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Protective effect of *N*-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine (PF 9601N), a novel MAO-B inhibitor, on dopamine-lesioned PC12 cultured cells

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Abstract

Oxidative stress may play a role in the pathogenesis of Parkinson's disease. We have standardised a new model of dopaminergic-cell toxicity that uses dopamine, which is able to generate free radicals, as a toxin. The effect of this catecholamine on cell viability (MTT staining) was dose-dependent, reaching 65% of cell loss at a dopamine concentration of 300 μM. In this model, the protective effect of a novel MAO-B inhibitor, *N*-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine (PF 9601N), was studied and compared with the effect of L-deprenyl assayed under the same experimental conditions. Whereas PF 9601N (50 μM and 100 μM) showed a significant protective effect, this was not the case with L-deprenyl. This different behaviour could be explained in terms of difference in antioxidant capacity. The toxicity induced in PC12 cells by 300 μM dopamine was partially reversed by incubating it in the presence of GBR-12909, a dopamine-transporter blocker. The results indicated that, besides the intracellular toxicity effect of dopamine, another non-specific extracellular mechanism could be involved.

Introduction

Parkinson's disease is characterised basically by the dysfunction of the dopaminergic nigro-striatal system, as a consequence of the degeneration of pigmented dopamine-containing cells of the zona compacta of the substantia nigra. However, the causes of dopaminergic neurodegeneration remain unclear. Various hypotheses assume that an accumulation of endogenous or exogenous toxins is the trigger of the disease. There is, however, increasing evidence that oxidative stress and free-radical formation may play a role in the pathogenesis of Parkinson's disease (Olanow, 1992). Oxidative stress refers to the cytotoxic consequences of a mismatch between the production of free radicals and the ability of the cell to defend against them. The substantia nigra in patients with Parkinson's disease has a higher level of iron, lower glutathione peroxidase activity and lower glutathione content than in control subjects, all of which may contribute to, or reflect, oxidative stress. Consequently a possible adjuvant therapy for this neurodegenerative disorder could be an antioxidant treatment.

L-Deprenyl (selegiline), a classic MAO-B inhibitor, has been used with levodopa in Parkinson's disease (Birkmayer et al 1975; Knoll 1986). It has been suggested that L-deprenyl, because of its antioxidant properties (Chiueh et al 1994) and its ability to block dopamine metabolism by monoamine oxidase (which generates hydrogen peroxide), may protect nigral neurons from the oxidative stress derived from increased dopamine turnover in the iron-rich basal ganglia (Cohen & Spina 1989).

A new series of acetylenic and allenic derivatives of tryptamine as MAO inhibitors has been described (Balsa et al 1990, 1994; Avila et al 1993; Perez et al 1996). Among these, the 5-benzyloxy derivatives were highly potent and selective MAO-B inhibitors (Perez et al 1999) and both acute and chronic treatment with *N*-(2-propynyl)-2-(5-benzyloxy-indolyl)methylamine (PF 9601N) was shown to have an ex-vivo neuroprotective effect in MPTP-lesioned C57/BL mice (Perez & Unzeta 2003). Additionally, these 5-benzyloxy-indol-alkylamine derivatives have shown in-vitro antioxidant properties (Romera & Unzeta 2000; Romera et al 2003).

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In this context, the aim of this work is to standardise a dopamine toxicity model using PC12 cultured cells, in which the lesion is induced by dopamine, and to determine in this experimental model the possible neuroprotective effect of PF 9601N, a novel MAO-B inhibitor. A comparative study will be assessed in parallel with L-deprenyl assayed under the same experimental conditions.

Materials and Methods

All experiments were performed on cultured PC12 cells during their exponential phase of growth. Cells were grown in 250-cm^3 tissue-culture flasks at 37 °C in an atmosphere of 5% CO_2 and 95% air in Dulbbecco's minimal essential medium (DMEM; GIBCO Laboratories, Life Technology, Barcelona, Spain), 6% horse serum (FONLAB, Barcelona, Spain) and 6% calf serum, (FONLAB, Barcelona, Spain) in a final volume of 20 mL. The medium was changed every 2 days. At 80–90% cell confluence, 30 000 cells/100 μL were plated in 96-well plastic dishes coated with poly-ornithine (10 mg mL^{-1}), to which they adhered firmly. In the dopamine toxicity experiments, cells were exposed to increasing concentrations of dopamine for 4 h and the cell viability was assessed by the MTT method. In studies on the protective effect of PF 9601N, different concentrations of this MAO-B inhibitor, L-deprenyl or the dopamine-transporter blocker GBR-12909 were incubated for 4 h in the presence of 300 μM dopamine.

Cell viability was assayed with the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay (Boix et al 1997). In this method, viable cells convert the soluble dye MTT to insoluble blue formazan crystals. MTT (100 μL; 0.5 mg mL^{-1}) was added to each well, and the cultures were incubated at 37 °C for 30 min. Cells were rinsed with dimethyl sulfoxide (DMSO) to remove excess MTT and the colour was read at 620 and 590 nm. The final absorbance value is taken as the difference between these readings. Cell viability is expressed as a percentage of the control values.

The 5-benzyloxy-indol-alkylamine derivatives were synthesised by previously published methods (Cruces et al 1991). L-Deprenyl (HCl) was from Sigma Chemical Co. (St Louis, MO) and GBR-12909 was from Research Biochemicals International (Natik, MA).

Regarding the statistical analysis, all values are expressed as the mean ± s.d. and analysed by one-way analysis of variance followed by the Scheffé test. The exact treatments under comparison and the number of replicates are shown in the footnote of the corresponding Tables. The level of significance was chosen as $P < 0.05$.

Results

The presence of dopamine in the incubation medium induced a decrease in viability of the PC12 cells, in a dose-dependent manner (Table 1). After 4 h incubation with 100 μM dopamine, a 10% decrease in cell viability was observed, and a 50% decrease was obtained when PC12 cells were incubated in the presence of 150 μM dopamine. Incubation with 500 μM dopamine resulted in no more than a 65% decrease in cell viability (data not shown). Above this, further increase in the dopamine concentration did not induce any significant cell death.

Table 1 Effects of dopamine on PC12 cell viability.

Dopamine (μM)	% Cell viability
0	100
100	90 ± 3
150	50 ± 2*
200	40 ± 3*
300	35 ± 1*

PC12 cells were incubated for 4 h with different concentrations of dopamine. Cell viability was established by the MTT assay. Data are expressed as a percentage of the control (0 μM dopamine) ± s.d. (n = 8 duplicate experiments). *$P < 0.05$, compared with control (Scheffé test).

To see whether the benzyloxy indole-alkylamine derivative, PF 9601N, and L-deprenyl protected PC12 cells against dopamine-induced toxicity, PC12 cells were incubated for 4 h in the presence of dopamine (300 μM) and increasing concentrations of PF 9601N (Table 2). No protective effect was observed at 0.1 μM or 1 μM of the MAO-B inhibitor. However, when incubating with 50 μM and 100 μM PF 9601N, the cell viability increased significantly to 57% and 70%, respectively, compared with the cells treated only with 300 μM dopamine. No protective effect was observed at the same concentration of L-deprenyl. Neither of these MAO-B inhibitors had any effect on cell viability in the absence of dopamine (data not shown). To elucidate whether the dopamine transporter mediated the toxic action of dopamine, PC12 cells were incubated with GBR-12909 (1 μM), a specific inhibitor of the dopamine transporter, in the presence of 300 μM dopamine. Cell viability, measured by MTT staining, was 65% of the control value (Table 2). The lack of total cell protection in this case suggested that dopamine also may act through an extracellular toxic mechanism.

Discussion

Since oxidative stress may be causative in Parkinson's disease, we have used dopamine as a more physiological toxin to generate an in-vitro model of dopaminergic toxicity. Dopamine is able to generate free radicals and quinone products that polymerise, generating melanin (Graham 1978). During this process, the free radicals generated can induce cellular death. This effect can be measured by the capacity of the metabolically active cells to reduce MTT to coloured formazan salts. When PC12 cultured cells were incubated with different concentrations of dopamine (100–300 μM) for 4 h, a dose-dependent loss

Table 2 Protective effect of PF 9601N and L-deprenyl against dopamine-induced toxicity in PC12 cells.

Compound	% Cell viability
Control	100
Dopamine (300 μM)	37 ± 4
PF 9601N 0.1 μM + dopamine (300 μM)	27.5 ± 2.5
PF 9601N 1 μM + dopamine (300 μM)	36.0 ± 2
PF 9601N 50 μM + dopamine (300 μM)	57.5 ± 0.5*
PF 9601N 100 μM + dopamine (300 μM)	70.0 ± 1.0*
L-Deprenyl 0.1 μM + dopamine (300 μM)	42.5 ± 0.5
L-Deprenyl 1 μM + dopamine (300 μM)	36.5 ± 3.5
L-Deprenyl 50 μM + dopamine (300 μM)	48.0 ± 4.0
L-Deprenyl 100 μM + dopamine (300 μM)	45.0 ± 4.0
GBR-12909 1 μM + dopamine (300 μM)	65 ± 0.1*

PC12 cells were incubated for 4 h with dopamine (300 μM) alone and in the presence of different concentrations of PF 601N, L-deprenyl or GBR-12909, and cell viability was determined by MTT assay. Data are expressed as a percentage of the control (0 μM dopamine) ± s.d. (n = 7 duplicate experiments). *$P < 0.05$ compared with PC12 cells incubated only with 300 μM dopamine (Scheffé test).

of cell viability was observed, with the lesion being greatest at 300 μM dopamine. No further loss of cell viability was observed above this concentration (data not shown), indicating a saturating toxic effect of dopamine on PC12 cells.

The protective effect of PF 9601N on PC12 cells lesioned with 300 μM dopamine was compared with the effect of L-deprenyl. Whereas PF 9601N at 50 and 100 μM resulted in a significantly increased cell viability after the lesion with dopamine, showing a clear protective effect, no increase in cell viability was observed with L-deprenyl assayed at identical experimental conditions. This protection against dopamine toxicity in PC12 cultured cells could be explained in terms of the antioxidant properties of the 5-benzyloxy-indol-alkylamine derivatives, which have previously been demonstrated in-vitro (Romera & Unzeta 2000) and its hydroxyl-radical scavenging properties (Romera et al 2003). Furthermore, it is worth remarking that dopamine is metabolized by MAO-A, the only isoform present in PC12 cells (Weingarten & Zhou 2001) generating hydrogen peroxide that can contribute to the cell death induced by the oxidative stress. In this context, PF 9601N, a potent and selective MAO-B inhibitor (Perez et al 1999), at high concentration, could lose selectivity and also inhibit MAO-A, contributing as well to the observed protective effect through the inhibition of the enzymatic free radicals formation. L-Deprenyl, assayed at the same concentration range, did not show any protective effect. A possible explanation could be that L-deprenyl showed less inhibitory potency of the autooxidation of dopamine when comparing with PF 9601N (Perez & Unzeta 2000).

Probably, a higher concentration (mM range) of L-deprenyl would be necessary to produce a similar protective effect.

The toxicity induced by dopamine in PC12 cells has mainly an intracellular origin, as demonstrated by the partial recovery of cell viability after dopamine transporter blockade with GBR-12909. However, inhibition of the dopamine transporter did not give total protection against dopamine toxicity, indicating that a non-specific extracellular toxic effect might also be involved, as has recently been observed in the case of 6-hydroxydopamine (Woodgate et al 1999).

This work presents a toxicity model in cultured PC12 cells, in which the lesion is induced by dopamine itself in a dose-dependent manner. This experimental model was used to demonstrate the protective effect of PF 9601N as a representative 5-benzyloxy-indol-alkylamine derivative, which probably acts through an antioxidant mechanism without precluding other possibilities. In contrast, L-deprenyl did not show any protective effect. Further experimental work will be necessary to elucidate the mechanisms underlying the different behaviour of these two MAO-B inhibitors and to determine the intracellular and extracellular mechanisms that contribute to the toxicity of dopamine in PC12 cells.

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