Biochemical and Biophysical Research Communications 411 (2011) 293–298

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Up-regulation of $K_{ir}2.1$ by ER stress facilitates cell death of brain capillary endothelial cells

Hiroaki Kito [a], Daiju Yamazaki [a,b,c], Susumu Ohya [a], Hisao Yamamura [a], Kiyofumi Asai [c], Yuji Imaizumi [a,*]

[a] Department of Molecular & Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan
[b] Department of Biological Chemistry, Kyoto University, Graduate School of Pharmaceutical Sciences, Kyoto, Japan
[c] Department of Molecular Neurobiology, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan

ARTICLE INFO

Article history:
Received 11 June 2011
Available online 25 June 2011

Keywords:
Brain capillary endothelial cell
Cell death
Tunicamycin
Membrane hyperpolarization
Inward rectifier K^+ channel
ER stress

ABSTRACT

Brain capillary endothelial cells (BCECs) form blood brain barrier (BBB) to maintain brain homeostasis. Cell turnover of BCECs by the balance of cell proliferation and cell death is critical for maintaining the integrity of BBB. Here we found that stimuli with tunicamycin, endoplasmic reticulum (ER) stress inducer, up-regulated inward rectifier K^+ channel ($K_{ir}2.1$) and facilitated cell death in t-BBEC117, a cell line derived from bovine BCECs. The activation of K_{ir} channels contributed to the establishment of deeply negative resting membrane potential in t-BBEC117. The deep resting membrane potential increased the resting intracellular Ca^{2+} concentration due to Ca^{2+} influx through non-selective cation channels and thereby partly but significantly regulated cell death in t-BBEC117. The present results suggest that the up-regulation of $K_{ir}2.1$ is, at least in part, responsible for cell death/cell turnover of BCECs induced by a variety of cellular stresses, particularly ER stress, under pathological conditions.

1. Introduction

Under physiological conditions, BBB restricts movements of various molecules and fluid, blocks the entry of noxious matter to brain and maintains homeostasis of central nervous system (CNS). BBB is composed of BCECs, which are structurally characterized by the presence of intercellular tight junctions and the association with surrounding astrocytes and pericytes [1]. A delicate balance between formation of new BCECs by proliferation and their elimination by cell death are essential for the integrity of BBB [2].

In BCECs, as well as in other types of cells, changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) regulate a variety of cell functions, including gene expression, cell proliferation, and cell death. The activation of K^+ channels is one of the main factors to generate apoptosis [3]. Membrane hyperpolarization induced by K^+ channel activation is supposed to increase the electromotive force of Ca^{2+} entry through non-selective cation channels in quiescent cells, including endothelial cells [4]. Sustained high $[Ca^{2+}]_i$ triggers mitochondrial Ca^{2+} overload and apoptosis in various cells [5]. ER is one of the major organelle regulating $[Ca^{2+}]_i$, folding of secreted and membrane proteins, and cell death. It has been reported that multiple stimuli and pathological conditions disturb ER homeostasis and result in ER stress [6]. Endothelial dysfunction is usually caused by endothelial cell injury or death, which result from environmental stresses including ER stress. Dysfunction of BCECs often promotes or exacerbates several diseases of the CNS: stroke, subarachnoid hemorrhage, and Alzheimer disease [7].

Our previous studies [8,9] have shown that anomalous membrane hyperpolarization is induced by ATP via the sequential activation of small conductance Ca^{2+}-activated K^+ channel (SK2) and inward rectifier K^+ channel ($K_{ir}2.1$) in t-BBEC117, which is an immortalized bovine BCEC cell line established by transfection with SV40 large T antigen [10]. ATP stimulation enhances cell proliferation via SK2 activation in the majority of the cells, but also facilitates cell death in a certain population expressing $K_{ir}2.1$ abundantly. The increased expression of $K_{ir}2.1$ may be a key event in anomalous membrane hyperpolarization and subsequent cell death.

In this study, we found that the activation of $K_{ir}2.1$ was induced in t-BBEC117 under stimuli with tunicamycin, which inhibits protein N-linked glycosylation to induce ER stress. In these cells, resting $[Ca^{2+}]_i$ was constantly increased and stress-induced cell death was, at least in part, dependent on the membrane hyperpolarization following $K_{ir}2.1$ induction. Our results suggest that $K_{ir}2.1$ plays significant roles in the regulation of cell death in BCECs under physiological and/or pathological conditions.

* Corresponding author. Address: Department of Molecular & Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuho-ku, Nagoya 467-8603, Japan. Fax: +81 52 836 3431.
E-mail address: yimaizum@phar.nagoya-cu.ac.jp (Y. Imaizumi).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.06.128

2. Materials and methods

2.1. Cell culture

The immortalized endothelial cell line t-BBEC117 was established from primary cultured bovine brain endothelial cells by the transfection of SV40 large T-antigen-expressing vector as described previously [10]. Before the application of stresses, cells were divided into following groups for treatment: (1) cultured normally; (2) cultured with tunicamycin (5 ng/ml) for 72 h.

2.2. Electrophysiological recording

Whole-cell voltage clamp were applied to a single t-BBEC117 with patch pipettes using a EPC7 amplifier (HEKA Electronics, Darmstadt, Germany), as has been reported previously [9]. Membrane currents and voltage signals were stored and analyzed using Clampex 10.2 and Clampfit 10.2 (Axon Instruments, Foster City, USA).

2.3. Western blot analysis

Proteins (30 μg/lane) of t-BBEC117 were subjected to SDS–PAGE (12%) and were then transferred to PVDF membrane. The blots were incubated with the primary antibody anti-$K_{ir}2.1$ (1:200 dilution, Alomon Lab, Jerusalem, Isreal). Resulting images were analyzed as previously reported [9].

2.4. Detection of nuclear morphology

t-BBEC117 were incubated with Hoechst 33342 (100 μg/ml) for 10 min and the intracellular accumulation of Hoechst33342 was observed with a laser scanning conforcal microscope (A1R, Nikon, Tokyo, Japan). DNA was prepared by dissolution of t-BBEC117. DNA sample was separated by 2.0% agarose gel and stained with ethidium bromide.

2.5. RNA extraction and real-time PCR

Total RNA was prepared using TriPure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Reverse transcription and real-time quantitative PCR was carried out as previously reported [9]. The utilized primers were as follows: $K_{ir}2.1$ primers, 1181–1281 (GenBank ID NM_174373, bovine); and GAPDH primers, 138–249 (GenBank ID NM_BC102589, bovine) for real-time PCR analysis and CHOP primers, 83–397 (GenBank ID NM_001078163, bovine) for RT-PCR.

2.6. Membrane potential measurements by voltage-sensitive fluorescent dye

Membrane potential was measured using $DiBAC_4(3)$ (DOJINDO, Kumamoto, Japan), a bis-barbituric acid oxonol dye with excitation maxima at approximately 488 nm, as has been reported previously [9]. Data were collected and analyzed using the ARGUS/HiSCA imaging system (Hamamatsu Photonics, Hamamatsu, Japan). The sampling interval of $DiBAC_4(3)$ fluorescence measurements was 5 s.

2.7. Flow cytometric analysis

Cell distribution based on the $DiBAC_4(3)$ fluorescence intensity were analyzed with a FACScan flow cytometer (BD LSR, Becton–Dickinson, Franklin Lakes, NJ, USA) acquiring at least 10,000 events. Data were analyzed using CellQuest software (Becton–Dickinson).

2.8. Measurement of intracellular Ca^{2+} concentrations

t-BBEC117 was incubated with 10 μM fura-2 AM (Invitrogen) in standard HEPES solution for 60 min at room temperature. Fura-2 fluorescent signals were measured using ARUGAS/HiSCA imaging system (Hamamatsu Photonics), as has been reported previously [9]. Cells loaded with fura-2 AM were alternatively illuminated at 340 and 380 nm wavelengths of light from a xenon lamp (Hamamatsu Photonics). The fluorescence emission (>520 nm) were captured with a charge-coupled device (CCD) camera (Hamamatsu Photonics).

2.9. MTT assay

Cell viability was measured by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich, St Louis, USA) assay as described previously [9]. MTT assay was performed against 6×10^3 cells/well.

2.10. Solution

For electrophysiology and Ca^{2+} imaging bath solutions, we used standard HEPES solution composed of the followings, in mM: 137 NaCl, 5.9 KCl, 2.2 $CaCl_2$, 1.2 $MgCl_2$, 14 glucose, 10 HEPES, NaOH to pH 7.4. Ca^{2+}-free solution was prepared by replacing 2.2 mM $CaCl_2$ by 5 mM EGTA. For $DiBAC_4(3)$ fluorescence imaging, we used 140 mM K^+ HEPES solution with the following composition, in mM: 2.9 NaCl, 140 KCl, 2.2 $CaCl_2$, 1.2 $MgCl_2$, 14 glucose, 10 HEPES. The pH of the solution was adjusted to 7.4 by the addition of 10 N NaOH solution. The pipette solution for electrical recordings contained, in mM: 140 KCl, 4 $MgCl_2$, 2 ATP-Na_2, 0.05 EGTA, 10 HEPES, KOH to pH 7.2.

2.11. Statistical analysis

Pooled data are expressed as means ± SE, and statistical significance was examined using Student's *t*-test for two groups and Tukey's test for more than three group after one-way analysis of variance. p values <0.05 were considered statistically significant.

2.12. Drug

The following compounds were used in this study: tunicamycin (Calbiochem, San Diego, USA), thapsigargin (Wako, Osaka, Japan) and barium chloride (Wako). Tunicamycin and thapsigargin were dissolved in dimethyl sulfoxide (DMSO) to make 10 mg/ml and 10 mM stock solution, respectively. Barium chloride was dissolved in distilled water to make 1 M stock solution.

3. Results

3.1. Upregulation of inward rectifier K^+ channel in t-BBEC117 by tunicamycin

Inward rectifier K^+ current was measured as a Ba^{2+}-sensitive membrane current component in single t-BBEC117, using whole cell patch-clamp recording. The component was up-regulated in t-BBEC117 treated with 5 ng/ml tunicamycin for 72 h than in the control group. In most cells under normal culture conditions, the current–voltage (I–V) relationship did not show clear inward rectification. However, in most tunicamycin-treated cells, large inward currents were detected at potentials negative to ~−80 mV. The application of 100 μM Ba^{2+} markedly reduced the inward current. Membrane currents were recorded in the absence (black line) and the presence (gray line) of Ba^{2+} under nor-