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Ischemic conditions and β-secretase activation: The impact of membrane cholesterol enrichment as triggering factor in rat brain endothelial cells

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ABSTRACT

Among harmful conditions damaging the blood–brain barrier, cerebral stroke and reperfusion injuries were proposed as contributing factors to Alzheimer's disease etiology. Indeed it was reported that ischemic conditions promote β-amyloid peptide production in brain endothelial cells, although implicated mechanisms are yet not fully understood.

Oxidative injury related to ischemia affects membrane-lipids profile by altering their biochemical properties and structural dynamics, which are also believed to play significant role in the amyloid precursor protein processing, suggesting a link between alterations in lipid membrane composition and β-amyloid peptide production enhancement.

Using brain microvascular endothelial cells, here we demonstrate how oxygen and glucose deprivation followed by normal conditions restoration, mimicking ischemic environment, increases cell cholesterol amount (+20%), reduces membrane fluidity and results in strong activation (+40%) of β-secretase 1 enzymatic activity. Moreover, we observed an increase of amyloid precursor protein and β-secretase 1 protein levels with altered localization in non-discrete (Triton X-100 soluble) membrane domains, leading to an enhanced production of amyloid precursor protein β-carboxyl-terminal fragment. Therefore, lipid alterations induced by oxygen and glucose deprivation enhance β-secretase 1 activity, favor its proximity to amyloid precursor protein and may concur to increased amyloidogenic cleavage. The latter, represents a detrimental event that may contribute to β-amyloid homeostasis impairment in the brain and to Alzheimer's disease-related BBB dysfunctions.

1. Introduction

Proper cerebral function necessitates a stable local environment able to maintain ions and nutrients concentration within a narrow range. Neurons and glial cells modulate the composition of bathing medium and interstitial fluid, while the blood–brain barrier (BBB), forming the interface between the central nervous system (CNS) and peripheral bloodstream, acts as selective barrier regulating ion balance and nutrient transport, as well as blocking potentially toxic molecules (Abbott and Friedman, 2012). Brain endothelium is particularly tight-packed, allowing the passive flux of small gaseous molecules but restricting permeation of hydrophilic compounds. However, many metabolic substances can enter the cerebral district through highly regulated mechanisms mediated by BBB membrane transporters (Abbott and Friedman, 2012; Enciu et al., 2013; Yang and Rosenberg, 2011). Brain endothelial cell membrane is organized in structural and functional microdomains, called lipid rafts,

Abbreviations: CNS, central nervous system; AD, Alzheimer's disease; RBE4, rat brain endothelial cell line; OGD, oxygen and glucose deprivation; ogR, oxygen and glucose Restoration; BACE1, amyloid precursor protein (APP) β-secretase; PSEN1, presenilin-1; Aβ, amyloid peptide; (α-CTF), APP α-carboxyl-terminal fragment; (β-CTF), APP β-carboxyl-terminal fragment; HIF1, hypoxia-inducible transcription factor 1; β-MCD, β-methylcyclodextrin; PMSF, Phenylmethylsulphonyl fluoride; FBS, fetal bovine serum; BSS, balanced salt solution; MEFs, membrane-enriched fraction; PNS, post nuclear supernatant; TS, Triton X-100 soluble fraction; TI, Triton X-100 and insoluble fraction; OP, organic phase; AP, aqueous phase; DPH, 1,6-diphenyl-1,2,5-hexatriene; ECL, enhanced chemiluminescence; RFU, relative fluorescence units; PL, phospholipids; GG, gangliosides; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; Pi, phospholipidic phosphorus.

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defined as small (10–200 nm) heterogeneous membrane domains enriched in cholesterol and sphingolipids (Di Paolo and Kim, 2011; Lingwood and Simons, 2010; Sagare et al., 2012; Sonnino and Prinetti, 2013). Given the importance of BBB in CNS homeostasis, barrier damages and dysfunctions are crucial in many cerebral pathologies (Iadecola, 2010; Jeynes and Provias, 2011; Sagare et al., 2012). Harmful conditions, such as trauma, hypoxia, stroke, infection, inflammation, as well as components of the diet, environmental toxins and genetic factors (Enciu et al., 2013; Kalaria et al., 2012; Lakhan et al., 2013) affect BBB morphology and structural organization.

In particular, cerebral stroke and reperfusion injuries, defined as an exacerbation of pathological processes initially caused by ischemia, are likely involved in BBB alterations and breakdown (Hein et al., 2013; Sutherland et al., 2012), contributing to CNS pathologies. Reduced brain blood perfusion (Ruitenberg et al., 2005) and silent infarcts (Vermeer et al., 2003), disrupting microvasculature integrity, seem increase risk of Alzheimer's disease (AD), characterized by brain deposition of amyloid peptide (Aβ) as senile plaques (Abbott and Friedman, 2012; Iadecola, 2010; Jeynes and Provias, 2011; Kalaria et al., 2012; Mayeux and Stern, 2012; Sagare et al., 2012). Molecular mechanisms involved are not clear yet, indeed whether BBB dysfunction and neuronal impairment occur simultaneously or as separated events of same detrimental cascade is under debate. Beside the well known increased microvascular permeability and disruption of tight junction proteins of brain endothelial cells, it has been proposed that, as response to ischemic insults, BBB may directly contribute to AD onset (Jabłoński et al., 2011; Sagare et al., 2012; Yang and Rosenberg, 2011). As recently demonstrated, ischemic conditions promotes Aβ peptide increase in brain endothelial cells (Bulbarelli et al., 2012). Since BACE1 and its substrates are membrane-resident proteins, the surrounding lipid environment represents a crucial factor in modulation of BACE1 enzymatic activity and of molecular events correlated to APP processing (Burg et al., 2013; Maulik et al., 2012; Zhang et al., 2011).

To deeply investigate mechanisms related to APP amyloidogenic processing enhancement in response to ischemic conditions, this work studies the role of membrane lipid composition and fluidity on BACE1 activity regulation and APP/BACE1 proteins localization in rat brain capillary endothelial (RBE4) cells subjected to oxygen and glucose deprivation (OGD).

2. Materials and methods

2.1. Materials

All commercial chemicals were of the highest available grade. All powdered reactants, solutions for electrophoresis, β-methylcyclodextrin (β-MCD) and anti-β-actin antibody, Sigma Chemical Co. (Milano, Italy). Solvent solutions, VWR International PBI (Milano, Italy). The 5% CO_2:95% N_2 gas cylinder, Sapio (Monza, Italy). Collagen I rat tail solution for RBE4 cell culture, anti-APP antibody, Invitrogen Corporation (Milano, Italy). Stock solutions for RBE4 cell culture: alpha-Mem medium with glutamax-1 and Nutrient Mix F-10 nutrient mixture (HAM), geneticin solution antibiotic, Euroclone (Milano, Italy). Complete protease inhibitor cocktail, Roche Diagnostics S.p.A (Milano, Italy). Anti-BACE1 antibody, Abcam (Cambridge Science park, UK), anti-Na^+/K^+ ATPase antibody, Millipore (Temecula, CA, USA), anti-CD81 antibody, Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-Presenili-1 antibody, cell signaling technology (Danvers, MA, USA). Secondary HRP-conjugated antibodies and ECL SuperSignal detection kit, Pierce (Rockford, IL, USA). β-Secretase activity assay kit, BioVision (Firenze, Italy).

2.2. Cell cultures

Rat brain endothelial (RBE4) clone shows typical endothelial morphology and retains many brain endothelial cell characteristics (Aschner et al., 2006; Balbuena et al., 2011; Faria et al., 2011; Roux and Couraud, 2005; Wilhelm et al., 2011). RBE4 cells, provided as a gift by Dr. M. Aschner (Department of Pediatrics, Vanderbilt Kennedy Center, Nashville, Tennessee, USA), were plated on collagen (50ug/mL in acetic acid 0.02 M) coated dishes or flasks and were grown in the presence of 44% alpha-MEM:44% F-10 Nutrient supplemented with 10% heat inactivated fetal bovine serum (FBS). RBE4 cells were plated in collagen-coated dishes (12,000 cells/cm^2) and maintained at 37 °C in a 5% CO_2 atmosphere for 3 days (80% confluence) before treatment.

2.3. Oxygen and glucose deprivation (OGD) treatment

RBE4 cells were subjected to oxygen and glucose deprivation (OGD) for 3 h, as previously described (Bulbarelli et al., 2012). After OGD, normoxic and normoglycemic conditions were restored for 1 h and 24 h. Cells were replaced in normal culture conditions (37 °C in a 5% CO_2 atmosphere) and BSS in each dish was supplemented with restoration solution containing 5 mM glucose and 10% FBS in culture medium. From here on, we will use the term "oxygen and glucose Restoration" (ogR) to indicate "normoxic and normoglycemic conditions restoration post-OGD". RBE4 untreated cells were maintained in normal culture conditions and collected together with treated cells subjected to ogR 24 h.

2.4. β-MCD treatment

β-MCD is commonly used to modulate cholesterol amount in cellular membranes (Guardia-Laguarta et al., 2009; Marquer et al., 2011; Murai et al., 2013). To deplete cholesterol from cellular membranes cells, after washing twice with medium w/o FBS, were incubated with β-MCD (2.5 mM) in medium w/o FBS at 37 °C for 60 min (Murai et al., 2013).

2.5. Membrane-enriched fractions (MEFs) isolation

At 1 and 24 h of ogR (with or without β-MCD pre-treatment), cells were scraped in PBS containing protease inhibitor cocktail. Pellets were homogenized with hypotonic solution (Tris–HCl pH 7.4 1 mM, EDTA pH 7.4 1 mM, KCl 15 mM, NaCl 30 mM, protease inhibitor cocktail), then an equal volume of isotonic solution 2× (sucrose 0.5 M, Tris–HCl pH 7.4 2 mM, EDTA pH 7.4 2 mM, protease inhibitor cocktail) was added. Samples were centrifuged 12 min at 800 × *g* in order to separate nuclei-enriched pellet and post nuclear supernatant (PNS). PNS was ultracentrifuged 1 h at 100,000 × *g*, the resultant membrane-enriched pellet was resuspended in MBST buffer (MES pH 6.5 25 mM, NaCl 150 mM, Triton X-100 1%, PMSF 1 mM, protease inhibitor cocktail). Membrane-enriched fractions (MEFs) were analyzed for lipid content, protein expression levels and protein activity dosage.

2.6. Triton X-100 soluble (TS) and insoluble (TI) membrane fractions separation

Cholesterol-enriched discrete membrane domains are insensitive to treatment with cold Triton X-100 (Lu and Chen, 2011; Patel and Insel, 2009). Thus, Triton X-100 soluble fraction (TS) and insoluble fraction (TI) were obtained as previously described (Donati et al., 2008) with opportune modifications. MEFs were kept in MBST buffer 30 min at 4 °C and successively were centrifuged 1 h at 100,000 × *g* to separate TS fraction (supernatant) and TI fraction (pellet). TI fractions were resuspended with MBST buffer in