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

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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

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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

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