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Extracellular ADP prevents neuronal apoptosis via activation of cell antioxidant enzymes and protection of mitochondrial ANT-1

A. Bobba [a], G. Amadoro [b], A. Azzariti [c], R. Pizzuto [d], A. Atlante [a,*]

[a] Institute of Biomembranes and Bioenergetics, CNR, Bari, Italy
[b] Institute of Translational Pharmacology, CNR, Roma, Italy
[c] Clinical and Preclinical Pharmacology Lab, National Cancer Research Centre, Istituto Tumori G. Paolo II, Bari, Italy
[d] Department of Health Sciences, University of Molise, Campobasso, Italy

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ABSTRACT

Apoptosis in neuronal tissue is an efficient mechanism which contributes to both normal cell development and pathological cell death. The present study explores the effects of extracellular ADP on low [K^+]-induced apoptosis in rat cerebellar granule cells. ADP, released into the extracellular space in brain by multiple mechanisms, can interact with its receptor or be converted, through the actions of ectoenzymes, to adenosine. The findings reported in this paper demonstrate that ADP inhibits the proapoptotic stimulus supposedly via: *i*) inhibition of ROS production during early stages of apoptosis, an effect mediated by its interaction with cell receptor/s. This conclusion is validated by the increase in SOD and catalase activities as well as by the GSSG/GSH ratio value decrease, in conjunction with the drop of ROS level and the prevention of the ADP protective effect by pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), a novel functionally selective antagonist of purine receptor; *ii*) safeguard of the functionality of the mitochondrial adenine nucleotide-1 translocator (ANT-1), which is early impaired during apoptosis. This effect is mediated by its plausible internalization into cell occurring as such or after its hydrolysis, by means of plasma membrane nucleotide metabolizing enzymes, and resynthesis into the cell. Moreover, the findings that ADP also protects ANT-1 from the toxic action of the two Alzheimer's disease peptides, i.e. Aβ1–42 and NH_2htau, which are known to be produced in apoptotic cerebellar neurons, further corroborate the molecular mechanism of neuroprotection by ADP, herein proposed.

1. Introduction

ATP is abundantly present in the central nervous system (CNS) [1] and is released into the extracellular space in response to stimulation-dependent neuronal activity [2] or from damaged and dying cells. The availability of extracellular ATP within the CNS is determined by the balance between its release and removal by means of ectonucleotidase-dependent degradation [3], which results in the generation of ADP and adenosine (ADO), two molecules that, acting at a level of cell membrane surface via purine-receptors [4–8], play a pivotal role in cell differentiation, growth and death affecting the development and the vital functions of different organs and apparatus [6,9–14].

It has been previously reported that when cerebellar granule cells (CGCs) are shifted to lethal conditions, i.e. exposed to a culture medium containing a low, more physiological, K^+ concentration (5 mM) [for refs see 15–17], in the presence of various purine receptor antagonists, 100 and 80% of neurons survived after 24 and 48 h [18], respectively. The antiapoptotic action of these molecules could be likely achieved via activation of the cellular antioxidant (AOX) system through the interaction of these ligands with their receptors in cultured cells [19,20]. Consistently, Suzuki's group [21] demonstrated that extracellular ATP has a preventive action on apoptotic cell death in differentiated PC12 cells, mainly via the activation of P2X2 receptors.

Taking advantage of the availability of a cell system, namely CGCs, in which the main apoptotic steps have been well characterized from a temporal and causative point of view [for refs see 17], we validated once for all the hypothesis that the antiapoptotic action of ADP is realized via activation of the AOX system, as suggested by the increase of

Abbreviations: Act D, actinomycin D; AD, Alzheimer's disease; ADK, adenylate kinase; ADO, adenosine; ADP, adenosine diphosphate; AMPCP, α,β-methyleneadenosine 5′-diphosphate; ANT-1, adenine nucleotide translocator; AOX, antioxidant; Ap5A, P1,P5-di(adenosine-50)penta-phosphate; ASC, ascorbate; ATP D.S., ATP detecting system; ATR, atractyloside; BME, basal medium Eagle; CGC, cerebellar granule cell; CNS, central nervous system; CsA, cyclosporine A; Cyt *c*, cytochrome c; DIV, days in vitro; Fe^{3+}-cyt c, ferricytochrome c; Fe^{2+}-cyt c, ferrocytochrome c; GDH, glutamate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, glutathione disulfide; h, hours; HK, hexokinase; MK801, (+/−)-5-methyl-10,11-dihydro-5H-dibenzo(a,d) cyclohepten-5,10-imine hydrogen maleate; NBMPR, S-(4-nitrobenzyl)-6-thioinosine; O_2^-, superoxide anion; PBS, phosphate-buffered saline medium; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; mPT, mitochondrial permeability transition; mPTP, mitochondrial permeability transition pore; RCR, respiratory control ratio; ROS, reactive oxygen species; S.D., standard deviation; S-K25 cells, control cells; S-K5 cells, apoptotic cells; 3h-S-K5 cells, apoptotic cells 3 h after the induction of apoptosis; SOD, superoxide dismutase; SUCC, succinate; z-VAD, z-VAD-fmk

* Corresponding author. Tel.: +39 080 5443364; fax: +39 080 5443317.
E-mail address: a.atlante@ibbe.cnr.it (A. Atlante).

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SOD and catalase activities as well as by the decrease of GSSG/GSH ratio value. Furthermore and for the first time, we demonstrated that extracellular ADP, after being internalized into the cells, prevents the impairment of the mitochondrial ANT-1 [see 17], a key protein in the death route, and rescues cells from death. Moreover, the ability of ADP to protect ANT-1 from the toxic action of the two Alzheimer's disease (AD) peptides, i.e. Aβ1–42 and NH_2htau, was also checked. On the assumption that neuropathies are the result of neuronal apoptosis, the identification of compounds that, like ADP, are able to protect neurons against apoptosis is highly desirable.

2. Materials and methods

2.1. Ethics statements

This study was performed in accordance with local ethics committee and with the principles contained in the Declaration of Helsinki as revised in 1996. All animals were handled and cared for in accordance with EEC guidelines (Directive 86/609/CEE). The animals were anesthetized and insensitive to pain throughout the procedure.

2.2. Reagents

Tissue culture medium and fetal calf serum were purchased from Gibco (Grand Island, NY, USA) and tissue culture dishes were from NUNC (Taastrup, Denmark). The antagonist of purine receptor (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, PPADS), the inhibitor of ectonucleotidase (α,β-methylene-adenosine-5′-diphosphate, AMPCP), the inhibitor of nucleoside transporter (S-(4-Nitrobenzyl)-6-thioinosine, NBMPR) and all other enzymes and biochemicals were from Sigma Chemical Co. (St Louis, MO, USA). The inhibitor z-VAD-fmk was purchased from Calbiochem (La Jolla, CA, USA). Fibrillar Aβ1–42 (Sigma Chemical Co., St. Louis, MO, USA) – sometimes called, for simplicity, Aβ – was prepared according to Eckert et al. [22] with minor modifications. The peptide was dissolved in deionized water at a concentration of 0.5 mM and stored at −20 °C. At occurrence, the stock solution was diluted in phosphate buffered saline (PBS) to a concentration of 0.1 mM and incubated at 37 °C, with gentle agitation, for 24 h to obtain aged, aggregated preparations of Aβ1–42. Synthetic NH_2-tau peptide, i.e. $NH_2$26–44 was synthesized by Sigma Genosys (Haverhill, UK), and purified to >95% homogeneity by reversed-phase high pressure liquid chromatography on C-18 silica columns with monitoring of A214 (peptide bonds).

2.3. Cell cultures

Primary cultures of CGCs were obtained from dissociated cerebellar of 7-day-old Wistar rats as in Levi et al. [23]. Cells were plated in basal medium Eagle (BME) supplemented with 10% fetal calf serum, 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamicin on dishes coated with poly-L-lysine. Arabinofuranosylcytosine (10 μM) was added to the culture medium 18–22 h after plating to prevent proliferation of non-neuronal cells.

2.4. Induction of apoptosis

Apoptosis was induced at 6–7 days in vitro (DIV): cells were washed and switched to a serum-free BME, containing 5 mM KCl and supplemented with 2 mM glutamine and 100 μg/ml gentamicin for the indicated times [15]. Apoptotic cells are referred to as S-K5 cells or as x-time-S-K5 to indicate the different 'x' time after apoptosis induction at which the cells are processed. In some experiments ADP (1 mM) was also added, at the indicated times, with exposure terminated by removal of the compound-containing medium, double washing of the cell layer and replacement with fresh media. Sister cultures prepared under the same conditions were used in each experiment. Control cells were treated identically but maintained in serum-free BME medium supplemented with 25 mM KCl for the indicated times; they are referred to as S-K25 cells. The occurrence of apoptosis was checked, as in [15,24], by measuring DNA laddering and prevention of death due to the addition of the transcriptional inhibitor actinomycin D (Act D).

2.5. Cell homogenate and mitochondria preparations

The culture medium was removed and the plated CGCs were repeatedly washed with phosphate-buffered saline (PBS), containing 138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 15 mM KH_2PO_4 pH 7.4, and then collected. Cell integrity was quantitatively assessed by the inability of cells to oxidize externally added succinate, and by the ability of ouabain to block glucose transport [25]. *Cell homogenate* was obtained from a cell suspension by 10 strokes with a Dounce homogenizer at room temperature. Cytosolic lactate dehydrogenase was released and subsequent treatment with Triton-X-100 did not cause further release. The functionality of the mitochondria was checked for their coupling by measuring the respiratory control index, i.e. (oxygen uptake rate after ADP addition) / (oxygen uptake rate before ADP addition) which reflects the ability of the mitochondria to produce ATP, and for their intactness by measuring in the post-mitochondrial supernatant the activities of adenylate kinase (ADK, E.C.2.7.4.3) and glutamate dehydrogenase (GDH, E.C.1.4.1.3), which are marker enzymes of the mitochondrial intermembrane space and matrix, respectively. The ADK reaction was assayed, essentially as in [17,24], at 25 °C and pH 7.2 to mimic intracellular pH in a standard coupled spectrophotometric assay, in which the ADK-catalyzed synthesis of ATP from ADP was measured by using glucose (2.5 mM), hexokinase (HK, 0.5 e.u.), glucose-6-phosphate dehydrogenase (G-6-PDH, 0.5 e.u.) and $NADP^+$ (0.2 mM). To prevent any ATP production via oxidative phosphorylation, 10 μg oligomycin and 20 μM ATR were also present to completely inhibit ATP synthase and ANT respectively. When determining the GDH activity at 25 °C the following substrates were used: 10 mM 2-oxoglutarate, 10 mM NH_4Cl and 0.2 mM NADH; the NADH oxidation was photometrically monitored at 340 nm as a function of time. Protein content was determined, according to [26], with bovine serum albumin used as a standard.

2.6. Assessment of neuronal viability

Viable CGCs were quantified by counting the number of intact nuclei in a hemocytometer, after lysing the cells in detergent-containing solution [27]. Cell counts were performed in triplicate and are reported as means ± standard deviation (SD). The data are expressed as the percentage of intact nuclei in the control cultures at each time point. Apoptosis was expressed as the percentage of intact cells with respect to control cells (%C) kept under the same respective experimental conditions. In control experiments 95–97% integrity was found after 24 h. In some experiments, a variety of compounds (including the inhibitors of ectonucleotidases, purine receptor and ADO transporter) were added at the induction time at the concentrations selected to avoid any possible interference with cell viability.

2.7. DNA fragmentation analysis

Fragmentation of DNA was performed as in [27]. Briefly CGCs (6×10^6) were plated in poly-L-lysine-coated 60 mm tissue culture dishes and collected with cold phosphate-buffered saline (PBS pH 7.2). After removal of the medium and washing once with cold PBS, CGCs were centrifuged at 3500 ×*g* for 5 min. The pellet was lysed in 10 mM Tris–HCl, 10 mM EDTA and 0.2% Triton X-100 (pH 7.5). After 30 min on ice, the lysates was centrifuged at 17,000 ×*g* for 10 min at 4 °C. The supernatant was digested with proteinase K and then extracted twice with phenol-chloroform/isoamyl alcohol (24:1). The aqueous phase, containing soluble DNA, was recovered and nucleic acids were

precipitated with sodium acetate and ethanol overnight. The pellet was washed with 70% ethanol, air-dried and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). After digestion with RNase A (50 ng/ml at 37 °C for 30 min), the sample was subjected to electrophoresis in a 1.8% agarose gel and visualized by ethidium bromide staining. Soluble DNA from equal numbers of cells was loaded in each lane.

2.8. Polarographic measurements

O_2 consumption was measured polarographically by means of a Gilson 5/6 oxygraph using a Clark electrode, as in [16,17]. In order to detect polarographically the cytochrome *c* (cyt *c*) presence in the extramitochondrial phase, the capability of cell homogenate to oxidize ascorbate (ASC) was checked [28 and the references therein]. Instrument sensitivity was such as to allow rates of O_2 uptake as low as 0.5 natoms min^{-1} mg^{-1} protein to be followed. The cell homogenate in PBS (about 0.2 mg protein) was incubated in a thermostated (25 °C) water-jacketed glass vessel (final volume equal to 1.5 ml).

2.9. Superoxide anion detection

Superoxide anion ($O_2^{-\bullet}$) was detected, as in [29], according to the Fe^{3+}-cyt c method. The newly formed $O_2^{-\bullet}$ gave an increase in absorbance at 550 ($\varepsilon_{550\ nm} = 37\ mM^{-1}\ cm^{-1}$) measured using a Perkin-Elmer LAMBDA-5 spectrophotometer equipped with a thermostated holder. A calibration curve was made by using an $O_2^{-\bullet}$ producing system, i.e. xanthine plus xanthine oxidase, and an $O_2^{-\bullet}$ detection system, i.e. Fe^{3+}-cyt c, that, in the presence of $O_2^{-\bullet}$, gave Fe^{2+}-cyt c with 1:1 stoichiometry.

2.10. Antioxidant enzyme activities

The activities of SOD and catalase were determined with the cell homogenate (about 0.1 mg cell protein). Superoxide dismutase (SOD, E.C.1.15.1.1) activity was measured by the inhibition of xanthine oxidase/cyt *c* system reaction, as described in [24]. In this assay xanthine oxidase, acting on xanthine in the presence of oxygen, generates superoxide anion, O_2^- which reduces cyt *c*, and this reduction is inhibited by SOD. One enzymatic unit of superoxide dismutase is the amount of enzyme required to inhibit the rate of reduction of cyt *c* by 50%: then the activity of SOD is expressed as the % inhibition of the control reaction. CGC homogenate was suspended at 25 °C in 1.5 ml PBS buffer in the presence of Fe^{3+}-cyt c (10 μM) plus xanthine (50 μM). The reaction was started with xanthine oxidase addition and the absorbance increase at 418 nm was monitored. Catalase (E.C.1.11.1.6) determination was performed by a spectrophotometric assay based on the catalyzed decomposition of H_2O_2 [for ref see 24]. The peroxide decomposition rate is directly proportional to the enzyme activity. To determine the catalase activity the cell homogenate was suspended at 25 °C in 1.5 ml PBS buffer in a quartz cuvette. After reading the initial absorbance at 240 nm, 100 μl H_2O_2 (final concentration 10 mM) was added and the decrease in absorbance monitored. The slope of the absorbance vs. time plot is directly proportional to the activity of the sample and is expressed as $\Delta A_{240}/min \times 10^6$ cells.

2.11. Glutathione disulfide (GSSG)/reduced glutathione (GSH) ratio measurement

GSH or GSSG was assayed in the cell homogenate, according to Akerboom and Sies [30]. Briefly, GSH in the presence of methylglyoxal (2 mM) and glyoxalase I (6 e.u.) was specifically converted into S-lactoyl-GSH which could be monitored directly at 240 nm; GSSG amount was assayed in the same cuvette by measuring the stoichiometric conversion of NADPH (10 μM) spectrophotometrically at 340 nm in the presence of glutathione reductase (1 e.u.).

2.12. Caspase-3 activity

Caspase activity was measured by using the Clontech ApoAlert Caspase-3 Assay Kit (Takara Bio Group, Otsu, Shiga, Japan) following manufacturer's instructions. DEVD-pNA 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 labeled caspase-3-specific substrate (DEVD-pNA).

2.13. ADP/ATP carrier measurement

The cell homogenate (0.1 mg protein), containing the mitochondria, was incubated at 25 °C in 2 ml of standard medium consisting of 200 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, and 20 mM HEPES–Tris pH 7.2. Appearance of ATP in the extramitochondrial phase, due to externally added ADP, was revealed as in [28,31,32], by using the ATP detecting system (ATP D.S.) consisting of glucose (2.5 mM), hexokinase (HK, 0.5 e.u.), glucose-6-phosphate dehydrogenase (G-6-PDH, 0.5 e.u.) and $NADP^+$ (0.2 mM) in the presence of P_1,P_5-Di(adenosine-5′)pentaphosphate (Ap5A), a specific inhibitor of adenylate kinase [for ref see 32]. The rate of $NADP^+$ reduction in the extramitochondrial phase was followed as absorbance increase at 334 nm, measured as the tangent to the initial part of the progress curve and expressed as nmol $NADP^+$ reduced/min × mg cell protein. Control experiments were carried out in the presence of ATR to ensure that the ADP/ATP exchange was mediated by the ADP/ATP carrier [see 32,33].

2.14. Measurement of the mitochondrial permeability transition pore opening

To visualize onset of mPT either mitochondrial swelling or adenylate kinase (ADK) release from the mitochondria was monitored, as in [17]. *Mitochondrial swelling* was monitored at 25 °C by following the absorbance decrease at 546 nm. The cell homogenate was suspended in a medium containing 250 mM sucrose, 2 mM HEPES pH 7.4, 0.5 mM K_2HPO_4, 1 μM oligomycin, 2 μM rotenone and 5 mM succinate. *ADK release* was monitored by assaying ADK activity at 25 °C, essentially as reported above, in post-mitochondrial supernatant of both control and S-K5 cells (about 30×10^6 cells/1 ml), obtained after homogenization in PBS and centrifugation at 15,000 ×*g* for 15 min.

2.15. Measurement of levels of both adenine nucleotides and AMP breakdown products

Concentrations of intracellular phosphorylated adenine nucleosides (ATP, ADP and AMP) and of AMP breakdown product, i.e. ADO, in neuronal extracts were determined by HPLC as previously described [28,34]. After 7 days in vitro, 6×10^6 CGCs were switched to high K^+ serum-free (S-K25) medium and incubated in the same medium for 1 h. After the incubation the cell monolayer was washed twice with 2 ml phosphate buffered saline (PBS, 120 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4). The cells were gently scraped off the plate with PBS and collected in a final volume of 1.4 ml. The cell suspension was immediately centrifuged at 2000 ×*g* for 5 min at 4 °C and the cell pellet, strictly 'dried out' with a stick of blotting paper, was resuspended in a final volume of 1.4 ml. This treatment was repeated two times. As a control, ADP was assayed in the supernatants – by means of a NADH enzyme-linked assay, i.e. Pyruvate kinase + Lactate dehydrogenase coupled reactions [see 35] – in order to verify that the washing procedure was able to remove all traces of the externally added ADP, which – if present – would invalidate the outcome of the experiment. Cell pellets were immediately subjected to acid extraction by adding 70 μl of ice-cooled 0.5 M $HClO_4$, vigorous shaking and incubation for 20 min in an ice bath. The acid extract was immediately frozen in liquid nitrogen and stored at −20 °C until the neutralization. The frozen

mixture was thawed and centrifuged (17,900 ×*g* for 15 min at 4 °C) to precipitate insoluble macromolecules; the supernatant (70–80 μl) was neutralized by 4/5 volume 0.5 M KOH plus 1/5 volume 1 M KH_2PO_4, pH 7.5, and left for 10–15 min on ice. The potassium perchlorate precipitate was finally removed by centrifugation (17,900 ×*g* for 15 min at 4 °C). The neutralized supernatant was either analyzed immediately or stored at −20 °C.

Results were expressed as nmol/mg cell protein. ADP (1 mM), in the absence or presence of different compounds (see legend to figure), when present, was added to the serum-free BME (see above) simultaneously with induction of apoptosis.

2.16. Statistical analysis and computing

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

Experimental plots were obtained using GraFit (Erithacus software).

3. Results

3.1. ADP prevents low-K^+ induced CGC apoptosis

Prior to investigating the mechanism responsible for the ADP-dependent prevention of apoptosis, we verified that ADP can rescue CGCs from death (see Materials and methods section), according to [14,18].

To this aim, 7 DIV neuron cultures were kept either in high potassium medium (S-K25 cells) or subjected to low potassium shift (S-K5 cells), which is responsible of triggering apoptosis [for ref see 15,24], in the absence or presence of ADP. That neurons died via apoptosis (Fig. 1) was confirmed by monitoring DNA laddering, which is a specific hallmark of apoptosis, and by the sensitivity of death to Act D. As shown in Fig. 1A, DNA laddering at 15 h after apoptosis induction occurs in S-K5 cells (*lane c*), but not in S-K25 (*lane b*), and, in agreement with [15], it was completely preserved in the presence of Act D (1 μg/ml) (*lane e*) [see 24]. In Fig. 1B, we confirmed that death occurred via apoptosis in S-K5 cells where survival was reduced by 60% ($p < 0.001$, six experiments) with respect to the control, at 24 h after potassium shift, in fairly good agreement with [15,17] and as shown by its complete prevention by Act D (1 μg/ml) (see [15]). Consistently, MK801 (1 μM), a selective NMDA receptor antagonist, which prevents glutamate-dependent necrosis, failed to rescue neurons (not shown).

ADP (0.01–1 mM) was proven to prevent death in a dose-dependent manner (Fig. 1C). The maximum protection was achieved in the presence of 1 mM ADP with an increase in cell survival up to about 90% ($p < 0.001$, 6 experiments) with respect to the 42% obtained in S-K5 samples. The choice of using ADP at a concentration equal to 1 mM in the forthcoming experiments was dictated by the fact that enzymes consuming ADP have a low affinity for ADP [36], and that this concentration, giving the largest protective effect, makes possible to highlight all differences, including the less significant, in the experiments in which use was made of compounds which prevent the ADP-mediated protection (see below). Consistently, ADP (1 mM), added to cell culture simultaneously with induction of apoptosis, completely prevented DNA laddering (Fig. 1A, *lane d*) as well as cell death (Fig. 1B). As a control, it was verified that a similar behavior is achieved in the presence of z-VAD (Fig. 1B), as already reported [24,37].

Interestingly, when ADP (1 mM) was added to cell culture at different times after induction of apoptosis with cell viability estimated at 48 h, the extent of cell survival varied depending on the time of ADP addition (Fig. 1D). Almost complete prevention of cell death occurs when ADP was added within the first 3 h after apoptotic stimulus administration, i.e. when maximum ATP level together with maximum increase of ROS production, AOX system activity and cyt *c* release was detected [24,34]. At 5 h, i.e. when both caspase activation and mitochondrial permeability transition pore (mPTP) opening start [17], and later at 8 h, under condition in which mitochondria uncoupling and caspase(s) activation are complete, ADP-mediated protection was strongly reduced and totally exhausted, respectively.

3.2. ADP effect on some mitochondria-linked steps in the process of CGC apoptosis

Having confirmed that ADP prevents apoptosis of CGCs, its effect on some mitochondria-linked steps in the process leading to apoptosis was further investigated. Briefly, we have already shown that in early apoptosis (0–3 h after induction) the rate of glucose oxidation decreases [16], the mitochondria are subjected to time-dependent uncoupling [16] and elevated production of ROS occurs [24,37]. Cyt *c* is released from the mitochondria while still coupled [37,38] and an increase in the ATP level occurs [34]. In late apoptosis (3–8 h after induction) – when the ROS level reaches steady state and ATP declines up to control levels – there is cyt *c*-dependent caspase(s) activation, the proteasome- and caspase(s)-dependent degradation of antioxidant system (AOS) as well as caspase(s)-dependent degradation of the released cytochrome *c* occur [24,37,38], and impairment of the adenine nucleotide translocator (ANT-1) takes place, with ANT-1 becoming a component of the mPTP [17].

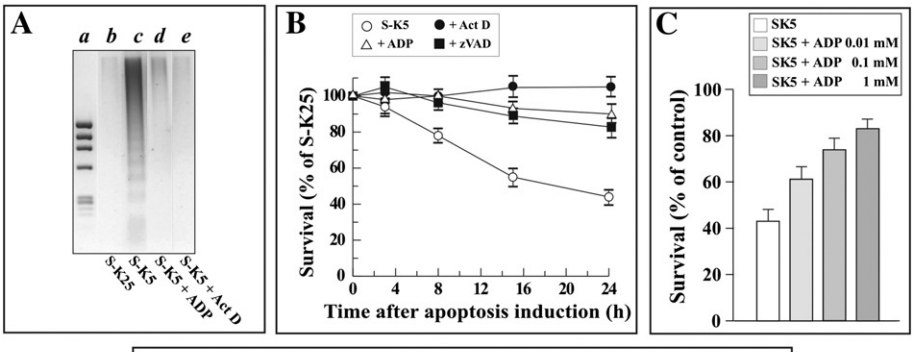
3.2.1. Mitochondrial coupling in ADP–S-K5 cells

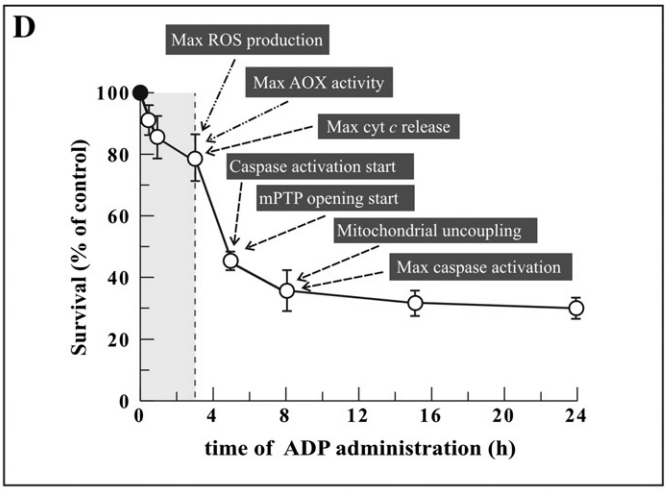
The ability of ADP (1 mM) to prevent impairment of mitochondrial function, which occurs in early apoptosis [16], was investigated by using the homogenates of either S-K25 or S-K5 CGCs, cultured in the absence or presence of ADP for 3 h (Fig. 2A). To achieve this, oxygen uptake was measured arising from the addition of succinate (SUCC) to the cell homogenate in either the absence (*state 2*) or presence (*state 3*) of ADP (1 mM) used to stimulate oxidation. The RCR values (oxygen uptake in *state 3*) / (oxygen uptake rate in *state 2*) which reflect the ability of the mitochondria to produce ATP, were about 5.1, 2.6 and 5,1 for S-K25, 3h-S-K5 and ADP–3h-S-K5 cells, respectively. When SOD (50 U/ml) was added to 3h-S-K5 cell culture, the RCR value was 4.5 (inset to Fig. 2A), in good accordance with [16]. Notice that *i*) the oxygen consumption rate remained constant in S-K25 CGCs up to 3 h; *ii*) the rate of oxygen uptake in *state 2* was not changed by treatment of culture cells with ADP, ruling out the possibility that this compound can uncouple the mitochondria.

3.2.2. Superoxide anion production and AOX system activity in ADP–S-K5 cells

Having established that ADP protects the mitochondria from the K^+-shift-dependent early uncoupling (see above), the ADP effect on other mitochondrial steps was investigated separately at the times of 3 h and/or 5 h after apoptosis induction, which reflect the *early* and the *late* phases of apoptosis, respectively (see [24]). In order to individuate the mechanism underlying the antiapoptotic action of the ADP, the experiments were performed in the absence or presence of a variety of compounds, such as purine receptor antagonist and/or ectonucleotidase and nucleoside transporter inhibitors.

In CGC apoptosis, ROS extra-production takes place [24,39 and the references therein] with all the apoptotic steps being mostly dependent on ROS level. Since the antiapoptotic effect of ADP could be achieved via inhibition of ROS production and/or activation of AOX enzymes, as suggested in [14,18], we investigated the effect of ADP on: *i*) $O_2^{-\bullet}$ level at 3 h apoptosis, when maximum superoxide detection occurs [see 24] (Fig. 2B) and *ii*) activity of AOX enzymes at 3 h apoptosis, when the maximum increase of enzyme activity takes place [24] (Fig. 2C).





E

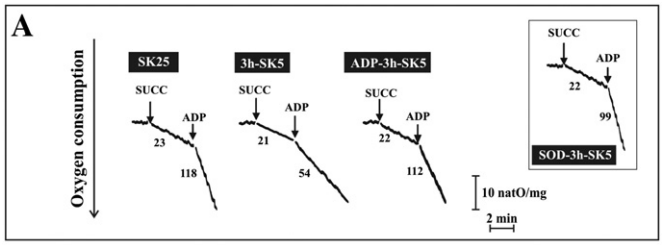
ADP effect on S-K5-CGC survival in the absence or presence of atractyloside

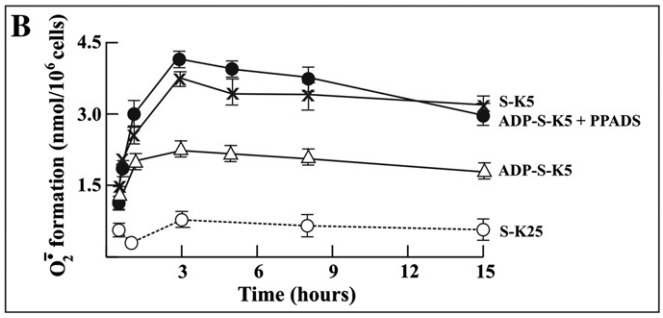
line	Treatment t = 0	Treatment t + 3h	Cell viability (% of S-K25)
1.	S-K5		30 ± 2
2.	S-K5 + ADP		90 ± 5**
3.	S-K5 + ATR		24 ± 3
4.	S-K5 + ADP + ATR		56 ± 4*
5.	S-K5	ADP	84 ± 4
6.	S-K5	ATR	25 ± 3§§
7.	S-K5 + ATR	ADP	48 ± 3§
8.	S-K5 + ADP	ATR	82 ± 5
9.	S-K5 + ActD		95±6

Fig. 1. DNA fragmentation and sensitivity of cell survival to ADP in cells undergoing apoptosis. (A) DNA fragmentation. Soluble DNA was extracted from either neurones switched to serum-free culture medium containing low K^+ (5 mM) in the absence (*lane c*) or presence of ADP (1 mM, *lane d*) or actinomycin D (Act D, 1 μg/ml; *lane e*). *Lane b* contains DNA from control cells maintained in high K^+ (25 mM) for the same apoptosis induction time, i.e. 15 h. DNA from equal numbers of plated cells (6×10^6) was loaded in each lane. Size marker was HaeIII-digested ΦX174 phage DNA (*lane a*). (B–E) Cell survival. In B), cells were switched to serum-free medium containing low K^+ in the absence (S-K5) or presence of ADP (1 mM). At different times, cell viability was determined by counting the number of intact nuclei. In C), ADP (0.01–1 mM) was added to cell culture simultaneously with the shift to low [K^+] and, 48 h later, viable cells were counted and compared to controls (% of Control). In D), ADP (1 mM) was added to cell culture at different times after apoptosis induction and cell viability estimated at 48 h. In E), ADP (1 mM) was added to culture cells at 2 times: *i*) t = 0, i.e. simultaneously with the shift to low [K+] and *ii*) t + 3 h, i.e. 3 h after apoptosis induction, when ADP is still able to protect cells from death (see above, panel D). Atractyloside, when present, was added at a concentration equal to 10 μM. Cell viability is expressed as the percentage of S-K25 cells to which a 100% value was given. Control values were 100 ± 5. Results are means ± standard deviations (S.D.) of triplicate measurements and representative of four different experiments carried out with different cell preparations from different groups of animals. Statistical analysis was by ANOVA and Bonferroni test: $^*p < 0.01$, $^{**}p < 0.001$ when comparing all samples (clear lines) with each other; $^§p < 0.01$, $^§p < 0.001$ when comparing all samples (shaded lines) with each other. The lack of asterisk indicates no statistically significant differences.

As shown in Fig. 2B, the superoxide level, which increases up to 3 h in CGCs undergoing apoptosis (S-K5) with about 4 nmol/10^6 cells ($p < 0.0001$, 4 exps), in agreement with [24], was strongly decreased (up to 2 nmol/10^6 cells) by the externally added ADP in S-K5 cells. As expected [29,40], treatment with SOD was found to partially prevent $O_2^{-\bullet}$ appearance (not shown). The ADP effect on $O_2^{-\bullet}$ level was almost completely prevented by 100 μM PPADS, a novel functionally selective antagonist of purine receptor [41], thus indicating that $O_2^{-\bullet}$ level modulation by ADP was strictly dependent on its interaction with receptor/s, as also suggested in [14,18].

Then, the effect of ADP on the activity of AOX enzymes was measured and reported in Fig. 2C as percentage of the activity found in S-K5 cells, taken as a control and to which a value of 100 was given. In ADP-treated S-K5 cells, 3 h after potassium shift, the activities of both SOD (136%) and catalase (124%) further increase while a consistent reduction of the GSSG/GSH ratio (62%), which is an index of





C

Antioxidant system	S-K5 (%)	ADP-S-K5 (% of S-K5)	ADP-S-K5 + PPADS (% of S-K5)
SOD Fe^{3+}cyt c reduction rate	72 ± 4 (100)	98 ± 7 (136)	78 ± 5 (108)
Catalase ΔA_{240}/ min x 10^6 cells	7.1 ± 0.3 (100)	8.8 ± 0.5 (124)	7.4 ± 0.4 (104)
GSSG/GSH	2.1 (100)	1.3 (62)	1.9 (90)

Fig. 2. Mitochondrial coupling, superoxide anion production and AOX system activity in ADP–S-K5 cells. (A) Mitochondrial coupling. Rat CGCs (2×10^6/well) at 7 DIV, switched from high potassium (25 mM) (S-K25) to low potassium (5 mM) serum-free culture medium in the absence (S-K5) or presence of 1 mM ADP (ADP–S-K5) for 3 h, were homogenized and incubated (0.2 mg cell protein) at 25 °C in a water-jacketed glass vessel and the consumption of O_2 was monitored polarographically. Succinate (SUCC, 5 mM) and ADP (1 mM) were added at the indicated times. In the inset, S-K5 cells were treated with SOD (50 U/ml) (SOD–S-K5). Numbers along the curves are rates of oxygen uptake expressed as natom O/min × mg cell protein. (B) Superoxide anion production. Rat CGCs (0.5×10^6/well) at 7 DIV were incubated either in high potassium (S-K25) or in low potassium (S-K5) serum-free culture medium added with Fe^{3+}-cyt c (10 μM) in the absence or presence of ADP (1 mM). Where present, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS, 100 μM), was added simultaneously with ADP. At different times after apoptosis induction, the culture solution was taken and the increase in absorbance at 550 nm, due to Fe^{2+}-cyt c formation, was determined. The experimental data are reported as nanomol ROS formed ± S.D./10^6 cells, calculated on the basis of the stoichiometry of the reaction using the extinction coefficient determined under our experimental conditions (see Materials and methods). The experiment was repeated four times with different cell preparations. (C) Antioxidant system activity and GSSG/GSH ratio. 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 in the absence or presence of 1 mM ADP. Where present, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS, 100 μM), was added simultaneously with ADP. At 3 h after apoptosis induction, cells were scraped, collected, homogenized and assayed for SOD, catalase and GSSG/GSH ratio (see Materials and methods). Each value represents the mean ± S.D. value of antioxidant system activity, expressed as percentage inhibition of Fe^{3+}-cyt c reduction rate for SOD and as ΔA_{240}/min for catalase, and of [GSSG]/[GSH] ratio value (see Materials and methods), obtained from five separate experiments carried out using different cell preparations. Values within the brackets indicate the percentage of increase/decrease with respect to S-K5 cells alone to which a value equal to 100 was given.

oxidative stress, was found. PPADS – which is without a direct effect on the AOX activities – strongly prevented the ADP-dependent activation of the cell AOX system in vivo.

3.2.3. The release of cytochrome c from the mitochondria and the caspase-3 activation in ADP–S-K5 cells

Since in CGC apoptosis, ROS production causes the release of cyt *c* from the mitochondria [for refs see 40,42] which, in its new cytoplasmic location, induces caspase activation by intervening in the formation of the apoptosome [for ref see 24], we investigated if the cyt *c* release is affected by ADP-treatment and hence if this compound influences the activation of caspase-3. To do this, as in [38,40], we turned to polarographic measurement of the activation of cyt *c*-dependent ASC oxidation, which itself confirmed that cyt *c* was released in an active form [38] (for details see the Materials and methods). In a typical experiment S-K25, S-K5 or ADP–S-K5 cells, at 3 h after apoptosis induction (time at which the maximum release of cyt *c* occurs), were compared to one another with respect to their ability to oxidize externally added ASC (5 mM) (Fig. 3A). No oxygen uptake was found in the S-K25 cell sample in the presence of ASC alone (*trace a*), showing both that the mitochondria were intact and that no release of cyt *c* had occurred. ASC addition to homogenates from S-K5 or ADP–S-K5 CGCs resulted in oxygen uptake at a rate of about 17 and 8 natom O/min × mg cell protein, respectively (*traces b–c*), thus showing that ADP is able to prevent the release of cyt *c* by about 50%. As a control (Fig. 3A, *column chart*), externally added SOD (6.5 ± 1.2 natom O/min × mg cell protein) was able to strongly prevent the release of cyt *c* as already reported [24]. When caspase-3 activity was measured at 8 h, time at which the maximum of activity was reached [37], a decrease of about 55% was obtained in the presence of ADP (0.085 a.u., $p < 0.001$, 5 experiments) with respect to S-K5 cells (0.17 a.u.) (Fig. 3B). As a control, we checked that ADP had no effect on the caspase activity in vitro, which instead is strongly inhibited by z-VAD and only partially by SOD (not shown).

3.2.4. ANT-1 activity, mPTP opening and ATP level in ADP–S-K5 cells

ATP is required for apoptosis to occur [43–45]. In early apoptosis, ATP is mostly produced in the mitochondria by oxidative phosphorylation [34] and is exported via the ANT-1 into the cytoplasm (for refs see [33]). The effect of ADP on the process that, starting with alteration of ANT-1, leads to the opening of mPTP [see above and 17] was investigated by measuring in parallel both ANT-1-mediated transport and the mPTP opening in S-K25, S-K5 and ADP–S-K5 cell homogenates (Fig. 4). Assays were carried out both at 3 h, when it was shown that ANT-1 function is impaired due to ROS, but no mPTP opening occurs, and at 5 h, when further caspase(s)-dependent ANT-1 impairment occurs and mPTP opening initiates [17]. When indicated, 1 mM ADP was added to the plate, i.e. ADP–S-K5. Since ADP itself might affect the outcome of the experiment, great care has been employed in the removal of ADP-containing media at the end of the incubation time by means of a thorough washing of the layer of adherent cells and replacing with fresh media. To measure the ANT-1 mediated transport, we resorted to a procedure (see [17]) that allows for the continuous monitoring of ATP efflux from the mitochondria incubated with ADP while mPTP opening was investigated as mitochondrial swelling, as in [17] (for details see Materials and methods), or as adenylate kinase (ADK) release. In a typical experiment cell homogenates were treated with AP_5A to inhibit adenylate kinase. In all cases the ATP concentration outside the mitochondria was negligible as no increase in the absorbance measured at 334 nm was found in the presence of the ATP D.S. As a result of addition of ADP (0.04 mM), an increase in the NADPH absorbance was found, indicating the appearance of ATP in the extramitochondrial phase. The explanation for this is as follows: ADP enters the mitochondria in exchange for endogenous ATP; inside the matrix ATP is synthesized by oxidative phosphorylation, and the newly synthesized ATP exits the mitochondria in exchange for further ADP via the ANT-1. That the rate of NADPH formation mirrored in all cases the rate of ADP/ATP exchange was confirmed by applying control flux analysis, as in [17] (not shown). The rates of ATP efflux were 10.8, 2.9 and 1.7 nmol $NADP^+$ reduced/min × mg cell protein for S-K25, 3h-S-K5 and 5h-S-K5 CGCs respectively (Fig. 4A–B). When SOD

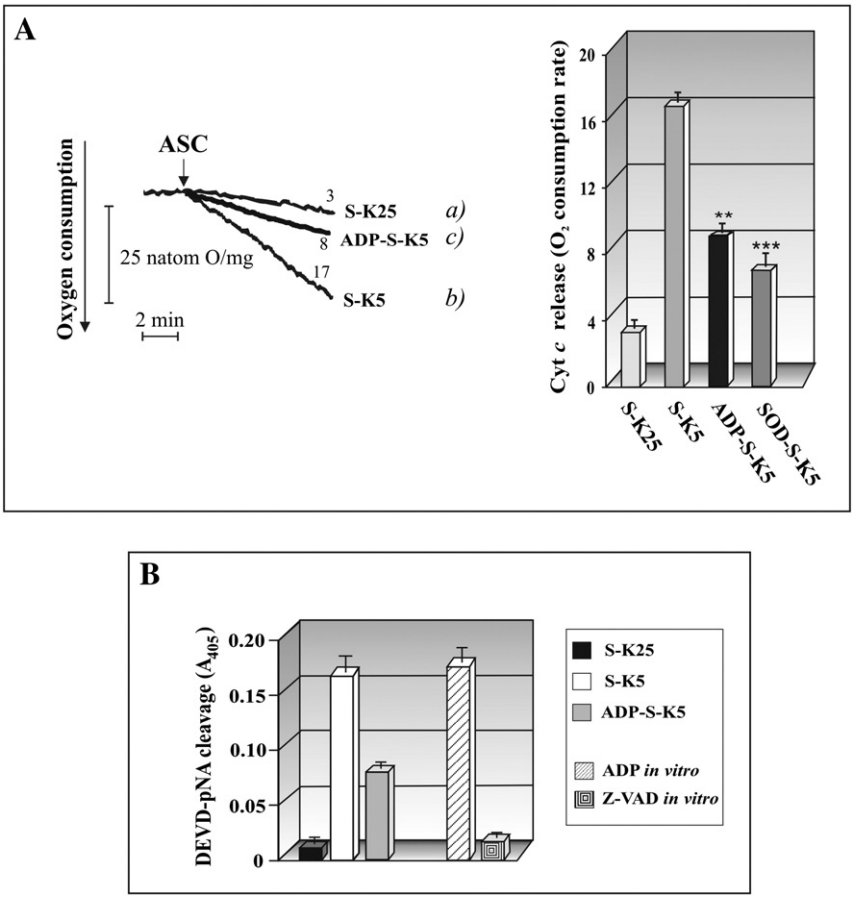


Fig. 3. Cytochrome *c* release from mitochondria and caspase-3 activation in ADP–S-K5 cells. (A) Cytochrome *c* release. Rat CGCs (2×10^6/well) at 7 DIV were incubated in either high potassium (S-K25) or low potassium serum-free culture medium in the absence (S-K5) or presence of 1 mM ADP (ADP–S-K5) or 50 U/ml SOD (SOD–S-K5). At 3 h after apoptosis induction, the cells were scraped, collected and homogenized. Cell homogenate (about 0.2 mg protein) was incubated at 25 °C in the presence of 3 μM rotenone, 0.8 μM antimycin and 6 μM myxothiazol in a water-jacketed glass vessel. Results, expressed as natoms O/min × mg cell protein, are the means ± S.D. of triplicate measurements and are representative of at least five different experiments carried out with different cell preparations obtained from different groups of animals (on the right). ANOVA and Bonferroni test: **,*** statistically significantly different with $p < 0.01$ and $p < 0.001$, respectively, when S-K5 cells were compared against the samples indicated with asterisk/s. (B) Caspase-3 activity. Cells (6×10^6/well) were incubated either in high potassium (S-K25) or in low potassium (S-K5) serum-free culture medium in the absence or presence of ADP (1 mM). At 8 h, cultures were lysed and assayed for DEVD-pNA cleavage. The increase in protease activity was determined by the spectrophotometric detection, at 405 nm, of the cleaved chromophore p-nitroanilide (pNA). Each value represents the mean ± S.D. values of DEVD-pNA cleavage, expressed as A_{405} value. The experiments were repeated five times. ADP/z-VAD in vitro indicates the effect of ADP or z-VAD (100 μM) on the caspase activity in vitro.

(50 U/ml) was added to cell cultures, almost complete prevention of ANT-1 impairment was found at 3 h after apoptosis induction (10.2 nmol $NADP^+$ reduced/min × mg cell); this confirmed that the decrease in ANT-1 efficiency depended on extra ROS production, in accordance with [17]. As expected, in 5h-S-K5 cells, where a progressive impairment in ANT-1 occurs mainly due to caspase(s) activation, the SOD prevention decreased to 6.1 nmol $NADP^+$ reduced/min × mg cell. In ADP–3h-S-K5 and ADP–5h-S-K5, the rates of ATP efflux were 10.6 and 10.7 nmol $NADP^+$ reduced/min × mg cell protein respectively (Fig. 4A–B). This shows that ADP completely prevents ANT impairment, not only at 3 h, as SOD does, but also surprisingly at 5 h. In a parallel experiment (Fig. 4C), mPTP opening was measured by monitoring mitochondrial swelling, as a change in absorbance at 546 nm of the CGC homogenate (see Materials and methods section). It should be noted that the decrease in light absorbance is a spontaneous process due to apoptosis and is not calcium-dependent. No mPTP opening was found at 3 h after induction of apoptosis (not shown), in accordance with [17]. In 5h-S-K5-CGCs, mPTP opening occurred which was fully prevented in the presence of 1 μM cyclosporine A (CsA), an inhibitor of mPTP [17,46]. In both SOD- and ADP–S-K5 cells, a decrease in both the rate and extent of mPTP opening was found (80 and 70%, respectively).

In the graphs reported in Fig. 4D, both ADP/ATP exchange (on the left) and mPTP opening (on the right), measured as ADK release into the extramitochondrial phase, were investigated as a function of time (0–8 h) of apoptosis. In early apoptosis (S-K5), up to 3 h, the rate of ADP/ATP exchange decreased gradually as compared to control cells (S-K25), which instead remained constant all over the time, with inhibition increasing with progression of apoptosis (about 55% inhibition found at 3 h, $p < 0.001$), essentially as in [17]; in the same time range, no or negligible mPTP opening was found in either S-K25 or S-K5 cell homogenates. In late apoptosis, between 3 and 8 h, a further decrease in the ADP/ATP exchange was found with about 10% residual ANT-dependent transport detected at 8 h. mPTP opening occurred progressively over the 3–8 h time range in S-K5 cells, confirming that during late apoptosis both a further decrease in the ANT-1 transport function and mPTP opening occur. Surprisingly, while SOD, externally added to S-K5 cells, was found to fully prevent ANT-1 impairment in the early apoptosis and only partially in the late stage up to 8 h, a complete prevention of ANT-1 impairment was observed in the presence of ADP throughout the considered time range, i.e. 0–8 h, suggesting that the decrease in ANT-1 efficiency also depends on other process/es in addition to the ROS-dependent ones, i.e. the receptor-dependent AOX enzyme activation (see Fig. 2C). Otherwise, no mPTP opening in the presence of either SOD or ADP was found in early apoptosis, showing that ROS cannot themselves cause mPT, which occurred only in the 3–8 h time range, although to a lesser extent than with S-K5 cells.

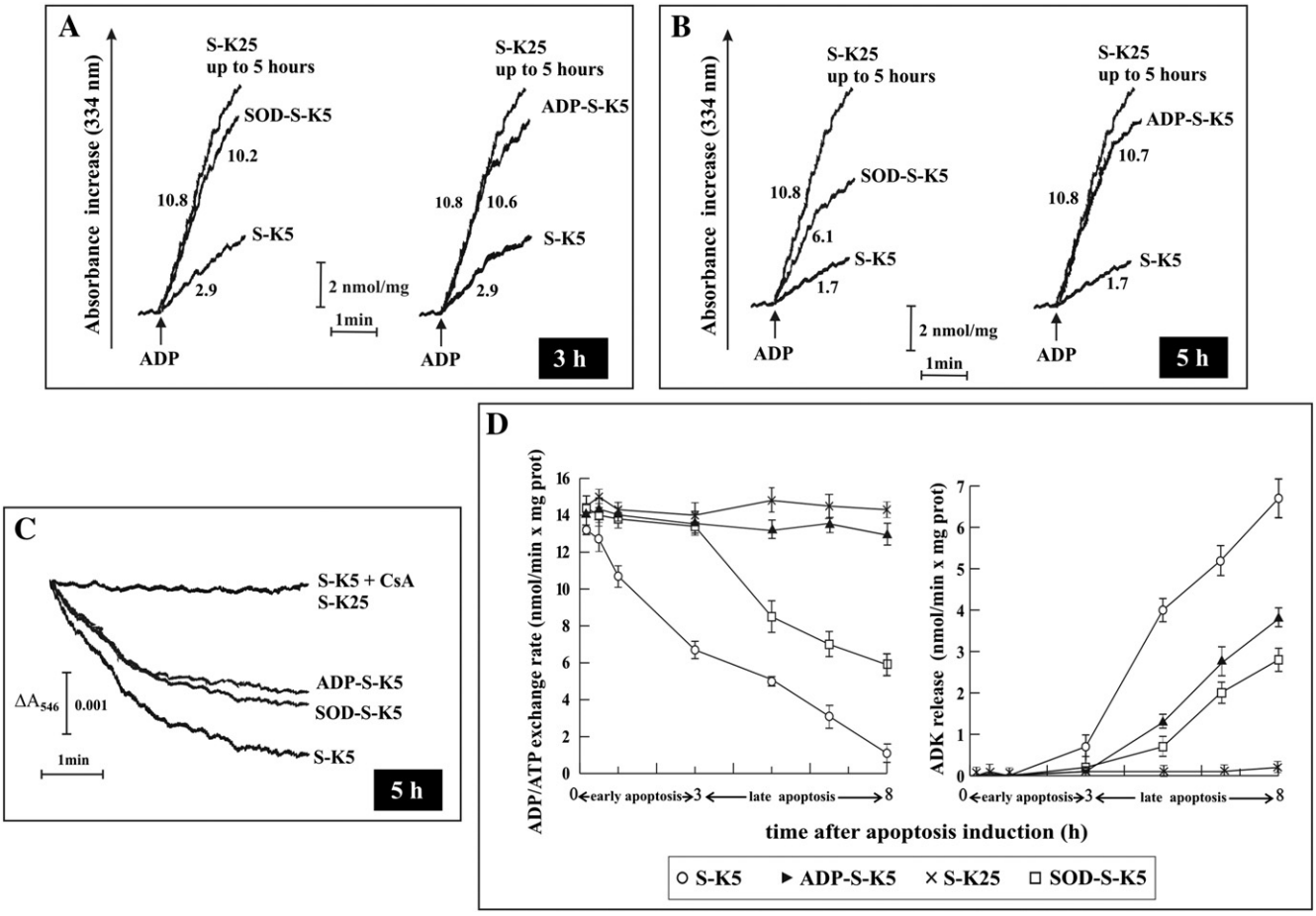


Fig. 4. ANT-1 activity and mPTP opening in ADP–S-K5 cells. (A–B) ANT-1 activity. Appearance of ATP due to the addition of ADP (0.04 mM) to CGC homogenate (0.1 mg protein) from S-K25 or S-K5 cells in the absence or presence of ADP (1 mM) (ADP–S-K5) or SOD (50 U/ml) (SOD–S-K5) at 3 (A) and 5 h (B) after apoptosis induction was monitored as described in the method section (Section 2.13). C) mPTP opening. Rat CGCs (15×10^6/well) at 7 DIV were incubated either in high potassium (S-K25) or low potassium (S-K5) serum-free culture medium in the absence or presence of ADP (1 mM) or SOD (50 U/ml) at 5 h after apoptosis induction. mPTP opening was monitored by mitochondrial swelling (see Materials and methods, Section 2.14). D) ADP/ATP exchange and mitochondrial permeability transition *en route* to apoptosis. Rat CGCs ($15 \times$ 106/well) at 7 DIV were incubated either in high potassium (S-K25) or in low potassium (S-K5) serum-free culture medium in the absence or presence of ADP (1 mM) (ADP–S-K5) or SOD (50 U/ml) (SOD–S-K5). At different times after apoptosis induction ADP/ATP exchange and mPTP formation *en route* to apoptosis were measured as in A) and by monitoring the adenylate kinase (ADK) release from mitochondria, respectively. Reported values are the mean of four independent neuronal preparations (with comparable results) each one in triplicate with the standard deviation. Statistical analysis was by ANOVA and Bonferroni test. ADP/ATP exchange: for S-K25 there were no statistically significant differences when comparing all the samples with each other in the whole time range; $p < 0.001$ when comparing S-K25/ADP–S-K5 vs S-K5 in the whole time rage; $p < 0.05$ when comparing S-K25/ADP–S-K5 vs SOD–S-K5 in the 3–8 h time range. Mitochondrial permeability transition: $p < 0.001$ when comparing each S-K25 vs S-K5; $p < 0.01$ when comparing S-K25/S-K5 vs ADP–S-K5/SOD–S-K5 in the 3–8 h time range.

In the light of the above results, i.e. the different effect of SOD and ADP on the mitochondrial translocator (Fig. 4D), and in order to having more clues on the ADP protective effect on cell processes, i.e. to clarify if it is only due to receptor-dependent antioxidant enzyme activation, as suggested in Fig. 2B,C, or if it also involves some strategic mitochondrial protein/s, such as ANT-1, we resorted to the use of ATR, a membrane-impermeant inhibitor of ANT which binds to the translocator from the cytosolic side with very high affinity [47]. The effect of ATR on cell viability was evaluated (Fig. 1E) by adding this compound to CGCs during apoptotic treatment, either in the absence or in the presence of ADP, under two experimental conditions: simultaneously with the apoptotic shift to low [K^+], lines 3 and 4 respectively, or after 3 h (lines 6 and 8), i.e. when the ADP is still able to protect cells from death (see above, Fig. 1D). No effect on cell viability was observed with respect to S-K5 sample (line 1) when ATR was incubated with S-K5 cells either simultaneously (line 3) or 3 h after the apoptosis induction (line 6). On the other hand, *i*) if ATR was added at time zero, both in the presence of ADP (line 4) and 3 h before ADP addition (line 7), partial prevention of ADP protection was observed (compare 56 and 48% with the 90% achieved in S-K5 + ADP sample, respectively); *ii*) if ATR was added 3 h after ADP addition (line 8) almost complete protection by ADP was confirmed (about 80%, compare lines 5 and 8), thus suggesting that the ADP-mediated cell protection is in part due to the ADP-binding to ANT-1. Act D, used as a control, completely prevents death (line 9).

These last results, more than others, raise the question whether the ADP effect is mediated only by its interaction with a cell purine receptor, or is also the consequence of its internalization into the cell. In the latter hypothesis, the ADP uptake into the cell can occur *i*) directly or *ii*) with a stepwise process consisting of ADP hydrolysis by means of plasma membrane nucleotide metabolizing enzymes, ADO uptake and finally resynthesis into the cell. To provide clues on this issue, the intracellular levels of ADP, ATP, AMP and ADO were measured by HPLC in the neutralized perchloric acid extracts of S-K25-CGCs incubated with ADP, in the absence or presence of the antagonist of purine receptor (PPADS), the inhibitor of ectonucleotidase (AMPCP) and the inhibitor of nucleoside transporter (NBMPR).

The results shown in Fig. 5 suggest that ADP internalization into cells also occurs. The intracellular levels of ATP, ADP, AMP and ADO in S-K25 cells, reported in the *No Addition* bars, were found to be 1, 1, 0.4 and 0.15 nmol/mg cell protein respectively, essentially as in [34]. These levels significantly increased when S-K25 cells were incubated with 1 mM ADP ranging from 25% in the case of ATP ($^\circ p < 0.05$, *+ADP* with respect to *No Addition*) up to 180% in the case of ADO ($^\circ p < 0.001$, *+ADP* with respect to *No Addition*). It should be noted that the

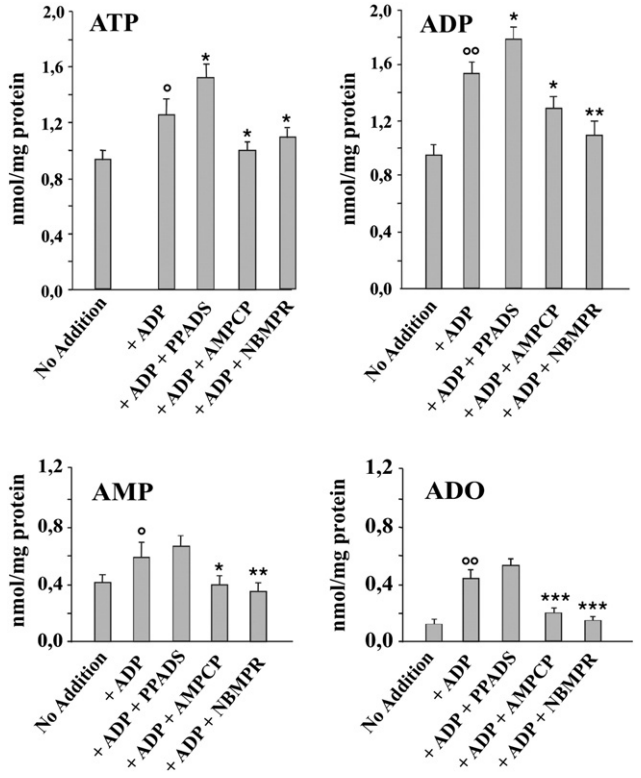


Fig. 5. ATP level in ADP–S-K25 cells. The amounts of ATP were measured by HPLC in neutralized perchloric acid extracts of S-K25 in the absence (*No Addition*) or presence of ADP (1 mM), added either alone (+*ADP*) or together with PPADS (100 μM), AMPCPX (100 μM), NBMPR (2 μM). ANOVA and Bonferroni test: °$p < 0.05$, °° $p < 0.001$ when comparing +*ADP* sample to S-K25 cells (*No Addition*); *, **, *** statistically significantly different with $p < 0.05$, $p < 0.01$ and $p < 0.001$ with respect to +*ADP* sample cells. The lack of asterisk indicates no statistically significant differences.

experimental procedure was carefully set so as to ensure that the residual externally added ADP has been completely removed before carrying out the measurements with HPLC (see Materials and methods for details). A further increase of intracellular ATP (up to 55%) and ADP (up to 80%) level occurs when S-K25 cells were treated with ADP in the presence of PPADS (100 μM) which was without effect on AMP and ADO amount. On the other hand, when AMPCP (100 μM) and NBMPR (2 μM), ectonucleotidase and nucleoside transporter inhibitors respectively [48,49], were added simultaneously with ADP to S-K25 cells, an almost complete prevention of the ADP-dependent intracellular increase of all the tested metabolites was observed and the levels of ATP, ADP, AMP and ADO become roughly similar to those obtained in S-K25 cells alone.

As a control, it was verified that *i*) the inhibitors had no effect on cells if ADP is not present (not shown) and *ii*) the ADP level was insignificant in medium – after the washing and resuspension of the cells previously treated with ADP – as verified by assaying ADP by means of a highly sensitive enzymatic reaction (not shown).

3.3. ADP effect on Abeta- or tau fragment-dependent ANT-1 impairment

Evidences show that, in S-K5 conditions, an amyloidogenic route early occurs in apoptotic CGCs in correlation with caspase(s)- and calpain-mediated tau cleavage generating one or more toxic NH_2-derived tau fragments which further contributes to the amplification of neuronal demise [for refs see 31,50]. Recently we have proved that two AD peptides – i.e. Aβ1–42 and the smaller tau peptide NH_2-26–44 (named NH_2htau) – added to CGC homogenate, individually inhibited the ANT-1 in a non-competitive and competitive manner, respectively, and together aggravated the mitochondrial dysfunction by exacerbating the ANT-1 impairment, thus leading to dysfunction in energy metabolism prior to induction of cell death [50,51]. Further, Aβ1–42, added first to the cell homogenate, was able to decrease the inhibitory effect of NH_2htau on ANT-1 activity.

In order to investigate on the capability of ADP to protect ANT-1 from the toxic '*attack*' of the two AD peptides, cell homogenates were prepared from S-K25 and ADP–S-K25 cells and the effect of Aβ1–42 and NH_2htau, either added individually or together, on ANT-1 activity was tested (Table 1). When either NH_2htau (line 3) or Aβ1–42 (line 5) or both of them (line 7) were incubated with S-K25 cell homogenates for 3 min, the reaction rates of ANT-1, expressed as percentage of the S-K25 sample (line 1) to which a value of 100 was given, were found to be 20, 77 and 14% respectively and essentially as in [32,50]. When ADP-treated cells were used, no NH_2htau inhibition was detected (comparing lines 2 and 4) (101 vs 96%). On the contrary, about 25% inhibition was confirmed when either Aβ1–42 or NH_2htau + Aβ1–42 was added to ADP-cells (lines 6 and 8, respectively). These findings suggest that the lack of the inhibitory effect of NH_2htau on ANT-1, which occurred if cells were treated with ADP, is likely due to an ADP-mediated protection of group/s involved – presumably at the ANT-1 active site – in the interaction of NH_2htau, but not of Aβ1–42 with ANT-1. The same applied to the effect of Aβ1–42 + NH_2htau (line 8), thus further confirming that the rate of NADPH synthesis was strictly dependent on the treatment of cells with ADP, regardless of the interaction between Aβ and NH_2htau and consistently with [50].

4. Discussion

The starting point of this paper is that ADP can prevent low potassium-dependent apoptosis in the cell model system of CGCs (Fig. 1), confirming its pro-survival effect and suggesting its use in neuroprotection. The added value of this paper lies in the fact that, for the first time, it has been possible to propose a mechanism of action which could explain, at the molecular level, the protective role of ADP towards apoptosis, this by studying the effect of ADP on the various apoptotic steps so far identified in the cell system herein employed (i.e. CGCs) and confirming that all the events, measured in the whole cellular context, respond in a consistent manner. Collectively our data are consistent with results by others [12–14,19,52], which demonstrate a cytoprotective action of adenosine during cell injuries, and with the finding that adenosine and ADP exert a specific and marked antiapoptotic action in cultured cerebellar granule cells [18]. On the other hand, the

Table 1
ADP effect on Abeta- or tau fragment-dependent ANT-1 impairment.

Line	Sample	Added peptide	ADP	V (% of S-K25)
1	S-K25	–	ADP	100
2	ADP–S-K25	–	ADP	101
3	S-K25	NH_2htau	ADP	20**
4	ADP–S-K25	NH_2htau	ADP	96
5	S-K25	A-beta	ADP	77*
6	ADP–S-K25	A-beta	ADP	73§
7	S-K25	A-beta + NH_2htau	ADP	14**
8	ADP–S-K25	A-beta + NH_2htau	ADP	75§

Rat CGCs (15×10^6/well) at 7 DIV were incubated in high potassium (S-K25) culture medium in the absence (*clear lines*) or presence of ADP (1 mM) (*shaded lines*). The ADP exposure was terminated by removing the compound-containing media, followed by washing twice and replacing with fresh media. Cell homogenates (0.1 mg protein), containing mitochondria, were incubated, at 25 °C, with either NH_2htau or Aβ1–42, at a concentration equal to 1 and 2 μM respectively, for 3 min, after which the suspension was centrifuged and the pellet, resuspended in 2.0 ml of standard medium (see Materials and methods), was incubated with the ATP detection system. NADPH formation was monitored after ADP addition, as described in the method section (Section 2.13). The reaction rate is expressed as percentage of S-K25 to which a value of 100 is given. Values are mean ± SD from four experiments, carried out using different cell preparations. Statistical analysis was by ANOVA and Bonferroni test: *$p < 0.01$, **$p < 0.001$ when comparing all the samples (*clear lines*) with each other; §$p < 0.01$ when comparing all the samples (*shaded lines*) with each other.

present work is not in line with previous findings from other cell systems [53–60].

First of all it is noteworthy that, in the same way in which the recovery from apoptosis in CGCs is accompanied by a parallel block of the functional impairment which occurs in certain steps of the mitochondrial pathway, so our results for the first time provide evidence that the effects of ADP involve mitochondria. Indeed several mitochondrial processes which occur during CGC apoptosis, i.e. impairment of mitochondrial coupling (Fig. 2A), release of cyt *c* (Fig. 3A), ANT-1 alteration and mPTP opening (Fig. 4), are prevented by ADP.

Moreover, as ADP was able to decrease ROS levels (Fig. 2B), which is in agreement with previous findings reporting that the ROS production takes place early during apoptosis in this neuronal model being upstream the mitochondrial pathway [16 and the references therein], we strongly suggest that the prevention of apoptosis by ADP is at least in part due to activation of the antioxidant enzymes.

Accordingly, we proved (Fig. 2C) that the up-regulation of antioxidant system activity occurring in ADP–S-K5 cells is significantly reduced when the receptor antagonist PPADS was added. The ADP-dependent fast increase in AOX activity comes in addition to the increase which *per sè* occurs in CGCs undergoing apoptosis: we have reported that the activity of SOD and catalase and the thiol oxidation state increase up to 3 h, a time at which cells are still all alive even though ROS production increases [see 24].

Furthermore, the finding that the ADP effect is mediated not only by the activation of the cellular AOX enzymes, but also by the interaction of ADP with the mitochondrial ANT-1, offers interesting clues of discussion. In fact, it proposes for the first time an internalization of ADP – as such or after its degradation outside the cell and re-synthesis inside – which, along with other purine base adenine, is present in vivo in the extracellular space and whose balance between release and removal by enzymatic degradation is influenced by several physiological or pathological stimuli [61–64].

The ADP internalization is clearly demonstrated by the results shown in Fig. 5 – providing that the external addition of ADP to cell culture causes an increase of intracellular ADP level as well as of its derivative products (i.e. ATP, AMP and ADO) – which support the hypothesis that ADP *i*) as such or *ii*) through its conversion to ADO, passes from outside to inside the cell. The first possibility is objected by numerous papers in which the uptake of ADP in cell apparently does not happen [65,66]. More evidences are in favor of the hypothesis that ADP is hydrolyzed via ecto-5′-nucleotidases with extracellular formation of adenosine which is subsequently re-uptaked via the ADO transporter [61,62,67,68]. Consistently with this hypothesis, the internalization process is sensitive to the antagonist of purine receptor and the inhibitors of ectonucleotidase and nucleoside transporter (see Fig. 5). Anyway, if ADP internalization is due to a direct ADP uptake process into the cell – this hypothesizing an ADP translocator – or to a metabolizing enzyme-dependent process remains to be established through a detailed study which is not pertinent with the focus of this paper. Unfortunately, at present we cannot still say which the real neuroprotective molecule is: whether the ADP per se or one of its degradation products. A more detailed work to clarify whether ADP exerts the protective action independently of its degradation to adenosine in the CGCs undergoing apoptosis is going to be addressed.

In Fig. 4, we show that, although ADP is essential to completely protect mitochondrial ANT-1, its effect on mPTP opening is only partial, thus suggesting that ANT-1 involvement is not the primary event leading to mitochondrial permeabilization and then to cell death, consistently with [17]. In this regard, a comparison made between loss of ANT transport function and mPTP opening reveals that the two processes occur simultaneously in S-K5 cells, either in the absence or presence of SOD, in the 5–8 h time range after apoptosis induction. This is not true with ADP–S-K5 cells for which the reciprocal relationship holds only up to 3 h but soon after the two processes follow separate paths supporting the hypothesis of some authors [17,69] whereby mPTP opening is dispensable to cell death. Consistently, the data shown in this study, i.e. the complete prevention of death, but the partial prevention of mPTP opening by ADP, confirm what we have previously suggested: in CGC apoptosis, death and mPTP are not strictly dependent on each other. There are two immediate feedbacks of these data: *i*) mPTP opening does not only require ANT impairment, somehow confirming what was sensationally suggested by Kokoszka et al. [69], who, by using the mitochondria from the liver of ANT knockout mice, observed that they still possess mPTP activity; *ii*) mPTP opening does not result in death, consistently with [17]. It is noteworthy, moreover, that in the light of the inverse overlapping sensitivity of ANT and mPTP both to the caspase inhibitor and to other inhibitors used to block cell systems participating in apoptosis, and because no change occurs either in the amount or the size of ANT, i.e. ANT itself was proved not to be the caspase target [17], we conclude that although it is sure that a functional alteration of ANT takes place resulting in ANT becoming a functional component of the pore responsible for the mPT, the lack of overlapping sensitivity of ANT and mPTP to ADP is probably due the failure of other proteins participating in the pore formation, as suggested in [17], and/or to other factor/s. The factor/s which can contribute to the prevention of mPTP opening by ADP may be kinetic parameters, concentrations of metabolite/s, exposure time to all those compounds/parameters, and all those ones that – unlike experiments conducted on isolated mitochondria from different organs, under specific and controlled experimental conditions [70–73] – cannot be kept under experimental control in a whole cellular context.

Shifting the focus towards the effect of ATR on the viability (see Fig. 1E), this selective ANT inhibitor prevents ADP protection, thus suggesting that the molecular mechanism responsible for the ADP protective effect is not unique. Since complete prevention of ADP protection occurred only when ATR and PPADS are added together (compare 29%, i.e. in the presence of PPADS + ATR, with 56%, in the presence of ATR alone) it is inevitable to assume that the ADP protective effect on cell death is mediated both by the activation of AOX system (see above) and by its direct interaction with the mitochondrial carrier that drives the ADP/ATP exchange. Nevertheless, it should also be considered that the activation of AOX enzymes results in depressed levels of ROS which in turn reduces the ANT-1 activity, as in [17].

Besides, we have recently established [31,50] that extracellular ADP protects ANT-1 from impairment produced by NH_2htau fragment which, either individually or even more when added together with Aβ1–42, inhibits the mitochondrial translocator. The ADP capability to protect the carrier from the toxic NH_2htau can be easily explained when considering that the inhibition exerted by this fragment on ANT-1 is competitive-like, that is, it interacts with the catalytic site to which the substrate binds. That ADP acts at the level of the catalytic site is further confirmed by data with ATR (Fig. 1E) and also by the mPTP prevention, knowing that ADP binding to the ADP/ATP translocase caused inhibition of the Ca^{2+}-induced permeability transition of the mitochondrial inner membrane [74,75].

Interestingly, it has been recently reported that the N-terminal region of tau protein has a putative site for ATP/ADP binding and that full-length tau is able to bind ATP. However, although the interaction tau/ATP induces in vitro self-assembly of the protein, no tau self-aggregation is found after incubation with ADP [76]. Therefore, it's possible to speculate that adenosine nucleotides can play a role in modulating tau toxicity during in vivo neurodegeneration. The interaction of tau with ATP not only could contribute to lower the cytosolic ATP pool in neurons but also could potentiate its inhibitory effect on ATP-producing mitochondria [77], which is in contrast blocked by ADP. An alternative but not mutually exclusive action of ADP could consist in restoring ATP levels which are generally reduced during apoptosis in several in vitro cell systems [for ref see 34] as well as in aging neurons and even more in Alzheimer's disease neurons [78]. However, the possible biological significance of this effect and of the mechanism through which it is reached still remains to be fully investigated.

Among the different possibilities that can be evoked to explain the antiapoptotic action of ADP, the restoration of Ca^{2+} homeostasis, which was imbalanced by the Ca_i^{2+} drop caused by the potassium withdrawal from culture medium [15], or the activation of alternative pathway/s occurring downstream the Ca_i^{2+} drop, could of course be considered. However, even though interesting, these topics fall outside the scope of our work at present.

Another issue that the complete protection by ADP calls into question – and on which there is a lot of discussion – is the 'point of no return', a decisive regulatory event also termed '*the commitment-to-die*'. This matter deserves special attention. Beyond the 'point of no return', the fate of the cell, undergoing apoptosis, is irreversibly marked: several studies suggested that the ultimate determinant of cell death could be the activation of caspase(s) [79–81], while others the loss of mitochondrial membrane potential directly governed by a subset of the BCL-2 family of proapoptotic proteins [82–84].

From the data of Fig. 1B, in which the viability at 48 h was evaluated in the presence of ADP, added at different times after apoptosis induction, it appears clear that the maximum rescue time within which it is possible to flip the fate of CGCs shifted to 5 mM K^+, is 3 h, that is when the ROS reaches the highest level and the AOX activity increases and cyt *c* release also occurs. It is important to notice that at this time, i.e. 3 h, caspase(s) is/are not yet active and mitochondrial membrane potential, which is early impaired [see 16,35] in apoptosis, can be recovered by the released cyt *c*, which can generate protonmotive force and drive ATP synthesis in the isolated mitochondria [36].

Throwing an arrow in favor of optimizing scientific research for therapeutic purposes, it could be assessed that this type of research brings close the possibility to block the degenerative process in an early presymptomatic stage or in a phase in which the neurons can still recover their functionality.

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References

[1] C. Volonté, S. Amadio, F. Cavaliere, N. D'Ambrosi, F. Vacca, G. Bernardi, Extracellular ATP and neurodegeneration, Curr. Drug Targets CNS Neurol. Disord. 2 (2003) 403–412 (Review).
[2] B. Sperlágh, E.S. Vizi, The role of extracellular adenosine in chemical neurotransmission in the hippocampus and basal ganglia: pharmacological and clinical aspects, Curr. Top. Med. Chem. 11 (2011) 1034–1046.
[3] M.L. He, A.E. Gonzalez-Iglesias, M. Tomic, S.S. Stojilkovic, Release and extracellular metabolism of ATP by ecto-nucleotidase eNTPDase 1–3 in hypothalamic and pituitary cells, Purinergic Signal 1 (2005) 135–144.
[4] T.K. Harden, J.L. Boyer, R.A. Nicholas, P2-purinergic receptors: subtype-associated signaling responses and structure, Annu. Rev. Pharmacol. Toxicol. 35 (1995) 541–579 (Review).
[5] M.E. Olah, G.L. Stiles, Adenosine receptor subtypes: characterization and therapeutic regulation, Annu. Rev. Pharmacol. Toxicol. 35 (1995) 581–606 (Review).
[6] P. Di Iorio, P. Ballerini, F. Caciagli, R. Ciccarelli, Purinoceptor-mediated modulation of purine and neurotransmitter release from nervous tissue, Pharmacol. Res. 37 (1998) 169–178.
[7] D.P. Westfal, L.D. Todorov, S.T. Mihaylova-Todorova, ATP as a cotransmitter in sympathetic nerves and its inactivation by releasable enzymes, J. Pharmacol. Exp. Ther. 303 (2002) 439–444.
[8] G. Burnstock, G.E. Knight, Cellular distribution and functions of P2 receptor subtypes in different systems, Int. Rev. Cytol. 240 (2004) 31–304.
[9] H. Zimmermann, Nucleotide signaling in nervous system development, Eur. J. Physiol. 452 (2006) 573–588.
[10] J.T. Barron, G.U. Liping, Energetic effects of adenosine on vascular smooth muscle, Am. J. Physiol. Heart Circ. Physiol. 278 (2000) H26–H32.
[11] R. Ciccarelli, P. Ballerini, G. Sabatino, M.P. Rathbone, M. D'Onofrio, F. Caciagli, P. Di Iorio, Involvement of astrocytes in purine-mediated reparative processes in the brain, Int. J. Dev. Neurosci. 19 (2001) 395–414.
[12] B.-K. Yoo, J.W. Choi, S.Y. Yoon, K.H. Ko, Protective effect of adenosine and purine nucleos(t)ides against the death by hydrogen peroxide and glucose deprivation in rat primary astrocytes, Neurosci. Res. 51 (2005) 39–44.
[13] M.V. Cohen, J.M. Downey, Adenosine: trigger and mediator of cardioprotection, Basic Res Cardiol. 103 (2008) 203–215.
[14] A.A. Fatokun, T.W. Stone, R.A. Smith, Cell death in rat cerebellar granule neurons induced by hydrogen peroxide in vitro: mechanisms and protection by adenosine receptor ligands, Brain Res. 1132 (2007) 193–202.
[15] S.R. D'Mello, C. Galli, M.T. Ciotti, P. Calissano, Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 10989–10993.
[16] A. Atlante, S. Gagliardi, E. Marra, P. Calissano, Neuronal apoptosis in rats is accompanied by rapid impairment of cellular respiration and is prevented by scavengers of reactive oxygen species, Neurosci. Lett. 254 (1998) 127–130.
[17] A. Atlante, A. Bobba, L. de Bari, F. Fontana, P. Calissano, E. Marra, S. Passarella, Caspase-dependent alteration of the ADP/ATP translocator triggers the mitochondrial permeability transition which is not required for the low-potassium dependent apoptosis of cerebellar granule cells, J. Neurochem. 97 (2006) 1166–1181.
[18] O.V. Vitolo, M.T. Ciotti, C. Galli, T. Borsello, P. Calissano, Adenosine and ADP prevent apoptosis in cultured rat cerebellar granule cells, Brain Res. 809 (1998) 297–301.
[19] C.G. Almeida, A. de Mendonça, R.A. Cunha, J.A. Ribeiro, Adenosine promotes neuronal recovery from reactive oxygen species induced lesion in rat hippocampal slices, Neurosci. Lett. 339 (2003) 127–130.
[20] L. Willems, K.J. Ashton, J.P. Headrick, Adenosine-mediated cardioprotection in the aging myocardium, Cardiovasc. Res. 66 (2005) 245–255.
[21] N. Fujita, M. Kakimi, Y. Ikeda, T. Hiramoto, K. Suzuki, Extracellular ATP inhibits starvation-induced apoptosis via P2X2 receptors in differentiated rat pheochromocytoma PC12 cells, Life Sci. 66 (2000) 1849–1859.
[22] A. Eckert, S. Hauptmann, I. Scherping, V. Rhein, F. Muller-Spahn, J. Götz, W.E. Muller, Soluble beta-amyloid leads to mitochondrial defects in amyloid precursor protein and tau transgenic mice, Neurodegener. Dis. 5 (2008) 157–159.
[23] G. Levi, F. Aloisi, M.T. Ciotti, V. Gallo, Autoradiographic localization and depolarization-induced release of acidic amino acids in differentiating cerebellar granule cell cultures, Brain Res. 290 (1984) 77–86.
[24] A. Atlante, A. Bobba, P. Calissano, S. Passarella, E. Marra, The apoptosis/necrosis transition in cerebellar granule cells depends on the mutual relationship of the antioxidant and the proteolytic systems which regulate ROS production and cytochrome c release en route to death, J. Neurochem. 84 (2003) 960–971.
[25] R.A. Sjodin, Measurement of Na^+–K^+ pump in muscle, Methods Enzymol. 173 (1989) 695–714.
[26] W.J. Waddell, C. Hill, A simple ultraviolet spectrophotometric method for the determination of protein, J. Lab. Clin. Med. 48 (1956) 311–314.
[27] C. Volonte`, M.T. Ciotti, L. Battistini, Development of a method for measuring cell number: application to CNS primary neuronal cultures, Cytometry 17 (1994) 274–276.
[28] A. Atlante, A. Bobba, G. Paventi, R. Pizzuto, S. Passarella, Genistein and daidzein prevent low potassium-dependent apoptosis of cerebellar granule cells, Biochem. Pharmacol. 79 (2010) 758–767.
[29] A. Atlante, S. Gagliardi, G.M. Minervini, M.T. Ciotti, E. Marra, P. Calissano, Glutamate neurotoxicity in rat cerebellar granule cells: a major role for xanthine oxidase in oxygen radical formation, J. Neurochem. 68 (1997) 2038–2045.
[30] T.P.M. Akerboom, H. Sies, Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples, Methods Enzymol. 77 (1981) 373–382.
[31] A. Atlante, G. Amadoro, A. Bobba, L. de Bari, V. Corsetti, G. Pappalardo, E. Marra, P. Calissano, S. Passarella, A peptide containing residues 26–44 of tau protein impairs mitochondrial oxidative phosphorylation acting at the level of the adenite nucleotide translocator, Biochim. Biophys. Acta 1777 (2008) 1289–1300.
[32] A. Bobba, G. Amadoro, V.A. Petragallo, P. Calissano, A. Atlante, Dissecting the molecular mechanism by which NH2htau and Aβ1–42 peptides impair mitochondrial ANT-1 in Alzheimer disease, Biochim. Biophys. Acta 1827 (2013) 848–860.
[33] S. Passarella, A. Atlante, D. Valenti, L. de Bari, The role of mitochondrial transport in energy metabolism, Mitochondrion 2 (2003) 319–343.
[34] A. Atlante, S. Giannattasio, A. Bobba, S. Gagliardi, V. Petragallo, P. Calissano, S. Passarella, An increase in the ATP levels occurs in cerebellar granule cells en route to apoptosis in which ATP derives from both oxidative phosphorylation and anaerobic glycolysis, Biochim. Biophys. Acta 1708 (2005) 50–62.
[35] E.M. De La Cruz, E.M. Ostap, Kinetic and equilibrium analysis of the myosin ATPase, Methods Enzymol. 455 (2009) 157–192.
[36] T.F. Wang, P.A. Rosenberg, G. Guidotti, Characterization of brain ecto-apyrase: evidence for only one ecto-apyrase (CD39) gene, Brain Res. Mol. Brain Res. 47 (1997) 295–302.
[37] A. Bobba, A. Atlante, S. Giannattasio, G. Sgaramella, P. Calissano, E. Marra, Early release and subsequent caspase-mediated degradation of cytochrome c in apoptotic cerebellar granule cells, FEBS Lett. 457 (1999) 126–130.
[38] A. Atlante, L. de Bari, A. Bobba, E. Marra, P. Calissano, S. Passarella, Cytochrome c, released from cerebellar granule cells undergoing apoptosis or excitotoxic death, can generate protonmotive force and drive ATP synthesis in isolated mitochondria, J. Neurochem. 86 (2003) 591–604.
[39] A. Valencia, J. Moran, Reactive oxygen species induce different cell death mechanisms in cultured neurons, Free Rad. Biol. Med. 36 (2004) 1112–1125.
[40] A. Atlante, P. Calissano, A. Bobba, A. Azzariti, E. Marra, S. Passarella, Cytochrome c is released from mitochondria in a reactive oxygen species (ROS)-dependent fashion and can operate as a ROS scavenger and as a respiratory substrate in cerebellar neurons undergoing excitotoxic death, J. Biol. Chem. 275 (2000) 37159–37166.
[41] G. Lambrecht, T. Friebe, U. Grimm, U. Windscheif, E. Bungardt, C. Hildebrandt, H.G. Bäumert, G. Spatz-Kümbel, E. Mutschler, PPADS, a novel functionally selective antagonist of P2 purinoceptor-mediated responses, Eur. J. Pharmacol. 217 (1992) 217–219.
[42] A. Bobba, A. Atlante, L. de Bari, S. Passarella, E. Marra, Apoptosis and cytochrome c release in cerebellar granule cells, In Vivo 18 (2004) 335–344.

[43] M. Leist, B. Single, A.F. Castoldi, S. Kühnle, P. Nicotera, Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis, J. Exp. Med. 185 (1997) 1481–1486.
[44] D.R. Green, J.C. Reed, Mitochondria and apoptosis, Science 1281 (1998) 1309–1312 (Review).
[45] C. Richter, M. Schweizer, A. Cossarizza, C. Franceschi, Control of apoptosis by the cellular ATP level, FEBS Lett. 378 (1996) 107–110 (Review).
[46] E. Basso, L. Fante, J. Fowlkes, V. Petronilli, M.A. Forte, P. Bernardi, Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D, J. Biol. Chem. 280 (2005) 18558–18561.
[47] A.P. Halestrap, C. Brenner, The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death, Curr. Med. Chem. 10 (2003) 1507–1525.
[48] I. Matsuoka, S. Ohkubo, J. Kimura, Y. Uezono, Adenine nucleotide-induced activation of adenosine A(2B) receptors expressed in *Xenopus laevis* oocytes: involvement of a rapid and localized adenosine formation by ectonucleotidases, Mol. Pharmacol. 61 (2002) 606–613.
[49] A. Bailey, H. Matthes, B. Kieffer, S. Slowe, S.M. Hourani, I. Kitchen, Quantitative autoradiography of adenosine receptors and NBTI-sensitive adenosine transporters in the brains and spinal cords of mice deficient in the mu-opioid receptor gene, Brain Res. 943 (2002) 68–79.
[50] G. Amadoro, V. Corsetti, A. Atlante, F. Florenzano, S. Capsoni, R. Bussani, D. Mercanti, P. Calissano, Interaction between NH(2)-tau fragment and Aβ in Alzheimer's disease mitochondria contributes to the synaptic deterioration, Neurobiol. Aging 33 (2012) 833.e1–833.e25.
[51] G. Amadoro, M.T. Ciotti, M. Costanzi, V. Cestari, P. Calissano, N. Canu, NMDA receptor mediates tau-induced neurotoxicity by calpain and ERK/MAPK activation, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 2892–2897.
[52] S.B. Maggirwar, D.N. Dhanraj, S.M. Somani, V. Ramkumar, Adenosine acts as an endogenous activator of the cellular antioxidant defense system, Biochem. Biophys. Res. Commun. 201 (1994) 508–515.
[53] L.M. Zheng, A. Zychlinsky, C.C. Liu, D.M. Ojcius, J.D. Young, Extracellular ATP as a trigger for apoptosis or programmed cell death, J. Cell Biol. 112 (1991) 279–288.
[54] D.D. Dawicki, D. Chatterjee, J. Wyche, S. Rounds, Extracellular ATP and adenosine cause apoptosis of pulmonary artery endothelial cells, Am. J. Physiol. 273 (1997) L485–L494.
[55] S. Rounds, W.L. Yee, D.D. Dawicki, E. Harrington, N. Parks, M.V. Cutaia, Mechanism of extracellular ATP- and adenosine-induced apoptosis of cultured pulmonary artery endothelial cells, Am. J. Physiol. 275 (1998) L379–L388.
[56] A.Y. Sun, Y.M. Chen, Extracellular ATP-induced apoptosis in PC12 cells, Adv. Exp. Med. Biol. 446 (1998) 73–83.
[57] M.L. Peyot, A.P. Gadeau, F. Dandré, I. Belloc, F. Dupuch, C. Desgranges, Extracellular adenosine induces apoptosis of human arterial smooth muscle cells via A(2b)-purinoceptor, Circ. Res. 86 (2000) 76–85.
[58] M. Aghaei, F. Karami-Tehrani, M. Panjehpour, S. Salami, F. Fallahian, Adenosine induces cell-cycle arrest and apoptosis in androgen-dependent and -independent prostate cancer cell lines, LNcap-FGC-10, DU-145, and PC3, Prostate 72 (2012) 361–375.
[59] L.F. Wu, G.P. Li, J.L. Feng, Z.J. Pu, Molecular mechanisms of adenosine-induced apoptosis in human HepG2 cells, Acta Pharmacol. Sin. 27 (2006) 477–484.
[60] L.T. Wen, A.F. Knowles, Extracellular ATP and adenosine induce cell apoptosis of human hepatoma Li-7A cells via the A3 adenosine receptor, Br. J. Pharmacol. 140 (2003) 1009–1118.
[61] F.E. Parkinson, C.J. Sinclair, T. Othman, N.J. Haughey, J.D. Geiger, Differences between rat primary cortical neurons and astrocytes in purine release evoked by ischemic conditions, Neuropharmacol. 43 (2002) 836–846.
[62] F.E. Parkinson, W. Xiong, Stimulus- and cell-type-specific release of purines in cultured rat forebrain astrocytes and neurons, J. Neurochem. 88 (2004) 1305–1312.
[63] F.E. Parkinson, W. Xiong, C.R. Zamzow, Astrocytes and neurons: different roles in regulating adenosine levels, Neurol. Res. 27 (2005) 153–160.
[64] S. Chu, W. Xiong, D. Zhang, H. Soylu, C. Sun, B.C. Albensi, F.E. Parkinson, Regulation of adenosine levels during cerebral ischemia, Acta Pharmacol. Sin. 34 (2013) 60–66.
[65] W.E. Jacobus, Theoretical support for the heart phosphocreatine energy transport shuttle based on the intracellular diffusion limited mobility of ADP, Biochem. Biophys. Res. Commun. 133 (1985) 1035–1041.
[66] O. Kongas, J.H. van Beek, Diffusion barriers for ADP in the cardiac cell, Mol. Biol. Rep. 29 (2002) 141–144.
[67] M.P. Rathbone, P. Middlemiss, C. Andrew, F. Caciagli, R. Ciccarelli, P. Di Iorio, R. Huang, The trophic effects of purines and purinergic signaling in pathologic reactions of astrocytes, Alzheimer Dis. Assoc. Disord. 12 (Suppl. 2) (1998) S36–S45.
[68] F.E. Parkinson, J. Ferguson, C.R. Zamzow, W. Xiong, Gene expression for enzymes and transporters involved in regulating adenosine and inosine levels in rat forebrain neurons, astrocytes and C6 glioma cells, J. Neurosci. Res. 84 (2006) 801–808.
[69] J.E. Kokoszka, K.G. Waymire, S.E. Levy, J.E. Sligh, J. Cai, D.P. Jones, G.R. MacGregor, D. C. Wallace, The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore, Nature 427 (2004) 461–465.
[70] M. Crompton, The mitochondrial permeability transition pore and its role in cell death, Biochem. J. 341 (1999) 233–249.
[71] G.P. McStay, S.J. Clarke, A.P. Halestrap, Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore, Biochem. J. 367 (2002) 541–548.
[72] A.P. Halestrap, G.P. McStay, S.J.B. Clarke, The permeability transition pore complex: another view, Biochimie 84 (2002) 153–166.
[73] N. Sokolova, S. Pan, S. Provazza, G. Beutner, M. Vendelin, R. Birkedal, S.S. Sheu, ADP protects cardiac mitochondria under severe oxidative stress, PLoS One 8 (2013) e83214.
[74] R.A. Haworth, D.R. Hunter, Control of the mitochondrial permeability transition pore by high-affinity ADP binding at the ADP/ATP translocase in permeabilized mitochondria, J. Bioenerg. Biomembr. 32 (2000) 91–96.
[75] Z.Z. Gizatullina, Y. Chen, S. Zierz, F.N. Gellerich, Effects of extramitochondrial ADP on permeability transition of mouse liver mitochondria, Biochim. Biophys. Acta 1706 (2005) 98–104.
[76] M. Farid, C.P. Corbo, A.D. Alonso, Tau binds ATP and induces its aggregation, Microsc. Res. Tech. 77 (2014) (2013) 133–137.
[77] V. García-Escudero, P. Martín-Maestro, G. Perry, J. Avila, Deconstructing mitochondrial dysfunction in Alzheimer disease, Oxidative Med. Cell. Longev. 2013 (2013) 162152 (Review).
[78] S. Hoyer, Oxidative energy metabolism in Alzheimer brain. Studies in early-onset and late-onset cases, Mol. Chem. Neuropathol. 16 (1992) 207–224.
[79] D.R. Green, G.P. Amarante-Mendes, The point of no return: mitochondria, caspases, and the commitment to cell death, Results Probl. Cell Differ. 24 (1998) 45–61.
[80] K.M. Boatright, G.S. Salvesen, Mechanisms of caspase activation, Curr. Opin. Cell Biol. 15 (2003) 725–731 (Review).
[81] S. Kothakota, T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T.J. McGarry, M.W. Kirschner, K. Koths, D.J. Kwiatkowski, L.T. Williams, Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis, Science (Wash DC) 278 (1997) 294–298.
[82] L.K. Chang, G.V. Putcha, M. Deshmukh, E.M. Johnson Jr., Mitochondrial involvement in the point of no return in neuronal apoptosis, Biochimie 84 (2002) 223–231.
[83] S.W. Tait, D.R. Green, Mitochondria and cell death: outer membrane permeabilization and beyond, Nat. Rev. Mol. Cell Biol. 11 (2010) 621–632.
[84] J.E. Chipuk, L. Bouchier-Hayes, D.R. Green, Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario, Cell Death Differ. 13 (2006) 1396–1402.