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Effect of hypoxia/reoxygenation on CD73 (ecto-5′-nucleotidase) in mouse microvessel endothelial cell lines

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Abstract

Cerebral ischemia and post-ischemic reperfusion commonly result in significant brain damage. Brain microvessel endothelial cells, the key target cells and regulating sites, can secrete adenosine which plays an important neuroprotective role in the ischemic brain. A primary determinant of localized production of adenosine at tissue interfaces is ecto-5′-nucleotidase (CD73). In our experiments, we used bEnd.3 cells, immortalized mouse brain microvessel endothelial cell lines, as the target cells to study the effect of hypoxia and posthypoxic reoxygenation on CD73 in brain microvessel endothelial cells. CD73 activity in bEnd.3 cells exposed to hypoxia significantly increased in time-dependent way. The upregulation of CD73 mRNA and protein expression induced by hypoxia in bEnd.3 cells were detected by RT-PCR and Western blot. However, for reoxygenation studies, CD73 activity, mRNA and protein expression decreased at the initial stages, but increased at prolonged reoxygenation. Our results suggest that hypoxia can induce upregulation of CD73 expression in brain microvessel endothelial cells, which can be reversed by reoxygenation of short duration. But CD73 expression increased gradually with the duration of reoxygenation. Then, we infer that CD73 in brain microvessel endothelial cells plays a very important role through forming adenosine during brain ischemia and reperfusion.
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Keywords: Hypoxia; Brain; Microvessel; Ecto-5′-nucleotidase; CD73; Adenosine; Endothelium

Introduction

Ischemic cerebrovascular disease is a major cause of death and disability in the world. The brain is highly sensitive to ischemic insults and requires a constant supply of oxygen and glucose and consequently is dependent on a consistent cerebral blood flow.

Adenosine plays an important role in mediating hypoxic increases in cerebral blood flow by effecting decreases in cerebrovascular resistance (Coney and Marshall, 1998; Blood et al., 2002, 2003). A low, nanomolar concentration of adenosine is normally present in the extracellular fluid, but it increases dramatically during hypoxia or ischemia in the central nervous system (CNS) (Wardas, 2002). In the brain, glutamate is the predominant excitatory neurotransmitter. During cerebral ischemia, the levels of adenosine increase up to 100-fold in the brain and exert a neuroprotective influence largely via the A1 receptor, which inhibits glutamate release and neuronal activity (Parkinson et al., 2002; Pearson et al., 2003). Adenosine (1 mM) significantly reduced hypoglycemia/hypoxia-induced glutamate release from the hippocampal slice (Corradetti et al., 1984; Kitagawa et al., 2002).

Adenosine has been known to be a potent vasodilator acting through purinergic receptors on both vascular smooth muscle and endothelial cells (Mosqueda-Garcia et al., 1991; Lo et al., 1998). Microvessel endothelial cells in the brain are known to have a crucial role in producing adenosine and modulating organ blood flow. Furthermore, endothelial cells of cerebral capillaries constitute the blood–brain barrier (BBB) which protects the CNS from any changes in homeostasis brought by pathological conditions including hypoxia and reoxygenation (Ralevic, 2002). In vivo, hypoxia is known to damage BBB leading to vasogenic brain edema (Fischer et al., 2002).

A primary determinant of localized production of adenosine at tissue interfaces is ecto-5′-nucleotidase (CD73) which is

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expressed throughout many different tissues. CD73 is a glycosyl-phosphatidyl-inositol (GPI)-anchored cell-surface glycoprotein. The main biological role of CD73 is catalyzing AMP and other nucleoside monophosphates into forms in which they can be utilized to meet the metabolic needs of cells, for example, adenosine and inosine. As a multifunctional ecto-enzyme, CD73 also plays important roles in the transduction of signals across the plasma membrane and in cell adhesion (Zimmermann, 1992). So far, some publications suggest that hypoxia-induced adenosine production on the endothelium is mediated by CD73 upregulation, notably in the cardiovascular system (Minamino et al., 1995; Synnestvedt et al., 2002). However, none have focused on the cerebrovascular endothelial cells. Therefore, it is the aim of the present study to find out how hypoxia/ischemia and posthypoxic reoxygenation regulate CD73 in the cerebral endothelial cells so as to influence adenosine production.

Materials and methods

Cell culture

Immortalized mouse brain endothelial cell lines, bEnd.3 cells, a gift from School of Life Sci., Fudan University, were maintained in culture using Dulbecco's modified Eagle's medium (Gibco, CA, USA) supplemented with 10% bovine serum at 37°C in a humidified atmosphere containing 5% CO_2 (Jeong et al., 2004).

Hypoxia and posthypoxic reoxygenation (H–R) treatment in vitro

bEnd.3 cells grown in 60-mm dishes were replaced with fresh medium with 2% bovine serum overnight before treatment. Confluent endothelial monolayers were exposed to hypoxia as follows: growth medium was replaced with fresh serum-free medium saturated with a mixture of 95% N_2 and 5% CO_2 for 10 min, and cells were placed in a modified hypoxic Chamber (GENBOX Jarre, France), which is an air-tight chamber with two resealable holes. The chamber was suffused with the above hypoxic gas mixture. Hypoxic cultures were then placed in a humidified tissue incubator at 37°C. During the experiment, continuous O_2% concentration was monitored (CYS-1 digital oxygen analyzer, China) and remained at 2% unchanged. For posthypoxic reoxygenation (H–R) experiments, after exposure in hypoxia of indicated time, the cells were returned to the incubator at normoxic conditions (5% CO_2 and 95% air) for more indicated time (Scorziello et al., 2001).

LDH assay

LDH released from damaged cells into culture medium has been assumed to represent a quantitative measurement of cell death. The LDH activity was measured with spectrophotometric method using an LDH test kit (Shanghai Fortune Long March Medical Science Co., Ltd.).

Measurement of surface 5′-ecto-nucleotidase activity

We assessed endothelial CD73 surface activity as described previously by measuring the conversion of etheno-AMP (E-AMP) (Sigma-Aldrich, St. Louis, MO, USA) to ethenoadenosine (E-ADO) (Sigma-Aldrich, St. Louis, MO, USA) (Bonitati et al., 1993; Narravula et al., 2000). Briefly, HBSS with absence or presence of α,β-methylene ADP (APCP, final concentration 100 μM) (Sigma-Aldrich, St. Louis, MO, USA) was added to bEnd.3 monolayers on six-well plates. After 10 min, E-AMP (final concentration 50 μM) was added for an additional 10 min, removed, acidified to pH 3.5 with HCl, spun (10,000 × *g* for 2 min, 4°C), filtered (0.45 μm) and frozen (−80°C) until analysis via high-performance liquid chromatography (HPLC) analysis. A high-performance liquid chromatograph (Agilent 1100 Series HPLC) with an HP 1100 diode array detector was used with reverse-phase HPLC column (Luna 5 μm C18, 250 × 4.60 mm, Phenomenex, Torrance, CA). E-AMP/E-ADO was measured with a 4–40% methanol/H_2O gradient mobile phase (1 ml/min over 20 min). CD73 activity was expressed as E-ADO production per mg protein for 10 min.

Total RNA extraction and semiquantitative RT-PCR analysis

bEnd.3 cells were treated with different conditions. Total RNA was isolated from cells using Trizol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RNA concentration was determined by absorbance at 260 nm, and its integrity was verified by electrophoresis on 1.2% denaturing agarose gels. cDNA was prepared by reverse transcription of 2 μg of total RNA using oligo$(dT)_{18}$ primer and reverse transcriptase. The aim genes were amplified by PCR. The CD73 PCR reaction contained 1 μM concentrations each of the sense primer (5′-GACCATCAAAGCAGACATTAACCAA-3′) and the antisense primer (5′-TTTGAGATGTATTCAGAAACCACGC-3′), 2 μL of 10× PCR buffer, 1.6 μL of $MgCl_2$, 0.2 mM dNTPs, 1 μL of previously synthesized cDNA and 1 unit of Taq DNA polymerase in a total volume of 20 μL. To define the optimal number of PCR cycles for linear amplification, an aliquot from each of the samples was removed at 21 cycles and thereafter at every one additional cycle until 30 cycles (data not shown). In all of our RT-PCR experiments, 25 cycles were used. After an initial denaturation step at 94°C for 3 min, the PCR profile for genes amplification with 25 cycles was: 94°C, 1 min; corresponding denature temperatures, 1 min; 72°C, 1 min; the prolonged step is at 72°C for 10 min. The PCR fragments were visualized on a 1.5% agarose gel containing 5 μg/ml ethidium bromide. The semiquantitative data are presented as the expression of the indicated gene product in relation to that of GAPDH. The sequence of GAPDH primer is as follows: sense primer: 5′-ACAGCCGCATCTTCTTGTGCAGTG-3′, antisense primer: 5′-GGCCTTGACTGTGCCGTTGAATTT-3′.

Cell surface biotinylation and CD73 immunoprecipitation

The surface proteins in bEnd.3 cells of different treatments were biotinylated (Henttinen et al., 2003). The cells were incubated for 40 min at room temperature in a final volume of 1 ml of phosphate-buffered saline containing 0.5 mg/ml sulfo-nHS-LC-Biotin (Pierce Biotechnology Inc., IL, USA), 0.1 mM $CaCl_2$ and 1.0 mM $MgSO_4$. Cells were solubilized in a lysis buffer containing 1% Triton X-100, 50 mM Tris pH 7.4, 150 mM Nacl, 20 mM iodoacetamide, 1 mM PMSF and 1% aprotinin. Cell debris was removed by centrifugation.

Lysates were precleared with 20 μL pre-equilibrated protein G–sepharose (Santa Cruz, CA, USA). Immunoprecipitation of CD73 was performed with mAb TY23 (Thompson et al., 1989) (a kind gift from Dr. Linda Thompson, Oklahoma Medical Research Foundation, Oklahoma City, OK) followed by addition of 50 μL pre-equilibrated protein G–sepharose and overnight incubation. Washed immunoprecipitates were boiled in 1× SDS reducing sample buffer (2.5% SDS, 0.38 M Tris, pH 6.8, 20% glycerol and 0.1% bromophenol blue), separated by SDS-PAGE, transferred to PVDF membrane and blocked 1 h at room temperature in blocking buffer with 5% BSA. Biotinylated proteins were labeled with streptavidin–peroxidase and visualized by enhanced chemiluminescence (ECL).

Statistical analysis

Mean values were calculated from data obtained from three or more separate experiments and reported as mean ± SEM. The significance of the difference between groups with multiple comparisons was estimated by one-way analysis of variance (ANOVA) followed by Student-Newman–Keuls test. Statistical significance was confirmed at $P < 0.05$.

Results

Effect of hypoxia on LDH release in bEnd.3 cells

As shown in Fig. 1, bEnd.3 cells exposed to brief hypoxia (2, 4, 8 h) did not demonstrate an increase in LDH release

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