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European Journal of Pharmacology 434 (2002) 17–20

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Short communication

Pergolide protects SH-SY5Y cells against neurodegeneration induced by H_2O_2

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Received 6 September 2001; received in revised form 15 November 2001; accepted 16 November 2001

Abstract

We found that pergolide, a dopamine D1/D2 receptor agonist used in the clinical therapy of Parkinson's disease, protects SH-SY5Y neuroblastoma cells from cell death induced by a brief pulse (15 min) of 1 mM H_2O_2. Neuroprotection was found when pergolide was added to the culture medium either simultaneously with (EC_{50} = 60 nM) or 2 h before (EC_{50} = 40 nM) H_2O_2 treatment. These effects were not blocked by different dopamine receptor antagonists. Our data suggest that pergolide, independently of dopamine receptor stimulation, may interfere with the early phases of the oxidative stress-induced neurotoxic process.

Keywords: Apoptosis; Glutathione; Neuroblastoma; Dopamine receptor; Parkinson's disease

1. Introduction

Parkinson's disease is a chronic and progressive neurological disease which is characterized by selective degeneration of dopamine neurons in the substantia nigra.

Several factors appear to contribute to the neurodegenerative process in the substantia nigra in Parkinson's disease. These include oxidative stress, mitochondrial dysfunction, excitotoxicity, inflammatory changes and apoptosis (Cadet and Brannok, 1998; Gassen and Youdim, 1999; Dunnet and Bjorklund, 1999). These mechanisms may all interact and amplify each other in a vicious cycle of toxicity leading to neuronal dysfunction and finally cell death. The progressive nature of the disease presents opportunities for therapeutic intervention aimed at blocking or slowing down the degenerative process. Indeed, it is generally believed that treatment of Parkinson's disease with drugs acting as agonists at a dopamine receptor level is symptomatic rather than neuroprotective or curative. However, increasing evidence indicates that, at least in vitro, some dopamine agonists have neuroprotective properties (Felten et al., 1992; Ogawa et al., 1994; Yoshikawa et al., 1994; Carvey et al., 1997; Kitamura et al., 1998; Gomez-Vargas et al., 1998; Gassen et al., 1998; Gassen and Youdim, 1999). It is very important to address this issue because, at present, no effective means are available for slowing down the progression of the disease.

We tested the hypothesis that pergolide, a dopaminergic drug used in the clinical therapy of Parkinson's disease, may have neuroprotective properties. Pergolide mesylate is a synthetic ergoline derivative endowed with dopamine agonist activity at both dopamine D1 and D2 receptors (Fuller et al., 1979; Wong and Reid, 1980). Several double-blind, controlled studies have demonstrated the efficacy of pergolide as adjunctive therapy in the treatment of Parkinson's disease (Ahlskog and Muenter, 1988). Recent studies have indicated pergolide monotherapy as an efficacious and well-tolerated first-line treatment in patients with early-stage Parkinson's disease (Barone et al., 1999).

In this study, human SH-SY5Y neuroblastoma cells that had differentiated into neuron-like cells after treatment with retinoic acid were used as experimental paradigm. These cells originate from a neuroblastoma subclone of the human SK-N-SH cell line and can be induced to differentiate by treatment with retinoic acid to acquire morphological, neurochemical and electrophysiological properties characteristic of neurons, including the expression of dopamine

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0014-2999/02/$ - see front matter
PII: S0014-2999(01)01537-0

receptors (Bielder et al., 1978; Cole et al., 1985; Farooqui, 1994; Itano and Nomura, 1995; Uberti et al., 1997). SH-SY5Y neuroblastoma cells are sensitive to several inducers of apoptosis, such as doxorubicin, *cis*-platinum, and hydrogen peroxide (H_2O_2).

We found that pergolide protects SH-SY5Y neuroblastoma cells from cell death specifically induced by H_2O_2, acting in very low concentrations (nanomolar range) and in very early stages of the neurotoxic intracellular process.

2. Materials and methods

2.1. Cell culture

Neuroblastoma cell line SH-SY5Y was routinely cultured in 1:1 Ham's F12:Dulbecco modified Eagle's medium supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 50 μg/ml penicillin, and 100 μg/ml streptomycin and were kept at 37 °C in humidified 5% CO_2/95% air. For differentiation, cultures were seeded at approximately 10^5 cells/dish and retinoic acid was added to a final concentration of 10 μM. Medium was changed every day and cultures were allowed to differentiate for 1 week.

2.2. Drug treatment

Differentiated SH-SY5Y cells were exposed to doxorubicin (50–100 nM) or *cis*-platinum (3–6 μg/ml) for 24 h. In the oxidative stress paradigm, cells were exposed to 1 mM H_2O_2 for 15 min, unless otherwise indicated. Pergolide (from 10 nM to 50 μM) was added to the culture medium simultaneously with, or 2 h before the addition of the cytotoxic agent, as indicated. Bromocryptine, apomorphine, dopamine, spiperone, (−)-sulpiride, phenoxybenzamine (all from Sigma), or SCH23390 (Tocris) was added to the culture medium 2 h before the H_2O_2 pulse. Pergolide was kindly supplied by Eli Lilly (Sesto Fiorentino, Italy).

2.3. Cell viability

Cell viability was evaluated 24 h after the addition of the cytotoxic agent to the medium by measuring lactate dehydrogenase (LDH) activity in culture-conditioned medium by using the Cytotoxic Detection Kit (LDH) (Roche Molecular Biochemicals) according to the instructions of the manufacturer. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity. Cytotoxicity was evaluated as percentage of total LDH activity.

In separate sets of dishes, cell viability was also established by a fluorescence method, according to Jones and Senft (1985). Briefly, cells were stained for 3 min with a mixture of fluorescein diacetate (150 μg/ml) and propidium iodide (80 μg/ml), and examined immediately with a standard epi-illumination fluorescence microscope (450 nm excitation, 520 nm barrier).

2.4. Glutathione contents

Total glutathione levels were determined according to Tietze (1985) with minor modifications. Briefly, cell pellets were resuspended in 25 mM Tris buffered saline (pH 7.4) and homogenized in 10% trichloracetic acid. Acid extracts were mixed with 10 mM phosphate buffer (pH 7.5), NADPH (4 mM), 5,5′ dithiobis(nitrobenzoic acid) (10 mM) and glutathione reductase (6 U/ml), and incubated for 5 min at 37 °C. Glutathione concentrations were determined by the rate of change of absorbance at 412 nm and comparison of the results with a glutathione standard curve.

2.5. Statistical evaluation

Results for cell viability are given as mean ± S.E.M. Statistical analysis of the data was carried out using analysis of variance (ANOVA) followed by Student's *t*-test, using $P < 0.05$ as the level of significance.

3. Results

3.1. Pergolide protects neuroblastoma cells from H_2O_2-induced neurotoxicity

Exposure of differentiated SH-SY5Y neuroblastoma cells to increasing concentrations of H_2O_2, ranging from 0.5 to 2 mM, for different periods of time, ranging from 1 to 20 min, resulted in concentration- and time-dependent cell death, which was easily detectable 24 h after the insult (data not shown). Based on the results obtained in the above-mentioned experiments, cells were routinely exposed to 1 mM H_2O_2 for 15 min to obtain submaximal cytotoxicity.

This experimental model of neurotoxicity was challenged by increasing concentrations of pergolide, ranging from 10 nM to 50 μM. The drug was added to the culture medium 2 h before or simultaneously with the H_2O_2 pulse. As shown in Fig. 1A, pergolide dose-dependently inhibited H_2O_2-induced cell death, as measured by LDH activity in cell-conditioned culture medium.

Similar results were obtained when neuroprotection was evaluated by fluorescein/ethidium iodide staining or Hoechst 33258 staining (data not shown). The calculated IC_{50} values of pergolide in protecting neuroblastoma cells from H_2O_2-induced cell death were about 40 and 60 nM, when the drug was added 2 h before or simultaneously with the H_2O_2 pulse, respectively. Incubation of the cells with 1 μM pergolide for 26 h did not affect cell viability.

3.2. Specificity of the neuroprotective effect of pergolide

The neuroprotective effect of pergolide on H_2O_2-induced neuronal cell death and apoptosis was shared by other dopamine receptor agonists. As shown in Fig. 1, dopamine, bromocryptine, or apomorphine was able to prevent H_2O_2-

induced cell death, as evaluated by measurement of LDH activity in cell-conditioned culture media, although with a lower potency than pergolide. Moreover, neuroprotection by pergolide was not prevented by preincubation of the cells with 10 μM phenoxybenzamine, 10 μM spiperone, 10 μM SCH 23390 or 10 μM (−)-sulpiride (Fig. 1).

The neuroprotective effect of pergolide was specific for H_2O_2, because doxorubicin- or *cis*-platinum-induced cell death was unaffected by the presence of the dopaminergic drug in the culture medium. As shown in Fig. 1B, cell death measured by and fluorescein/ethidium iodide staining induced by maximally effective concentrations of the anthracycline antibiotic doxorubicin (100 nM) or the antineoplastic drug *cis*-platinum (6 μg/ml) was 78% and 92%

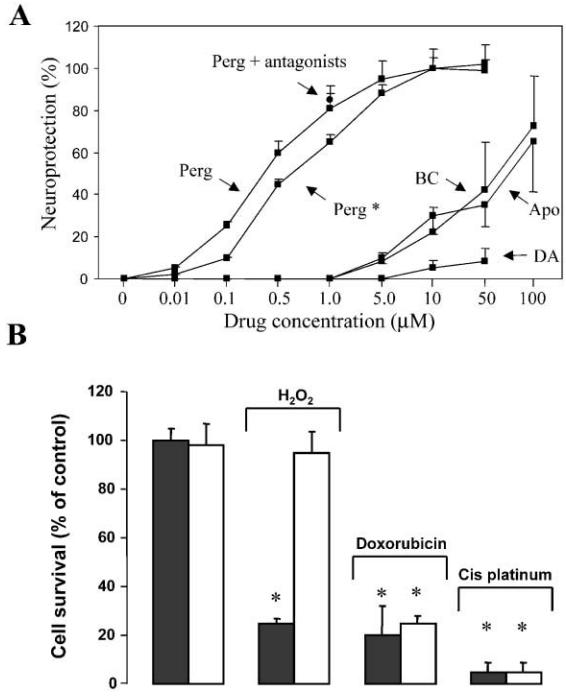

Fig. 1. Effects of pergolide and other dopamine receptor agonists on SH-SY5Y neuroblastoma cell death induced by different agents. Panel A: Cells were exposed to 1 mM H_2O_2 for 15 min in the absence or presence of increasing concentrations of different dopamine agonists, as indicated on the bottom of the bars. Drugs were added to the culture medium 2 h before incubation with H_2O_2 with the exception of pergolide (perg *), which was also tested after simultaneous addition with H_2O_2. Cell viability was determined from LDH release in the culture medium. Values represent % of protection and are means ± S.E.M. of at least three different experiments and are from three separate cell preparations. Drug concentrations are indicated on the bottom of the graph. Perg, pergolide; Perg *, pergolide added simultaneously with H_2O_2; DA, dopamine; BC, bromocryptine; Apo, apomorphine. Antagonists referred to are (−)-sulpiride, SCH 23390, phenoxybenzamine, and spiperone, all at 10 μM concentration. Panel B: Cells were exposed to different inducers of apoptosis in the absence (grey bars) or presence (open bars) of pergolide, as indicated. Cell viability was determined by fluorescein diacetate/propidium iodide staining. Bars represent means ± S.E.M. of at least three different experiments and are from three separate cell preparations. Drug concentrations: Pergolide, 100 nM; doxorubicin, 100 nM; *cis*-platinum, 6 μg/ml; H_2O_2, 1 mM. Pergolide was added to the culture media 2 h before H_2O_2, doxorubicin, or *cis*-platinum.
* $P<0.01$ vs. the corresponding control values.

Table 1
Effects of pergolide on H_2O_2-induced glutathione depletion

Time	0	10 min	30 min
H_2O_2	3.81 ± 0.48	1.33 ± 0.31[a]	4.27 ± 0.48
Pergolide	3.90 ± 0.35	2.90 ± 0.21[a,b]	4.02 ± 0.49

Cells were exposed to 1 mM H_2O_2 for 15 min in the absence or presence of 100 nM pergolide. Glutathione levels were measured at different time points after the H_2O_2 pulse, as indicated. Values are expressed as nmol/mg of protein and represent means ± S.E.M. of at least three different experiments and are from three separate cell preparations.
[a] $P<0.01$ compared with control values (H_2O_2 at 0 time point).
[b] $P<0.05$ compared with values at the same time point.

over control, respectively. Preincubation of the cells with 100 nM pergolide for 2 h before the cytotoxic agent did not affect the neurotoxic effect of either doxorubicin or *cis*-platinum. Similar results were obtained by measuring LDH release in the cell-conditioned medium (data not shown). A lack of neuroprotection by pergolide was also found when doxorubicin or *cis*-platinum was used at submaximally effective concentrations (20 nM and 3 μg/ml, respectively) (data not shown).

Treatment of SH-SY5Y cells with H_2O_2 resulted in a fast and short-lasting depletion of glutathione (Table 1). This effect is believed to reflect an increase in levels of reduced glutathione (GSH) as a compensatory antioxidant defence of the cells in response to reactive oxygen species generation. The glutathione depletion induced by H_2O_2 in cells pretreated with pergolide was lower, although statistically significant, than that measured in absence of the dopaminergic drug (−80% without and −40% with pergolide).

4. Discussion

This study shows that pergolide treatment protects SH-SY5Y neuroblastoma cells from H_2O_2-induced cell death. This effect is (i) concentration-related, (ii) detectable over a nanomolar concentration range, and (iii) independent of dopamine receptor stimulation.

The molecular mechanisms by which H_2O_2 induces, and pergolide protects against, cell death are not completely understood. In this particular experimental model of neurotoxicity, the generation of reactive oxygen species may play a relevant role. Lipid peroxidation, mitochondrial dysfunction, and DNA damage are indeed among the intracellular effects triggered by an abnormal production of reactive oxygen species. As a consequence, cells may activate a protective response which involves either antioxidant defences or activation of a restricted number of transcription factors, or both.

When neurodegeneration was triggered by agents acting downstream of reactive oxygen species generation, such as the topoisomerase II inhibitor doxorubicin or the direct DNA damaging inducer *cis*-platinum, pergolide, as well as other dopamine agonists (unpublished results), was found to

be ineffective. These data suggest that pergolide may interfere with the early phases of the cell death programme, including free radical scavenging and antioxidant actions (Gomez-Vargas et al., 1998).

Finally, the neuroprotective effects of pergolide appeared to be independent of dopamine receptor activity because different dopamine receptor antagonists, such as spiperone, SCH 23390 and (−)-sulpiride, and the α-adrenoceptor antagonist phenoxybenzamine, were unable to inhibit pergolide-mediated neuroprotection.

In conclusion, our data indicate that pergolide is neuroprotective against cell death induced by oxidative stress. This effect may represent an additional pharmacological property of this drug that contributes to its clinical efficacy in Parkinson's disease.

Acknowledgements

This work was financially supported by grants from Ely Lilly-Italia and COFIN-MURST 2000.

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