Pyruvate protects cerebellar granular cells from 6-hydroxydopamine-induced cytotoxicity by activating the Akt signaling pathway and increasing glutathione peroxidase expression


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Parkinson disease (PD) is the second-most common age-related neurodegenerative disease and is characterized by the selective destruction of dopaminergic neurons. Increasing evidence indicates that oxidative stress plays a crucial role in the pathogenesis of idiopathic PD. Anti-oxidant agents including catalase, manganese porphyrin and pyruvate confer cytoprotection to different cell cultures when challenged with 6-hydroxydopamine (6-OHDA). Herein we used rat cerebellar granular cell cultures to ascertain the plausible cellular pathways involved in pyruvate-induced cytoprotection against 0.1 mM 6-OHDA. Pyruvate provided cytoprotection in a concentration-dependent manner (2–10 mM). Consistent with its well-established anti-oxidant capacity, pyruvate (10 mM) prevented 6-OHDA-induced lipid peroxidation by blocking the rise in intracellular peroxides and maintaining the intracellular reduced glutathione (GSH) levels. Further experiments revealed that pyruvate increased Akt, but not extracellular signal-regulated kinase phosphorylation. Moreover, phosphatidylinositol 3-kinase (PI3K) inhibitors attenuated pyruvate-induced cytoprotection indicating that PI3K-mediated Akt activation is necessary for pyruvate to induce cytoprotection. On the other hand, pyruvate also up-regulated glutathione peroxidase mRNA levels, but not those of the anti-oxidant enzymes superoxide dismutase-1 and -2, catalase or the anti-oxidative oncoproteins Bcl-2 or Bcl-xL. In summary, our results strongly suggest that pyruvate, besides the anti-oxidant properties related to its structure, exerts cytoprotective actions by activating different anti-apoptotic routes that include gene regulation and Akt pathway activation.

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Parkinson’s disease (PD) is a neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (Noelker et al., 2005), with the resulting loss of nerve terminals accompanied by a dopamine (DA) deficiency in the striatum (Hornykiewicz, 1966). 6-Hydroxydopamine (6-OHDA), an endogenously generated metabolite of dopamine oxidation (Fornstedt et al., 1986), is extensively utilized in experimental models of PD because it causes cell death in different cell types, including human neuroblastoma SK-N-SH (Shimizu et al., 2002), SH-SY5Y (Von Coelln et al., 2001; Jordan et al., 2004) and pheochromocytoma PC12 cells (Nie et al., 2002), besides of being a specific neurotoxin of dopaminergic neurons in vivo (Noelker et al., 2005; Blum et al., 2001). Indeed, if reactive oxygen species (ROS) are not properly detoxified, they can damage cell lipids, proteins or DNA, impairing normal cell function. Uncoupling of mitochondrial oxidative phosphorylation resulting in energy deprivation and by-products resulting from 6-OHDA auto-oxidation such as quinones and hydrogen peroxide (H₂O₂) are involved in these cytotoxic processes (Galindo et al., 2003; Thakar and Hassan, 1988). Interestingly, both mitochondrial dysfunction and oxidative stress appear to play a central role in the pathogenesis of PD (Beal, 2003; Blum et al., 2001; Mazzio et al., 2004). In fact, anti-oxidant agents, such as catalase, vitamin E, N-acetyl cysteine, ascorbic acid and pyruvate are known to elicit cytoprotection against 6-OHDA in experimental models (Jordan et al., 2004; Galindo et al., 2003; Lai and Yu, 1997).

Alpha-ketoacid structures like pyruvate have been shown to confer cytoprotection against different noxious stimuli including transient forebrain ischemia, hemorrhagic shock, β-amyloid, exogenous H₂O₂ and menadione (2-methyl-1,4-naphthoquinone)
transduction (Song et al., 2005; D’Emelo et al., 2003). The activation of this pathway is known to protect lesion (Perovic et al., 2005). We have found that pyruvate blocks architecture were detectable following unilateral striatal 6-OHDA

granular cell cultures from rat cerebellum, a region where cytoprotective effects of pyruvate on 6-OHDA-induced toxicity in 1993).

role in apoptosis inhibition (Yoon and Seger, 2006; Pages et al., 2004; Love et al., 2002; Lai et al., 1997). The PI3K pathway has required (Chung et al., 2004). In this manner, pyruvate seems to be the critical component to explain the trophic activity of glia-conditioned media for central nervous system neurons (Selak et al., 1985). On the other hand, pyruvate might act as a ROS scavenger due to its anti-oxidant capacity. For example, pyruvate is able to undergo nonenzymatic decarboxylation in the presence of H2O2 and thus prevent •OH formation by the so-called Fenton reaction (Alvarez et al., 2003).

Anti-oxidant systems and intracellular signaling pathways, including phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK), have been shown to be important in the regulation of cell survival. Superoxide dismutase, catalase, glutathione peroxidase and non-enzymatic molecules such as vitamin E, glutathione and alpha-ketoacid compounds are anti-oxidant systems that neutralize and eliminate ROS and confer cytoprotection versus 6-OHDA-induced cell death (Jordan et al., 2004; Love et al., 2002; Lai et al., 1997). The PI3K pathway has emerged as one of the critical factors in anti-apoptotical signal transduction (Song et al., 2005; D’Mello et al., 1997; Franke et al., 2003). The activation of this pathway is known to protect cells from several apoptotical stimuli (Kennedy et al., 1997). The ERK (p42/p44 mitogen-activated protein kinase; MAPK) cascade is a central pathway that transmits signals from many extracellular agents to regulate cellular processes. It is thought to play a pivotal role in the integration and transmission of transmembrane signals required for growth and differentiation. ERK activation is essential for cell growth and plays a crucial role in apoptosis inhibition (Yoon and Seger, 2006; Pages et al., 1993).

Here we analyzed the possible mechanisms underlying the cytoprotective effects of pyruvate on 6-OHDA-induced toxicity in granular cell cultures from rat cerebellum, a region where structural reorganization, alterations in neuronal activity and architecture were detectable following unilateral striatal 6-OHDA lesion (Perovic et al., 2005). We have found that pyruvate blocks 6-OHDA-induced toxicity in a concentration-dependent manner without affecting the auto-oxidation process of 6-OHDA. Further experiments revealed that pyruvate prevented the 6-OHDA-induced, intracellular peroxide rise and blocked the depletion of GSH levels. The activation of the Akt pathway and the increase in the expression of glutathione peroxidase also participated in the cytoprotective mechanism activated by this alpha-ketoacid. Finally, the metabolic product, malate, did not protect cell cultures against 6-OHDA toxicity and failed to activate the abovementioned cytoprotective pathways.

Materials and methods

Cell culture

Primary cultures of cerebellar granular neurons were obtained from dissociated cerebella of 7- to 8-day-old rats (Fernandez-Gomez et al., 2005a). Dissection and dissociation were carried out in Basal Medium Eagle (BME; Life Technology). Tissues were incubated with trypsin for 20 min at 37°C and dissociated by trituration in a medium containing DNase and trypsin. Cells were plated on 96 plastic well dishes or on 60-mm plastic Petri dishes pre-coated with poly-c-lysine (10 g/ml) at a concentration of 8×10^6 cells/ml in BME containing 25 mM KCl, 10% de-complemented fetal calf serum (FCS; Life Technology), glutamine and antibiotics. Cytosine-β-D-arabinofuranoside (Ara-C) (10 μM) was added at 3 days in vitro (DIV) to prevent the growth of non-neuronal cells. All experiments were carried out after 7 days in culture.

Intracellular generation of reactive oxygen species

We used the oxidation-sensitive fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to measure the production of reactive oxygen species (ROS), mainly hydrogen peroxide and hydroxyl radicals. DCFH-DA is deacetylated by esterases to dichlorofluorescein (DCFH). This non-fluorescent product is then converted by reactive species into DCF, which can easily be visualized by strong fluorescence at 530 nm when excited at 485 nm. Cells seeded in 96-well culture plates were incubated with DCFH-DA (10 μg/ml) for 5 min, treated with or without 10 mM pyruvate before adding either 0.1 mM 6-OHDA or vehicle and 4 h later fluorescence intensity was measured in a Spectra Max Gemini XS (Molecular Devices). The average relative percent ROS production from four wells of at least three separate cultures was determined. Results are expressed as mean±SD values, and significance was determined by Student’s t test. Statistical significance was considered at the p<0.05 level.

Measurement of glutathione levels

Levels of glutathione were determined by using monochlorobimane (mBCl) fluorescence. Glutathione (GSH) is specifically conjugated with mBCl to form a fluorescent bimane-GSH adduct in a reaction catalyzed by glutathione S-transferases (Shrieve et al., 1988). The concentration of the bimane-GSH adduct increases during the initial 10 to 12 min period of this reaction with first order kinetics, before leveling off (Young et al., 1990). Culture medium was removed and cells were washed twice with 0.2 ml Krebs and fluorescence was later measured in a Spectra Max Gemini XS (Molecular Devices). The average relative percent ROS production from four wells of at least three separate cultures was determined. Results are expressed as mean±SD values, and significance was determined by Student’s t test. Statistical significance was considered at the p<0.05 level.

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<td>Sequences of the oligonucleotide primer pairs used for real-time PCR</td>
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measured at an excitation wavelength of 340 nm and emission wavelength of 460 nm in a Spectra Max Gemini XS (Molecular Devices). The average relative percent reduced GSH levels from at least three separate cultures was determined. Results are expressed as mean ± SD values, and significance was determined by Student’s t test. Statistical significance was considered at the $p < 0.05$ level.

**Lipid peroxidation**

Lipid peroxidation was measured by determining malondialdehyde (MDA) levels. Each sample ($8 \times 10^6$ cells) was collected in 100 μL of ice-cold 20 mM BTris-HCl buffer, pH 7.4, and sonicated. Amounts of MDA were determined in the cellular extracts using a Lipid Peroxidation Assay Kit from Calbiochem.

![Fig. 1](image-url)

**Fig. 1.** Protective effects of pyruvate against 6-OHDA-induced cell death. (A–C) Phase contrast. Control cultures (A) or cells challenged during 24 h with 0.1 mM 6-OHDA pre-treated (C) or not (B) for 1 h with 10 mM pyruvate. (D) Cell viability was assayed 24 h after 6-OHDA addition by using the MTT test. Pyruvate (0.5–10 mM) was added 1 h before 6-OHDA (0.1 mM) and maintained until the end of the experiment. (E) Pyruvate (10 mM) additions were performed 1 h before (−1), at the time (0) or 0.5, 1, 3 or 6 h after 6-OHDA treatment. Cell viability was measured 24 h after 6-OHDA addition. Data represent the mean ± SD of three independent experiments. ***$p < 0.001$ vs. control conditions (0 pyruvate), $t$ test significantly different from 6-OHDA alone.
Based on the condensation reaction of the chromogene 1-methyl-2-phenylindole with MDA. The stable chromophores were determined using a VERSAmax Lunable microplate reader (Molecular Devices) with absorbance at 586 nm. Results are expressed as micromole MDA per mg protein.

**Formation of quinoidal products by 6-OHDA auto-oxidation**

The formation of quinoidal products by 6-OHDA auto-oxidation was determined using a cell-free system. Briefly, 6-OHDA (0.1 mM) was incubated at room temperature (22–25°C)
for 15 min in culture medium with or without pyruvate, and the formation of quinoidal products of 6-OHDA auto-oxidation was monitored by absorbance at 490 nm as previously described (Tiffany-Castiglioni et al., 1982) using a VERSAmax Lunable microplate reader (Molecular Devices).

**Fluorescence measurements of H$_2$O$_2$ production from 6-OHDA auto-oxidation**

Hydrogen peroxide generation was measured fluorimetrically as previously described (Votyakova and Reynolds, 2001). Briefly, 6-OHDA was added, at room temperature, to a standard incubation buffer that contained: 125 mM KCl, 2 mM K$_2$HPO$_4$, 5 mM MgCl$_2$, 10 mM HEPES (pH adjusted to pH 7.0 with KOH), 10 μM EGTA and scopoletin (2 μM) in the presence of 1 U/ml horseradish peroxidase. Scopoletin fluorescence was monitored at excitation/emission wavelengths of 365 nm/460 nm in a Spectra Max Gemini XS (Molecular Devices).

**Cell viability**

The cells were exposed to 6-OHDA (0.1 mM) and cell viability was measured 24 h later by the ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the blue formazan product. After removal of culture medium, cells were incubated with 1 mg/ml MTT in regular BME for 2 h at 37°C. BME was then aspirated and the formazan dissolved in 200 μL DMSO. Absorbance at 570 nm was measured in a VERSAmax Lunable microplate reader (Molecular Devices), and the absorbance of control conditions was used as 100% cell culture viability.

**Western blot analysis**

For immunoblotting of phosphorylated forms of Akt or ERK in total cell lysates, 30 μg of total protein was resolved in SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride Immobilon-P transfer membrane filters (Millipore, Hucoa-Erlos, Barcelona) using an Amersham Biosciences semidyery Trans-Blot (Amersham Biosciences, Barcelona). Membranes were blotted with an anti-phospho-Akt-specific antibody: anti-P-Ser473, or an anti-phospho-p44/42 MAPK antibody: anti-P-ERK (New England Biolabs, Servicios Hospitalarios, Barcelona) following the instructions of the provider. To control the contents of the specific protein in each lane, membranes were re-probed with a monoclonal anti-α-tubulin antibody (Sigma) or with anti-Akt antibody (C-20) (Santa Cruz Biotechnology, Quimigranel, Barcelona). Blots were developed using the ECL Advance Western Blotting Detection Kit chemiluminescent substrate (Amersham Biosciences). Band intensity was estimated densitometrically on a GS-800 calibrated densitometer (Bio-Rad Quantity One).

**RNA isolation**

Total RNA was obtained with Trizol® Reagent (Invitrogen) following the manufacturer’s indications. Eight million cells were used per milliliter of Trizol. The isolated RNA was then subsequently treated with DNase (Promega) to remove any genomic DNA contamination. The integrity of RNA was always checked by running an aliquot in an agarose gel.

**Real-time-PCR**

cDNA was synthesized from 10 μg total RNA in 100 μl volume containing 1× RT Buffer (Applied BioSystems), 500 μM dNTPs, 2.5 μM random hexamers and 1.25 U/μl MultiScribe Reverse Transcriptase. Reaction was performed in a thermal Cycler at 48°C for 30 min. Samples were then kept at −20°C until their utilization. PCR amplification was performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems), using the SYBR Green PCR Master Mix (Applied Biosystems). One microliter cDNA was used for each reaction. PCR amplifications were always performed in triplicate wells, using 40 two-temperature cycles (15 s at 94°C and 1 min at 60°C). Once demonstrated that the efficiency for the different primer combinations was similar, the quantification was performed by the comparative cycle threshold method (Livak and Schmittgen, 2001), using GAPDH as internal control. Primers for all target sequences (Table 1) were designed using the computer Primer

![Fig. 3.](image-url)
**Statistical analysis**

The data are expressed as the mean±SD and were analyzed using the two-tailed unpaired Student’s t test. Treatment differences were considered statistically significant at \( p < 0.05 \). Data analyses were performed using the Statistical Program for the Social Sciences (SPSS for Windows, 11.0).

**Results**

**Pyruvate protects against 6-OHDA-induced cell death**

The results shown here confirm and expand the observations that 6-OHDA induces cell death in different models (Galindo et al., 2003; Mazzio et al., 2004; Blum et al., 2001). As shown in Fig. 1, 6-OHDA induced a marked reduction in cell viability of 7 DIV cerebellar granular cultures. To analyze the effects of pyruvate on 6-OHDA-induced cytotoxicity, cells were pre-treated with 0.5–10 mM pyruvate for 1 h before the addition of 0.1 mM 6-OHDA. By 24 h after 6-OHDA addition, pyruvate was observed to afford cytoprotection to the cell cultures in a concentration-dependent manner at concentrations higher than 2 mM (Fig. 1D). Morphological analysis under light microscopy showed that 6-OHDA (0.1 mM) induced severe cell damage as evidenced by cell size and loss of the bright refringent halo around cell bodies. Pre-treatment with pyruvate (10 mM) for 1 h resulted in the blockades of morphological changes caused by 6-OHDA (Figs. 1A–C).

In order to have a better idea about how long we can postpone the supplementation of pyruvate to afford cytoprotection, we performed an additional experiment in which 10 mM pyruvate was added to the cerebellar granule cells at different times before and after 6-OHDA treatment. As shown in Fig. 1E, pyruvate-
conferred protection was evident only when pyruvate was added to the cells either before or at the same time as 6-OHDA, but delayed treatment with pyruvate was no longer able to reverse the cytotoxicity. Moreover, if pyruvate was removed from the culture medium just before the 6-OHDA addition, it lacked its cytoprotective effects.

Pyruvate exerts anti-oxidant actions in cerebellar granular cell cultures

Previous studies have shown that pyruvate might afford cytoprotection through a mechanism that implies a ROS scavenger capacity (Alvarez et al., 2003; Salahudeen et al., 1991). Additionally, it has also been proposed that 6-OHDA requires oxidation in order to induce cytotoxicity (Clement et al., 2002). The 6-OHDA auto-oxidation yields ROS, mainly H$_2$O$_2$, and quinones that might react with different proteins to produce quinoproteins (Tiffany-Castiglioni et al., 1982).

To determine whether pyruvate modulates the endogenous production of H$_2$O$_2$-derived oxidants in cerebellar granular cells, we measured the oxidation of DCFH, a non-fluorescent probe, to a fluorescent dichlorofluorescein (DCF). Consistent with previous reports (Jordan et al., 2004), 6-OHDA induced intracellular peroxide-like radical production in cerebellar granular cells. As shown in Fig. 2A, 4 h after the addition of 0.1 mM 6-OHDA, we observed a 1.7-fold increase in the intracellular peroxide levels. To analyze the effect of pyruvate on this event, we pre-treated cellular cultures for 1 h with pyruvate (0.5–10 mM) before 6-OHDA addition.
addition. Under these conditions pyruvate prevented intracellular 6-OHDA-induced peroxide increase in a concentration-dependent manner (Fig. 2A). It is well established that oxidative stress in various cells usually leads to accumulation of potent, cytotoxic lipid peroxides such as MDA (Estebauer et al., 1991). Therefore, we studied the effects of pyruvate on the accumulation of MDA as an indicator of lipid peroxidation. By 24 h, the levels of MDA in cells challenged with 6-OHDA was higher than in control or cultures pre-treated with 10 mM pyruvate (Fig. 2B).

When cerebellar granular cells were treated with 6-OHDA, the culture medium turned its colour to orange-brown as result of its auto-oxidation. To ascertain if the pyruvate anti-oxidant effectiveness is mediated by preventing 6-OHDA auto-oxidation, we explored quinone formation levels in the presence of pyruvate. To do so, we measured the formation of quinones spectrophotometrically at 490 nm in fresh culture medium. The presence of 10 mM pyruvate failed to prevent the formation of quinones from 0.1 mM 6-OHDA (Figs. 2C and D). We found no difference in A490 in cell culture medium after 24 h of 6-OHDA addition. Furthermore, we determined whether pyruvate modifies the amount of peroxides generated by 0.1 mM 6-OHDA auto-oxidation by using the peroxide-sensitive dye scopoletin. As shown in Fig. 2E and F, pyruvate did not modify peroxide formation from 6-OHDA auto-oxidation at any of the concentrations tested. After consideration of these data, we have concluded that the anti-oxidant effect of pyruvate is not mediated by the inhibition of 6-OHDA auto-oxidation.

**Pyruvate prevents 6-OHDA-induced glutathione depletion**

The tripeptide GSH plays an important role in detoxifying oxygen radicals and its relevance in 6-OHDA-induced damage has been reported (Jordan et al., 2004; Sian et al., 1994). This finding prompted us to measure cellular glutathione levels after pyruvate treatment. Intracellular levels of reduced GSH were determined by using monochlorobimane (Fernandez-Gomez et al., 2005b). In resting conditions, treatment with pyruvate (0.5–10 mM) for 24 h did not modify intracellular GSH levels. However, cerebellar granular cells challenged with 6-OHDA (0.1 mM) for 24 h showed a severe depletion of the intracellular GSH, which represented around 30% of untreated control levels (Fig. 3A). Under these stress conditions, pyruvate pre-treatment (10 mM; 1 h) widely prevented 6-OHDA-induced intracellular GSH level depletion (Fig. 3A). To ascertain the relevance of GSH levels in pyruvate-affected cytoprotection, we pre-treated the cultures with i-buthionine (S, R)–sulfoximine (BSO), a specific gamma-glutamlycysteine synthetase inhibitor, to deplete intracellular GSH levels. Consistent with an anti-apoptotic role for GSH in this model, pyruvate-affected cytoprotection from 6-OHDA was abolished in cell cultures pre-treated for 12 h with 100 μM BSO (Fig. 3B).

**Effect of pyruvate on the AKT pathway**

Akt and ERK participate in intracellular pathways related to the survival effects of different neuronal stimuli, such as neurotrophic factor treatment or high potassium stimulated cells (Signore et al., 2006; D’Mello et al., 1997 Kennedy et al., 1997). In the first set of experiments, we were interested in finding out whether 0.1 mM 6-OHDA modulated p-AKT levels. As shown in Fig. 4A, 0.1 mM 6-OHDA failed to induce p-AKT levels. To analyze the involvement of these kinases in pyruvate-induced cell protection, we analyzed Akt and ERK phosphorylation levels in cerebellar granular cell supplemented with pyruvate. To this end, cell cultures were serum-deprived overnight to reduce basal p-AKT levels, and challenged with 10 mM pyruvate. Cells were collected at different times: 10 min, 30 min, 1 h or 6 h, lysed and total cellular lysates were analyzed by immunoblotting using anti-phospho-Ser473/Akt antibody (P-Akt) or an anti-phospho-ERK antibody (P-ERK). As shown in Fig. 4B, an increase of in Akt phosphorylation in 30 min pyruvate-treated cells was observed when compared with non-stimulated cells. The same increase in Akt phosphorylation was present in 1-h and 6-h treated cells, indicating that the presence of pyruvate in the culture medium induced a maintained Akt phosphorylation. However, no changes in ERK phosphorylation level were observed in the same cell extracts when compared with non-stimulated cultures (Fig. 4B).

Akt is a well-known downstream PI 3-kinase effector (Alessi et al., 1996). In the next set of experiments, we used chemical inhibitors LY294002 and wortmannin to study the relevance of PI3-kinase-Akt signaling pathway in pyruvate-induced cytoprotective effects. The efficiency of blocking pyruvate-induced Akt phosphorylation by these two drugs at the concentrations used was corroborated by measuring P-Akt in pyruvate challenged cells pre-treated with inhibitors for 30 min (Fig. 5A). The pre-treatment for 30 min with LY294002 (5 μM) or wortmannin (1 μM) blocked around 30% of the protective effects afforded by 10 mM pyruvate (Fig. 5B). These results together suggest that pyruvate induces PI 3-kinase/Akt pathway activation, which is partially involved in the survival promoting effect of 6-OHDA-treated cells.

**Effects of pyruvate on anti-apoptotic protein levels**

Anti-oxidant enzymes including catalase, superoxide dismutase (SOD-1 and -2), and glutathione peroxidase (Gpx) belong to the defense machinery that the cells have to scavenge reactive oxygen species and avoid oxidative stress. They have been shown to be increased after neuroprotective drug treatments and their overexpression might lead to cytoprotection against several insults.
To ascertain whether pyruvate might modulate the expression of some of these enzymes, we utilized quantitative real time PCR to measure the level of expression of those enzymes in cerebellar granular cells treated with 10 mM pyruvate for 1 h, time by which pyruvate treatment prevents 6-OHDA-induced cell death. The results, shown in Fig. 6, revealed that pyruvate induces a strong up-regulation of GPX1, whereas no modifications were observed in catalase or SOD family member levels.

In a previous study we observed that the overexpression of Bcl-xL prevented SH-SY5Y cells from 6-OHDA toxicity (Jordan et al., 2004). Thus, we tested whether pyruvate cytoprotective effects were mediated throughout changes in the expression levels of Bcl-2 or Bcl-xL. As shown in Fig. 6, by 1 h after 10 mM pyruvate addition, the expression levels of either anti-apoptotic oncogenes were not modified in cerebellar granular cells.

**Malate did not prevent 6-OHDA-induced cell death**

Next we examined the neuroprotective capacity of the energy substrate malate to ascertain the relevance of improving mitochondrial function. As shown in Fig. 7A, malate, at the doses tested (0.5–10 mM), did not afford any protection to cerebellar granular cell cultures challenged with 6-OHDA (0.1 mM). Consistent with this lack of cytoprotective effect, malate failed to block the effect of 6-OHDA on ROS increases (Fig. 7B) and GSH decreases (data not shown). Finally, malate did not modulate the phosphorylation of Akt in cerebellar granular cell cultures (Fig. 7C).

**Fig. 7. Malate does not afford cytoprotection against 6-OHDA.** (A) Cell viability was assayed 24 h after 6-OHDA addition by using the MTT test. Malate (0.5–10 mM) was added 1 h before 6-OHDA (0.1 mM) and was maintained until the end of the experiment. Data represent the mean±SD of 3 independent experiments. (B) Malate failed to block intracellular ROS-production. Cerebellar granular cells were treated with 6-OHDA once pre-treated for 1 h with malate at different concentrations. Four hours later green fluorescence characteristic of DCF was measured in a Spectra Max Gemini XS microplate reader. Data represent the mean±SD of 3 independent experiments. (C) Malate did not induce Akt, or ERK, phosphorylation. After malate (10 mM) treatment (10 min, 30 min, 1 h, 3 h and 6 h) cells were collected and protein extracts were analyzed by western blot using anti-P-Akt and anti-P-ERK-specific antibodies. Protein loading amount was checked by re-probing the filters with the anti-Akt (C-20) and anti-tubulin antibodies. Histogram shows the relative values from the densitometric analysis. Protein levels were expressed as fold of induction over the values obtained in untreated cells. Treatments with the same lowercase letters are not significantly different within each column (p>0.05).
Discussion

The results presented in this study are consistent with the hypothesis that pyruvate affords cytoprotection by an anti-oxidant mechanism. Although this is a well-known fact, we have demonstrated for the first time that this alpha-ketoacid confers cytosecution to cerebellar granular cell cultures challenged with 6-OHDA through a mechanism that involves the up-regulation of glutathione peroxidase expression and the activation of the PI3K/Akt signaling pathway.

Consistent with the cytoprotective capacity of pyruvate shown in several experimental models where reactive oxygen species are involved, pyruvate, in a concentration-dependent manner, protected rat cerebellar granular cell cultures and prevented the rise in lipid peroxidation induced by 24 h exposure to 6-OHDA (0.1 mM). According to our data, this anti-oxidant action of pyruvate is related to its ability to block the increase of intracellular peroxide levels induced by 6-OHDA rather than by avoiding 6-OHDA auto-oxidation. Thus, pyruvate at the highest dose tested, 10 mM, failed to scavenge the peroxides or to prevent the quinone formation resulting from 6-OHDA auto-oxidation in a cell-free system. Although pyruvate did not increase GSH levels in resting conditions, it maintained the reduced glutathione levels after 6-OHDA treatment, revealing a possible mechanism for the prevention of peroxide formation. Consistent with a key role for this tripeptide in our model, pyruvate-induced protection against 6-OHDA toxicity disappeared when we depleted intracellular GSH levels by using the GSH synthesis inhibitor 1-buthionine sulfoximine. GSH is an efficient anti-oxidant and a free radical scavenger whose levels have been found to be altered in the basal ganglia of PD patients (Sian et al., 1994). How pyruvate avoids GSH depletion after 6-OHDA remains unknown. Although not addressed in this study, it has been demonstrated that pyruvate induces ATP and NAD(P)H formation (De Andrade et al., 2004). It is therefore conceivable that pyruvate in cerebellar granular cells increases NAD(P)H levels and subsequently GSH levels.

The fact that pyruvate lost its cytoprotective effect when added after the 6-OHDA insult together with the inability of malate to afford cytoprotection to cerebellar granular cells prompted us to investigate the requirement of the activation of an intracellular signaling pathway. To achieve our goal, we used quantitative real-time PCR to measure the levels of expression of several anti-oxidant enzymes by using the constitutively active Akt, these authors demonstrated the importance of Akt in preserving dopaminergic cells exposed to 6-OHDA. It is noteworthy that pre-treatment with LY294002, but not wortmannin, predisposes cell cultures to 6-OHDA-induced toxicity, suggesting that the PI3K-Akt pathway participates in the physiological anti-apoptotic cellular machinery. Although both inhibitors are considered to be specific PI3K inhibitors, it has been recently reported that LY294002 might also exert other effects despite inhibiting PI3K (Yamaguchi et al., 2006). We do not know why these two inhibitors present different effects. Further investigation is warranted to delineate the likely distinct mechanisms by which these drugs act.

In summary, our results appear to indicate that pyruvate-induced cytoprotection against 6-OHDA is mediated by an increase in the anti-oxidant capacity of cells due to the up-regulation of glutathione peroxidase expression rather than to its anti-oxidant capacity derived from its alpha-ketoacid structure or to its energetic substrate role. Furthermore, our data also show that the activation of the PI3K-Akt signaling pathway plays a significant role in the protection afforded by pyruvate against 6-OHDA toxicity.

Acknowledgments

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References


Blum, D., Torch, S., Lambeng, N., Nissou, M., Benabid, A.L., Sadoul, R.,


