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Glucose-6-phosphate tips the balance in modulating apoptosis in cerebellar granule cells

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ABSTRACT

A metabolic shift from oxidative phosphorylation to glycolysis (*i.e.* the Warburg effect) occurs in Alzheimer's disease accompanied by an increase of both activity and level of HK-I. The findings reported here demonstrate that in the early phase of apoptosis VDAC1 activity, but not its protein level, progressively decreases, in concomitance with the physical interaction of HK-I with VDAC1. In the late phase of apoptosis, glucose-6-phosphate accumulation in the cell causes the dissociation of the two proteins, the re-opening of the channel and the recovery of VDAC1 function, resulting in a reawakening of the mitochondrial function, thus inevitably leading to cell death.

1. Introduction

Proper cell activity requires an efficient exchange of molecules between mitochondria and cytoplasm. Lying in the mitochondrial outer membrane (MOM), voltage-dependent anion channel (VDAC) – the most abundant, ancient and highly-conserved protein, previously considered to be responsible for the almost free permeability of the MOM [1–4] or a large mesh sieve [5] – unexpectedly soars as gatekeeper for the entry and exit of mitochondrial metabolites, thereby controlling cross-talk between mitochondria and the rest of the cell [3–8]. In higher eukaryotes, three VDAC isoforms – encoded by three separate genes – have been characterized, *i.e.* VDAC1, VDAC2 and VDAC3 [9], being VDAC1 the most abundant in most cells (see [10]). Its location at the boundary between the mitochondria and the cytosol enables VDAC1 to interact with proteins that mediate and regulate the interrelationship between mitochondria and other cellular activities. Along with regulating cellular energy production and metabolism, VDAC1 is also a key protein in mitochondria-mediated apoptosis, participating in the release of apoptotic proteins and interacting with antiapoptotic proteins. Between the mitochondria-interacting proteins which utilize VDAC1 as anchor point, the one of major interest is hexokinase (HK). Several studies concerning HK claim that, in cells undergoing apoptosis, this enzyme is up-regulated [11], as it happens in cancer cells which are characterized by a high rate of glycolysis [12,13] – *i.e.* the known Warburg effect – and also that the binding of HK to VDAC1 is somehow involved in the protection against proapoptotic stimuli [14–18].

Although some evidences supporting the concept that VDAC1 role is cardinal in Alzheimer's disease (AD) progression exist [19–23], its precise involvement as a tool to regulate cell bioenergetics and apoptosis is unknown. Here, we evaluated, in the early as well in the late phase of apoptosis, the expression level and the activity of VDAC1 before examining the interaction of VDAC1 with HK-I. Compelling evidences suggest that glucose-6-phosphate (G6P)

Abbreviations: AD, Alzheimer's disease; ANT-1, adenine nucleotide translocator-1; Ap5A, P1,P5-di(adenosine-5′)pentaphosphate; ATP D.S., ATP detecting system; ATR, atractyloside; BME, basal medium Eagle; CGCs, cerebellar granule cells; COX, cytochrome c oxidase; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; GLU, glucose; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; HK, hexokinase; IMS, inter-membrane space; MOM, mitochondrial outer membrane; O_2, molecular oxygen; PBS, phosphate buffer saline medium; PHLO, phloretin; PnAc, cis-parinaric acid; mRC, mitochondrial respiratory chain; ROS, reactive oxygen species; S.D., standard deviation; SK5 cells, apoptotic cells; S-K25 cells, control cells; SOD, superoxide dismutase; VDAC, voltage-dependent anion channel

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disrupts the physical interaction between the two proteins, then assuming the attractive role of tip the scales for modulating mitochondrial dynamics in AD.

2. Materials and methods

2.1. Cell culture, suspension and homogenate preparation

Primary cultures of cerebellar granule cells (CGCs) were obtained from dissociated cerebella of 7-day-old Wistar rats, as in [24]. CGC suspension and homogenate were obtained by (i) removing the culture medium, (ii) repeatedly washing plated cells with phosphate-buffered saline (PBS), (iii) scraping cells, (iv) collecting them to have the cell suspension [25] and then (v) breaking up this last by about 10 strokes with a Dounce homogeniser at room temperature [see 26,27] to obtain cell homogenate. For protein content, see [28].

2.2. Induction of apoptosis and assessment of neuronal viability

Apoptosis was induced at 6–7 days in vitro, as reported in [29]. Apoptotic and control cells are referred to as S-K5 and S-K25 cells, respectively. Viable CGCs were quantified as in [30].

2.3. Mitochondrial function measurements

Oxygen consumption was measured by means of a Gilson 5/6 oxygraph using a Clark electrode, as in [27]. ANT-1 and VDAC1 activities were estimated by following the exchange ADP/ATP across mitochondrial membranes measured as in [27], in the absence or in the presence of either atractyloside (ATR) or 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), *i.e.* cell permeable blockers of ANT and VDAC1 [31–33], respectively. The rationale for this approach is described in the Results section. Cytochrome oxidase activity was assayed essentially as in [34,35].

2.4. Lipid peroxidation

Lipid peroxidation was detected by using the sensitive technique of *cis*-parinaric acid (PnAc) fluorescence loss, as in [35].

2.5. HK assays

The activity of HK was assayed spectrophotometrically at 340 nm under V_{max} conditions, as in [11].

2.6. Determination of G6P in cells incubated with glucose

Glucose (GLU), at concentrations and for times reported in the legend, was incubated with plated cells. When the incubation time was terminated, removal of the glucose-containing medium, double washing of the cell layer and replacement with fresh medium were made. Immediately after, apoptosis was induced (see above). G6P was assayed, essentially as in [36], in cell homogenate either before apoptosis induction, *i.e.* time = 0, or after the apoptosis induction time, *i.e.* 3 h. Our care has been ensuring that the accumulation of G6P in cell does not substantially change, *i.e.* G6P level does not undergo an obvious metabolism-dependent decline, during the time of apoptosis induction. The use of inhibitor/s of G6PDH was avoided to prevent uncontrollable side effects and so operate under conditions closest to those physiological.

2.7. Western blot analysis

Cell lysis and Western blot analysis were performed mainly as in [37]. Briefly, equal amounts of protein were subjected to SDS–PAGE on 11% Tricine–SDS–polyacrylamide gels [38], blotted onto PVDF membranes and then probed with polyclonal anti-HK-I (AB3543, Millipore, Temecula, CA, USA) and monoclonal anti-β-actin antibodies (A4700, Sigma Chemical Co., St. Louis, MO, USA). HRP-conjugated secondary antibodies were used for detection followed by enhanced chemiluminescence development.

2.8. Co-immunoprecipitation

Cells were lysed in 1 % Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT pH 7.2, for 10 min at 4 °C in the presence of protease and phosphatase inhibitor cocktails. Lysates were then centrifuged at 4 °C for 10 min at 960×*g*. The protein extracts (500 μg) were immunoprecipitated by Protein A/G PLUS-Agarose according to the manufacturer's instructions using 7 μg of polyclonal anti-HK-I antibody. After overnight incubation at 4 °C, the immunocomplexes were eluted with Laemmli buffer 2× and were next analyzed by immunoblotting with the monoclonal anti-VDAC1 antibody (MABN504, Millipore, Temecula, CA, USA).

3. Results and discussion

3.1. VDAC1 activity and protein level in cells undergoing apoptosis

VDAC1 translocates a variety of metabolites, including ATP and ADP, across the mitochondrial outer membrane. In order to estimate its activity, an experimental strategy allowed us to follow the ADP/ATP exchange measuring VDAC1 and ANT-1 activities in the same experiment. To start with, the measurement of ADP/ATP exchange was carried out in homogenates from S-K25 cells in the absence or presence of compounds designed to block VDAC1, *i.e.* DIDS (20 μM), or ANT-1, *i.e.* ATR (2 μM). In a typical experiment, cell homogenate was treated with AP_5A (10 μM) to inhibit adenylate kinase [for Ref. see 21], thus preventing mitochondrial ATP synthesis in a manner not dependent on oxidative phosphorylation, and then incubated in the presence of an ATP detecting system (see Ref. [27] for methodological details). The ATP concentration in the extramitochondrial phase of the homogenates was negligible as shown by the fact that no increase in the absorbance measured at 340 nm was found in the presence of glucose, hexokinase, glucose-6-phosphate dehydrogenase and $NADP^+$. As a result of ADP addition (0.04 mM), an increase in the NADPH absorbance was observed, indicating the appearance of ATP in the extramitochondrial phase. The rate of NADPH formation was approximately 10 nmol $NADP^+$ reduced/min mg cell protein (Fig. 1Aa), in good agreement with values obtained by Atlante et al. [26]. NADPH formation derives from (i) ADP uptake into mitochondria in exchange for endogenous ATP, (ii) ATP synthesis from imported ADP via ATP synthase and (iii) efflux of the newly synthesized ATP from the mitochondria in exchange for further ADP. A decrease of NADPH formation (Fig. 1Ab-c) was recorded in the presence of DIDS or ATR (3.2 and 5.5 natoms/min × mg protein, respectively), showing that (i) the exchange of ADPext with ATPint exclusively occurs through the outer and inner mitochondrial membranes, respectively; (ii) both the membranes are intact and (iii) the exchange is mediated by protein/s.

Applying the control strength criterion and using DIDS at different concentrations (Fig. 1B), we observed that the rate of absorbance increase, *i.e.* ADP/ATP exchange, in the absence of inhibitor was lower than the value corresponding to the straight line that intercepts the *Y*-axis at zero inhibitor concentration – obtained by interpolating the experimental points of absorbance increase rate in the presence of DIDS – thus showing that (i) the inhibited step of measured processes, *i.e.* the ADP/ATP exchange across MOM via VDAC1, is not the limiting step and (ii) the reciprocal