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Cabergoline protects SH-SY5Y neuronal cells in an in vitro model of ischemia

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Abstract

Dopamine receptor agonists are protective in different models of neurodegeneration by both receptor-dependent and -independent mechanisms. We used SH-SY5Y cells, differentiated into neuron-like type, to evaluate if cabergoline, a dopamine D2 receptor agonist endowed with anti-oxidant activity, protects the cells against ischemia (oxygen–glucose deprivation model). Cabergoline protected the cells from ischemia-induced cell death in a concentration-dependent manner (EC_{50} = 1.2 μM), as demonstrated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, lactate dehydrogenase (LDH) release, and fluorescein diacetate–propidium iodide staining. This effect, observed even when the drug was added after oxygen–glucose deprivation, was not mediated by either dopamine D2 receptor activation or anti-apoptotic Bcl-2 protein over-expression (Western blotting analysis), but was linked to a reduction in cellular free radical loading (2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) staining) and membrane lipid peroxidation (thiobarbituric acid-reacting test). In conclusion, cabergoline protects in vitro neurons against ischemia-induced cell death, suggesting its possible use in the therapy of other neurodegenerative diseases in addition to Parkinson's disease.

Keywords: Dopamine receptor; Neuroprotection; Oxygen–glucose deprivation; Stroke; Free radical; Anti-oxidant

1. Introduction

The biochemical and cellular events leading to ischemia-induced neuronal degeneration have been extensively studied (Siesjo, 1992a,b; Lipton, 1999; Zheng et al., 2003). Dopamine, the first neurotransmitter to be identified as having a role in ischemic damage (Lust et al., 1975; Lavyne et al., 1975), is released during ischemia by both Ca^{2+}-dependent exocytosis and reversal transport (Kim et al., 1995). Released dopamine may be oxidised by non-enzymatic or enzymatic (monoamine oxidases) reactions, both processes generating free radicals. Moreover, dopamine and/or its precursor L-3,4-dihydroxyphenylalanine (L-DOPA) may induce glutamate release (Misu et al., 2002; Marti et al., 2002), and glutamate itself may cause an overloading of intracellular Ca^{2+} (Choi, 1992) leading to excitotoxicity (Olney, 1986), free radical production (Pellegrini-Giampietro et al., 1988), and dopamine release, creating a dramatic vicious circle (Wang, 1991; Desce et al., 1992; Hoyt et al., 1997).

Although dopamine has an important role in determining ischemia-induced brain damage, it has been shown that pharmacological stimulation of the dopaminergic system may also be clinically useful in stroke patients (Mukand et al., 2001; Scheidtmann et al., 2001). More recently, it was shown that structurally different molecules (ergot- or non-ergot-related compounds), endowed with agonist activity at the dopamine D2 receptor subfamily, are also protective in several in vitro and in vivo models of neurodegeneration, such as intrastriatal injection of 6-hydroxydopamine, oxidative stress and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced striatal dopamine reduction (Lombardi et al., 2002; Schapira, 2002; Kitamura et al., 2003). The mechanisms of action underlying these neuroprotective effects may involve activation of the dopamine receptors, but anti-oxidant or radical scavenger activity could be involved as well (Lombardi et al., 2002; Kitamura et al., 2003).

Possible neuroprotective effects of such compounds in in vitro models of ischemia have not yet been studied, but

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previous in vivo results do suggest neuroprotective effects (Liu et al., 1995; Hall et al., 1996; O'Neill et al., 1998). We decided to study if cabergoline, an ergoline derivative endowed with a potent, selective, long-lasting agonist action at the dopamine D2 receptor subfamily (Fariello, 1998) and clinically effective in the therapy of Parkinson's disease (Baas and Schueler, 2001), possibly protects neurons against ischemia-induced cell death. Cabergoline was previously demonstrated by our group to be experimentally useful in protecting neuronal cells from oxidative stress by a non-receptor-mediated mechanism linked to its anti-oxidant activity (Lombardi et al., 2002). For the experiments here reported, we used an in vitro model (oxygen–glucose deprivation) of ischemia–reperfusion on SH-SY5Y human neuroblastoma cells, differentiated into neuron-like type by treatment with retinoic acid. These cells originate from a neuroblastoma subclone of the human SK-N-SH cell line and after retinoic acid treatment acquire morphological, neurochemical and electrophysiological properties of neurons, including the expression of dopamine receptors (Biedler et al., 1978; Farooqui, 1994; Itano and Nomura, 1995; Uberti et al., 2002).

Taken together, our results demonstrate that in vitro cabergoline is able to protect neurons against ischemia-induced cell death, even if applied to the cell after the ischemic insult. These effects do not seem to be mediated by dopamine D2 receptor activation, but are, instead, linked to the anti-oxidant, radical scavenger properties of cabergoline.

2. Materials and methods

2.1. Cell culture

SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture Ham's F-12 (1:1) media, supplemented with 10% foetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM); the cell culture medium was replaced every 2 days. The cultures were maintained at 37 °C in 95% air–5% CO_2 in a humidified incubator.

The cells were differentiated into neuron-like type by treatment with retinoic acid (10 μM) that was added to the cell culture medium every day for 2 weeks. The day before the experiments, differentiated cells were plated in a six-well culture plate (1×10^6 cells/well).

2.2. In vitro model of ischemia

The in vitro model of ischemia we used was achieved maintaining the cells under oxygen–glucose deprivation for 5 h, followed by 20 h of recovery. Briefly, the standard culture medium was replaced with a glucose-free buffer (oxygen–glucose deprivation buffer) (in mM: 154 NaCl, 5.6 KCl, 5.0 HEPES, 3.6 $NaHCO_3$, 2.3 $CaCl_2$), pH 7.4, bubbled with an anaerobic gas mixture (95% N_2, 5% CO_2) for at least 2 h before use, with or without various concentrations of drugs. The culture plates were then placed into an anaerobic chamber (Oxoid, Basinstoke, Hampshire, UK), flushed with the anaerobic gas mixture and maintained at 37 °C for the appropriate time. During this time, the oxygen partial pressure (pO_2) was constantly monitored using an oxygen meter (Microelectrodes, MI-730) microelectrode and was maintained at 60 mm Hg. After the appropriate time, the culture plates were removed from the anaerobic chamber, returned to the standard culture medium and placed in a humidified incubator at 37 °C for 20 h of recovery before being analysed. When different oxygen–glucose deprivation or recovery times were used in the experiments, they are specifically mentioned in the text.

2.3. Drug treatments

Cabergoline and vitamin E (α-tocopherol) were dissolved in ethanol; haloperidol was dissolved in dimethylulfoxide. Final drug concentrations were obtained by dilution of stock solutions in experimental media. Final concentration of organic solvents were always less than 0.01%, and they have no effects on cell viability. Drugs were added to the experimental buffer 2 h before oxygen–glucose deprivation, during oxygen–glucose deprivation, during recovery or for the entire experiment. Cells exposed to solvent alone are considered as controls (drug-untreated ischemic samples).

2.4. Cell viability

Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Absorbance was measured at 570–630 nm using an Ultramark microplate reader (Bio-Rad Laboratories, Milan, Italy). The percentage of neuroprotection was calculated as follows: percentage of neuroprotection = $100 - [(x - z)/(x - y) \times 100]$, where x is the absorbance read in non-ischemic samples, y is absorbance read in drug-untreated (solvent alone) ischemic samples, and z is absorbance read in drug-treated ischemic samples.

Cell death was determined by both fluorescein diacetate–propidium iodide staining (Jones and Senft, 1985), counting the number of dead (red) and vital (green) cells using a fluorescent microscope (Nikon Eclipse E-600, Nikon Instruments, Italy), and by measuring the lactate dehydrogenase (LDH) activity in the culture media (Murphy et al., 1993).

2.5. Determination of free radical production and lipid peroxidation

Free radical production was measured by incubating the cells with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Tammariello et al., 2000). DCFH-DA freely crosses the cell membranes and it is hydrolysed by cellular esterases to 2′,7′-dichlorodihydrofluorescein ($DCFH_2$), a non-fluorescent molecule that can be oxidised to the fluorescent 2′,7′-dichlorofluorescein (DCF) in the pres-

ence of peroxides. Accumulation of DCF in the cells was measured as an increase in fluorescence at 525 nm, when the sample was excited at 488 nm using a Jasco (FP-777) spectrofluorometer (Jasco International, Tokyo, Japan).

Lipid peroxidation was evaluated by measuring the thiobarbituric acid-reacting substances (TBARS) in the cells at the end of the experiments (Ohkawa et al., 1979). Briefly, the cells were washed and harvested with ice-cold 50 mM phosphate buffer, pH 7.4, then 500 μl of 1% thiobarbituric acid and 500 μl of 8 N HCl were added to each sample. The samples were boiled for 20 min and subsequently cooled with tap water. 1-Butanol, 1.5 ml, was then added to the samples and the mixture was shaken for 2 min. After centrifugation at 2000 × *g* for 10 min, the fluorescence intensity at 550 nm (excitation) and 532 nm (emission) in the butanol phase was measured by a Jasco (FP-777) spectrofluorometer.

2.6. Western blotting analysis

SH-SY5Y cells were lysed with ice-cold protein lysis buffer (in mM: 10 Tris–HCl, 50 NaCl, 5 ethylenediaminetetraacetic acid, plus 1% Triton X-100) containing proteases and phosphatases inhibitors aprotinin (10 μM), leupeptin (10 μM), pepstatin (100 μM), phenylmethylsulfonyl fluoride (1 mM), NaF (10 mM) and sodium orthovanadate (1 mM) for 20 min. Samples (50 μg proteins) were then electrophoresed in a 15% polyacrylamide gel and electroblotted overnight on a nitrocellulose membrane at 4 °C. The membrane was then incubated in 5% milk powder in 0.1% Tween 20–phosphate-buffered saline for 1 h. The blot was incubated with the polyclonal anti-Bcl-2 (1:200) or monoclonal anti-Hsp-90 (1:1000) primary antibody for 2 h, and then with the anti-rabbit (1:10000) or anti-mouse (1:8000) horseradish peroxidase-labeled secondary antibody for 1 h. The protein bands were visualized by the chemiluminescence reaction (ECL detection kit), and then densitometrically analysed by an imaging system (Fluor-S™ multimanager, Bio-Rad Laboratories).

2.7. Materials

Dulbecco's Modified Eagle Medium/Nutrient Mixture Ham's F-12 (1:1) media and fetal bovine serum were obtained from Invitrogen (Milan, Italy). Penicillin, streptomycin, L-glutamine, retinoic acid, haloperidol, vitamin E, MTT, fluorescein diacetate, propidium iodide, DCFH-DA, ethylenediaminetetraacetic acid, L-ascorbic acid, thiobarbituric acid, aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, sodium orthovanadate, and Triton X-100 were purchased from Sigma-Aldrich (Milan, Italy), cabergoline was obtained from Pharmacia Italia (Milan, Italy). LDH activity was quantified using the Cytotoxicity Detection Kit (LDH) from Roche Diagnostic (Mannheim, Germany). Anti-Bcl-2 (ΔC21) was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-Hsp-90 was obtained from BD Biosciences (Milan, Italy). Secondary antibodies and the ECL detection kit were obtained from Amersham Biosciences Europe (Cologno Monzese, Italy). All other reagents and solvents were from Merck (Darmstadt, Germany).

2.8. Data analysis

Results are expressed as means ± S.E.M. of at least six experiments. Statistical significance was evaluated by Student's *t* test for paired varieties. Differences were considered statistically significant when $P < 0.05$. Origin version 6.0 (Microcal Software, Northampton, MA, USA) was used as a non-linear regression model for analysis of the concentration–response data to obtain a 50% effective concentration (EC_{50}).

3. Results

3.1. Time course of ischemia-induced cell death

Exposure of SH-SY5Y cells to oxygen–glucose deprivation leads to ischemia-induced cell death, which was a function of the duration time (h) of oxygen–glucose deprivation (Fig. 1). A significant ($P < 0.05$) percentage of cell death was measured starting at 4 h of oxygen–glucose deprivation (25 ± 2%, in comparison with non-ischemic cells) and it progressively increased with time exposure,

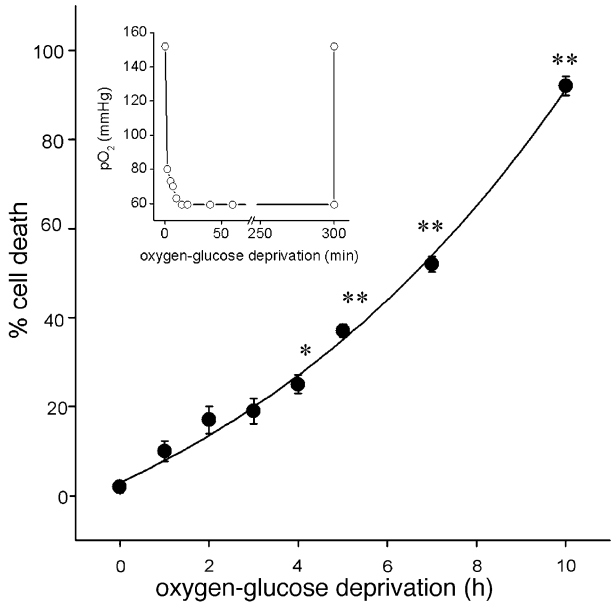

Fig. 1. Time course of oxygen–glucose deprivation-induced cell death. The cells were maintained under oxygen–glucose deprivation for different times (1–10 h) followed by 20 h of recovery. The values represent the percentage of cell death (MTT assay) in comparison with control cells not exposed to oxygen–glucose deprivation. The data are the means ± S.E.M. of at least six experiments run in triplicate. Insert panel shows the pO_2 (mm Hg) values measured at different oxygen–glucose deprivation time (0–300 min). *$P < 0.05$; **$P < 0.01$ vs. control cells not exposed to oxygen–glucose deprivation.

reaching almost 100% of cell death after 10 h of oxygen–glucose deprivation. For our experiments, we selected 5 h oxygen–glucose deprivation because at that time the percentage of cell death (37 ± 1%) may still be pharmacologically modulated. At the beginning of our studies, the partial pressure of oxygen into the anaerobic chamber was measured by an oxygen meter at different times (min) during oxygen–glucose deprivation and it was 60 ± 2 mm Hg after 15 min of oxygen–glucose deprivation (see insert panel in Fig. 1).

3.2. Neuroprotective effects of vitamin E

Since oxidative stress is considered to have a key role in ischemia-induced cell death, we studied if increasing concentrations of vitamin E, a well-known anti-oxidant molecule (Burton and Ingold, 1989; van Acker et al., 1993) are able to protect human SH-SY5Y cells against oxygen–glucose deprivation insult. As shown in Fig. 2, when cells were exposed to this compound during oxygen–glucose deprivation (B), during recovery (C), or during the entire experiment (D), significant ($P < 0.01$) neuroprotection was measured (mean of neuroprotection: 70 ± 6% at 50 μM, in comparison with vitamin E-untreated cells). In all these conditions, the EC_{50s} calculated were identical and were 1.5 μM. When vitamin E was present only before oxygen–glucose deprivation (A), we did not observe the neuroprotective effects.

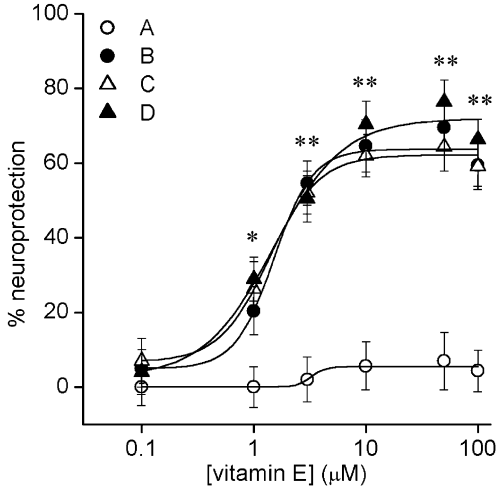

Fig. 2. Concentration–response curves of the neuroprotective effects of vitamin E on oxygen–glucose deprivation-induced cell death. Vitamin E (0.1–100 μM) was added to the cells: only before oxygen–glucose deprivation (A), during oxygen–glucose deprivation (B), during recovery (C), or during the entire experiment (D). The values represent the percentage of neuroprotection, in comparison with vitamin E-untreated ischemic cells (for the calculation of neuroprotection, see Materials and methods). Cell death (MTT assay) was evaluated at the end of the recovery period. The EC_{50s} calculated for the B, C, and D curves were identical and were all 1.5 μM. The data represent the means ± S.E.M. of at least six experiments run in triplicate. *$P < 0.05$; **$P < 0.01$ vs. drug-untreated cells.

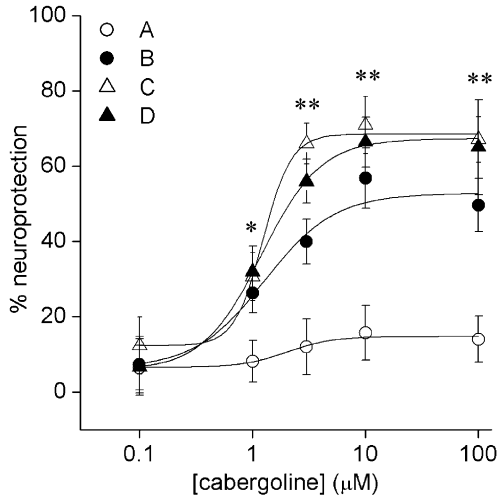

Fig. 3. Concentration–response curves of the neuroprotective effects of cabergoline on oxygen–glucose deprivation-induced cell death. Cabergoline (0.1–100 μM) was added to the cells: only before oxygen–glucose deprivation (A), during oxygen–glucose deprivation (B), during recovery (C), or during the entire experiment (D). The values represent the percentage of neuroprotection in comparison with cabergoline-untreated ischemic cells (for the calculation of neuroprotection, see Materials and methods). Cell death (MTT assay) was evaluated at the end of the recovery period. The EC_{50s} calculated for the B, C, and D curves were identical and were all 1.2 μM. The data represent the means ± S.E.M. of at least six experiments run in triplicate. *$P < 0.05$; **$P < 0.01$ vs. drug-untreated cells.

These results confirmed that in the oxygen–glucose deprivation model of ischemia we used, oxidative stress is an important contributor to cell death, which may still be pharmacologically modulated. This allowed us to utilise this model to characterise the neuroprotective effects of other drugs.

3.3. Concentration–response curves of the neuroprotective effects of cabergoline

To study if cabergoline may protect neurons against ischemia-induced cell death, increasing concentrations (0.1–100 μM) of this drug were added to the ischemic buffer.

Table 1
Effects of cabergoline and vitamin E on ischemia-induced cell death and LDH release

	% Cell death	% LDH release
Ischemic cells	22.5 ± 1.0	59.7 ± 2.0
Ischemic cells plus cabergoline (10 μM)	4.16 ± 2.0[a]	36.8 ± 0.67[a]
Ischemic cells plus vitamin E (50 μM)	5.83 ± 2.0[a]	28.1 ± 1.2[a]

Cells were exposed to oxygen–glucose deprivation (see Materials and methods). Cabergoline or vitamin E were added after oxygen glucose deprivation and kept in the buffer until the end of experiment. Dead neurons were counted (four separate field-dish) by fluorescein diacetate–propidium iodide staining, and expressed as percentage of control, non-ischemic cells. The amount of LDH released into the experimental medium was measured, and expressed as percentage of control, non-ischemic cells. Background LDH release was measured in control non-ischemic cells and subtracted from all experimental values.

[a] $P < 0.01$ vs. drug-untreated ischemic cells.

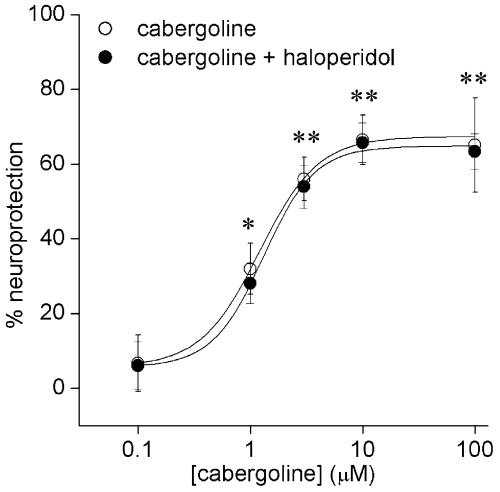

Fig. 4. Effects of haloperidol on cabergoline-induced neuroprotection. The cells were exposed to 1 μM haloperidol 5 min before treatments with cabergoline (0.1–100 μM). The drugs were maintained during the entire experiment. The values represent the percentage of neuroprotection, in comparison with cabergoline untreated ischemic cells (for the calculation of neuroprotection, see Materials and methods). Cell death (MTT assay) was evaluated at the end of the recovery period. The EC_{50s} calculated in the presence or in the absence of haloperidol were identical and were 1.3 μM. The data represent the means ± S.E.M. of at least six experiments run in triplicate. *$P<0.05$; **$P<0.01$ vs. drug-untreated cells.

Fig. 3 shows that cabergoline significantly ($P<0.01$) prevented cell death (mean of neuroprotection: 65 ± 8% at 10 μM, in comparison with cabergoline-untreated cells) if present during oxygen–glucose deprivation (B), during recovery (C), or during the entire experiment (D). In all these conditions, the EC_{50}s calculated were identical and were 1.2 μM. On the contrary, the neuroprotective effects were lost when cabergoline was present only 2 h before oxygen–glucose deprivation (A). For all the experiments reported below, the cells were exposed to cabergoline (10 μM) immediately after oxygen–glucose deprivation.

A similar extent of neuroprotection by cabergoline was also measured when the ischemia-induced cell death was studied both morphologically, by counting the number of dead cells (fluorescein diacetate–propidium iodide staining), and biochemically, by measuring the amount of LDH enzyme released by dead cells into the experimental medium (Table 1). In both cases, the neuroprotective effects of cabergoline were similar to that obtained by vitamin E (50 μM) in the same experimental condition.

3.4. Pharmacological characterization of the neuroprotective effects of cabergoline

The neuroprotective effects of cabergoline against ischemia-induced cell death were not mediated by its action at the dopamine D2 receptors. In fact, the concentration–response curve of cabergoline was not affected by the simultaneous presence of haloperidol (1 μM), a selective dopamine D2 receptor antagonist (Fig. 4), and the EC_{50s} calculated in the presence or in the absence of haloperidol were identical and were 1.3 μM.

3.5. Anti-oxidant effects of cabergoline

To evaluate the mechanisms underlying the neuroprotective effects of cabergoline, the amount of free radicals overloading into the ischemic cells, exposed to different duration times of recovery (h) in the presence of cabergoline, was measured by using the fluorescent probe DCFH-DA (Fig. 5A). Cabergoline significantly ($P<0.01$) inhibited the levels of free radicals in the cells, with the maximum effect after 6 h of recovery (33 ± 1% of inhibition over cabergoline-untreated ischemic cells).

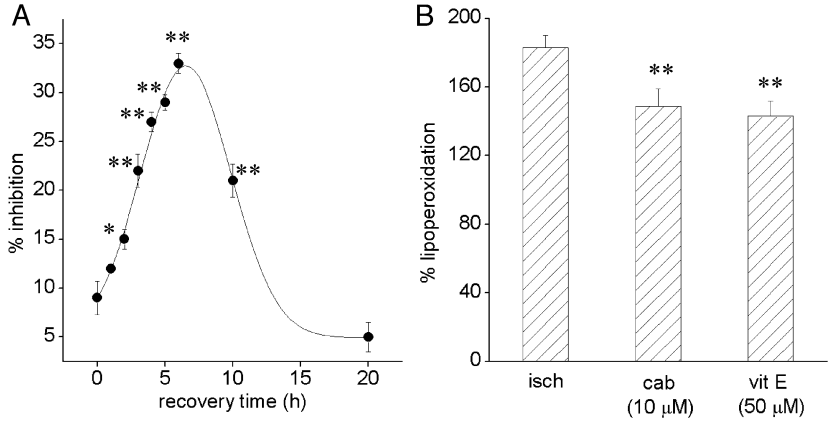

Fig. 5. Anti-oxidant effects of cabergoline. (A) The cabergoline-induced inhibitory effect on free radical accumulation (see Materials and methods) was measured at different times during recovery (0–20 h), and expressed as percentage of inhibition over cabergoline-untreated ischemic cells. (B) Membrane lipoperoxidation was measured as TBARS levels (see Materials and methods). The bars represent the mean ± S.E.M. percentage of lipoperoxidation, calculated of ischemic (isch)-, cabergoline (cab; 10 μM)-, vitamin E (vit E; 50 μM)-treated cells over non-ischemic cells in the same experimental condition. *$P<0.05$; **$P<0.01$ vs. drug-untreated ischemic cells.

Moreover, the levels of TBARS, which are products of membrane lipid peroxidation, were also significantly ($P < 0.01$) decreased ($-19 \pm 5\%$ over cabergoline-untreated ischemic cells) by cabergoline (Fig. 5B). Vitamin E (50 μM) gave similar results ($-22 \pm 4\%$) in the same experimental condition.

3.6. Effects of cabergoline on anti-apoptotic Bcl-2 protein expression

Since it has been reported that several dopamine D2 receptor agonists may induce an up-regulation of anti-apoptotic Bcl-2 protein expression (Kihara et al., 2002; Kitamura et al., 2003), we studied if the neuroprotective effects of cabergoline, observed in our model, were also due to an over-expression of this protein. Fig. 6 shows that cabergoline was not able to modulate the expression of Bcl-2 protein if added during oxygen–glucose deprivation (Fig. 6A) or during 5 or 20 h of recovery (Fig. 6B and C, respectively).

4. Discussion

For the above-described experiments, oxygen–glucose deprivation was used as in vitro model of the in vivo ischemia–reperfusion insult. This model was devised by Goldberg and Choi (1993) and then extensively used in many studies (Moroni et al., 2001; Wang et al., 2002; Pei and Cheung, 2003). It represents a simple tool both for studying damage processes and testing neuroprotective drugs. In the literature, many authors used primary neuronal cell cultures

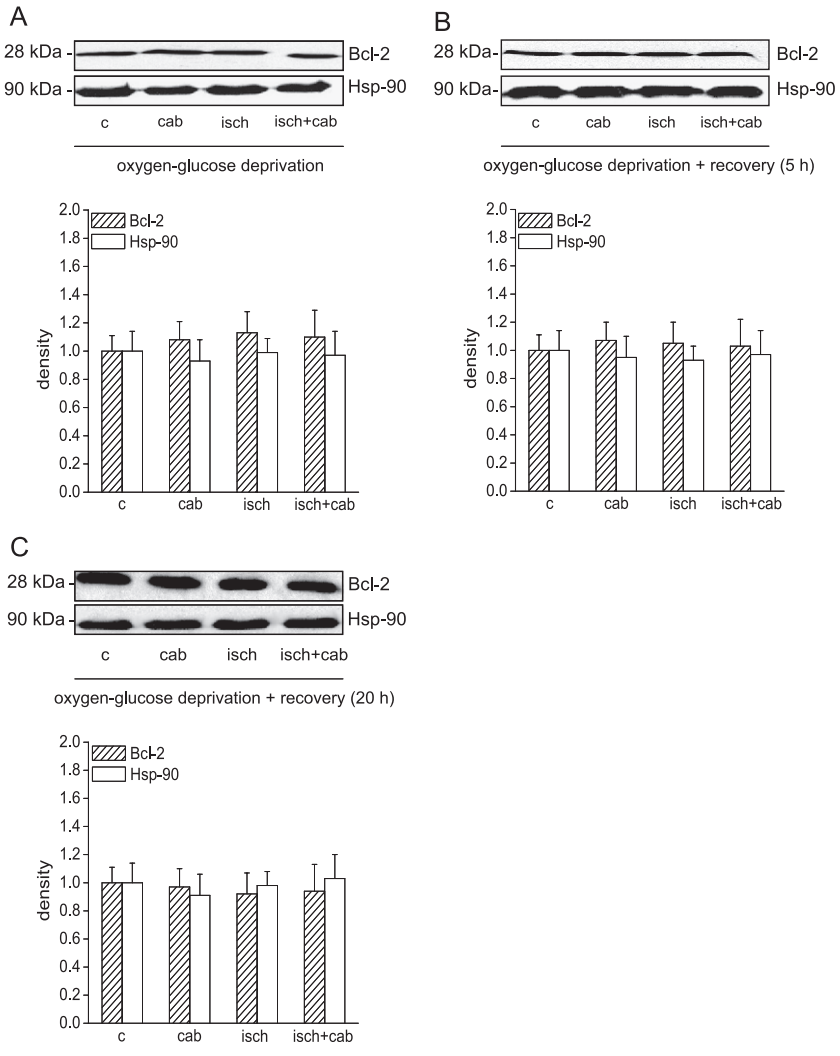

Fig. 6. No changes in protein levels of anti-apoptotic Bcl-2 protein. The level of the proteins were detected by Western blotting (see Materials and methods), in non-ischemic cells (c), in cabergoline-treated non-ischemic cells (cab), in ischemic cells (isch), and in cabergoline-treated ischemic cells (isch + cab). Cells were treated with cabergoline (10 μM) during oxygen–glucose deprivation (A), during oxygen–glucose deprivation and 5 h of recovery (B), or during oxygen–glucose deprivation and 20 h of recovery (C). To determine whether equal amounts of proteins were loaded onto gels, membranes were reblotted with anti-Hsp-90 monoclonal antibodies. Immunoreactivity was quantified as described in the Materials and methods and densities of Bcl-2 and Hsp-90 bands were given as the mean ± S.E.M. at least of three determinations, based on the density of the band in non-ischemic cells (c) as 1.

for oxygen–glucose deprivation models, but from our previous experiences (data not shown), cell vulnerability often depends both on the selected cell type (i.e., cortical, hippocampal, cerebellar) and the specific intrinsic factors of the primary cell culture preparation (i.e., cell density, purity, age, medium). Therefore, for the studies reported here, we used cell lines. SH-SY5Y human neuroblastoma cell line was chosen because the cells are sensitive to oxidative stress (Amoroso et al., 1999; Uberti et al., 2002; Lombardi et al., 2002) and express glutamate receptor proteins (*N*-methyl-D-aspartate receptor and metabotropic glutamate receptors) (Nair et al., 1996; Sun and Murali, 1998). Free radical accumulation and excitotoxicity are in fact the major contributors to ischemic damage, and the utilisation of cells, responsive to both of these events, may offer a better understanding of this complex phenomenon.

Since undifferentiated tumour cells, having lost control over proliferation and having their anti-apoptotic machinery switched on, are less vulnerable to the ion and chemical changes resulting from ischemic insults (Benedetti et al., 1984), we used SH-SY5Y cells differentiated into neuronal type by 15 days of retinoic acid treatment. The oxygen–glucose deprivation time (5 h) we used was consequently shorter than that previously published by other authors (10–16 h) (Wang et al., 2002), who used undifferentiated SH-SY5Y cells. On the other hand, other authors (Pei and Cheung, 2003) used 1 h of oxygen–glucose deprivation to obtain similar levels of ischemia-induced cell death in undifferentiated SH-SY5Y cells. In our opinion, these methodological discrepancies may be linked to differences existing between cell clones and aging (Martin et al., 1998). In fact, previous experiments performed in our laboratory showed that SH-SY5Y cells increase their resistance to ischemic insult with aging. Thus, for the studies here reported, we always used 15th–20th subcultures.

Since ischemia-induced neurodegeneration and other neurological disorders (i.e., Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis) share many similar biochemical and cellular events leading to cell death (i.e., oxidative stress, energy failure and excitotoxicity), our hypothesis was that a neuroprotective drug, clinically effective in Parkinson's disease therapy, might also have beneficial effects against ischemic insult. We decided to study the possible neuroprotective effects of cabergoline, a lipophilic compound that easily enters into the central nervous system. Cabergoline is a potent and long-lasting dopamine D2 receptor agonist, is clinically effective in Parkinson's disease therapy (Baas and Schueler, 2001) and is endowed with anti-oxidant activities (Lombardi et al., 2002; Yoshida et al., 2002; Yoshioka et al., 2002). The results we obtained confirmed our hypothesis: cabergoline protects in vitro neurons against ischemia-induced cell death in a concentration-dependent manner, even if applied to the cells after the ischemic insult.

The mechanisms of action underlying the neuroprotective effects of cabergoline were not related to its action at dopamine D2 receptors, but are probably linked to its anti-oxidant activity, in a similar manner to vitamin E. However, whereas vitamin E and L-ascorbic acid, another well-known anti-oxidant compound, are pro-oxidants at high concentrations (Lai and Yu, 1997; Dyatlov et al., 1998), cabergoline (concentration up to 1 mM) did not enhance cell vulnerability (data not shown). These data suggest that cabergoline, in spite of its high volume of distribution owing to tissue accumulation (Fariello, 1998), is never neurotoxic, as is also demonstrated by its long-term clinical use in the therapy of Parkinson's disease (Baas and Schueler, 2001). The results here reported are consistent with those taken from the literature, demonstrating that cabergoline is able to reduce lipid peroxidation both in vivo (Finotti et al., 2000) and in vitro (Lombardi et al., 2002), directly scavenges free radicals (Yoshida et al., 2002) and increases GSH intracellular levels (Lombardi et al., 2002; Yoshioka et al., 2002). Moreover, in our model, cabergoline did not modulate the expression of the anti-apoptotic Bcl-2 protein, whose over-expression protects cells against oxidative stress, anoxia and ischemic damage (Howard et al., 2002; Saitoh et al., 2003; Zhao et al., 2003). Kitamura et al. (1998) and Takata et al. (2000) described an over-expression of Bcl-2 protein in un-differentiated SH-SY5Y cells after 4 days of treatment with talipexole and pramipexole, but not with bromocriptine, whereas Kihara et al. (2002) reported an up-regulation after 24 h of treatment with bromocriptine in cortical neurones. These different results obtained with various dopamine D2 receptor agonists could be a consequence of differences existing between cell types and time of drug treatments. Our results do not rule out the possibility that by increasing the time of drug exposure, cabergoline may be active in modulating the expression of Bcl-2 or other Bcl-2 family members, and through this mechanism may be effective in reducing ischemia-induced cell death.

In conclusion, our results demonstrate that cabergoline protects SH-SY5Y cells in an in vitro model of ischemia–reperfusion by a mechanism neither receptor-mediated nor associated to Bcl-2 modulation, but probably linked to its anti-oxidant radical scavenger activity. It is noteworthy that the neuroprotective effects of cabergoline were also observed when the drug was added after ischemic insult, during the recovery period. The overall results encourage future studies on cabergoline effects in other in vitro and in vivo models of ischemia and suggest possible use of this drug in the pharmacotherapy of other neurodegenerative diseases, in addition to Parkinson's disease.

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