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Adrenomedullin protects rat cerebral endothelial cells from oxidant damage in vitro

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Abstract

Increased permeability and reduced cerebral endothelial cell (CEC) viability induced by oxidative stress are the hallmarks of the blood–brain barrier disruption. In our experiments hydrogen peroxide (H_2O_2, 0.5 mM) induced a continuous decrease of the transendothelial electrical resistance (TEER) and resulted in intercellular gap formations in cultured rat CECs. Adrenomedullin (AM) increased TEER, enhanced peripheral localization of F-actin bands and attenuated the increased permeability induced by H_2O_2. Furthermore, AM treatment preserved mitochondrial membrane potential, attenuated cytochrome c release, and consequently improved CEC viability in H_2O_2 treated cultures. These results suggest that AM treatment protects CECs against oxidative injury.

Keywords: Adrenomedullin; Rat cerebral endothelial cell; Hydrogen peroxide; Permeability; Viability

1. Introduction

Cerebral endothelial cells (CECs) play an important role in the formation of the blood–brain barrier (BBB) which separates the brain microenvironment from the circulating blood [1]. CECs are characterized by the formation of tight junctions, low number of pinocytotic vesicles and the presence of specialized transport systems which are responsible for the maintenance of the ionic and metabolic homeostasis of the brain parenchyma [2]. Disruption of BBB by reactive oxygen species (ROS) occurs under many pathological situations, such as diabetes [3], ischemia/reperfusion injury [4] and stroke [5].

Since the brain has a high nutritional and oxygen demand, it is not surprising that CECs have 2–5 times more mitochondria than peripheral endothelial cells in order to meet the transport associated energy requirements of CECs [6]. The higher number of active mitochondria and the augmented oxygen flux through the cerebral capillaries also means that the CECs are potentially exposed to higher levels of free radicals.

Adrenomedullin (AM) is a multifunctional peptide [7] and elevated AM levels have been observed under multiple pathologic situations such as hypertension [8], myocardial infarction [9] and subarachnoid hemorrhage [10]. Accumulating evidence suggests that AM has beneficial effect against pathological changes in these disorders [11,12]. Our previous studies showed that CECs have approximate one magnitude higher AM production than other cell types [13] and AM is an important autocrine/paracrine regulator of BBB functions [14,15]. However, the precise effects and mechanisms of AM actions in the cerebral microvessels under pathological situations remain unclear. Kato et al. [16] demonstrated that AM protects myocardial cells against ischemia/reperfusion injury via suppression of oxidative stress-induced activation of the proapoptotic factor Bax and

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activation of the antiapoptotic Akt-Bad-Bcl-2 signaling pathway. In the present study, we examined the effects and mechanisms of AM against hydrogen peroxide (H_2O_2)-induced CEC injury and BBB disruption in an in vitro model of BBB.

2. Materials and methods

2.1. Cell cultures

Wistar rats (Seac Yoshitomi, Kitakyushu, Japan) were used for our cell culture experiments with the permission of the Ethics Committee of Animal care and Experimentation, University of Occupational and Environmental Health, Japan. Primary rat CECs were isolated as previously described [13,17] and were seeded onto collagen type IV and fibronectin coated dishes. The endothelial culture medium consisted of Dulbecco's modified Eagles medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 20% FETALCLONE® I serum (HyClone, Logan, UT, USA), 2 mM glutamine, 1 ng/mL basic fibroblast growth factor, 100 μg/mL heparin, 5 μg/mL vitamin C, and antibiotics. Confluent cultures (4th–5th day in vitro) consisted of more than 95% of rat CECs verified by positive immunocytochemistry for von Willebrand factor, and negative immunochemistry for glial fibrillary acidic protein (GFAP) and α-smooth muscle actin.

Rat cerebral astrocyte cultures were prepared from neonatal Wistar rats [13]. Meninges were removed and cortical pieces were mechanically dissociated in astrocyte culture medium (DMEM supplemented with 10% fetal bovine serum and antibiotics). Dissociated cells were seeded into cell culture flasks. In order to obtain type I astroglia, confluent cultures were shaken at 37 °C overnight. The purity of astrocytes was checked by immunostaining for GFAP, and the cells were used at passage 2.

For astrocyte–CEC co-culture studies, rat CECs were seeded onto collagen type IV and fibronectin coated Transwell inserts (diameter 24 mm, pore size 3 μm; Corning), which were placed into 6-well plates containing confluent layers of astrocytes. Transendothelial electrical resistance (TEER) was measured with an EVOM resistance meter (World Precision Instruments, Saratoga, FL, USA). Experiments were carried out on filters where the TEER reached at least 300 Ω cm^2. After each experiment a high concentration of H_2O_2 (1 M; Santoku Chemical Industries Co. Ltd, Tokyo, Japan) was applied for 15 min to the CECs to completely disrupt the BBB properties of the cell monolayer, then the resistance of each filter was measured and these values were regarded as the background resistance of the filter itself and were subtracted from the measured TEER values to obtain the net resistance of the CEC monolayer.

2.2. F-actin and mitochondrial staining

CECs were cultured on collagen-coated glass coverslips (Becton Dickinson, Bedford, MA, USA) until confluence. The cells were pretreated with different concentrations of rat AM (Peptides Institute, Osaka, Japan) for 15 min then H_2O_2 (0.5 mM) was added and the cells were incubated for 6 h. After the incubation period the medium was replaced and the cells were incubated for 20 min at 37 °C in the regular culture medium containing 200 nM of the mitochondria specific dye CMXRos (Molecular Probes, Eugene, OR, USA). Then the CECs were washed twice with phosphate buffered saline (PBS), fixed in 10% formaldehyde, permeabilized with 0.1% Triton X-100 in PBS, blocked with 1% BSA and stained with FITC-labeled Phalloidin (500 nM), (Molecular Probes, Eugene, OR, USA) in PBS for 15 min followed by nuclear counterstaining with DAPI (1.5 μg/mL), (Molecular Probes, Eugene, OR, USA) for 10 min. Cellular fluorescence was acquired on a NIKON ECLIPSE E600 fluorescence microscope (NIKON, Tokyo, Japan) using appropriate filter sets (CMXRos: λ_{ex}=540–580 nm and λ_{em}=600–660 nm; FITC: λ_{ex}=465–495 nm and λ_{em}=515–555 nm; DAPI: λ_{ex}=340–380 nm and λ_{em}=435–485 nm).

2.3. Analysis of mitochondrial membrane potential

CECs were cultured on 48-well plates (Falcon, New Jersey, USA). Confluent cultures were pretreated with AM for 15 min then H_2O_2 were applied and the cells were incubated for different periods of time (30 min, 3 h and 24 h). After the incubation period the medium was replaced and the cells were incubated for 10 min at 37 °C in the regular culture medium containing 10 μM of the mitochondrial membrane potential sensitive dye JC-1 (Molecular Probes). JC-1 produces green fluorescence in the cytoplasm and red-orange fluorescence when aggregated in the respiring mitochondria. The cells were washed with PBS and cellular fluorescence was measured at 20 °C with a Fluoscan Ascent FL fluorescent plate reader (Thermo Electron Corporation, Vantaa, Finland) using λ_{ex}=544 nm/λ_{em}=590 nm and λ_{ex}=485 nm/λ_{em}=538 nm filter sets. After subtraction of the background values, red/green fluorescence ratios were calculated.

2.4. Cell viability assay

CECs were trypsinized off the culture dishes and were seeded to 96-well plates at a density of 5000 cells/well. The cells were pretreated with AM for 15 min then H_2O_2 (0.5 mM) was administered to the cells and they were incubated for 3 h, then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Molecular Probes) were added to each well (final concentration 1 mg/mL)