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# Purine and pyrimidine nucleosides preserve human astrocytoma cell adenylate energy charge under ischemic conditions

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**Abstract**

The brain depends on both glycolysis and mitochondrial oxidative phosphorylation for maintenance of ATP pools. Astrocytes play an integral role in brain functions providing trophic supports and energy substrates for neurons. In this paper, we report that human astrocytoma cells (ADF) undergoing ischemic conditions may use both purine and pyrimidine nucleosides as energy source to slow down cellular damage. The cells are subjected to metabolic stress conditions by exclusion of glucose and incubation with oligomycin (an inhibitor of oxidative phosphorylation). This treatment brings about a depletion of the ATP pool, with a concomitant increase in the AMP levels, which results in a significant decrease of the adenylate energy charge. The presence of purine nucleosides in the culture medium preserves the adenylate energy charge, and improves cell viability. Besides purine nucleosides, also pyrimidine nucleosides, such as uridine and, to a lesser extent, cytidine, are able to preserve the ATP pool. The determination of lactate in the incubation medium indicates that nucleosides can preserve the ATP pool through anaerobic glycolysis, thus pointing to a relevant role of the phosphorolytic cleavage of the *N*-glycosidic bond of nucleosides which generates, without energy expense, the phosphorylated pentose, which through the pentose phosphate pathway and glycolysis can be converted to energetic intermediates also in the absence of oxygen. In fact, ADF cells possess both purine nucleoside phosphorylase and uridine phosphorylase activities.


*Keywords:* Neuroprotection; Human astrocytoma; Purine and pyrimidine nucleosides; Oligomycin

## 1. Introduction

The brain depends on both glycolysis and mitochondrial oxidative phosphorylation for the maintenance of ATP levels. In fact, several neurodegenerative disorders, such as Alzheimer's, Huntington's and Parkinson's diseases have been related to mitochondrial disfuntion and oxidative stress (Beal, 1992). A release of adenine nucleotides, which are subsequently hydrolyzed to adenosine, or adenosine itself has been reported for astrocytes and neurons, respectively, under ATP-depleting conditions (Parkinson and Xiong, 2004). Furthermore, in ischemic/hypoxic conditions, the turnover of endogenous nucleotides stemming from dead cells may possibly supply a discrete amount of purine (Uemura et al., 1991; Rudolphi and Shubert, 1995; Ciccarelli et al., 1999) and pyrimidine nucleosides. Indeed, a number of reports indicate that purines are likely to exert trophic effects on neuronal cells following traumatic events. While the effect of adenosine appears to be, at least in a number of cases, receptor-mediated (Kobayashi et al., 1998; Tomaselli et al., 2005), the finding that nucleosides different from adenosine exert a protective effect as well, points to a different receptor-independent mechanism. A beneficial effect of purine nucleosides, especially marked with inosine, has been observed on the neurite outgrowth of primary rat cerebellar granule cells after hypoxia (Böcklinger et al., 2004). Haun et al. (1996) have reported that adenosine in order to exert its neuroprotective effect on rat astrocyte cultures subjected to combined glucose–oxygen deprivation must be intracellularly converted into inosine. However, the authors, without measuring the level of ATP, postulate that the preservation of the adenine nucleotide pool is not the mechanism by which inosine protects: indeed, the authors, neglecting the fate of the sugar

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moiety of the nucleoside, to support their conclusions, assess that hypoxanthine, the nucleobase produced by inosine phosphorolysis, has no effect on astroglial injury, and indicate a possible involvement of hitherto unknown intracellular signalling pathways. Still neglecting the destiny of the ribose moiety, the reduction of ischemic brain injury in rats by inosine has been ascribed to the effect of the nucleoside on A3 receptors (Shen et al., 2005). On the other hand, the correlation between protection and ATP preservation observed during glucose deprivation and mitochondrial inhibition in glial cells treated with purine nucleosides (Jurkowitz et al., 1998) has been explained by a mechanism that requires a prior phosphorolysis of the nucleosides, with generation of ribose-1-phosphate (Rib-1-P), which after conversion to ribose-5-phosphate (Rib-5-P), enters the pentose phosphate pathway and is converted to glycolytic intermediates, yielding a net production of ATP. A similar mechanism of purine nucleoside protection has been postulated by Litsky et al. (1999) by using primary mixed cultures of murine embryonic spinal cord neurons and glia. A receptor-independent protective effect of adenosine and purine nucleos(t)ides against the death induced by hydrogen peroxide and glucose deprivation in rat primary astrocytes has been also reported (Yoo et al., 2005). Recently, it has been demonstrated that in conditions of energy depletion induced by mitochondrial inhibition, human colon carcinoma cells not only utilize nucleosides to restore the ATP pool, but, accordingly with the role of these compounds as energy source alternative to glucose, also their uptake is increased (Giannecchini et al., 2005). Indeed, although glucose represents the major energy source for most cell types, when the glucose supply is lowered and the oxygen is not sufficient to support mitochondrial respiration (ischemic and/or hypoxic conditions), nucleosides may become one of the major energy sources for the cell.

## 2. Materials and methods

### 2.1. Materials

RPMI medium 1640, Dulbecco's modified Eagle medium without glucose (DMEM) and foetal bovine serum (FBS) were from Gibco (Berlin, Germany). [2-$^{14}$C]uridine (53 mCi/mmol), [2-$^{14}$C]cytidine (53.8 mCi/mmol), oligomycin, nitrobenzylthioinosine (NBTI), 8-cyclopentenyl-1,3-dipropylxanthine (DPCPX), xanthine oxidase (EC 1.1.3.22), alcohol dehydrogenase (EC 1.1.1.1) and lactic dehydrogenase (EC 1.1.1.27) were purchased from Sigma (St Louis, MO, USA). Glutamate–pyruvate transaminase (EC 2.6.1.2) was from ICN Biomedicals (Irvine, CA, USA). Human astrocytoma cells (ADF) were a kind gift of Dr. W. Malorni, Istituto Superiore di Sanità (Roma, Italy). All other chemicals were of reagent grade.

### 2.2. Treatment of cells

Cells were routinely grown in RPMI medium with 10% FBS and 2% antibiotics at 37 °C in a humidified 5% $CO_2$/95% air atmosphere. The experiments were performed with cells (approximately 800,000) kept in 35-mm plates for 10 min in 0.5 mL of serum-free DMEM medium in the absence or presence of 0.1 μM oligomycin. When NBTI or DPCPX were present in the incubation mixture, their final concentration was 10 and 100 μM, respectively. Cells were washed twice with 2 mL cold physiological solution. Some plates were treated as described in Section 2.3 for the extraction of nucleotides whereas 0.5 mL of DMEM medium either alone or containing the following metabolites were added to other plates: glucose (from 0.5 to 10 mM), inosine (from 0.25 to 10 mM), adenosine (from 0.25 to 10 mM), uridine (from 1 to 10 mM), guanosine, cytidine, hypoxantine, ribose, and deoxyinosine at a final concentration of 10 mM. When NBTI or DPCPX was present their final concentration was 10 and 100 μM, respectively. After 30 min incubation, medium was collected and kept at −20 °C for the determination of lactate (see Section 2.4); cells were then treated as described below for the extraction of nucleotides.

### 2.3. Extraction and measurement of intracellular adenine nucleotides

Cells were subjected to the treatment described in the previous section. After treatment, the incubation medium was removed, and cells were washed twice with 2 mL of cold physiological solution. For the extraction of nucleotides, 0.1 mL of ice-cold 0.6 M perchloric acid were added to each plate and kept for 15 min; cells were harvested with a cell scraper, and the suspension was centrifuged in an Eppendorf Microfuge. The supernatant was neutralized with 15 μL of 3.5 M $K_2CO_3$. After centrifugation in a Microfuge, the supernatant was analyzed by HPLC according to Micheli et al. (1999).

### 2.4. Determination of lactate

The culture medium, collected as described in Section 2.2, was subjected to the spectrophotometric assay for the determination of lactate (Martì et al., 1997). The reaction mixture contained in a final volume of 0.5 mL, 200 mM glycylglycine–40 mM glutamate (pH 10), 4 mM $NAD^+$, 27 U of lactic dehydrogenase (5248 U/mL), 7 U of glutamate–pyruvate transaminase (1773 U/mL), 90 μL of culture medium, and 20 mM Tris–HCl pH 7.4.

### 2.5. Enzyme assays

Extracts for the determination of enzyme activities, obtained from astrocytoma cells both before (control) and after treatment for 10 min with 0.1 μM oligomycin (ischemic cells), were prepared essentially as previously described (Giorgelli et al., 2000). All enzyme assays were performed at 37 °C. For each sample, at least 2 determinations with different amounts of cell extract were carried out. Linearity with time and protein concentration was observed for all enzyme assays. A spectrophotometric method was applied for the assay of purine nucleoside phosphorylase (EC 2.4.2.1), adenosine deaminase (EC 3.5.4.4) and deoxyriboaldolase (EC 4.1.2.4). For purine nucleoside phosphorylase, the reaction mixture (0.5 mL) contained 100 mM phosphate buffer, pH 7.4, 0.3 mM inosine, 0.02 U of xanthine oxidase (1.8 U/mL), and 30–70 μg of cell extract. The change in optical density at 293 nm was followed against a reference cuvette in which inosine was substituted by water. For adenosine deaminase, the reaction mixture (0.5 mL) contained 100 mM Tris–HCl, pH 7.4, 0.08 mM adenosine, and 30–70 μg of cell extract. The change in optical density at 265 nm was followed against a cuvette in which adenosine was substituted by water. For the assay of deoxyriboaldolase the reaction mixture (0.5 mL) contained 70 mM Tris–HCl, pH 7.4, 1.5 mM dithiothreitol, 0.22 mM NADH, 11 U of alcohol dehydrogenase (1098 U/mL), 2 mM deoxyRib-5-P, and from 0.3 to 0.6 mg of cell extract. The assay was performed spectrophotometrically, by monitoring the change in optical density at 340 nm, against a reference cuvette in which deoxyRib-5-P was substituted by water. A radioenzymatic assay was applied to measure the activity of uridine phosphorylase (EC 2.4.2.3) and cytidine deaminase (EC 3.5.4.5). For the assay of uridine phosphorylase, the reaction mixture (50 μL) contained 5 mM phosphate buffer, pH 7.4, 1 mM [2-$^{14}$C]uridine (3720 DPM/nmol), from 75 to 150 μg of cell extract, and 30 mM Tris–HCl, pH 7.4. For cytidine deaminase, the reaction mixture (50 μL) contained 60 mM Tris–HCl, pH 7.4, 3 mM dithiothreitol, 0.25 mM [2-$^{14}$C]cytidine (6072 DPM/nmol), 5 mM $ZnSO_4$, and from 10 to 20 μg of cell extract. At 0, 10, 20, and 30 min incubation, 10 μL aliquots were withdrawn and spotted on a PEI-cellulose plate which was developed overnight with *n*-propanol/TCA (100% saturation)/$NH_3$/$H_2O$ (75/0.7/5/20, v/v). The spots corresponding to uracil (for uridine phosphorylase) and uridine (for cytidine deaminase) were cut and the radioactivity counted, after addition of 8 mL of liquid scintillation counter.

One unit of enzyme activity is the amount of enzyme which catalyzes the formation of 1 μmol of product/min under the adopted assay conditions.