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AMPK is activated early in cerebellar granule cells undergoing apoptosis and influences VADC1 phosphorylation status and activity

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Abstract The neurodegeneration of cerebellar granule cells, after low potassium induced apoptosis, is known to be temporally divided into an early and a late phase. Voltage-dependent anion channel-1 (VDAC1) protein, changing from the closed inactive state to the active open state, is central to the switch between the early and late phase. It is also known that: (i) VDAC1 can undergo phosphorylation events and (ii) AMP-activated protein kinase (AMPK), the sensor of cellular stress, may have a role in neuronal homeostasis. In the view of this, the involvement of AMPK activation and its correlation with VDAC1 status and activity has been investigated in the course of cerebellar granule cells apoptosis. The results reported in this study show that an increased level of the phosphorylated, active, isoform of AMPK occurs in the early phase, peaks at 3 h and guarantees an increase in the phosphorylation status of VDCA1, resulting in a reduced activity of this latter. However this situation is transient in nature, since, in the late phase, AMPK activation decreases as well as the level of phosphorylated VDAC1. In a less phosphorylated status, VDAC1 fully recovers its gating activity and drives cells along the death route.

Keywords Cerebellar granule cells · Apoptosis · AMPK · VDAC

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Introduction

Cerebellar granule cells (CGCs), which undergo apoptosis when deprived of potassium, are an in vitro model for neurodegeneration since several events, resembling those happening in the course of Alzheimer's Disease (AD), take place in this experimental system (see [1] and refs. therein). We have demonstrated that the apoptotic pathway can be formally divided into an early (0–3 h) and a late (3–8 h) phase, each one characterized by specific events acting sequentially [2]. In early-apoptosis, different neuroprotective defense mechanisms prevail [3–5], mainly sustained by a Warburg-like response. This is characterized by increased expression and activity of some glycolytic enzymes (i.e. glucose transporters 1 and 3 (GLUT1 and GLUT3), hexokinase (HK), phosphofructokinase and lactate dehydrogenase), HK interaction with the voltage-dependent anion channel-1 (VDAC1), closure of VDAC1 and consequent numbness of mitochondria [5]. In the late phase, the increase in glucose-6P induces HK dissociation from VDAC1. The activity of VDAC1 increases and, due to the opening of the channel protein, mitochondria restart working at full regime, increasing ROS production and leading cell to death [6].

AMP-activated protein kinase (AMPK), a hetero-trimeric complex composed of a catalytic alpha subunit and two regulatory beta and gamma subunits, is a serine/threonine kinase critical for maintaining cellular energy homeostasis. AMPK is a sensor of cellular stress, maximally activated when phosphorylated at Thr172 on its catalytic alpha subunit (P-AMPK) by one of the upstream kinases, liver kinase B1 (LK1) or calcium/calmodulin-dependent protein kinase kinase β (CamKKβ) [7]. Once activated, by phosphorylating key enzymes involved in different metabolic pathways, AMPK triggers catabolic reactions and simultaneously represses

anabolic pathways in order to maintain energetic homeostasis [8]. Notwithstanding AMPK is highly expressed in neurons, its role in neuronal homeostasis and during neurodegeneration is still under debate with AMPK apparently endowed with the dual effect to promote either death or survival. The activation of AMPK has been shown to induce neuronal apoptosis by increasing the expression of the pro-apoptotic molecule Bim [9] or to be neuroprotective by increasing GLUT3 expression [8]. The kinetics of AMPK activation, as well as the cellular context in which it occurs, seems to be important factors for determining its cellular responses. While a transient activation of AMPK was described to promote neuroprotection, sustained AMPK activation induced apoptosis either in vivo and in vitro [10]. Furthermore, AMPK deregulation is central for the metabolic changes occurring in major neurodegenerative diseases, including Huntington, Parkinson as well as AD, albeit with a controversial role [11]. The active isoform of AMPK is highly elevated in postmortem AD brain [12] and downregulation of AMPK activity reduced the Aβ-induced loss of synapses [13]. On the other hand, it was described that AMPK activation repressed amyloidogenic route and tau phosphorylation in neurons as well as Aβ levels and amyloid deposition in transgenic mice [14, 15].

VDAC1, the most abundant VDAC isoform, is a protein considered to be the gate-keeper of mitochondria, responsible for the flux of small metabolites and ions across mitochondrial membranes. We have recently identified, in this protein, the cellular switch regulating the progression from the early to the late phase of CGC apoptosis [6]. It is known that VDAC1 can undergo post-translational modifications that may alter its activity and interaction with other proteins as well as influence its proteolytic degradation [16]. In particular, phosphorylation at threonine, serine and tyrosine residues has been described in various physiopathological conditions.

The aim of this study was to determine whether AMPK activation is involved in the apoptotic process of CGCs. It is known that VDAC1 can be regulated by post-translation modification such as phosphorylation, thus we investigated whether a link exists between AMPK activation and VDAC1 phosphorylation status in the course of neuronal apoptosis. Results obtained in this study suggest that AMPK is activated early in CGCs undergoing apoptosis peaking at 3 h, and its activation strictly correlates with the phosphorylation status of VDAC1 and hence with VDAC1 activity.

Materials and methods

Reagents

Compound C was obtained from Enzo Life Sciences (Farmingdale, NY, USA), 5′-aminoimidazole-4-carboxamide riboside (AICAR) was from SIGMA (St Louise, MO, USA) as well as all other reagents unless indicated otherwise.

Cell cultures and induction of apoptosis

Primary cultures of CGCs were obtained from dissociated cerebellar of 7-day-old Wistar rats as in [17]. Cells were plated in basal medium Eagle (BME; Life technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum, 25 mM KCl, 2 mM glutamine and 100 µg/ml gentamicin on dishes (Nunc, Roskilde, Denmark) coated with poly-L-lysine. 1b-Arabinofuranosylcytosine (10 µM) was added to the culture medium 18–22 h after plating to prevent proliferation of non-neuronal cells. All experiments were performed with fully differentiated neurons (7–8 DIV). To induce apoptosis, cells were washed twice and switched to serum-free BME, containing 5mM KCl (K5) supplemented with glutamine and gentamicin. Apoptotic cells are then referred to as S-K5 cells. Control cells were treated identically but maintained in serum-free BME medium supplemented with 25 mM KCl (K25) and then referred to as S-K25 cells. Cultures were treated with different substances at the concentrations and for the times specified in the figure legends.

Assessment of neuronal viability

Viable CGCs were quantified by counting the number of intact nuclei after lysing the cells in detergent-containing solution [18]. This method has been shown to be reproducible and accurate and to correlate well with other methods of assessing cell survival-death [19]. Cell counts were performed in triplicate and are reported as means ± SD.

Caspase-3 activity

Caspase activity was measured by using the Clontech ApoAlert Caspase-3 Assay Kit following manufacturer's instructions (Clontech Laboratories Inc., Mountain View, CA, USA). DEVDpNA was used as a colorimetric substrate. The increase in protease activity was determined by the spectrophotometric detection at 405 nm of the chromophore *p*-nitroanilide (pNA) after its cleavage by caspase-3 from the labelled caspase-3-specific substrate (DEVDpNA).

Western blotting analysis

Cell lysis and Western blot analysis were performed mainly as in [3, 4]. Briefly, equal amounts of protein were subjected to SDS-PAGE on 11% Tricine-SDS-polyacrylamide gels, blotted onto PVDF membranes and then probed with polyclonal anti-AMPK and anti-Phospho-AMPK (Thr172)

(Millipore, Temecula, CA, USA), and monoclonal anti-GADPH and anti-PARP antibodies (Sigma Chemical Co. St Louis, MO, USA). HRP-conjugated secondary antibodies were used for detection followed by enhanced chemiluminescence development.

Co-immunoprecipitation

Cells were lysed in 1% Triton X-100, 150 mM NaCl, 20 mM TrisHCl, 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-VDAC antibody (Santa Cruz Biotechnology). After overnight incubation at 4 °C, the immunocomplexes were eluted with Laemmli buffer 2× and were next analyzed by immunoblotting with the monoclonal anti-Phospho-Threonine (P-Thr) and anti-VDCA1 antibodies (Millipore, Temecula, CA, USA).

VDAC1 activity assay

VDAC1 activity assay was performed, as described in [6], using an experimental strategy which allows to follow the ADP/ATP exchange measuring VDAC1 and adenine nucleotide translocator (ANT-1) activities in the same experiment. Briefly, the measurement was carried out in homogenates from 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). Appearance of ATP, in the exchange with ADP added to homogenate, was monitored by using the ATP detecting system—consisting of glucose (2.5 mM), HK (0.5 e.u.), G6PDH (0.5 e.u.) and $NADP^+$ (0.2 mM)—and the rate of $NADP^+$ reduction in the extramitochondrial phase was followed as the absorbance increase at 334 nm. NADPH formation decrease is recorded in the presence of DIDS or ATR, 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. Then, the determination of VDAC1 and ANT-1 activities is made possible by applying the control strength criterion and using the inhibitors at different concentrations. The channel activity was expressed as percent of S-K25 control cells.

Statistical analysis

All statistical analyses in this study were performed by using SPSS software (SPSS, Chicago, IL, USA). The data were representative of at least three independent neuronal preparations (with comparable results) each one in independent measurements and are reported as the mean with the standard deviation (SD). Statistical significance of the data was evaluated using the one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni test. $p < 0.05$ was considered as significant for all analyses.

Results

AMPK is activated in CGC apoptosis

It is well known that CGCs, referred to as S-K25 cells, can be maintained in culture in the absence of serum but in the presence of 25 mM KCl [20, 21]. On the other hand, CGCs undergo massive apoptosis if the potassium concentration is lowered to 5 mM [20–23]. Apoptotic CGCs, referred to as S-K5, show typical hallmarks of apoptosis as well as degenerative changes and neurite retraction [20, 22, 24]. We used this experimental model for all the experiments, and the pictures of both control (S-K25) and apoptotic (S-K5) cells were shown in Fig. 1a. In Fig. 1b the activity of caspase-3, an apoptotic hallmark, was measured at 8 h after apoptosis induction showing a sharp increase in S-K5 cells as compared to S-K25 cells. The cleavage of the nuclear protein PARP (116 kDa), often used to assess the apoptotic nature of the death [25–27], was shown in Fig. 1c and identified by Western blots as the appearance of the 85 kDa band in S-K5 cells (see also [3]). Used as a control, the broad range caspase inhibitor, z-VAD, proved to be effective in preventing both caspase-3 activation [see also 2, 28] and PARP cleavage (see also [3]).

In Fig. 2 cell viability of control and apoptotic cells was measured either in the presence or absence of Compound C (CC), a widely used AMPK inhibitor [29] and AICAR, a drug known to activate AMPK [30–32]. As shown in Fig. 2a, the survival of S-K5 cells decreases up to 42 ± 5% after 24 h in low potassium as compared to the 94 ± 2% observed in S-K25 cells. When increasing concentrations of CC were added to S-K5 cells, at the time of apoptosis induction, a progressive improvement of cell survival was obtained with a full recovery reached at 2 µM CC and about 95 ± 3% living cells. The same inhibitor concentration (2 µM) proved not to be toxic on S-K25 cells. On the other hand, further increasing CC concentration up to 15 µM, dramatically worsened cell viability both in control and apoptotic cells and survival dropped to 28 ± 2 and 9 ± 2% respectively (Fig. 2a). Then, CC 2 µM was chosen for further experiments, being the only concentration able to fully rescue apoptotic cells and non-toxic for control cells.

In a second set of experiments the AMPK inhibitor was added at increasing time after apoptosis induction—i.e.

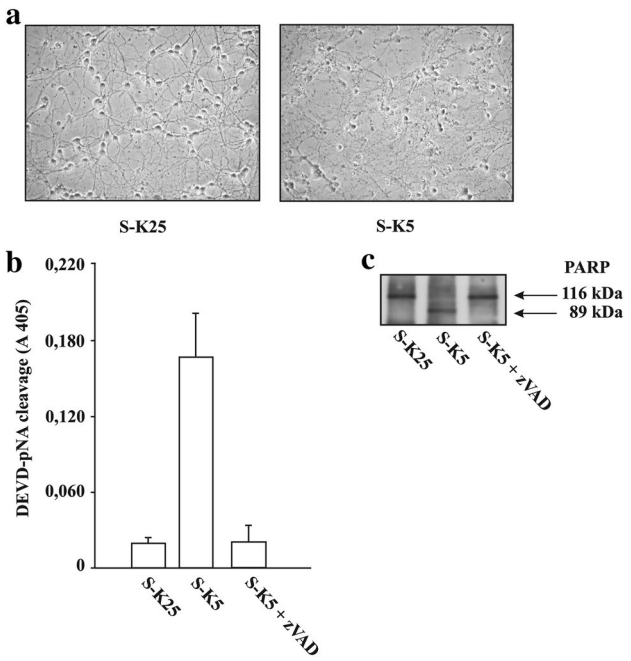

Fig. 1 CGCs undergo apoptosis in the absence of serum and in the presence of 5 mM KCl. **a** Phase-contrast micrographs showing CGCs maintained for 24 h in serum-free medium containing either 25 mM KCl (S-K25) or 5 mM KCl (S-K5). The phase contrast micrograph was taken using a NIKON DIAPHOT TS-100 epifluorescence microscope (equipped with a ×40 objective). Images were digitized by using a Nikon Digital Video Camera E995 (Coolpix 995). **b** Caspase-3 activity was determined after 8 h by spectrophotometric detection, at 405 nm, of DEVD-pNA cleavage. When indicated, 100 μMz-VAD has been added to S-K5 cells. **c** PARP cleavage was determined after 15 h by Western blot analysis of particulate neuronal lysates generated as described in [3]. When indicated, 100 μMz-VAD has been added to S-K5 cells. The *arrows* indicate the native PARP protein (116 k kDa) and the 85 kDa cleavage product of PARP

after 1, 3, 6 and 8 h from the potassium withdrawn—in order to delineate the time-window of the apoptotic process in which it exerts the prosurvival effect. As shown in Fig. 2b, CC completely rescues cells from death only when added at time 0. On the other hand, the pro-survival effect of CC progressively decreases when added after 1, 3 or 6 h after apoptosis induction and at 8 h it is no longer apparent.

The effect of the AMPK activator, AICAR, is then checked on S-K25 and S-K5 cells. At the concentration of 100 μM, AICAR was proved to induce a 15% cell death in control cells, as compared to S-K25 cells alone, and even more in apoptotic cells where cell survival decreased from 50 ± 5%, observed in untreated S-K5 cells, to 28 ± 3% in the presence of the activator (Fig. 2c).

P-AMPK level early increases in apoptotic CGCs

Increased phosphorylation of AMPK at Thr172 is a marker of AMPK activation. Then, to evaluate whether AMPK activity was modulated in the course of CGC apoptosis, total AMPK and P-AMPK were measured by Western blot analysis of CGC extracts taken at different time after apoptosis induction. As shown in Fig. 3a, a significant increase in P-AMPK was detected with apoptosis progression, starting as soon as 1 h after potassium withdrawn and reaching a maximum after 3 h. Later, the level of P-AMPK decreased, being roughly similar to that of control cells after 24 h. Densitometric analysis and calculation of the P-AMPK/AMPK ratio confirmed these results (Fig. 3b). Having previously verified that AMPK appears to be involved in the early phase of CGC apoptosis, the effect of both inhibitor and activator of AMPK was then checked at 1 and 3 h. At both times, AICAR was proved to be able to further increase the level of P-AMPK while CC reduced it (Fig. 3a, b).

VDAC is phosphorylated within the first 3 h of CGC apoptosis and kept partially inactive

The level of phosphorylated VDCA1 (P-VDAC1) was assessed with coimmunoprecipitation experiments and results were reported in Fig. 4a, b. In S-K5 cells, VDAC1 appeared to be in a phosphorylated status after 3 h of apoptosis with about 30% increase over S-K25 cells. After 8 h the level of P-VADC1 decreased up to that of control cells. When the effect of CC on S-K5 cells was verified (Fig. 4a), the level of P-VDAC1 decreased after 3 h of apoptosis from 137 ± 8 to 106 ± 9%. No significant variations were revealed after 8 h of apoptosis. As a control, the same experiment was performed in the presence of LiCl, a well-known inhibitor of the GSK3β kinase, which is able to phosphorylate VDAC and which totally rescues S-K5 from death (data not shown and [33]). Similar to the results obtained in the presence of CC, also 20 mM LiCl was able to reduce the level of P-VADC1 at 3 h but had no effect at 8 h (Fig. 4b). On the other hand, AICAR was able to further increase the level of P-VDAC1 after only 1 h of apoptosis rising up its value from 135 ± 7 to 175 ± 12% (Fig. 4c).

With the aim to verify whether the phosphorylated status of VDAC1 could be responsible for the modulation of its activity in the course of CGC apoptosis, the activity of VDAC1 was assessed as a function of time both in presence and absence of CC, LiCl and AICAR. Results are reported in Fig. 5 and expressed as percent of S-K25 cells. In S-K5 cells, at 1 and 3 h, VDAC1 activity was lower than in control cells, being 65 ± 4 and 53 ± 6% respectively, as already found in our previous paper [6]. In presence of CC, i.e. when the level of P-VDAC1 decreased, VDAC1 activity partially recovered rising up to 76 ± 5 and 73 ± 3%, after 1 and 3 h of apoptosis. LiCl produced similar results. Conversely, AICAR reduced the activity of VDAC1 of about 12% at both times. When the assay was performed after

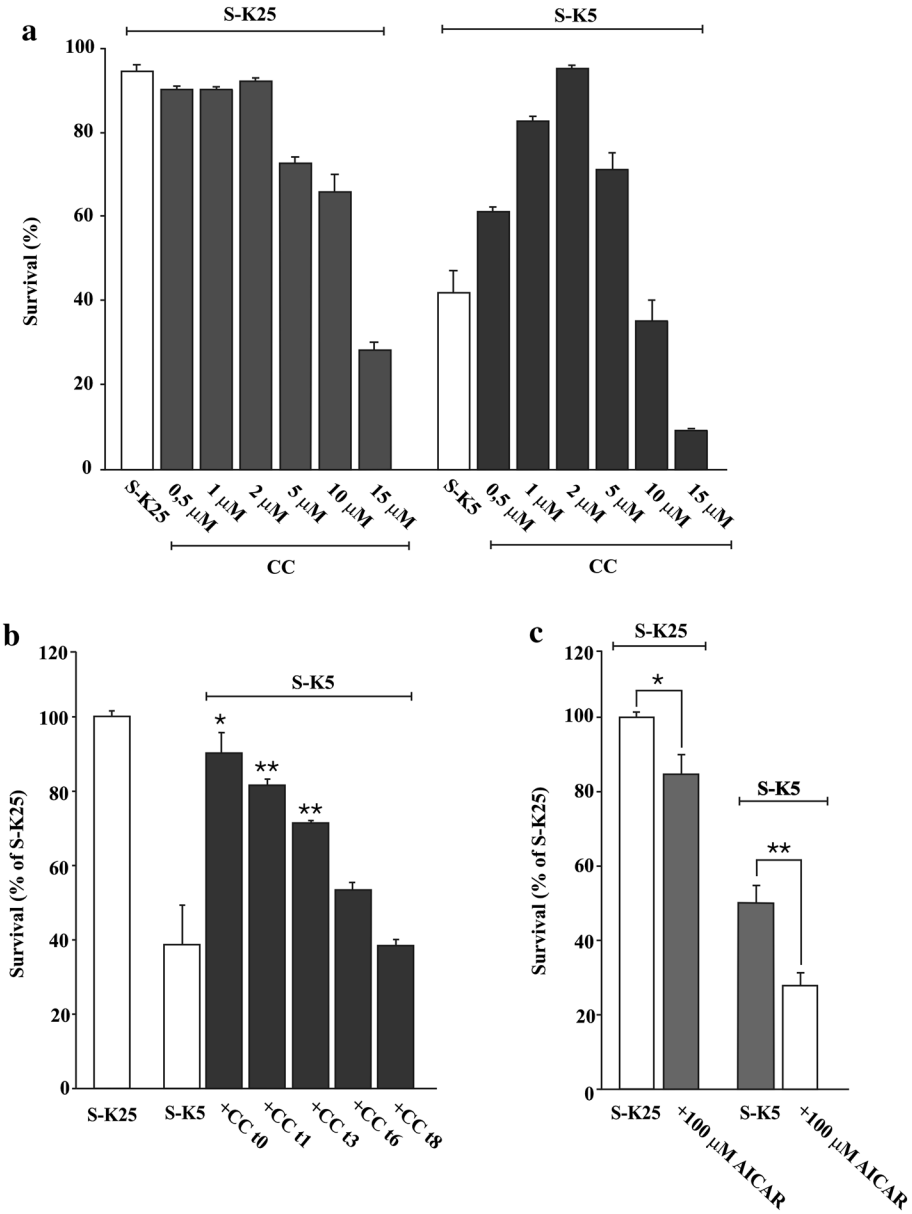

Fig. 2 AMPK is involved in CGC apoptosis: sensitivity of CGC survival to Compound C and AICAR. **a** Increasing concentration of CC were added to control cells (S-K25) or to cell culture simultaneously with the shift to low $[K^+]$ (S-K5) and, 24 h later, viable cells were counted and expressed as %. **b** CC (2 μM) was added to apoptotic cells (S-K5) concomitantly to the shift to low $[K^+]$ (t = 0) or after 1, 3, 6 and 8 (t1, t3, t6 and t8 respectively). Viable cells were counted 24 h later and expressed as % of S-K25 cells. *Asterisks* indicate values that are significantly different with respect to S-K5 (*$p < 0{,}001$, **$p < 0{,}01$). **c** AICAR (100 μM) was added to apoptotic cells (S-K5) concomitantly to the shift to low $[K^+]$. Viable cells were counted as stated above. *Asterisks* indicate values that are significantly different with respect to S-K25 (*$p < 0{,}05$) and S-K5 (**$p < 0{,}001$) respectively

8 h of apoptosis, i.e. when the P-VADC1 level in S-K5 cells decreased to a value similar to that of control cells, the activity of VDAC1 increased up to 84 ± 4% and both CC and LiCl proved to be ineffective to further increase it. Instead AICAR was still effective to keep constantly low VDAC1 activity.

Discussion

The AMPK role in influencing neuronal homeostasis is often contradictory: AMPK activation could induce cell death [9, 13, 34–36] as well as promote neuroprotection. [37–39]. Since the kinetics of AMPK activation might be crucial to determine its role inside neurons, whether in favor or in detriment to neuronal survival [8, 9, 40], it is intriguing to verify the involvement of AMPK in the time course of the apoptotic process of CGCs.

In the present study we have demonstrated that AMPK activation is an upstream event in CGC apoptosis. In S-K5 cells treated with the AMPK inhibitor, CC, a full recovery in survival is obtained only when CC is added simultaneously with apoptosis induction. On the other hand, CC—when added to S-K5 cells in subsequent times with respect to apoptosis induction—proves to progressively lose its pro-survival effect, suggesting that CC targets a crucial step in the very early phase of the apoptotic time-course. Consistently, analysis of the expression level of AMPK isoforms has shown a clear but transient increase in P-AMPK, which is peaked at 3 h, soon after apoptosis induction. Later, in

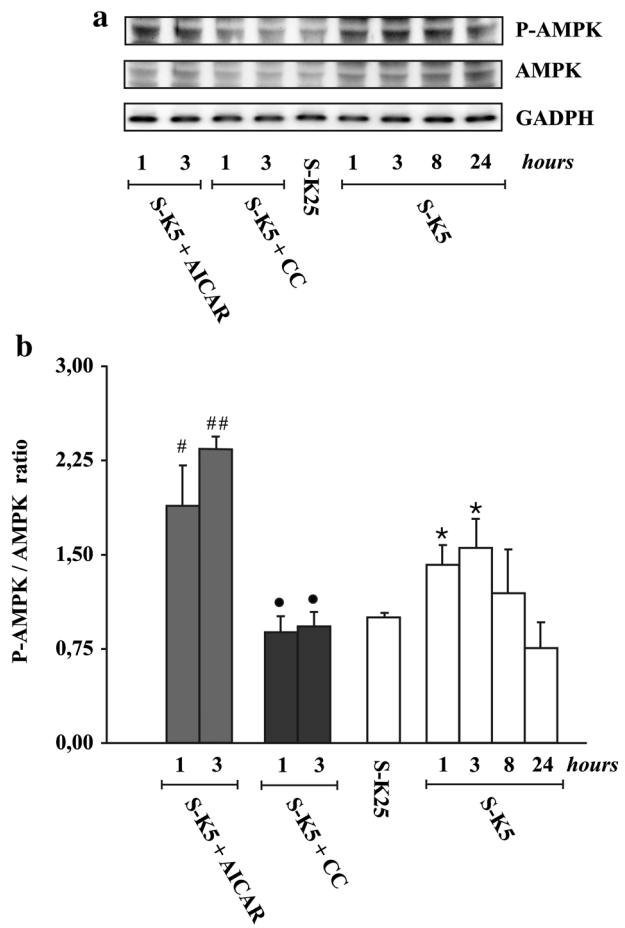

Fig. 3 Upregulation of phosphorylated AMPK protein in CGC undergoing apoptosis. **a** Representative Western Blot analysis with anti-P-AMPK and anti-AMPK antibodies. Cell lysates from either control (S-K25) or apoptotic CGCs (S-K5) were analysed by Western blotting, as described in "Materials and methods". Antibodies against GADPH were used to normalize the protein amount loaded onto the gel. **b** P-AMPK/AMPK ratio was calculated after densitometric analysis of the immunoreactive band. Values represent the mean (±SD) of six independent measurements. *Symbols* indicate values that are significantly different: S-K5 + AICAR versus S-K5 samples at the correspondent time ($^{\#}p < 0.01$ and $^{\#\#}p < 0.001$); S-K5 + CC versus S-K5 samples at the correspondent time ($^{\bullet}p < 0.001$); S-K5 versus S-K25 ($^{*}p < 0.0001$)

the route leading to death, P-AMPK level decreases even below the level observed in control cells after 24 h. This trend clearly suggests the involvement of other control mechanisms such as dephosphorylation mediated by phosphatases, whose involvement has been documented in cell death regulation as well as in AD [41, 42]. In line with our findings, it was also recently demonstrated that the activation of AMPK signaling occurs in CGCs undergoing glutamate excitotoxicity [43]. Herein, the AMPK-dependent compensatory mechanisms (i.e. increased expression of GLUT3 at the plasma membrane and increased intracellular ATP levels) sustain the neuroprotective role of the FCCP-induced mild mitochondrial uncoupling against glutamate excitotoxicity and support the notion that AMPK could actually be activated prior to or at very early phase of cell death in order to prevent energy loss via upregulation of cellular energetic flux.

As far as CGCs undergoing low potassium induced-apoptosis are concerned, at a first glance AMPK activation seems to be detrimental to neuronal survival. In fact CC is able to fully rescue neurons from death, as measured 24 h after apoptosis induction, as well as to keep low the level of P-AMPK protein in the initial phase of the apoptotic process. In parallel experiments, AICAR, the AMPK activator, which further increases P-AMPK/AMPK ratio, induces only a slight worsen in cell survival. However, when analyzing these results, it should be kept in mind that neurodegeneration is a complex process, encompassing several molecular events that take place in the time interval between the early activation of AMPK (observed between 1 and 3 h) and the late detection of cell death (measured after 24 h). It is likely that—in this time frame—AMPK could phosphorylate different substrates that could have conflicting effects. In this contest, AMPK has been reported to be an activator of glucose uptake and glycolysis, two events that have been correlated with its neuroprotective nature [8]. Recently, we have also demonstrated that apoptotic CGCs undergo an early metabolic reprogramming characterized by increased activities of some glycolytic enzymes. This Warburg effect-like mechanism is one of the earliest attempt that CGCs put in place to resist the death process, at least until the accumulating glucose-6P doesn't induce HK detachment from VDAC. At this point, the consequent increased activity of VDAC, the gate-keeper that controls mitochondrial function, behaves as a cellular switch that accelerates the progression from the early-to the late-phase of apoptosis [5, 6]. Therefore, it is tempting to speculate that the early activation of AMPK could be involved in, if not responsible for, the onset of the Warburg effect described in apoptotic CGCs. In this context, it is noteworthy that the pharmacological inhibition of VDAC proved to activate AMPK signaling pathway in endothelial cells [44].

Due to the central role played by VDAC1 and since it is known that, at least in vitro, VDAC1 can be phosphorylated by different kinases, we investigated whether VDAC1 phosphorylation occurs in the course of CGC apoptosis and whether it is somehow dependent on AMPK activation. Herein, we provide evidence that, parallel to the increased level of P-AMPK, the level of P-VDAC1 increases too and becomes higher in early apoptosis, at 3 h, than at 8 h. The inhibitor and the activator of AMPK—i.e. CC and AICAR—alter the phosphorylation status of VDAC1, by reducing or increasing it respectively, and thus influencing the activity of the channel protein. LiCl, the inhibitor of the GSK3β kinase which is known to phosphorylate VDAC1

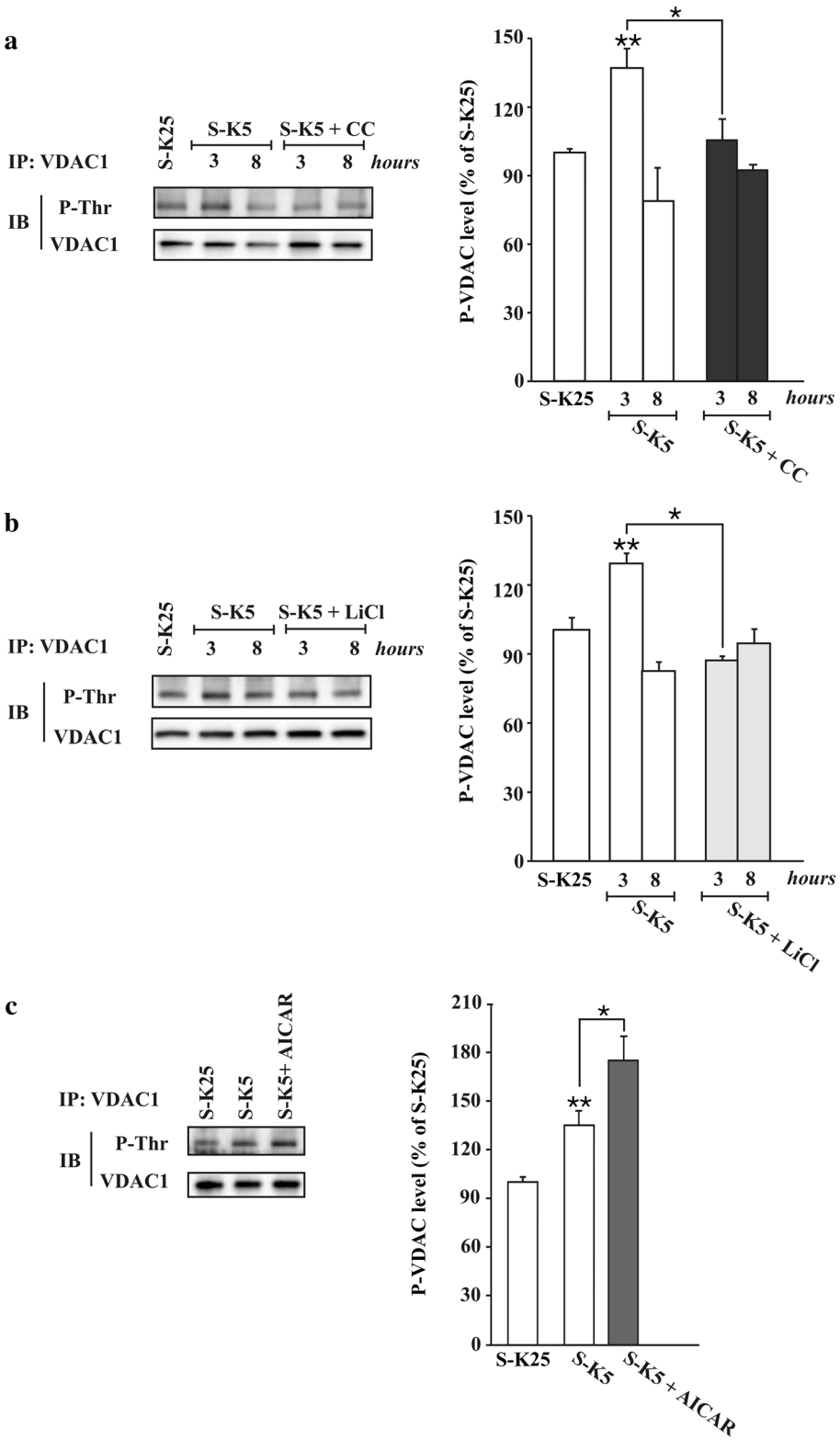

Fig. 4 Increased P-VDAC1 level in early-apoptosis Lysates of either control (S-K25) or apoptotic CGCs (S-K5), at 3 and 8 h, were subjected to immunoprecipitation by anti-VDAC1 antibody and immunoprecipitated samples were probed with anti-phosphothreonine monoclonal antibody (P-Thr) or with an anti-VDAC1 antibody as a loading control. In **a** when indicated, 2 μM CC was added to S-K5 samples at the time of apoptosis induction. P-VDAC1 level is determined by densitometric analysis of the immunoreactive bands and expressed as % of S-K25 cells. *Asterisks* indicate values that are significantly different with respect to S-K25 (**$p < 0.001$) and to S-K5 at 3 h (*$p < 0,01$). In **b** when indicated 20 mM LiCl was added to S-K5 samples at the time of apoptosis induction. P-VDAC1 level is determined by densitometric analysis of the immunoreactive bands and expressed as % of S-K25 cells. *Asterisks* indicate values that are significantly different with respect to S-K25 (**$p < 0.001$) and to S-K5 at 3 h (*$p < 0.01$) In **c** 100 μM AICAR was added at the time of apoptosis induction and after 1 h samples were processed as described above. *Asterisks* indicate values that are significantly different with respect to S-K25 (**$p < 0.001$) and to S-K5 (*$p < 0.01$)

[45, 46], has been used as a positive control. During the first 3 h of apoptosis, when VDAC1 is in a phosphorylated status, its activity decreases as compared to control S-K25 cells suggesting that phosphorylation partially closes the channel protein and limits its activity. Consistently, in the presence of AICAR, while P-VDAC1 level increases, VDAC1 activity further decreases remaining constantly low at all the times analyzed. On the other hand, CC, as

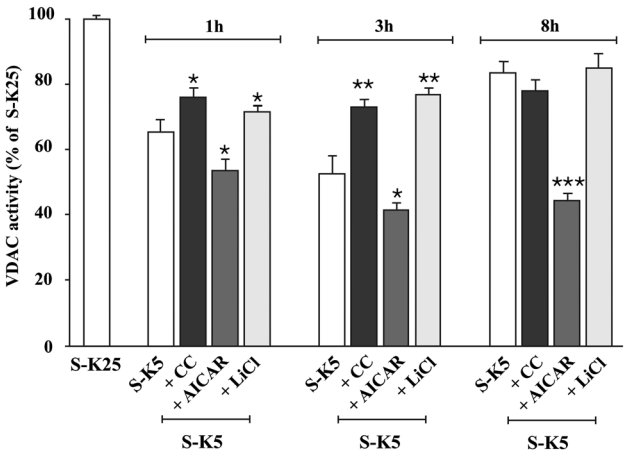

Fig. 5 Modulation of VDAC1 activity during apoptosis of CGCs. The activity of VDAC1, calculated at different times after apoptosis induction, is expressed as % of control. When indicated 2 μM CC, 100 μM AICAR or 20 mM LiCl were added to S-K5 samples at time of apoptosis induction. Within each time group, *asterisks* indicate values that are significantly different with respect to correspondent S-K5 samples (*$p < 0.0$; **$p < 0.01$; **$p < 0.001$)

well as LiCl, is able to reduce P-VDAC1 level and recover VADC1 activity at level even higher than that observed in apoptotic cells, at 1 and 3 h. At 8 h, VDAC1 activity increases in apoptotic cells, but both CC and LiCl were not able to induce further increment, presumably because VDAC1 phosphorylation has reached a plateau level and/or come into play other regulatory mechanisms that contribute to control VDAC1 opening, i.e. activity.

At present, the role and the effect of VDAC1 phosphorylation on signal transduction pathways and on cell fate is still not clear nothwidstanding a number of different kinases have been described to participate in the regulation of VDAC1 [47, 48], In AD brain, the level of VDAC1 dephosphorylation, presumably due to phosphatase activation, was found to correlate with the severity of the disease [49] and amyloid beta (Aβ) was reported either to induce dephosphorylation of VDAC1 in neuronal lipid rafts [49] or VDAC1 phosphorylation by GSK3β kinase [50]. Similarly, by using in vitro approaches, the phosphorylated state of VDAC1 was correlated with either the closed state of the protein [51, 52] or the open state [52] and the effect of phosphorylation has been interpreted as to be either proapoptotic [51] or antiapoptotic [52].

Herein, by using an in vivo approach, we hypothesize that AMPK could be involved in VDAC phosphorylation, thus contributing to maintain VDAC1 in a partially closed (i.e. inactive) state during early-apoptosis in CGCs. In support of this hypothesis, Strogolova et al. [54] reported, for the first time, a direct interaction between Snf1 protein kinase—the AMPK-homolog in *Saccharomyces cerevisiae*—with the yeast mitochondrial porin (Por 1) and suggested that Por1/VDAC can be as a sort of "functional niche" that blocks Snf1/AMPK close to mitochondria, i.e. in the better position to monitor glucose/energy failure. However, as far as the results herein reported are concerned, due the inherent limitations in the use of inhibitors, whose side effects must be taken into account, we cannot exclude a priori that AMPK, besides directly acting on VDAC, could also indirectly regulate its phosphorylation.

In conclusion, we suggest that AMPK is activated early in CGCs undergoing apoptosis and its activation strictly correlates with the phosphorylation status of VDAC1 and hence with VDAC1 activity. Furthermore, results obtained make sense that the controversial role on the activation/inhibition of AMPK in apoptosis could be a matter of time. In early-apoptosis, AMPK activation is endowed with a neuroprotective intent since AMPK could mediated, directly or indirectly, the phosphorylation of VADC1 thus contributing to keep its activity low and to sustain the pro-survival Warburg effect-like. However, in late-apoptosis, the ability of AMPK to support the cellular resistance to death decreases and while AMPK is no more able to sustain adequate level of VDAC1 phosphorylation, other AMPK-substrates can progressively disclose their toxicity. Future research aim will be to seek out and identify other substrates of AMPK that may play a key role in the route of CGC neurodegeneration.

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