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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

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Abstract

Although it is recognized that ATP plays a part in apoptosis, whether and how its level changes en route to apoptosis as well as how ATP is synthesized has not been fully investigated. We have addressed these questions using cultured cerebellar granule cells. In particular, we measured the content of ATP, ADP, AMP, IMP, inosine, adenosine and L-lactate in cells undergoing apoptosis during the commitment phase (0–8 h) in the absence or presence of oligomycin or/and of citrate, which can inhibit totally the mitochondrial oxidative phosphorylation and largely the substrate-level phosphorylation in glycolysis, respectively. In the absence of inhibitors, apoptosis was accompanied by an increase in ATP and a decrease in ADP with 1:1 stoichiometry, with maximum ATP level found at 3 h apoptosis, but with no change in levels of AMP and its breakdown products and with a relatively low level of L-lactate production. Consistently, there was an increase in the cell energy charge and in the ratio ([ATP][AMP])/[ADP]^2. When the oxidative phosphorylation was completely blocked by oligomycin, a decrease of the ATP content was found both in control cells and in cells undergoing apoptosis, but nonetheless cells still died by apoptosis, as shown by checking DNA laddering and by death prevention due to actinomycin D. In this case, ATP was provided by anaerobic glycolysis, as suggested by the large increase of L-lactate production. On the other hand, citrate itself caused a small decrease in ATP level together with a huge decrease in L-lactate production, but it had no effect on cell survival. When ATP level was further decreased due to the presence of both oligomycin and citrate, death occurred via necrosis at 8 h, as shown by the lack of DNA laddering and by death prevention found due to the NMDA receptor antagonist MK801. However, at a longer time, when ATP level was further decreased, cells died neither via apoptosis nor via glutamate-dependent necrosis, in a manner similar to something like to energy catastrophe. Our results shows that cellular ATP content increases in cerebellar granule cell apoptosis, that the role of oxidative phosphorylation is facultative, i.e. ATP can also derive from anaerobic glycolysis, and that the type of cell death depends on the ATP availability.

Keywords: Cerebellar granule cell; Apoptosis; ATP content; L-lactate; Mitochondria

Abbreviations: Act D, actinomycin D; AQ_{ADK}, ADK quotient activity; DIV, days in vitro; BME, basal medium Eagle; CGCs, cerebellar granule cells; CITR, citrate; cyt *c*, cytochrome *c*; FCS, fetal calf serum; HPLC, high-performance liquid chromatography; L-LACT, L-lactate; MK801, (+)-5methyl-10,11dihydro-5*H*-dibenzo(a,d)cyclohepten-5,10-imine hydrogen maleate; NMDA, *N*-methyl-D-aspartate; O_2, molecular oxygen; OLIGO, oligomycin; PBS, phosphate buffer saline medium; PFK, phosphofructokinase; S-K25 cells, control cells; S-K5 cells, apoptotic cells; ROS, reactive oxygen species

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1. Introductory statement

In vitro cultured rat cerebellar granule cells (CGCs) die via necrosis when subjected to excessive and prolonged glutamate exposure [1,2] and via apoptosis when deprived of serum and depolarizing levels (25 mM) of extracellular potassium [3]. Therefore, these neurons constitute a versatile system to dissect the mechanisms which are common to apoptosis and necrosis and those which are unique. Furthermore, both death conditions are not induced by toxic or harmful manipulations as in other experimental paradigms [4–7], but, rather, by events which may occur at any time during the course of the life span of an organism. Thus, glutamate-evoked massive release is the most rapid event following neuronal oxygen shortage, while potassium deprivation causing apoptosis is the in vitro counterpart of in vivo deafferentation [8,9].

The processes leading to apoptosis have been investigated in detail and a role identified for an antioxidant system, a proteolytic system including proteasome and caspases, and for released cytochrome *c* (cyt *c*) [10–15]. In particular, we showed that released cyt *c*, working as a ROS scavenger and electron donor to cytochrome *c* oxidase, can generate an electrochemical proton gradient and drive the synthesis of ATP that is required for apoptosis, but not necrosis, to occur (see Refs. [15,16]).

It is clear that ATP-dependent steps take place in apoptotic signal transduction [17] including: (i) apoptosome complex formation [18,19] and processing of pro-caspase-9 [20]; (ii) chromatin condensation and apoptotic body formation [21]; (iii) phosphorylation of kinases and other pro-apoptotic proteins [22–24]; (iv) externalization of phosphatidylserine [25]. Moreover ATP concentration has been reported as a switch in the decision between apoptosis and necrosis [17,26,27].

It is not, however, known whether and how the ATP level can change and how ATP is synthesized en route to apoptosis. Thus, in this work we assayed the content of both the adenine nucleotides and AMP breakdown products as a function of time after induction of apoptosis. We found that ATP content increases en route to apoptosis with an increase in cell energy charge and in the activity quotient of the adenylate kinase reaction. Moreover we found that ATP itself is needed for apoptosis to occur in CGCs, and that it can be supplied by anaerobic glycolysis when oxidative phosphorylation is totally impaired.

2. Materials and methods

2.1. Reagents

Tissue culture medium and fetal calf serum were purchased from GIBCO (Grand Island, NY) and tissue culture dishes were from NUNC (Taastrup, Denmark). All enzymes and chemicals were from Sigma Chemicals Co. (St Louis, MO, USA).

2.2. Cell cultures

Primary cultures of CGCs were obtained from dissociated cerebellar of 7-day-old Wistar rats as in [28]. Cells were plated in basal medium Eagle (BME) supplemented with 10% fetal calf serum (FCS), 25 mM KCl, 2 mM glutamine and 100 μg/ml gentamicin on dishes coated with poly-L-lysine. Cells were plated at 2×10^6 per 35 mm dish, 6×10^6 per 60 mm dish, or 15×10^6 per 90 mm dish. Arabinofuranosylcytosine (10 μM) was added to the culture medium 18–22 h after plating to prevent proliferation of non-neuronal cells.

2.3. Induction of apoptosis

Apoptosis was induced as in [3]. After 6–7 days in vitro (DIV), cells were washed twice and switched to serum-free BME (S-), containing 5 mM KCl and supplemented with 2 mM glutamine and 100 μg/ml gentamicin for the times reported in the figure legends. Apoptotic cells are referred to as S-K5 cells. 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.

2.4. Glutamate neurotoxicity induction

Glutamate exposure was performed 7 days after plating. Primary cultures were exposed for 30 min to glutamate (100 μM) at 25 °C in Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, 10 mM Hepes pH 7.4) in the presence of 1 μM glycine added in order to fully activate NMDA-sensitive glutamate recognition sites [29]. Cells were then replenished with BME containing 25 mM KCl, 2 mM glutamine and gentamicin (100 μg/ml) and put in the incubator. For the quantitative assessment of glutamate neurotoxicity (GNT), cell integrity and count were measured, as described below, after 12–24 h. Glutamate-treated cells and control cells are referred to as GNT- and C-GNT cells respectively.

2.5. Cell suspension and homogenate preparations

Before each experiment, the culture medium was removed and the plated CGCs were 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 by gentle scraping into a volume of PBS depending on the particular experiment. Although suspended granule cells lacked the morphological organization observed in culture dishes such as cell–cell and cell–substrate contacts, and had no neurites, they showed full viability. Cell integrity, which remained essentially constant