparent permeability coefficient (Papp) as reported [27]. Papp (in centimeters per second) = $dQ/(dt \times A \times \text{Co})$, where dQ is the amount of FITC-dextran getting into the abluminal compartment, dt is the FITC-dextran incubation time (in s), A is the luminal surface area of insert (in

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