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Down-regulation of the mitochondrial aspartate-glutamate carrier isoform 1 AGC1 inhibits proliferation and N-acetylaspartate synthesis in Neuro2A cells

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ABSTRACT

The mitochondrial aspartate-glutamate carrier isoform 1 (AGC1) catalyzes a Ca^{2+}-stimulated export of aspartate to the cytosol in exchange for glutamate, and is a key component of the malate-aspartate shuttle which transfers NADH reducing equivalents from the cytosol to mitochondria. By sustaining the complete glucose oxidation, AGC1 is thought to be important in providing energy for cells, in particular in the CNS and muscle where this protein is mainly expressed. Defects in the AGC1 gene cause AGC1 deficiency, an infantile encephalopathy with delayed myelination and reduced brain N-acetylaspartate (NAA) levels, the precursor of myelin synthesis in the CNS. Here, we show that undifferentiated Neuro2A cells with down-regulated AGC1 display a significant proliferation deficit associated with reduced mitochondrial respiration, and are unable to synthesize NAA properly. In the presence of high glutamine oxidation, cells with reduced AGC1 restore cell proliferation, although oxidative stress increases and NAA synthesis deficit persists. Our data suggest that the cellular energetic deficit due to AGC1 impairment is associated with inappropriate aspartate levels to support neuronal proliferation when glutamine is not used as metabolic substrate, and we propose that delayed myelination in AGC1 deficiency patients could be attributable, at least in part, to neuronal loss combined with lack of NAA synthesis occurring during the nervous system development.

1. Introduction

The mitochondrial aspartate-glutamate carrier (AGC) catalyzes the unidirectional export of mitochondrial aspartate in exchange with cytosolic glutamate plus a proton across the inner mitochondrial membrane [1]. Stimulated by cytosolic calcium [1,2], AGC is a fundamental component of the malate-aspartate shuttle (MAS) which is the main biochemical pathway to transfer NADH reducing equivalents from the cytosol to mitochondria along with the glycerol-3-phosphate shuttle. These processes occur in the various cell types with different efficiency and are indispensable for the complete aerobic oxidation of glucose, preserving the cellular redox state with higher energy yield and efficient ATP production. In humans, AGC exists as two isoforms, AGC1 and AGC2 encoded by *SLC25A12* and *SLC25A13* genes, respectively [1]. Both isoforms share very similar substrate specificity and affinities, although AGC2 show higher transport rates than AGC1 [1]. Mutations in AGC1 and AGC2 are associated with two human diseases [3]. AGC1 deficiency is a severe infantile-onset encephalopathy with epilepsy, global developmental delay, abnormal myelination, and reduced cerebral

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N-acetylaspartate (NAA) content [4,5]. Defects in AGC2 cause adult-onset type 2 citrullinemia [6]. The two disorders have very different clinical pictures related to the higher expression of AGC1 and AGC2 in brain and liver, respectively [7], and are both associated with impaired MAS activity [4,8]. However, biochemical hallmarks of these pathologies appeared not merely linked to a bioenergetic deficit. In type 2 citrullinemia, the loss of aspartate efflux from mitochondria due to AGC2 transport defects induces a liver-specific impairment of argininosuccinate synthetase, a cytosolic enzyme of the urea cycle [6]. Furthermore, hypomyelination in AGC1 deficiency could be secondary to reduced levels of neuronal-generated NAA [5], a precursor for myelin synthesis produced from aspartate and acetyl-CoA by the enzyme aspartate-N-acetyltransferase [9]. Should this evidence demonstrate the physiological role of AGC, AGC isoforms (AGCs) might potentially act as crucial means to supply aspartate from mitochondria for biosynthetic purposes, with respect of the specific function of the cells where the two isoforms are expressed. However, a detailed description of the relative contribution of AGCs in the various tissues is still elusive. This is particularly true in the brain where a controversial expression pattern of AGCs has been hitherto depicted: AGC1 is generally thought to be the main isoform in neurons [10–11], where AGC2 is likewise expressed, although only in restricted brain areas and at low levels [12]. In glial cells, conflicting results did not clarify the expression profile of AGCs in astrocytes [13–16], whereas both isoforms have been reported to be expressed in oligodendrocytes and oligodendrocyte progenitors [17]. The clinical manifestations in AGC1 deficiency suggest that AGC1 should have a predominant role at least in discrete brain cells most likely during brain development.

In the present study, AGC1 was identified as the sole AGC isoform in proliferating mouse neuroblastoma Neuro2A (N2A) cells, while AGC2 is present only during differentiation. Consistently with the importance of AGC in MAS, we showed that AGC1 down-regulation is detrimental to the survival of undifferentiated N2A cells when exclusively fed with substrates producing NADH in the cytosol. By contrast, addition of glutamine rescued the proliferation of N2A cells with reduced AGC1: in these cells, we demonstrated increased glutamine oxidation accompanied by higher mitochondrial ROS production, strongly suggesting the activation of adaptive mechanisms which might favor the generation of substrates supporting proliferation, such as aspartate [18], otherwise impeded by AGC1 inactivation. Furthermore, we demonstrated that NAA levels in undifferentiated N2A neuroblastoma cells are strongly reduced if AGC1 is down-regulated, a defect that can be ascribed not only to insufficient aspartate, but also to the limited pyruvate oxidation subsequent to MAS impairment which prevents the formation of appropriate acetyl-CoA levels for NAA synthesis.

2. Materials and methods

2.1. Cell culture and reagents

Neuro2A cells were purchased from ATCC-LGC Standards (Italy) and cultured at 37 °C in a humidified atmosphere with 5% CO_2 in high glucose DMEM (D6546 SIGMA - Italy) supplemented with 10% fetal bovine serum, 50 U of penicillin G/ml and 50 μg of streptomycin sulfate. In this study, cells were used between #3 and #20 passages. Unless otherwise indicated, cell incubations in minimal growth medium were accomplished by using Minimal Essential Medium (MEM, M5650 SIGMA - Italy). Cell counting was performed using the Scepter™ Automated Cell Counter (Merck Millipore, Germany) according to the manufacturer's instructions, in parallel to trypan blue visualization to exclude significant differences in live/dead trypsinized cells ratio among the tested cells.

2.2. AGC1-silencing shRNA design and lentiviral construct generation for stable transduction of N2A cells.

Two shRNA cassettes 5′-TGCTTGTTCGAAAGATCTATAGCTCGAGC TATAGATCTTTCGAACAAGCTTTTT-3′ and 5′-TGCTTGCAGACCTATATAA TGCCTCGAGGCATTATATAGGTCTGCAAGCTTTTT-3′ were designed as a hairpin-loop structure from the mouse Slc25a12 cDNA sequence encoding AGC1 protein according to the guidelines described in http://sirna.wi.mit.edu/ [19] and *Age*I/*Eco*RI cloned into the pLKO.1 vector (Sigma - Italy), as previously described [20]. In parallel, a mismatch shRNA 5′-TACAACCAACGCACGCTAATCTCGAGATTAGCGTGCGTTGGT TGTTTTTT-3′ was cloned into the same vector to generate unsilencing control plasmid. The resulting constructs were sequenced and used to transfect HEK293T cells with the Lentiviral Packaging mix (SIGMA - Italy) according to the manufacturer's instructions for the recombinant generation of control LVshMM, and AGC1-silencing LVshAGC1.1 and LVshAGC1.2 lentiviral particles. 4 days after transfection, cell conditioned media containing the virus were harvested, their titer estimated and used for the transduction. N2A cells were transduced with the recombinant LVshMM and LVshAGC1 particles at a viral titer of ~5 plaque-forming units/ml for 24 h at 37 °C and treated with 1 μg/ml puromycin for stable selection.

2.3. Immunoblotting

Total cell extracts or mitochondrion-enriched fractions obtained with Potter-Elvehjem homogenization and serial centrifugations were lysed in RIPA buffer, solubilized in the presence of 10 mM Tris/HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol and subjected to 15% SDS-polyacrylamide gel for subsequent western blot analysis. AGC1, AGC2, UCP2, α-actin and NFL antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX USA). AAC antibody was purchased from Mitoscience (Eugene, OR USA). Caspase 3 and PARP-1 antibodies were purchased from Cell Signaling Technology (Danvers, MA USA). Homemade antibodies raised in rabbit against mitochondrial carriers for dicarboxylates (DIC), citrate (CIC), glutamate (GC), phosphate (PiC) and 2-oxoglutarate/malate (OGC) were used. Densitometric analyses were accomplished by using the Image Lab™ Touch software (Bio-Rad Laboratories, CA USA).

2.4. Mitochondrial carriers transport activity measurements

30 μg of isolated mitochondria from N2A cells were solubilized (0.5 mg/ml) in a buffer containing 3% TX-114, 1 mM EDTA, 10 mM PIPES, pH 7.0 for 45 min on ice, and reconstituted in liposomes, as previously described [21]. Transport measurements at 25 °C were initiated by adding radioactive substrates at the indicated concentrations to liposomes reconstituted with mitochondrial extracts containing 20 mM unlabeled substrates and terminated by adding 10 mM pyridoxal 5′-phosphate and 8 mM bathophenanthroline, according to the inhibitor-stop method [1,22]. The incorporated radioactivity was quantified by a LS 6500 liquid scintlilation counter (Beckman Coulter, CA USA).

2.5. Measurements of oxygen consumption (OCR) and extracellular acidification rates (ECAR)

OCR and ECAR were simultaneously measured with XF^{96} Extracellular Flux analyzer (Seahorse Bioscience, MA USA). Respiration parameters were also determined through the high resolution O2k respirometer (OROBOROS Instruments, Austria). In Seahorse experiments, 25.000 cells/well were washed three times with unbuffered XF base medium (Seahorse Bioscience, MA USA) without added substrates and then incubated for 1 h in humidified incubator at 37 °C in the presence of unbuffered XF base medium supplemented with 1 g/l glucose + 1 mM pyruvate or 5 mM lactate ± 2 mM glutamine. After incubation, basal OCR/ECAR were recorded three times for total 12 min prior to the sequential injections of 2 μM oligomycin, as inhibitor of ATP synthase (three measurements for total 25 min), 0.2 μM FCCP, as mitochondrial uncoupler (three measurements for total 25 min), and 1 μM antimycin A + 1 μM rotenone, as mitochondrial respiratory chain inhibitors (three measurements for total 25 min). In O2k oxygraphy experiments, 1 × 10