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Pharmacological approaches to counter the toxicity of dopa

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Summary. Dopa and related catecholamines and their degradation products have been demonstrated to have neurotoxic potential in a number of cellular and *in vivo* experiments. Several mechanisms have been hypothesized to be involved including generation of prooxidant products that subsequently oxidize membrane lipids and exposed macromolecules. We have utilized a neuronal culture of cerebellar granule cells to study the toxicity of Dopa and the ability of various neuroprotective and antiparkinsonian compounds to offer protection therefrom. This model is apparently based on the ability of Dopa to non-enzymatically induce an oxidative injury to the neuronal cultures. Evidence for this arises from the equal neurotoxic potency of L- and D-Dopa in these cells and the ability of catalase, superoxide dismutase and glutathione to protect the neurons from this toxicity. Further, we found that the neuroprotective antioxidant, PNU-101033 is more effective and potent than vitamin E and deprenyl in this regard. Similarly the D_2/D_3 agonist, pramipexole is also capable of blocking Dopa toxicity in this model and this effect is independent of dopamine receptor affinity as both enantiomers are equally potent in this assay but disparate in receptor affinity. Also the protection by pramipexole is accompanied by the preservation of reduced glutathione. Thus, this activity seems to be a function of the oxidation potential of pramipexole and it's consequent antioxidant property. Potent antioxidants are effective blockers of Dopa toxicity. If the mechanisms involved in this toxicity have relevance to the progression of Parkinson's pathology in Dopa treated (or untreated) patients, these compounds have the potential to alter the course of the illness.

Keywords: Parkinson's disease – Neurotoxicity – Antioxidant – Pyrroloprimidine – Pramipexole – Vitamin E – Deprenyl

Introduction

Although Dopa has become the standard of treatment for Parkinson's disease, questions still remain as to the possibility of long term neurotoxic effects of chronic dosing with gram amounts of this amino acid. For example, emergent signs and symptoms occur reproducibly in patients treated for months and years with Dopa. Whether these are simply related to the pathologic state of

the patient or are representative of drug induced toxic changes is not known. However, in animal and cellular studies toxic effects of Dopa are readily demonstrable at Dopa concentrations similar to those achieved in patients treated with the drug. For example, Ling et al. (1996) have demonstrated that D- and L-Dopa are equally effective and potent in the ability to kill tyrosine hydroxylase positive and negative neurons in mesencephalic cultures. The death of neurons in these conditions may be in part due to the generation of quinone products of Dopa as well as the uptake and subsequent metabolism of Dopa in dopaminergic cells (Pardo et al., 1995). However, the Dopa induced apoptotic death of cultured neurons (eg PC12 cells) does not seem to require the uptake of Dopa but is reversed by antioxidants (Walkinshaw and Waters, 1995). Further evidence for oxidative toxicity mechanism of Dopa in these cells has been presented by Basma and coworkers (1995); catalase and superoxide dismutase individually and in combination partially and completely protect against Dopa respectively. However, no such protection was afforded by interfering with Dopa decarboxylation or subsequent deamination. Again these results suggest that the products of Dopa autoxidation may be the primary mediators of the neurotoxicity of this amino acid.

In the present experiments, we sought to validate and use cultures of cerebellar granule cells to study the phenomenon of Dopa toxicity as it relates to oxidative mechanisms and the ability of putative neuroprotective agents to protect against this toxicity.

Materials and methods

Primary cultures of cerebellar granular cells

Primary cultures of cerebellar granular cells were prepared from 8-day-old Sprague Dawley rats (Charles River, Portage, MI) as described previously (Novelli et al., 1988). Neurons were grown on 6-well, 35 mm culture dishes (Nunc, Denmark) for 8–9 days at 1×10^6 cells/ml, 2 ml/well. Glial cell proliferation was prevented by adding cytosine-arabinofuranoside-monophosphate (Sigma, St. Louis, MO), 18–20 hrs after plating at a final concentration of 10 μM. Cultures generated by this method have been characterized and contain more than 90% neurons (Nicolletti et al., 1986).

Cell toxicity models

Eight day-old cultures of cells were washed 2 × with serum-free medium leaving a final volume of 2 ml. Deprenyl (RBI, Natick, MA) Pramipexole, catalase (Calbiochem, La Jolla, CA), superoxide dismutase (Calbiochem, La Jolla, CA) or reduced glutathione (Sigma, St. Louis, MO) solubilized in serum-free BME medium, or serum-free medium vehicle was added 1:100 to the cells for 5 min. Vitamin E (Aldrich, Milwaukee, WI) or PNU-101033E emulsions or corresponding blank lipid emulsions (without drug) were applied to cells for 1 hr followed by a 3 × wash with the final 2 ml wash remaining in the wells. The emulsions were prepared as follows: Vitamin E was solubilized in absolute ethanol and PNU-101033E was solubilized in 0.1 N HCl. These stocks were diluted 1:10 into 20% Intralipid® fat emulsion (Pharmacia, Clayton, NC). These emulsion stock solutions were then diluted 1:20 into serum-free medium and this solution was applied directly to the cells. Following the drug applications, L-Dopa (Sigma, St. Louis, MO), solubilzed in 0.01 N HCl with sonication and diluted 1:10 in serum-free medium, or corresponding vehicle was added 1:10 to the cells. The L-Dopa treatment was allowed to

continue for 24–28 hrs. For viability measurements cells were then washed and pulsed with 0.5 μCi/ml of 2-amino[1-^{14}C]isobutyric acid (Amersham, Arlington Heights, IL) in Lockes buffer for 1 hr. Cells were solubilized, following a wash, with 0.5% Triton X-100. The solubilized cells were then counted for ^{14}C on a scintillation counter. Data was expressed as the mean of triplicates ± S.D. for each point and statistical analysis was done using ANOVA.

GSH/total protein measurements

Reduced cellular GSH levels were obtained by removing the cells with 0.1N HCl and assayed by HPLC with fluorometric detection of glutathione-orthophthaldialdehyde (Sigma, St. Louis, MO) adducts generated as previously described (Neuschwander-Tetri and Roll, 1989). Total protein was assayed using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL). The cellular GSH level data was divided by the corresponding total protein amount for each sample and was expressed as a ratio of GSH/total protein as the mean of triplicates ± S.D. Statistical analysis of the GSH/total protein fitted curves was done using linear regression with 95% confidence limits.

Results

The toxic effects of L- or D-Dopa on primary cultures of cerebellar granule cells are shown in Fig. 1. Cell viability was determined by measurements of the uptake of radio labeled amino isobutyric acid (AIB), a small neutral amino acid which is effectively taken up by cells functioning normally. All cell viability data reported herein were determined using this method. The toxicity curves of the two optical isomers are superimposable and result in a TC_{50} value of approximately 100 μM. This suggests that uptake of this amino acid is not necessary for the toxicity in this model. At the two highest concentrations

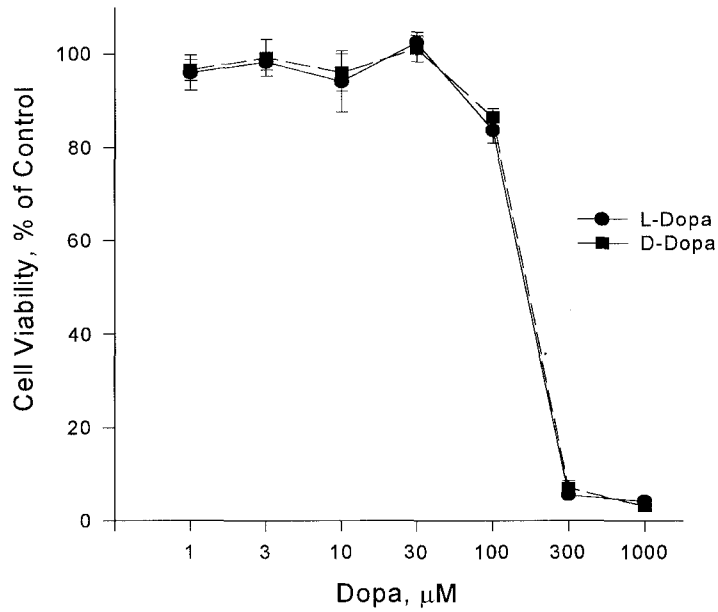

Fig. 1. Effects of L-Dopa and D-Dopa 24–28 hr treatments on cerbellar granule cells. Values are means of triplicates from a single experiment ± S.D.

(300 μM and 1 mM), following the 24–28 hr incubation, we observed a darkened color in the cell medium. Presumably this was due to the formation of semi-quinones and/or quinones following auto-oxidation of dopa. The effects of three different exogenously added reactive oxygen scavengers on L-Dopa toxicity are shown in Fig. 2. Treatment of the cells with 100 μM L-Dopa alone resulted in a 45–55% loss of cell viability. The addition of 30 u/ml catalase, 50 u/ml SOD or 100 μM GSH provided complete neuronal protection against this toxicity suggesting the involvement of hydrogen peroxide, superoxide and possible compromise of endogenous GSH. Figure 3 shows the concentration-dependent neuronal protective effects of the monoamine oxidase B inhibitor, R(-)-deprenyl, the D_2/D_3 dopamine agonist pramipexole (PPx) and the antioxidants vitamin E or PNU-101033E. Percent neuronal protection is defined as the percent increase of cell survival within a level of toxicity for a given experiment. In Fig. 3 this level of toxicity was a 54% loss of cell viability induced by the 24–28 hr L-Dopa treatment. The order of neuronal protective potency, from least to most potent, observed was: deprenyl, vitamin E, pramipexole and PNU-101033E. Deprenyl only achieved a maximum of 45% neuronal protection, vitamin E and PPx achieved maximum neuronal protection of 80% and PNU-101033E achieved complete (100%) neuronal protection. These highest levels of protection were observed at 100 μM in each case although PPx seemed to reach its optimal effect at 30 μM and consequently level off. Electrochemically, the oxidation potentials of the four drugs coincides with their neuronal protection potency with the slight exception of PPx (data not shown). Vitamin E and PNU-101033E have relatively low and

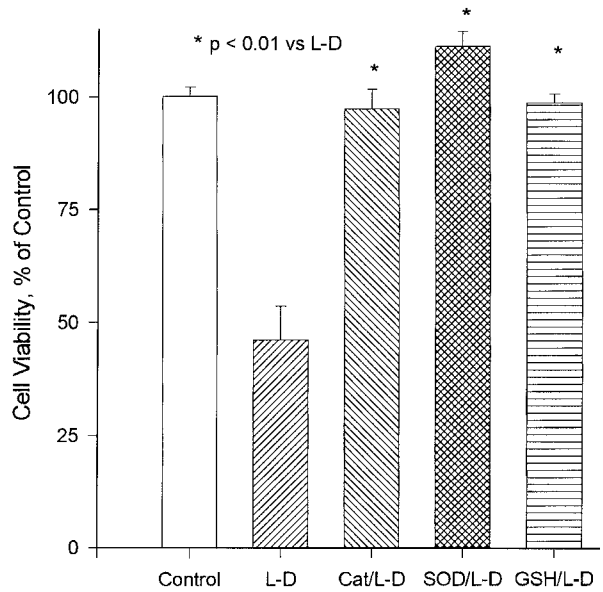

Fig. 2. Effects of 30 u/ml catalase (*Cat*), 50 u/ml superoxide dismutase (*SOD*) of 100 μM reduced glutathione (*GSH*) on 24–28 hr 100 μM L-Dopa (*L-D*) toxicity in cerebellar granule cells. Values are means of triplicates from two separate experiments ± S.D.

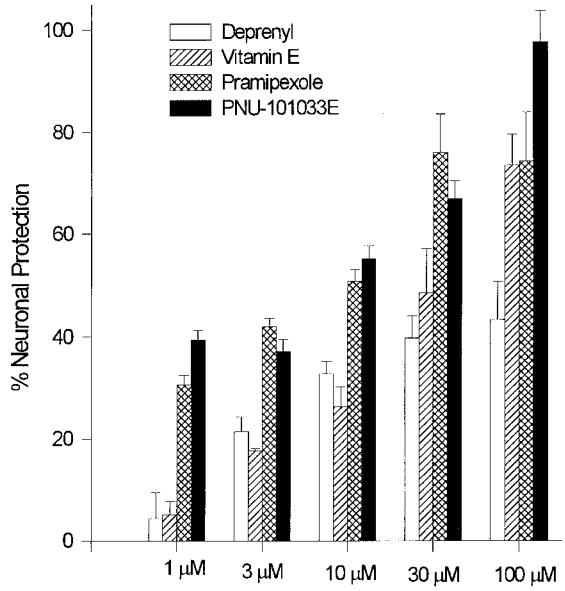

Fig. 3. Effects of R(-)-deprenyl, vitamin E, pramipexole or PNU-101033E on 24–28 hr 100 μM L-Dopa toxicity in cerebellar granule cells. Values are means of triplicates from a single experiment ± S.D.

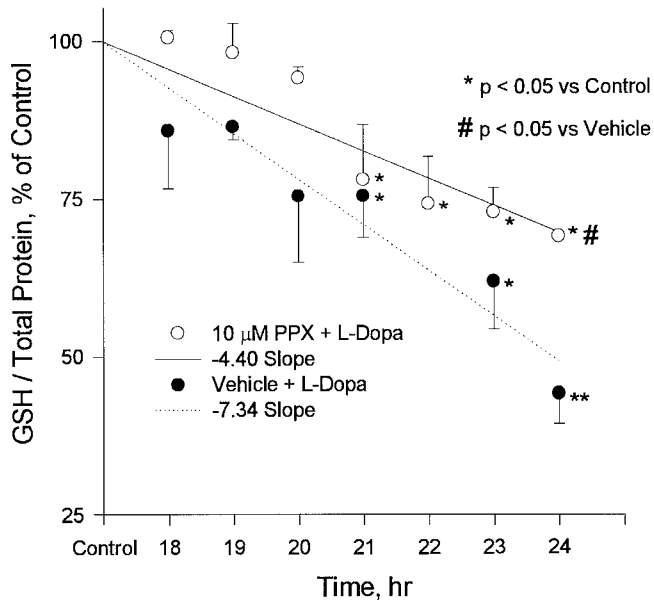

Fig. 4. Effects of pramipexole (PPx) on 18–24 hr 100 μM L-Dopa-mediated reduced glutathione (*GSH*) loss in cerebellar granule cells. Values are means of triplicates from a single experiment ± S.D.

similar oxidation potentials, deprenyl has a relatively high oxidation potential whereas the oxidation potential of PPx is intermediate. Overall, the electrochemical antioxidant characteristics of the compounds seem to be an important factor in their neuronal protection in this model. The (+) enantiomer of PPx which has much higher K_i values with respect to D_2 family dopamine receptor binding shows neuronal protection equipotent to that of the (–) entantiomer (data not shown). This suggests that neuronal protection afforded by PPx in this model is independent of dopamine receptor binding. The GSH levels that were determined in cells treated with 100 μM L-Dopa, with or without 10 μM PPx, at 18 to 24 hrs of exposure to the drugs are shown in Fig. 4. GSH levels were normalized to the total protein content of the cell samples. The timepoints correspond to the time in which the cells are just beginning to show signs of death based on morphological observations by light microscopy. Cells not treated with either drug (control) were harvested at 24 hrs. All other data was plotted relative to these GSH control measurements. Although only the 24 hr timepoint showed a significant difference between the PPx-treated and non PPx-treated samples, the confidence limits (at 95%) of the slopes of the two fitted curves did not overlap. This indicates a significant difference ($p < 0.05$) of GSH loss between PPx-treated and the non PPx-treated cells.

Discussion

As early as 1977 it was postulated that chronic treatment of L-Dopa in Parkinson's disease (PD) could contribute to the progression of the disease (Marsden and Parkes, 1977). Both metabolism and autooxidation of Dopa have been shown to be neurotoxic to dopaminergic and non-dopaminergic cells in culture (Ling et al., 1996, Pardo et al., 1995, Walkinshaw and Waters, 1995). We found dopa toxicity in cerebellar granule cells to be a model of chemical oxidative stress based on the identical toxicity of L- or D-dopa and the complete neuronal protection achieved with added catalase, superoxide dismutase or reduced glutathione. We therefore used this model to investigate the neuroprotective effects of a pyrrolopyrimidine antioxidant (PNU-101033E) and a D_2 /D_3 agonist with putative antioxidant properties (pramipexole; PPx). These were compared directly to deprenyl and vitamin E, which have undergone clinical trial testing for Parkinson's disease (PD). In the DATATOP clinical trial deprenyl (selegiline), a monoamine oxidase type B inhibitor, showed the possible altering of the natural course of PD while vitamin E (alpha-tocopherol) conferred no neuroprotection (Lewitt, 1994). Based on these results we speculated that an antioxidant that is more effective and diverse than vitamin E could lead to new neuroprotective therapeutic strategies for PD exceeding the minimal protective actions of deprenyl. PNU-101033E (or a similar pyrrolopyrimidine antioxidant) which was significantly more potent than vitamin E in our model, and also has been shown to be readily bioavailable and blood brain barrier penetrating (Hall et al., 1997), may lead to a new class of therapeutics for treating the progression of PD.

The oxidation of catecholamines can form metabolites which can then react with the nucleophilic sulfhydryl groups of cysteinyl residues (Hastings and Zigmond, 1994). Catecholamines (L-dopa and dopamine) have also been shown to react with superoxide to form semiquinone radicals which react with and deplete GSH (Spencer et al., 1995) and GSH levels in the substantia nigra of patients with PD have been shown to be decreased by 40% (Sian et al., 1994). In cybrids containing mitochondrial DNA from platelets of PD subjects there has been found a 20% decrement in complex I activity and an increase in oxygen radical production (Swerdlow et al., 1996). This along with the decrease in GSH may play an important role in the neurodegeneration associated with PD and may increase these subjects' susceptibility to mitochondrial toxins. These observations are consistent with the idea of antioxidants being effective for the treatment of PD. In our cellular model, GSH levels are time-dependently decreased by L-Dopa treatment and this decrease is lessened with PPx. The ability of PPx to preserve GSH as well as its low oxidation potential (Hall et al., 1996) suggests that PPx may protect neurons by antioxidant mechanisms. This action may compliment the dopamine agonist activity of PPx in the treatment of PD.

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