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Oxygen glucose deprivation-induced astrocyte dysfunction provokes neuronal death through oxidative stress

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ABSTRACT

Understanding the role of astrocytes in stroke is assuming increasing prominence, not only as an important component on its own within the neurovascular unit, but also because astrocytes can influence neuronal outcome. Ischemia may induce astrogliosis and other phenotypic changes, but these remain poorly understood, in part due to limitations in reproducing these changes *in vitro*. Dibutyryl cyclic AMP-differentiated cultured astrocytes are more representative of the *in vivo* astroglial cell phenotype, and were much more susceptible than undifferentiated astrocytes to an ischemic-like stress, oxygen-glucose deprivation (OGD). OGD altered the expression/distribution and activity of glial glutamate transporters, impaired cellular glutamate uptake and decreased intracellular levels of glutathione preferentially in differentiated astrocytes. Resistance to OGD was conferred by inhibiting caspase-3 with DEVD-CHO and oxidative stress by the antioxidant N-acetylcysteine (NAC). The resistance of undifferentiated astrocytes to OGD may result from a transient but selective morphological transformation into Alzheimer type II astrocytes, an intermediary stage prior to transforming into reactive astrocytes. Co-culture of neurons with OGD-exposed astrocytes resulted in neurotoxicity, but at surprisingly lower levels with dying differentiated astrocytes. The antioxidant NAC or the 5-LOX inhibitor AA861 added upon co-culture delayed (day 1) but did not prevent neurotoxicity (day 3). Astrocytes undergoing apoptosis as a result of ischemia may represent a transient neuroprotective mechanism *via* ischemia-induced release of glutathione, but oxidative stress was responsible for neuronal demise when ischemia compromised astrocyte supportive functions.

Abbreviations: AA861, selective 5-lipoxygenase inhibitor; AIIA, Alzheimer type II astrocytes; AMP, adenosine monophosphate; ANOVA, analysis of variance; ATP, adenosine triphosphate; dBcAMP, dibutyryl cyclic AMP; BSA, bovine serum albumin; BSS, balanced salt solution; DEVD-CHO, highly specific, potent and reversible inhibitor of caspase-3; CNS, central nervous system; DAPI, 4,6-diamidino-2-phenylindole; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; EAAC1, excitatory amino-acid carrier 1; EAATs, excitatory amino-acid transporters; E15, embryonic day 15; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GLAST, glutamate/aspartate transporter; GLT-1, glutamate transporter 1; GSH, glutathione; HPLC, high-performance liquid chromatography; HS, horse serum; LDH, lactate deshydrogenase assay; 5-LOX, 5-lipoxygenase; MAP-2, microtubule associated protein-2; mBCl, monochlorobimane; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NAC, N-acetylcysteine; NMDA, N-methyl-D-aspartate; NMDARs, N-methyl-D-aspartate receptors; OGD, oxygen and glucose deprivation; PDC, L-trans-pyrrolidine-2,4-dicarboxylate; PBS, phosphate buffered saline; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; S.E.M., standard error of the mean; TBOA, DL-threo-β-benzyl-oxy-aspartate; TNF-α, tumor necrosis factor alpha; tPA, tissue plasminogen activator; TTBS, Tween 20 and Tris buffered solution; U.S.A., United States of America.

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1. Introduction

Stroke is the second leading cause of death, due to its high incidence, the severity of the insult, and lack of cell-targeted therapeutics. Stroke annually afflicts approximately 780 000 Americans, costing approximately 74 billion dollars in 2010 [1–3]. Given the severity of this neurological condition, the lack of neuroprotective agents approved for clinical practice and, with only ~5% of patients receiving the 'clot-buster' agent tPA, identification of targets and therapeutic approaches remains acutely required. The clinical translational failure of many drugs or targets identified through ischemia models in cultured cells and in rodents may be partly attributed to a lack of full understanding of the interactions of the different cells comprising the afflicted brain region. Although neurons are the most susceptible to ischemia, glia and the vasculature comprising the neurovascular unit are also susceptible to ischemic injury. Within minutes of a severe interruption of blood flow, the sharp energy deficit occurring in the ischemic core leads to rapid cell death; in the ischemic penumbra, ion homeostasis of brain cells is drastically altered leading to neurotransmitter release which, in concert with glial glutamate reuptake alteration, generates neuronal glutamate-mediated excitotoxicity, and eventually delayed glial death [4].

The perception of the role of astrocytes in the brain has transformed from one of being passive supporting cells to being highly active regulators of neuron formation, connectivity and activity [5]. The role of glial dysfunction or death in neurological conditions has not been strongly investigated, due in part to the dogma that most astrocytes were thought to resist or to even proliferate after neurodegenerative insults [6]. However, an increasing number of reports indicate demise of even quiescent astrocytes in either acute or chronic insults where glutamate transport is altered [7–9]. In addition, some astrocytes – known as Alzheimer type II astrocytes (AIIA), presenting altered cytoplasmic and nuclear morphology – have been described in the process of transformation into reactive astrocytes in ischemic injury [10]. Thus, it becomes important to utilize the advantages of an *in vitro* approach, but by examination of the astroglial cell phenotype observed *in vivo*. Dibutyryl cyclic AMP-differentiated astrocytes – also known as quiescent astrocytes – may more faithfully reproduce astroglial features such as stellate morphology and levels of glutamate transporters, compared to undifferentiated astrocytes.

In the ischemic brain, glutamate neurotransmitter release due to peri-infarct depolarizations in concert with impairment or reversal of glial excitatory amino acid transporters (EAATs) generates toxic concentrations of extracellular glutamate [11]. Although both neurons and glial possess glutamate transporters, a common view is that the EAATs reverse primarily in neurons [12], since glutamate content is not decreased in astrocytes and may even increase short term in ischemia models or under severe ATP depletion [13,14]. Elucidating the specific mechanisms that mediate responses of different astrocyte types to brain injury and their consequences for neuronal viability is of potential importance, since preventing such responses could provide neuroprotection and promote recovery. As well, astrocytes may form the basis of restorative therapy, promoting post-stroke recovery therapy by bone marrow derived stromal cells.

We previously demonstrated that differentiated astrocytes are more vulnerable to the EAATs substrate inhibitor L-trans-pyrrolidine-2,4-dicarboxylate (PDC), since cellular uptake of PDC fosters glutamate release through hetero-exchange [15–17]. Substantial glutamate is released from differentiated astrocytes before any detectable decrease is detected in intracellular glutamate levels [15,16]. Moreover, PDC treatment resulted in a decline in antioxidant capacity and delayed oxidative death of dBcAMP-differentiated astrocytes, but not in undifferentiated astrocytes [15,16]. Determining the effect of compromised astrocytes for neuronal viability is of importance, as a decline in antioxidant defenses may increase neuronal vulnerability to excitotoxicity, particularly given that increased generation of ROS, including nitric oxide, superoxide, and peroxynitrite are hallmarks of cellular response to ischemia [18].

To provide insight into the role of quiescent astrocytes and reactive astrogliosis in an ischemic-like insult, we explore if the preferential vulnerability of differentiated astrocytes to EAAT dysfunction extends to OGD. Striatal cultures were chosen for examination due to a heightened sensitivity to ischemia. We extended this examination to consider co-cultures of differentiated or undifferentiated OGD-exposed astrocytes with neurons, in order to determine neuronal fate.

2. Materials and methods

2.1. Materials

dBcAMP, MTT, N-acetylcysteine, 4,6-diamidino-2-phenylindole (DAPI), insulin, Lactate Dehydrogenase Assay Kit (TOX-7) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Dubelcco's Modified Eagle's Modified (DMEM) and glucose were purchased from Gibco (Invitrogen, Cergy Pontoise). Fetal calf serum, horse serum, glutamine, and penicillin–streptomycin were obtained from Cambrex (Vervier, Belgium). The EAATs substrate inhibitor L-trans-pyrrolidine-2,4-dicarboxylate (PDC) was purchased from Tocris (Bristol, UK). Monochlorobimane (mBCl), and rabbit and mouse secondary antibodies coupled to Alexa 488/555 fluorophores were purchased from Invitrogen (Eugene, OR, USA). Primary rabbit anti-GLT1 and GLAST antibodies were kindly provided by Pr. J.D. Rothstein and anti-cleaved caspase 3 antibodies was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Poly-D-lysine-coated glass coverslips were purchased from Becton Dickinson (Le Pont de Claix, France). The caspase 3 cell permeable inhibitor DEVD-CHO was purchased from Calbiochem (France).

2.2. Animals

All experiments were performed according to policies on the care and use of laboratory animals of European Community Legislation. Experiments were approved by the local Ethics Committee. All efforts were made to minimize animal suffering and usage. Adult female Cd1 mice were maintained under approved conditions (agreement N° 13-055-21 and N° C06-152-5) and had free access to standard rodent diet and tap water.

2.3. Striatal murine astrocyte cultures

Astrocyte cultures were prepared from dissected striata as we previously reported [19]. After mechanical dissociation, the cell suspension was diluted in DMEM supplemented with 10% fetal calf serum, 4 mM glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin and seeded in 12-well uncoated plates (Falcon, Elvetec, France) (600 000 cells per 1 mL per well). Culture medium was changed 3 days after seeding and twice a week thereafter. After attaining confluence, cultures were maintained in a defined medium suitable for astrocytes; *i.e.*, Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine, 5 μg/mL insulin, 0.5 mg/mL bovine serum albumin fatty acid free, 50 U/mL penicillin and 50 μg/mL streptomycin. This medium switch was performed to avoid any bias due to the influence of serum on astrocyte morphology and response to drugs [20]. Differentiated astrocytes were