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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

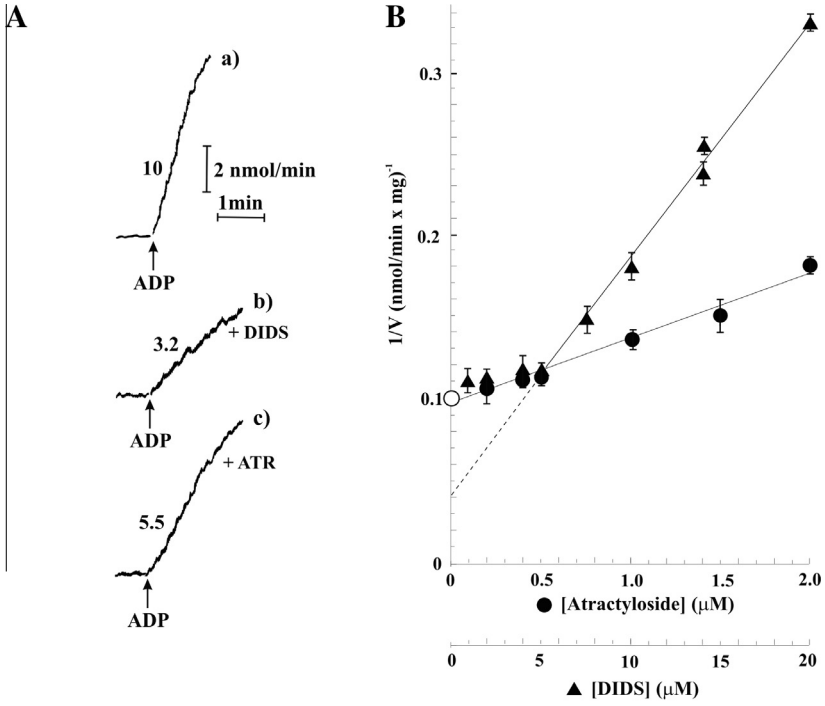
Cells were lysed in 1 % Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT pH 7.2, for 10 min at 4 °C in the presence of protease and phosphatase inhibitor cocktails. Lysates were then centrifuged at 4 °C for 10 min at 960×*g*. The protein extracts (500 μg) were immunoprecipitated 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



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Time after apoptosis induction	VDAC activity (% of Control)
10’	95 ± 5
30’	83 ± 3
45’	72 ± 4
1h	66 ± 6
3h	60 ± 4
5h	70 ± 5
8h	86 ± 4

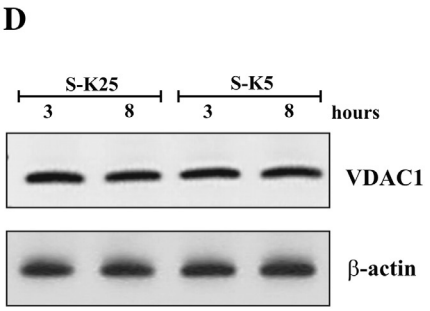


Fig. 1. VDAC1 activity and protein level in cells undergoing apoptosis. (A) Appearance of ATP due to addition of ADP (0.04 mM) to CGC homogenate (0.1 mg protein) from S-K25 cells was monitored, either in the absence (a) or in the presence of 20 μM DIDS (b) and 2 μM ATR (c), added to cell homogenate 1 min before ADP addition, by using an ATP detecting system consisting of glucose (2.5 mM), HK (0.5 e.u.), G6PDH (0.5 e.u.) and $NADP^+$ (0.2 mM) in the presence of P1,P5-di(adenosine-5′)penta-phosphate (AP5A), a specific inhibitor of ADK. The rate of $NADP^+$ reduction in the extramitochondrial phase was followed as the absorbance increase at 334 nm, measured as the tangent to the initial part of the progress curve and expressed as nmoles $NADP^+$ reduced per min per mg cell protein. (B) Appearance of ATP due to addition of ADP (0.04 mM) to CGC homogenate (0.1 mg protein) from S-K25 cells was monitored, as in A, either in the absence (○) or in the presence of DIDS (▲) and ATR (●) at the indicated concentrations. Reported values are the mean of three independent neuronal preparations (with comparable results), each one in triplicate, with the standard deviation. (C) The values of VDAC1 activity, calculated at different times after apoptosis induction and expressed as % of control, are reported (for details see Section 3). (D) VDAC1 protein expression. Cell lysates from either control (S-K25) or apoptotic CGCs (S-K5), at 3 and 8 h, were analyzed by Western blotting analysis with anti-VDAC1 antibody, as described in Section 2. Antibodies against β-actin were used to normalize the protein amount loaded onto the gel.

value of the intercepts to the ordinate axis is measure of the inhibited process, *i.e.* VDAC1 activity. This was true also for all the considered times of apoptosis (not shown).

In contrast, the intercept of the *Y*-axis in the case of the ADP/ATP exchange, inhibited by ATR, coincides with the rate value in the absence of inhibitor, according to [27].

Then, to determine the activities of VDAC1 in S-K5 cells at different times after low potassium-apoptosis induction, the reciprocals of the intercept to the *Y*-axis were analyzed, essentially as reported in [39,11], and showed a decrease in activity which starts as soon as 10 min after apoptosis induction (Fig. 1C). The maximum enzyme activity decrease in S-K5 cell homogenate (about 40%) was measured 3 h after potassium shift, time at which VDAC1 activity undergoes a sharp reversal trend, with progressive increase up to 8 h, when it returns to values similar to control. Worthy of note is the fact that here, for the first time, the activity of VDAC1 has been measured in conditions close to the physiological ones. In fact, as for any membrane protein responsible for exchange of solutes across a membrane, the functional properties of VDAC have always been examined in reconstituted systems with artificially prepared phospholipid bilayers [see 40]. As expected [27], a progressive decrease of activity of ANT-1 in cell undergoing apoptosis was observed (not shown). Importantly, variations in VDAC1 activity take place in cell undergoing apoptosis (at 3 and 8 h after K^+-shift) regardless of the relative abundance of the protein, as checked by Western blotting analysis on whole-cell extracts (Fig. 1D), proving thus that its regulation under our in vitro conditions was not attributable to differences in intracellular expression levels but

rather to a modulation in enzymatic activity. Conversely, in post-mortem brain of AD patients, VDAC1 and/or VDAC2 levels are significantly reduced or elevated in different brain regions [19], as well VDAC1 is overexpressed in the hippocampus from amyloidogenic AD transgenic mice models [21].

3.2. VDAC1 interacts with HK-I. Effect of DIDS

We have recently demonstrated that, in the early phase of apoptosis, glucose metabolism is enhanced with up-regulation of key proteins which internalize and metabolize glucose, such as HK-I, while a parallel decrease in oxygen consumption by mitochondria occurs [11].

In order to investigate whether in our cell model of apoptosis HK-I interacts with VDAC1 at the MOM – as it occurs in cancer cells [see 9] – coimmunoprecipitation experiments on whole cell extracts were performed from apoptotic samples at 3 and 8 h after K^+-shift – and relative controls – using polyclonal anti-HK-I antibody as bait-antibody, followed by immunoblotting with a monoclonal antibody which specifically interacts with VDAC1. As shown in Fig. 2A, VDAC1 coimmunoprecipitated with HK-I suggesting that the VDAC1/HK-I complex was present in SK5 cells at 3 h after apoptosis induction. Surprisingly, in 8 h-S-K5 cells the VDCA1 signal dramatically decreased and became roughly similar to the faint signal detected in control cells, at both 3 and 8 h, thus suggesting that the stability of VDAC1/HK-I complex was inversely associated with apoptosis progression. In this regard, Azoulay-Zohar et al. [41] showed that HK-I directly interacts with bilayer-reconstituted VDAC to induce closure of the VDAC channel in a manner that is reversed by G6P. In addition, HK-I and VDAC have been co-immunoprecipitated [42]. Moreover, mutagenesis studies revealed that a single mutation in VDAC1 prevented HK-I-mediated protection against apoptosis and channel closure [43], while N-terminally truncated VDAC1 lost its HK-I binding capacity [44].

In the experiments reported in Fig. 2B, both VDAC1 (▲) and HK-I (●) activities, measured as in Fig. 1 and in [11] respectively, were reported as a function of time (0–8 h) of apoptosis. As shown, the rate of ADP/ATP exchange, via VDAC1, at 0.04 mM ADP, decreased up to 3 h; in the same time range, HK-I increased. After 3 h a reverse trend of the activities occurred: the activity of VDAC1

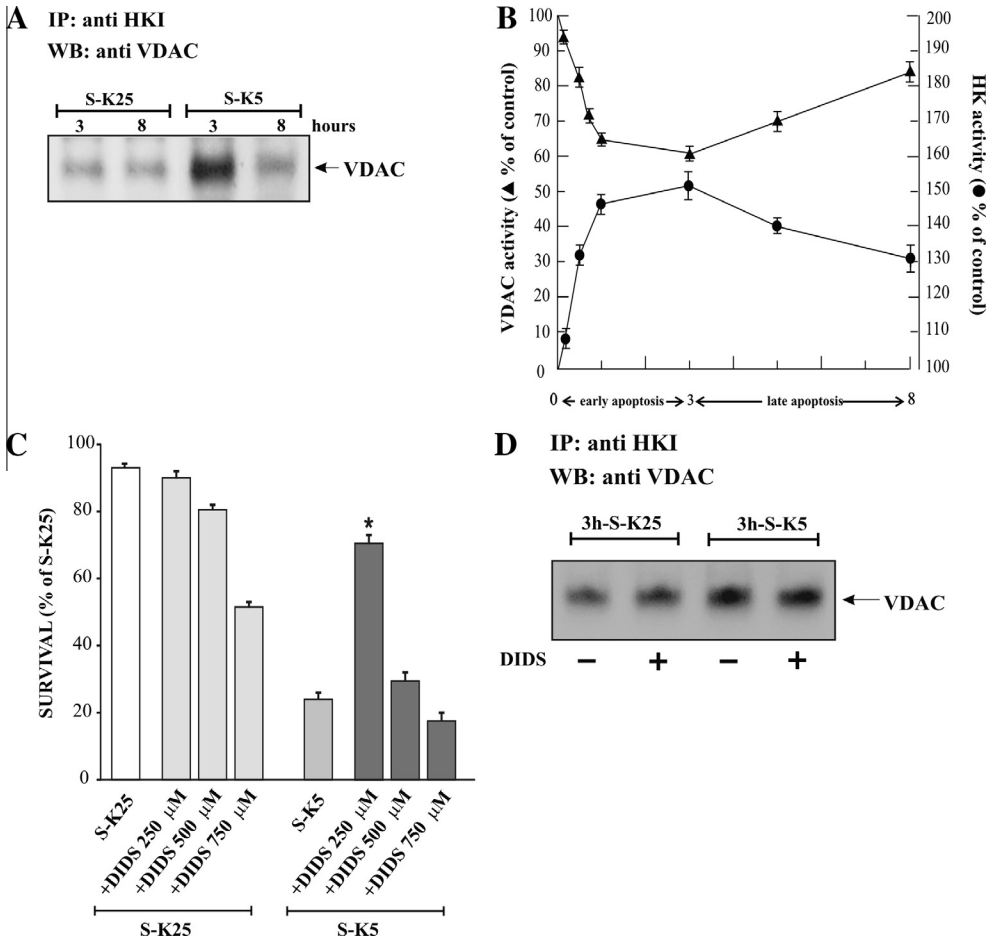


Fig. 2. VDAC1 interacts with HK-I. Effect of DIDS. (A) Lysates of either control (S-K25) or apoptotic CGCs (S-K5), at 3 and 8 h, were subjected to immunoprecipitation by anti-HK-I antibody and immunoprecipitated samples were probed for VDAC1. (B) The activities of VDAC1 and HK-I, calculated at different times after apoptosis induction, are expressed as % of control (for details see Section 3). All assays, performed at least in duplicate, were at 25 °C and pH 7.2 to mimic intracellular pH. (C) Sensitivity of cell survival to DIDS in cells undergoing apoptosis. Rat CGCs (2×10^6/well) at 7 DIV were incubated either in high potassium (S-K25) or in low potassium (S-K5) serum-free culture medium. Where indicated DIDS, at different concentrations, was added to cultured S-K25 or S-K5 CGCs for 120 min, with exposure terminated by (i) removal of the compound-containing medium, (ii) twice washing of cell layers and (iii) S-K25- or S-K5-medium replacement. Cell survival, measured after 24 h by counting the number of intact nuclei, is expressed as the percentage of the S-K25 cells to which a value of 100% has been given. Results are means ± S.D. of triplicate measurements and representative of six different experiments carried out with different cell preparations from different groups of animals. Statistical analysis was by ANOVA and Bonferroni test: $^*p < 0.001$ when compared the sample with S-K5 cell. The lack of asterisk indicates no statistically significant differences. (D) Lysates of either control (S-K25) or apoptotic CGCs (S-K5), at 3 and 8 h, treated or not with 250 μM DIDS for 120 min as described above, were subjected to immunoprecipitation by anti-HK-I antibody and immunoprecipitated were probed for VDAC1.

was broadly restored, while that of HK-I is lowered. At 8 h, time at which VDAC1/HK-I complex was destroyed (Fig. 2A), the activities of both enzymes tended to the control values. Then all the results suggest that the activity of VDAC1 strictly depends on physical interaction with HK-I. It is low when HK-I, bound to the cytosolic face of VDAC1, acts as a gate, affecting mitochondrial and overall cellular bioenergetics, while it tends to rise when the binding is destroyed. Since this disruption favors a VDAC1 conformational state more prone to the release of proapoptotic factors [see 9], regulation of the function of this channel may be a promising therapeutic approach to combat AD. Consistently, some reports have shown that in tumors the HK–VDAC1 interaction prevents apoptosis [17,41,43,45], while disruption of HK–VDAC1 binding via mutagenesis of key amino acids on VDAC, significantly enhances induction of apoptosis [46].

Before investigating the role of G6P – whose level increases in cell undergoing apoptosis [11] – which, as known, acts as allosteric inhibitor of HK, we have examined the effect of DIDS on both cell viability and VDAC1–HK-I binding (Fig. 2C and D). When the responsiveness of S-K5 cells to increasing DIDS concentrations was checked at 24 h, a strong prevention of death was observed in the presence of 250 μM DIDS (Fig. 2C), which was without effect on the survival of S-K25 control cells, consistently with [31]. On the other hand, the same DIDS concentration (250 μM) had no effect on the VDAC1–HK-I binding since it did not attenuate the band intensity at 3 h which was comparable to that obtained in the absence of the VDAC blocker (Fig. 2D). These results are noteworthy and confirm the above Fig. 2A and B: knock out the VDAC1, either by the interaction with HK-I or with the channel blocker, is an advantage for the cell, which attempts to oppose the impending catastrophe, and confers an anti-apoptotic role to HK-I when bound to VDAC1. Consistently, disruption of HK binding to VDAC1 by mutation in VDAC1 or by addition of VDAC1-based peptides decreased the survival of cells [47], data supported by the finding that cisplatin-induced apoptosis is inhibited in cells silenced for VDAC1 expression [23,48,49], in which, for more, the mitochondrial maintenance and function is improved and may protect against toxicities of AD-related genes.

3.3. G6P accumulation detaches HK-I from VDAC1 and restores mitochondrial function

In a recent paper [11], we show that CGCs upregulate Warburg effect-enzymes in a manner reminiscent of cancer cells, in the early phase of apoptosis, thus mimicking the apoptotic resistance mechanism of surviving neurons in the AD brain. This situation is characterized by (i) high aerobic glycolysis and suppression of mitochondrial respiration, (ii) high expression of HK-I which binds to the MOM via a specific association with VDAC1 [see 50 and results of Fig. 2] and (iii) G6P accumulation, whose level gradually rises in S-K5 cells. In this context, G6P, the product of HK reaction, antagonizes binding to VDAC1 and leads to release of HK-I from mitochondria [see 50], then opening VDAC1 and allowing mitochondria to resume their normal aerobic metabolism, including uptake of ADP, Pi and respiratory substrates, oxidative phosphorylation, and release of ATP into the cytosol.

We have collected several and compelling evidences proving that HK-I, clinging to VDAC1, inhibits the channel in cells simulating an AD-like situation. In order to investigate the action of G6P in neurons undergoing apoptosis, we incubated plated cells with GLU with the aim of researching the experimental condition that ensures (i) an accumulation of G6P into cell and that (ii) G6P high levels remain as such in the time range required to perform the measurements. Fig. 3A′ and A″ reports G6P assay in cells added with (i) GLU at different concentrations for 30 min (A′) and (ii) GLU (25 mM) for different incubation times (A″). As a control, phloretin (PHLO, 50 μM), a specific inhibitor of glucose transport [51], prevents G6P accumulation (Fig. 3A′). In Fig. 3A‴, cytosolic G6P level has been monitored in the times following the incubation period with 25 mM GLU: G6P remained roughly high up to 3 h (about 80–85% with respect to time = 0, *i.e.* immediately after the 30 min incubation of cells with GLU), thus confirming that G6P accumulation lasts for all the time required by the experimental conditions.

To determine whether mitochondrial function is affected in G6P-loaded-S-K5 cells, we measured VDAC1 activity, oxygen consumption, lipid peroxidation and cytochrome oxidase activity in cells at 3 h after K^+-shift, time at which (i) the maximum decrease of VDAC1 activity and (ii) the change of trend of the VDAC1 activity occurs, as compared to unloaded S-K5 cells. VDAC1 activity increases (27%) with respect to the control (Fig. 3B), suggesting that G6P reverses blockade of VDAC1 conductance. Control was made that G6P was without effect when added to the cuvette in which VDAC1 activity was assayed (not shown). This result simply allows mitochondria to resume operation: oxygen consumption by glucose increases (30%) (Fig. 3C), due to the mitochondrial respiratory chain (mRC) that resumes to work at a high rate. As expected, a greater loss of fluorescence of cis-Parinaric acid (PnAc), *i.e.* a higher membrane lipid peroxidation, is observed (20%) in G6P-loaded-S-K5 cells, indicating that the G6P-dependent resumption of VDAC1 may enhance the lipid peroxidation (Fig. 3D), likely due to increased production of mitochondrial superoxide anions at the mRC level. To confirm this hypothesis, about 25% G6P-induced COX inhibition was observed, almost completely prevented by either 5 U/ml SOD or 250 μM DIDS (Fig. 3E), thus allowing to propose that the effect of G6P on COX activity – which, as known is cardiolipin-dependent [35] – is ROS-mediated. As expected, a HK-I activity decrease (35%) was found under these experimental conditions, *i.e.* in G6P-loaded cells (Fig. 3F). All these data strongly suggest that G6P, accumulated into cells before apoptosis starts, partially prevents the HK-dependent closure of VDAC1, and that the HK anchorage at VDAC1 is a reversible process in cell acting not only as the "guardian of mitochondria" but, more interestingly, as a promising target for developing novel therapeutic approaches against neurodegeneration.

4. Conclusions

Our results suggest that HK-I regulates apoptotic cell death via interaction with and closure of VDAC1 in a fruitless attempt to promote cell survival. Therefore, as a result, VDAC1 stands acting as a 'governator' of mitochondrial function, establishing set points for global mitochondrial activity that change in response to cellular needs and metabolic stresses. Within this scenario, a leading figure is that of G6P, whose level, along with L-lactate and NADH/NAD^+ ratio (see [11]), raises in cells which – when subjected to an apoptotic stimulus – activate a Warburg effect-like mechanism [11]. The overall process occurring in the course of CGC apoptosis, is the sum of the consequent steps illustrated in Scheme 1:

(i) high level of HK-I [11] causes its physical anchorage to VDAC1 (Fig. 2A), whose activity, but not the protein level, decreases in the early phase of apoptosis (Fig. 1A and B);
(ii) mitochondrial function is then impaired, while glycolysis speeds up [11];
(iii) no Mitochondrial respiration, no ROS production, no ROS leakage: the HK-I–VDAC1 binding keeps low the oxidative stress in cell;
(iv) inhibition or down-regulation of a metabolic glycolysis step [see 11] causes higher level of G6P;

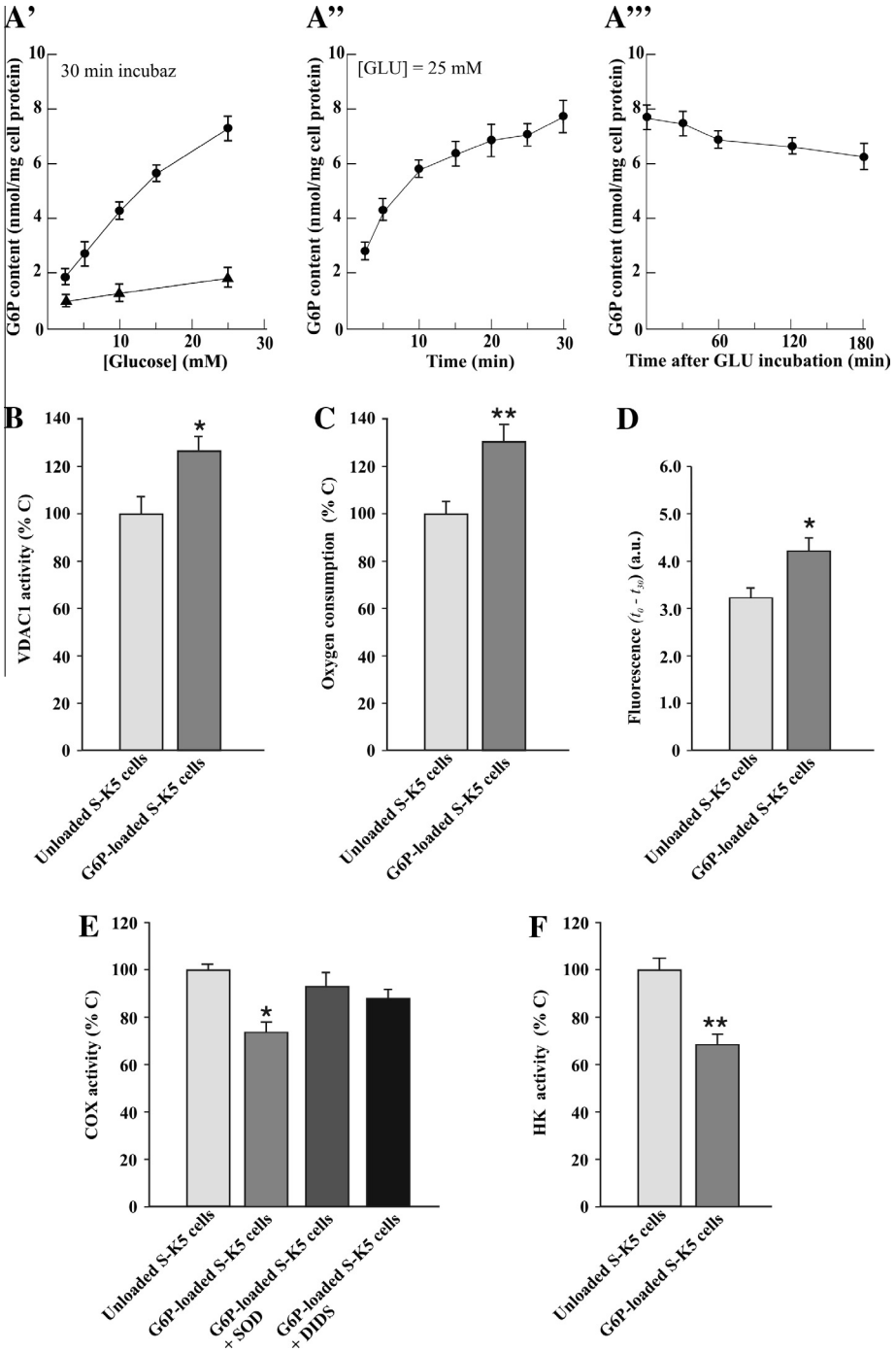
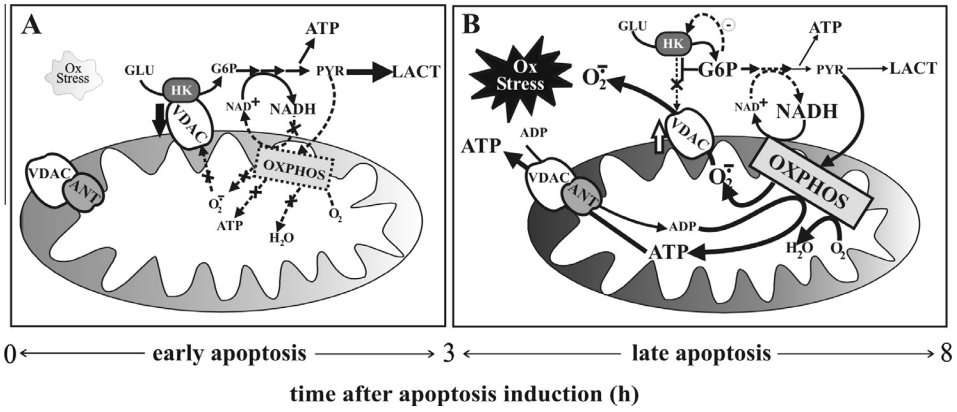


Fig. 3. Cell G6P accumulation restores mitochondrial function. (A)G6P accumulation inside the cells. In A′-A″, glucose (GLU) was added to plated cells at different concentrations (A′), either in the absence (●) or in the presence of phloretin (▲, 50 μM) for 30 min or at 25 mM for different times (A″). At the indicated time, cells were lysed and G6P was assayed (see Section 2). In A‴, after the 30 min incubation period with GLU, G6P level was assayed at different times up to 3 h, time of apoptosis. (B) VDAC1 activity was assayed, as reported in Fig. 1, both in unloaded (control) and in G6P-loaded S-K5 cells, and expressed as % of control. (C) Oxygen consumption. Externally added glucose (5 mM) to the cell suspension causes oxygen consumption (v = 25 natom O/min × mg cell protein) as a result of a multistep process consisting of glucose transport across the cell membrane, entry into the glycolytic pathway, pyruvate uptake by mitochondria where oxidative decarboxylation via the PDC occurs, citric cycle and oxidative phosphorylation. The rate of oxygen consumption was expressed as % of control. (D) Lipid peroxidation. Unloaded- or G6P-loaded S-K5 cell homogenate (0.25 mg/ml), containing mitochondria, were preincubated with 5 μM cis-Parinaric acid for 15 min in the dark with stirring at 25 °C. Then the suspensions were centrifuged and the pellets were resuspended in 2.0 ml of PBS (see Section 2). The fluorescence (318 nm excitation and 410 nm emission) in arbitrary units was recorded for 30 min. Fluorescence values at the beginning of the reaction (t_0) minus the value measured after 30 min (t_{30}) are reported as an index of lipid peroxidation (a.u.). (E) COX activity was determined by measuring O_2 consumption polarographically in the oxygen electrode chamber where cell homogenate was incubated at 25 °C in 2.0 ml respiration medium (210 mM mannitol, 70 mM sucrose, 20 mM Tris/HCl, 5 mM KH_2PO4/K_2HPO_4, pH 7.4, 3 mM $MgCl_2$). The activity reaction was initiated by adding 5 mM ascorbate plus 0.2 mM tetramethyl p-phenylenediamine to the cell CGC homogenate (0.1 mg protein) of unloaded- or G6P-loaded S-K5, subjected to three freeze–thaw cycles to disrupt membranes and expose enzymes, and expressed as % of control. SOD (5 U/ml)or DIDS (250 μM) were present where indicated. (F) HK-I activity was assayed, as reported in Section 2, both in unloaded- and in G6P-loaded S-K5 cells, and expressed as % of control. Statistical analysis was by ANOVA and Bonferroni test: $^{*}p < 0.05$, $^{**}p < 0.01$ when compared G6P-loaded with unloaded cells. The lack of asterisk indicates no statistically significant differences.



Scheme 1. Time course of apoptosis: the protective effect of HK-I binding to VDAC1 is lost in time with apoptosis progression. (A) In the early phase of apoptosis, glycolysis speeds up, VDAC1 activity decreases due to HK-I binding, mitochondria operate at low capacity and oxidative stress is hold back. (B) In the late phase of apoptosis, G6P level increases and detaches HK-I from VDAC1 which restores its full activity. Mitochondria work at full capacity, oxidative stress grows up and cell irreversibly proceed to death. For details see text.

(v) G6P accumulation (Fig. 3A′–A‴) (i) "causes" detachment of HK-I from VDAC1, which then recovers its function, and (ii) functions as an alarm clock for mitochondrial function (Fig. 3B–F), which, as reported in [11], was apparently numb;

(vi) then, in the late phase of apoptosis, a functional VDAC1 is required for the progression of apoptosis, both of them blocked by DIDS (Table to Figs. 1A and 2D).

In conclusion, these results suggest that both glucose phosphorylation and mitochondrial binding to VDAC1 contribute to the protective effects of HK-I, consistently with [41–47,52 and refs therein], showing that HK-I binding to the mitochondria inhibits apoptosis.

Future research will be needed to determine the merits of this hypothesis and investigate on the molecular mechanism by which VDAC1 channelling may be also modulated by AD proteins, such as tau fragment and Aβ. In this regard, recent research revealed that VDAC1 interacts with phosphorylated tau and Aβ and that these interactions progressively increased with disease progression [53].

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