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Protein kinase C-mediated impairment of glutamine outward transport and SN1 transporter distribution by ammonia in mouse cortical astrocytes

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ABSTRACT

SN1, a system N amino acid transporter specific for astrocytes, is mainly responsible for export of newly synthesized L-glutamine from the cells. Astrocytic retention of L-glutamine which plays a critical role in ammonia-induced astrocytic swelling resulting in brain edema, could be tentatively attributed to the impaired L-glutamine export from astrocytes. The present study demonstrates that treatment of cultured mouse cortical astrocytes for 24 h with 5 mM ammonium chloride ("ammonia") inhibits the system N-mediated L-glutamine transport out of the cell, and that this inhibition is related to the reduced presence of the SN1 transporter on the cell membrane. Ammonia decreased total protein kinase C (PKC) activity in the absence but not in the presence of PKC activator, phorbol 12-myristate 13-acetate (PMA), and activation of PKC by PMA reversed both the ammonia-induced decrease of system N-mediated L-glutamine release and ammonia-induced SN1 deficit in the membrane fraction. However, while ammonia did not change the protein level of PKCα isoform, it decreased the protein content of PKCδ. Moreover, ammonia treatment increased the cell surface expression of SN1 in cells with silenced PKCα and PKCδ. Silencing of PKCδ abrogated the decrease of system N (SN1)-mediated L-glutamine release by ammonia. The results implicate the involvement of PKCδ in the inhibition of SN1 membrane expression and activity by ammonia.

1. Introduction

L-glutamine is the most ubiquitous amino acid in all mammalian tissues and body fluids, including the central nervous system (CNS), where its concentration is at least one order of magnitude higher than of any other amino acid (Albrecht et al., 2007; Cynober, 2002; Pithon-Curi et al., 2002). In the brain L-glutamine is formed in astrocytes from L-glutamate and ammonia in an ATP-consuming reaction catalyzed by glutamine synthetase (Albrecht et al., 2010a). A significant proportion of synthesized L-glutamine exits astrocytes and enters neurons, to give rise to the excitatory neurotransmitter amino acid L-glutamate (Albrecht et al., 2010a; Suárez et al., 2002), and the inhibitory neurotransmitter γ-aminobutyric acid (Albrecht et al., 2010a), reflecting the neuronal leg of the glutamate/glutamine cycle (Albrecht et al., 2010a; Waniewski and Martin, 1986). A certain proportion of astrocyte-derived L-glutamine leaves the CNS *via* the cerebral capillary endothelial cells forming the blood-brain barrier (Albrecht et al., 2010b; Lee et al., 1998).

In CNS diseases associated with hyperammonemia including hepatic encephalopathy, excessive L-glutamine synthesis and its accumulation in astrocytes resulting from detoxification of excess of ammonia is considered to be deleterious to brain function. Astrocytes are the locus of glutamine synthetase in the brain (Suárez et al., 2002), which renders them a primary target of excess ammonia. Specifically, excess of newly synthesized L-glutamine contributes to astrocytic swelling which results from its interference with mitochondrial function and from osmotic action (Kruczek et al., 2011; Sinke et al., 2008). In turn, astrocytic swelling is the primary cause of brain edema, a frequent fatal complication of hepatic encephalopathy (Blei and Larsen, 1999; Häussinger et al., 2000).

Astrocytic L-glutamine transport is mediated by a Na^+-coupled amino acid transport system N, represented by three carriers SN1, SN2, and SN7 (SLC38A3, SLC38A5 and SLC38A7, respectively) (Pochini et al., 2014). SN1, coded by the *Snat3* gene identified and annotated on the chromosome 3p21.31 is the most abundant system N transporter in astrocytes, which is mainly responsible for export of newly synthesized L-glutamine from the cells (Bröer et al., 2004). Recently, silencing of transporters SN1 and SN2 in cultured mouse astrocytes has been reported to cause L-glutamine retention in these cells (Zielińska et al., 2016). Those observations prompted a hypothesis that ammonia may contribute to intra-astrocytic L-glutamine retention by interfering with SN1-mediated glutamine efflux. Having confirmed this hypothesis, we

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asked about the mechanism by which ammonia alters the expression and activity of SN1.

Phosphorylation by protein kinase C (PKC) is the principal mechanism which controls intracellular distribution and activity of different membrane proteins (Nissen-Meyer and Chaudhry, 2013). SN1 appears to be regulated by this kinase as was documented by other groups (Balkrishna et al., 2010; Nissen-Meyer and Chaudhry, 2013; Nissen-Meyer et al., 2011; Sidoryk-Wegrzynowicz et al., 2011). PKC activation by phorbol esters (PMA) leads to internalization of SN1, a process correlated with its decreased expression and transport activity as shown in *X. laevis* oocytes and in cultured rat astrocytes *in vitro* (Balkrishna et al., 2010; Nissen-Meyer et al., 2011; Sidoryk-Wegrzynowicz et al., 2011). In turn, SN1 interactions with different PKC isozymes including α, γ and δ isoforms were shown in cultured rat astrocytes (Nissen-Meyer and Chaudhry, 2013; Nissen-Meyer et al., 2011; Sidoryk-Wegrzynowicz et al., 2011). Basing on the above considerations we hypothesized that the mechanism by which ammonia inhibits L-glutamine efflux from astrocytes during hyperammonemia may be related to altered interaction of PKC with SN1 and the ensuing modulation of its activity. Down this valley, we endeavored the role of PKCδ and α isoforms. To test this hypothesis we analyzed mutual relations between activation of total PKC or silencing the particular PKC isozymes, and the expression and cell membrane distribution of SN1 transporter in cultured mouse cortical astrocytes treated or not with ammonia. Analysis of the effect of activation of PKC by phorbol esters also included the rate of [^{3}H]glutamine release from astrocytes with silenced distinct PKC isoforms.

2. Materials and methods

2.1. Materials

Plastic tissue culture dishes were purchased from Corning Costar (Sigma-Aldrich, St. Louis, MO, USA), culture medium from Sigma-Aldrich (St. Louis, MO, USA), fetal bovine serum (FBS) from Biosera (Nuaillé, France), antibiotic antimycotic from Gibco (Thermofisher Scientific, USA) and HiPerfect Transfection Reagent (Qiagen, Germany). All other chemicals of the purest grade were purchased from available commercials sources.

2.2. Astrocyte cultures

Cortical astrocytes were isolated from 7-day-old C57BL6/J mice of both sexes and cultured as described earlier (Hertz et al., 1989). The C57BL6/J mice were obtained from the animal colony of the Mossakowski Medical Research Centre, Polish Academy of Sciences in Warsaw. All experiments were performed according to institutional guidelines for animals, and all efforts were made to minimize the number of animals used (institutional approval no. 55/2015). Seven day after birth the pups were removed from their dams, anesthetized and killed by rapid decapitation. Dissected neocortical tissues were used for the preparation of primary astrocytic cell cultures. Briefly, cortices were passed through Nitex nylon netting (80 μm pore size) into Dulbecco's modified Eagle's medium containing 20% (v/v) FBS. Medium was changed 2 days after plating and subsequently twice a week gradually changing to 10% FBS. In the third week of culturing, dBcAMP was added to the culture medium to promote morphological differentiation. Cells were grown at 37 °C in the atmosphere of 95% O_2 and 5% CO_2, on 24-well, 6-well plates or on 60 mm and 100 mm dishes. Experiments were performed on 3-week-old astrocytes. Astrocytes were treated with 5 mM ammonium chloride ("ammonia") which was added into cell culture medium for 24 h (1 M stock solutions of ammonium chloride were stored at -20° C and added at indicated concentration to culture medium).

2.3. PMA and BisI treatment

Cultured astrocytes were treated with PKC activator, phorbol 12-myristate 13-acetate (PMA; 200 nM; Sigma-Aldrich, USA) and/or PKC inhibitor, bisindolylmaleimide I (BisI; 1 μM; Calbiochem, USA) for 24 h. Co-treatment with BisI and PMA was performed by adding PMA after 10–15 min incubation of cells with BisI.

2.4. Real-time qPCR analysis

Total RNA from astrocytes was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA). RNA concentration was measured by NanoDrop1000 Spectrophotometer (ThermoFisher) and 1 μg of RNA was reversely transcribed using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Warrington, UK). Real-time PCR was performed on 96-well plates with The Applied Biosystems 7500 Fast Real-Time PCR System using the minor groove binder (MGB) Taqman probe assay. Primers and probes for SN1, PKCδ, PKCα and endogenous control β-actin (Mm0120670_m1, Mm00440891_m1, Mm00440858_m1 and Mm00607939_s1 respectively) were purchased from Applied Biosystems. Each reaction contained 5 μl TaqMan Fast Universal PCR Master Mix (Applied Biosystems) in a total volume of 10 μl, and 1.5 μl of cDNA. The real-time PCR reactions were performed at 95 °C for 20 s followed by 45 cycles of 3 s at 95 °C and 30 s at 60 °C. The results of the analysis were calculated and expressed according to an equation ($2^{-\Delta\Delta Ct}$) that gives the amount of the target, normalized to an endogenous control (β-actin). Ct is a threshold cycle for target amplification (Livak and Schmittgen, 2001).

2.5. Cell membrane isolation

Three-week-old astrocytes were washed twice with cold PBS, scrapped off and centrifuged at 2500 g for 5 min at 4 °C. Pellets were homogenized in lysis buffer (15 mM Tris-HCl, pH 7.6, 0.25 M sacharose, 1 mM DTT, 0.5 mM PMSF) containing protease (concentration 1:200, Sigma-Aldrich, St. Louis, MO, USA) and phosphatase (concentration 1:100, Sigma-Aldrich, St. Louis, MO, USA) inhibitors by sonication and subsequently centrifuged for 10 min at 1000 g at 4 °C. Supernatant was collected in the new Eppendorf tubes and the procedure was repeated. Supernatant was added to the previously collected and centrifuged for 20 min at 14000 g at 4 °C. Pellet was dissolved in 40 μl of lysis buffer and subjected to Western Blot analysis.

2.6. Protein isolation and western blot

Astrocytes were washed with PBS, scrapped off and centrifuged at 1000 g for 5 min at 4 °C. Pellets were homogenized in RIPA buffer containing protease (concentration 1:200, Sigma-Aldrich, St. Louis, MO, USA) and phosphatase (concentration 1:100, Sigma-Aldrich, St. Louis, MO, USA) inhibitors and 50 mM sodium fluoride (Fluka, Sigma-Aldrich, Switzerland) by sonication and subsequently centrifuged for 10 min at 10000 g at 4 °C. Supernatant was collected and subjected to Western blot analysis. Protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientific, Thermofischer). Cell lysates containing 30 μg of protein were denatured by boiling in SDS-Page loading buffer for 10 min at 95 °C, separated on SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membranes were blocked in 5% BSA in TBS-T buffer. Incubation with antibodies against SN1 (1:800, ProteinTech, Manchester, UK), PKCδ, PKCα (1:900, ProteinTech, Manchester, UK) was done in 1% BSA in TBS-T buffer overnight at 4 °C followed by 1-h incubation with HRP-conjugated-antirabbit IgG (1:3000 for SN1 and 1:4500 for PKC isoforms; Sigma-Aldrich, USA) for detection by Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). The antibodies were stripped of with 0.1 M glycine, pH 2.9, and the membranes were incubated with HRP-conjugated antibody against Glyceraldehyde 3-