Oxygen–glucose deprivation increases the enzymatic activity and the microvesicle-mediated release of ectonucleotidases in the cells composing the blood–brain barrier

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ABSTRACT

The blood–brain barrier (BBB), the dynamic interface between the nervous tissue and the blood, is composed by endothelial cells, pericytes and astrocytes. Extracellular nucleotides and nucleosides and their receptors (the purinergic system) constitute a widely diffused signaling system involved in many pathophysiological processes. However, the role of this system in controlling BBB functions is still largely unknown. By using cultures of these three cell types grown separately and a BBB in vitro model consisting of triple co-cultures, we studied for the first time the expression and distribution of the ecto-enzymes nucleoside triphosphate diphosphohydrolases (NTPDases, the enzymes which hydrolyze extracellular nucleotides) under control and ischemic (oxygen–glucose deprivation in vitro; OGD) conditions. NTP-Dase1 was detected in all three cell types, whereas NTPDase2 was expressed by astrocytes and pericytes and, to a lesser extent, by endothelial cells. Endothelial cells were extremely susceptible to cell death when OGD was applied to mimic in vitro the cytotoxicity induced by ischemia, whereas astrocytes and pericytes were more resistant. A semi-quantitative assay highlighted markedly increased e-ATPase activity following exposure to OGD in all three cell types, either when grown separately or when co-cultured together to resemble the composition of the BBB. Moreover, electron microscopy analysis showed that both endothelial cells and astrocytes shed microvesicles containing NTPDases from their membrane, which may suggest a novel mechanism to increase the breakdown of ATP released to toxic levels by damaged BBB cells. We hypothesize that this phenomenon could have a protective and/or modulatory effect for brain parenchymal cells. This in vitro model is therefore useful to study the role of extracellular nucleotides in modulating BBB responses to ischemic events, and to develop new effective purinergic-based approaches for brain ischemia.

1. Introduction

The blood–brain barrier (BBB) is the dynamic interface between the blood flow and the central nervous system (CNS), consisting of endothelial cells of brain capillaries, pericytes and astrocyte end-feet. The four major roles played by the BBB are (i) the creation of a regulated microenvironment for reliable neuronal signaling within the CNS, (ii) the supply of nutrients, (iii) the defense from toxic substances and (iv) the control of the communication between the CNS and the periphery (Abbott et al., 2006). Overall, the BBB acts as a physical, transport and metabolic barrier for the nervous system (Zlokovic, 2008).

Brain endothelial cells are a specialized type of epithelial cells, which form the inner layer of cerebral blood vessels (Joó, 1996). They are connected with each other through tight junctions and constitute a physical barrier to restrict the flux of cells and molecules between the blood and the brain (Deli, 2009). The morphology and the molecular composition of these junctions are controlled by the brain microenvironment (Hawkins and Davis, 2005), the specific properties of brain endothelial cells being also induced and maintained by a cross-talk with neighboring cells. The crucial role of astrocytes in preserving the integrity of the BBB was discovered first (as reviewed in Deli et al. (2005) and

Haseloff et al. (2005); see below); later, pericytes were also identified as key player in the regulation of barrier properties (Nakagawa et al., 2007).

Pericytes, also known as mural cells, perivascular cells or vascular smooth muscle cells, are fundamental for the correct development and functionality of the BBB, both during embryogenesis and adulthood (Armulik et al., 2010; Daneman et al., 2010). They indeed influence the growth of endothelial cells (McIlroy et al., 2006), play an essential role in the integrity and topography of the structural vessels (Kim et al., 2006), actively control the diameter of brain capillaries (Cardoso et al., 2010) and can protect from BBB disruption induced by hypoxia in vitro (Krueger and Bechmann, 2010). The loss of pericytes, among others, leads indeed to edema in the adult brain and is lethal at embryonic stages (Bjarnegard et al., 2004).

Astrocytes, besides being responsible for a wide range of functions (from modulating synaptic functions, Fellin, 2009, to providing trophic support for neuronal cells), have also a special role in the development of BBB through the induction of barrier-relevant molecules, including tight junction proteins (Abbott et al., 2006; Haseloff et al., 2005).

The development of the BBB is tightly regulated, and can be dramatically hampered by chronic perinatal stressful conditions, with yet unexplored consequences on neurogenesis, synaptogenesis and myelination (Gómez-Gonzáles and Escobar, 2009). Indeed, the active physiological protective role of the BBB is lost following its breakdown, which occurs in many acute and chronic CNS diseases such as edema (Spatz, 2010), Alzheimer's disease (Bell and Zlokovic, 2009) and cerebral ischemia (Weiss et al., 2009). The latter, also called stroke, is one of the most diffuse and severe neurovascular diseases, representing the third cause of death in the western world after cardiovascular pathologies and tumors (Feigin, 2005). It is provoked by the interruption of the cerebral blood flow, which causes a rapid reduction in the levels of oxygen and glucose in the intercellular milieu with accumulation of toxic metabolites, leading to cell sufferance and death (Weiss et al., 2009). These events contribute to increase the permeability of BBB and consequently provoke plasma extravasation and edema, worsening the clinical consequences of the original trigger event. Due to the severe consequences of ischemia and to the lack of effective therapies, the study of the mechanisms involved in BBB deregulation following an ischemic episode is of paramount importance for the identification of effective pharmacological approaches aimed at ameliorating its clinical outcome. For example, administration of progesterone to rats previously subjected to middle cerebral artery occlusion was neuroprotective by increasing the integrity of endothelial cell tight junctions, and by reducing the total brain water content (Jiang et al., 2009).

Among the possible signaling pathways controlling the functionality of cells composing the BBB, the purinergic signaling is a likely candidate, due to its well-established role in the control of cell-to-cell communication both in physiological and pathological conditions (Abbracchio et al., 2009). Membrane receptors for adenosine (named P1 receptors) and nucleotides (named P2 receptors, and activated by ATP, ADP, UTP, UDP and nucleotide sugars) were recognized on the basis of their mechanism of action, pharmacology and molecular cloning (Burnstock, 2008). The various members of the purinergic family of neurotransmitters are derived from one another thanks to sequential hydrolysis steps. A family of ectonucleotidases, named nucleoside triphosphate diphosphohydrolases (NTPDases), represents the dominant part of a complex cell surface-located machinery for nucleotides hydrolysis and interconversion, and is responsible for the hydrolysis of tri- and di-phosphate nucleotides (for review, see Robson et al., 2006). The sequential steps of dephosphorylation of ATP finally lead to the generation of adenosine, through the action of the ecto-5'-nucleotidase enzyme, and adenosine can be further degraded to inactive inosine by adenosine deaminase (Yegutkin, 2008). Purinergic receptors have a clear pharmacological specificity for their natural ligands, and their biological activities are quite different and often opposite to each other. It is therefore evident that the nucleotide/nucleoside metabolizing enzymes play a fundamental role in controlling the relative concentrations of the various purinergic transmitters, which in turn can influence the overall biological response of the affected tissue.

Under ischemic/hypoxic conditions extracellular nucleotide concentrations are dramatically increased (Jurányi et al., 1999; Melani et al., 2005), thus suggesting that the contribution of metabolizing enzymes in controlling purinergic signaling in cell-to-cell communication can be further enhanced in case of pathological conditions. For example, astrocytes express different NTPDases which are crucially involved in the modulation of reactive astrogliosis, the astrocytic response to brain injuries (Wink et al., 2006). Also endothelial cells express various NTPDases (Robson et al., 2006), and nucleotides have been recognized as mediators of vascular inflammation, thrombosis and of regional control of the vascular tone (Robson et al., 2005). Indeed, infusion of exogenous soluble ectonucleotidases can protect ENTPD1/CD39-deficient mice from ischemia/reperfusion injury, thus highlighting a crucial role for these enzymes in brain disorders (Pinsky et al., 2002). Conversely, nothing is currently known on the expression and role of NTPDases in controlling the physiopathological functions of pericytes, and very few studies have addressed this issue also on the BBB as a whole, especially under hypoxic conditions (Simard et al., 2003).

Thus, the aims of our present work have been to examine the expression and distribution of NTPDases 1 and 2, the two most abundant brain members of this family of enzymes (Robson et al., 2006), in rat brain microvessel endothelial cells, pericytes and astrocytes, as well as in an in vitro BBB model, composed of the three cell types grown together as co-cultures (Nakagawa et al., 2009). We especially focused on the changes in the activity of NTPDases following exposure to oxygen–glucose deprivation (OGD) in vitro, which closely mimics an ischemic/hypoxic event occurring in vivo.

2. Materials and methods

2.1. Preparation of primary cell cultures

For the preparation of primary cell cultures, experiments were performed in accordance with the National and International Guidelines on the use of animals in research. The number of animals utilized was kept to the minimum, and every effort was made to minimize animal suffering.

Primary cultures of rat brain capillary endothelial cells were prepared as previously published (Veszelka et al., 2007). Briefly, 10 male 2-week-old Sprague–Dawley rats (Charles River Lab, Calco, Italy) were decapitated. Meninges were carefully removed from the forebrains and the gray matter was minced into small pieces of approximately 1 mm³ in ice-cold Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, Milan, Italy) (containing 50 μg/ml gentamicin, Sigma–Aldrich, and 2 mM glutamine, Euroclone, Milan, Italy). Following dissociation by 25-times of up- and down-strokes with a 5 ml-pipette, the tissue was digested in DMEM containing 1 mg/ml collagenase 2 and 15 μg/ml DNaseI (Sigma–Aldrich) on a shaker for 1.5 h at 37 °C. After centrifugation (1000g, 8 min) the pellet was resuspended in 20% bovine serum albumin–DMEM and centrifuged at 1000g for 20 min in order to remove the myelin layer. The pellet containing the microvessels was further digested with 1 mg/ml collagenase-dispase (Roche,