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Neuroprotective effects of nicergoline in immortalized neurons

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Abstract

We studied the potential neuroprotective action of nicergoline in immortalized hypothalamic GT1-7 cells exposed to agents which deplete levels of reduced glutathione, thus causing oxidative stress and cell death. Treatment with diethylmaleate (1 mM), buthionine sulfoximine (500 μM) or menadione (10–50 μM) caused diffuse GT1-7 cell degeneration, as assessed by using either the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay or the fluorescent dyes fluorescein diacetate and propidium iodide. Pre- and/or co-exposure of the cells to nicergoline significantly prevented diethylmaleate- or buthionine sulfoximine-induced neuronal death, whereas nicergoline was ineffective against menadione-induced toxicity. This effect was concentration-dependent and was mimicked by the classical antioxidants idebenone and vitamin E, and did not depend on interference with protein kinase C. Interestingly, the antineurodegenerative activity of nicergoline and vitamin E or idebenone was not additive, suggesting that these compounds share some intracellular mechanism(s) responsible for their protective effects. In conclusion, the present data indicate that nicergoline has neuroprotective activity, possibly mediated by the antioxidant activity of the molecule, and give support to the potential use of nicergoline in the prevention and therapy of neurodegenerative diseases.

Keywords: Nicergoline; Diethylmaleate; Buthionine sulfoximine; Free radicals; GT1-7 cell

1. Introduction

Nicergoline is an ergoline derivative indicated in the treatment of senile cognitive deterioration syndromes (of either vascular or degenerative origin). Initial pharmacological investigations focused on its vasodilator effects. It is now well established, however, that the primary pharmacological effects of nicergoline in the central nervous system are related not to α-adrenoceptor blockade but to other mechanisms (reviewed in Canonico et al., 1996). Extensive preclinical studies have in fact shown other pharmacological effects of nicergoline, many of which are probably involved in the therapeutic efficacy of the compound in neurodegenerative diseases. Nicergoline can improve cerebral metabolism under conditions of hypoxia and/or ischemia (Le Poncin-Lafitte et al., 1984; Groo et al., 1988; Shintomi, 1991) and increase glucose uptake and utilization (Benzi et al., 1971; Le Poncin-Lafitte et al., 1984), protein and nucleic acid synthesis (Paul and Chandra, 1979) and oxygen utilization (Benzi et al., 1971). It also increases cerebral ATP levels (Benzi et al., 1971) and inhibits lipid peroxidation in brain homogenates of hypoxic rats. In vitro, nicergoline also antagonizes the cyanate-induced inhibition of cytochrome oxidase activity in mouse brain homogenates (Shintomi et al., 1986). These data may be particularly relevant for a possible antidegenerative effect of nicergoline. However, no data are available demonstrating a direct neuroprotective action of nicergoline.

The GT1 cell line, developed by targeted tumorigenesis in transgenic mice (Mellon et al., 1990), is one of the few examples of central nervous system immortalized neurons at present available. For this reason, besides their use for the investigation of hypothalamic secretory activity (reviewed in Stojilkovic et al., 1994), GT1 cells represent a suitable model for the direct study of cytotoxic and cytoprotective mechanisms in vitro (Kane et al., 1993; Ellerby et al., 1996; Sortino and Canonico, 1996). GT1-7 cells exhibit a high sensitivity to oxidative stress induced by

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agents that decrease levels of reduced glutathione (GSH), which, in this cell line, leads to necrotic cell death (Kane et al., 1993). This effect can be prevented by inducing the expression of the protooncogene bcl-2 (Kane et al., 1993), or by specific treatment with growth factors, such as insulin-like growth factor I (IGF-I) and fibroblast growth factor (Sortino and Canonico, 1996), or with classical antioxidants (i.e., idebenone) (Sortino and Canonico, 1996).

Here we report that nicergoline is effective in protecting GT1-7 neurons against oxidative injury induced by different pharmacological tools that cause intracellular GSH depletion.

2. Materials and methods

2.1. Cell cultures

GTl-7 cells (kindly provided by Dr. R.I. Weiner at the University of California at San Francisco) were grown under sterile conditions in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum, 5% horse serum, and antibiotics (all from GIBCO) at 37°C in a humidified atmosphere of 5% CO_2. Cells were routinely passaged using a 0.01% trypsin solution, and only cells between passages 12 and 31 were used in the present study. All experiments were carried out in static cultures of cell monolayers plated on poly-L-lysine (Sigma, St. Louis, MO)-coated plates. For cell viability studies [i.e., MTT assay], GT1-7 cells were seeded into 96-well multiwell plates (Falcon, Oxford, UK) at a density of 5×10^4 cells/well and used for the assay 3–4 days after plating. Microscopical evaluation of cell viability was carried out in GT1-7 cells plated into 35-mm dishes (Nunc, Roskilde, Denmark) at an initial density of 5×10^5 cells/dish.

2.2. Treatments

Nicergoline (10α-methoxy-1,6-dimethylergoline-8β-methanol-5-bromonicotinate) (a gift from Pharmacia & Upjohn, Milano, Italy) was dissolved in distilled water by grinding the compound together with an equimolar weight of tartaric acid crystals. Fresh solutions were used in all experiments. Control cultures were always treated with comparable amounts of tartaric acid. The initial solution of diethylmaleate and phorbol 12-myristate 13-acetate (PMA) (both from Sigma) was made in ethanol. Final solutions were made in culture medium. Menadione (Sigma) was dissolved in dimethylsulfoxide.

2.3. MTT cell viability assay

The MTT assay is based on the conversion of a diphenyltetrazolium salt into blue formazan detectable in an ELISA plate reader. After exposure to various treatments, medium was removed and GT1-7 cells were incubated with the dye solution (MTT, 0.9 mg/ml final concentration; Sigma) for 4 h at 37°C. The solubilization solution, containing 20% sodium dodecyl sulfate, was then added overnight and formazan production was evaluated the day after in a plate reader (absorbance = 560 nm).

2.4. Fluorescein diacetate / propidium iodide staining

After treatment with diethylmaleate for 3 h, GT1-7 cells pretreated or not with nicergoline were incubated with

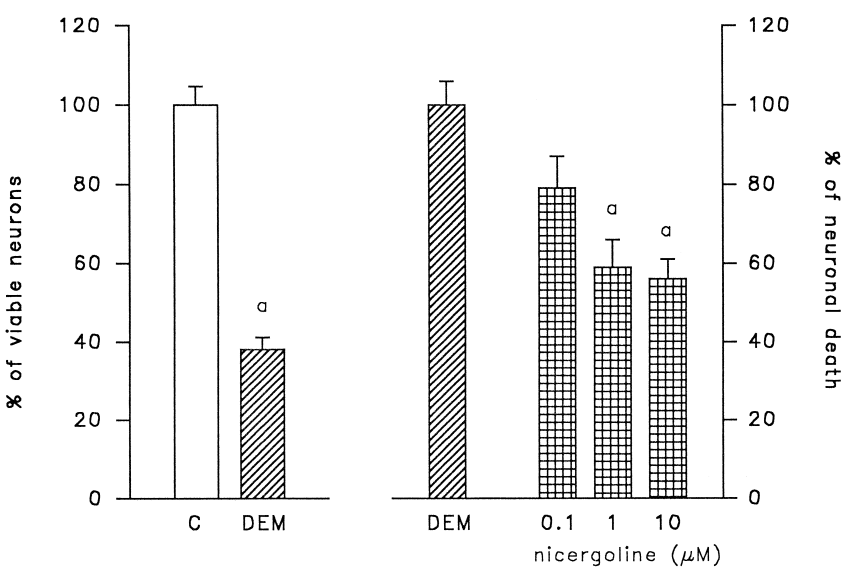

Fig. 1. Concentration–response curve for the neuroprotective effect or nicergoline on diethylmaleate (DEM)-induced neuronal death as assessed by the MTT assay. Cells were exposed to different nicergoline concentrations for 24 h and to 1 mM diethylmaleate for the last 3 h of incubation. The total number of dead cells after diethylmaleate treatment (left panel) is reported as 100% in the right panel. The mean ± S.E.M. of three experiments is shown. [a] $P < 0.01$ vs. control (left panel) and vs. diethylmaleate-treated (right panel) groups.

fluorescein diacetate and propidium iodide (both from Sigma) for 3 min, rapidly washed with phosphate buffer, and examined under a fluorescence microscope. Fluorescein diacetate is hydrolyzed by intracellular esterases of vital neurons to produce a green–yellow fluorescence, whereas propidium iodide interacts only with the DNA of injured cells to yield a red fluorescence. The percentage of viable neurons was counted in four separate fields per culture dish.

2.5. Statistical analysis

Results are expressed as the means ± S.E.M. of three independent experiments performed in quadruplicate. Data were analyzed by Student's *t*-test and, when appropriate, one-way analysis of variance (ANOVA) followed by Newman–Keuls *t*-test for significance.

3. Results

As already reported (Kane et al., 1993; Sortino and Canonico, 1996), incubation of GT1-7 cells with 1 mM diethylmaleate, a compound that binds to the free sulfhydryl groups of GSH (Ku and Billings, 1986), produced diffuse neuronal death (30–40% of viable neurons). Pretreatment of GT1-7 cells for 24 h with increasing concentrations of nicergoline preserved neuronal viability (as assessed by the MTT assay), with a significant effect being detected at concentrations of 1–10 μM (Fig. 1).

Treatment of GT1-7 cells for 24 h with buthionine sulfoximine (500 μM), a specific inhibitor of GSH synthesis (Griffith, 1982), reduced neuronal viability by about 55–60%. This effect was present only after the cells were allowed to recover for 24 h, whereas no significant neuronal death was observed soon after buthionine sulfoximine removal. Co-treatment of GT1-7 cells with nicergoline during the 24-h exposure to buthionine sulfoximine and during the 24-h recovery period resulted in a significant, concentration-dependent prevention of neuronal death that ranged between 15–20% and 35–45% at nicergoline concentrations of 0.1 and 10 μM, respectively (Fig. 2).

A comparable extent of neuroprotection by nicergoline was also observed when diethylmaleate- or buthionine

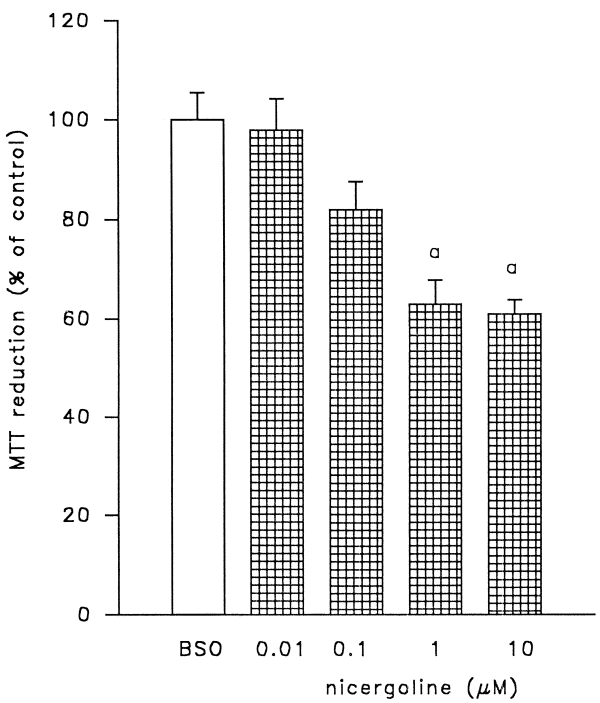

Fig. 2. Protective effect of nicergoline on buthionine sulfoximine (BSO)-induced neuronal death. The number of viable GT1-7 cells as measured by the MTT assay was reduced by a 24-h treatment with buthionine sulfoximine by about 55–60%. Neuronal death induced by buthionine sulfoximine (that is reported as 100% in the figure) was partially prevented by co-treatment of GT1-7 cells with nicergoline during the exposure to buthionine sulfoximine and during the 24-h recovery period. Results are expressed as the means ± S.E.M. of three independent experiments performed in quadruplicate. [a] $P < 0.01$ vs. buthionine sulfoximine-treated group.

Table 1
Nicergoline protects GT1-7 cells from diethylmaleate- or buthionine sulfoximine-induced death as assessed by staining with the fluorescent dyes fluorescein diacetate and propidium iodide

	% viable neurons
Experiment I	
Vehicle	95 ± 2.1
Nicergoline (10 μM)	93 ± 2.9
Diethylmaleate (1 mM)	35 ± 4.1[a]
Nicergoline + diethylmaleate	57 ± 3.3[a,b]
Experiment II	
Vehicle	93 ± 2.5
Nicergoline (10 μM)	94 ± 2.7
Buthionine sulfoximine (500 μM)	42 ± 2.6[a]
Nicergoline + buthionine sulfoximine	60 ± 3.1[a,b]

[a] $P < 0.01$ vs. vehicle and nicergoline alone.
[b] $P < 0.01$ vs. diethylmaleate (Experiment I) or buthionine sulfoximine (Experiment II) (one-way ANOVA followed by Newman–Keuls test for significance).

Table 2
Nicergoline does not modify menadione-induced GT1-7 cell death

	MTT reduction (% of control)
Vehicle	100 ± 3.1
Menadione (10 μM)	45 ± 2.8[a]
Menadione (50 μM)	17 ± 3.2[a]
Nicergoline (10 μM)	98 ± 2.1
Nicergoline + 10 μM menadione	48 ± 3.3[a]
Nicergoline + 50 μM menadione	15 ± 3.6[a]

GT1-7 cells pretreated for 24 h with nicergoline were challenged with 10 or 50 μM menadione for 2 h. Data represent the means ± S.E.M. of three experiments performed in quadruplicate.
[a] $P < 0.01$ if compared to vehicle-treated group.

Table 3
Protective effect of nicergoline, idebenone and vitamin E on diethylmaleate-induced GT1-7 cell death

	MTT reduction (% of control)
Vehicle	100 ± 2.5
Diethylmaleate (1 mM)	39 ± 3.2[a]
Nicergoline (10 μM)	96 ± 2.2
Nicergoline + diethylmaleate	64 ± 3.1[a,b]
Idebenone (1 μM)	97 ± 1.5
Idebenone + diethylmaleate	69 ± 4.1[a,b]
Vitamin E (1 μM)	98 ± 2
Vitamin E + diethylmaleate	70 ± 3.1[a,b]
Nicergoline + idebenone + diethylmaleate	68 ± 2.4[a,b]
Nicergoline + vitamin E + diethylmaleate	73 ± 2.9[a,b]

Cells were pre-exposed to nicergoline, idebenone and/or vitamin E for 24 h before the 3 h of incubation with diethylmaleate. Viable cells were assessed by using the MTT cytotoxicity assay. Values represent the means ± S.E.M. of three independent studies performed in quadruplicate.
[a] $P < 0.01$ vs. vehicle.
[b] $P < 0.01$ vs. diethylmaleate-treated group.

sulfoximine-induced neuronal death was evaluated by assessing the number of GT1-7 cells stained with fluorescein diacetate and propidium iodide (Table 1). By contrast, nicergoline treatment did not affect menadione-induced neurotoxicity. Menadione, 10 and 50 μM (2 h treatment), produced extensive GT1-7 cell death [(80–90% neuronal death at 50 μM menadione)]. Preincubation of GT1-7 cells for 24 h (Table 2) or co-incubation for 2 h (not shown) with 10 μM nicergoline did not have a significant effect in preventing menadione-induced neuronal death.

The protective effect of nicergoline in GT1-7 cells against diethylmaleate-induced neurotoxicity was similar to that induced by the antioxidants vitamin E or idebenone (which acts both as a radical scavenger and an electron-trapping agent) (Miyamoto et al., 1989; Bruno et al., 1993) (Table 3). Interestingly, co-treatment of GT1-7 cells with maximally effective concentrations of nicergoline (10 μM) and vitamin E (1 μM) or idebenone (1 μM) did not modify significantly the effect produced by each single treatment (Table 3).

The effect of nicergoline does not appear to be mediated by activation of protein kinase C. Treatment of GT1-7 cells with PMA (100 nM) (added to the incubation medium together with—not shown—or 6 h prior to the exposure to 1 mM diethylmaleate) did not modify cell viability either under basal conditions or in the presence of the toxic agent (Table 4). In addition, the protein kinase C inhibitor, staurosporine, did not antagonize the prevention by nicergoline of diethylmaleate-induced GT1-7 cell death (Table 4).

4. Discussion

Increased exposure to free radicals is recognized to represent a major cause of neuronal cell death (reviewed in Perez-Polo, 1991; Choi, 1992). Thus, one of the pharmacological strategies currently used to slow down the progression of neurodegenerative diseases is to antagonize oxygen free radical production and/or action (Sano et al., 1997). We show here that nicergoline, a drug with complex, multifaceted actions (Canonico et al., 1996), is effective in preventing neuronal cell death induced by oxidative damage.

Exposure of GT1-7 cells to 1 mM diethylmaleate, which in this cell line dramatically reduces the intracellular GSH concentration (Kane et al., 1993), produced extensive cell death (55–60%) within a few hours of incubation. A concomitant treatment with nicergoline produced a concentration-dependent enhancement of cell survival. This protective effect of nicergoline was also present when neu-

Table 4
Activation of protein kinase C is not involved in the neuroprotective effect of nicergoline on diethylmaleate-induced neuronal death as assessed by the MTT assay

	MTT reduction (% of viable cells)
Vehicle	100 ± 1.8
Diethylmaleate (1 mM)	42 ± 3.7[a]
Nicergoline (10 μM)	96 ± 2.1
Nicergoline + diethylmaleate	61 ± 2.4[a,b]
PMA (100 nM)	97 ± 2.3
PMA + diethylmaleate	39 ± 2.8[a]
Nicergoline + PMA + diethylmaleate	59 ± 1.8[a,b]
Staurosporine (10 nM)	98 ± 3.6
Staurosporine + nicergoline	97 ± 3.3
Staurosporine + diethylmaleate	44 ± 2.8[a]
Staurosporine + nicergoline + diethylmaleate	62 ± 4.1[a,b]

Cells were exposed to nicergoline and/or staurosporine for 24 h and to 1 mM diethylmaleate for the last 3 h of incubation. PMA was added for 6 h prior to diethylmaleate.
[a] $P < 0.01$ vs. vehicle.
[b] $P < 0.01$ vs. diethylmaleate-treated group.

ronal death was induced in GT1-7 cells by exposure for 24 h to buthionine sulfoximine, a treatment that causes a massive decrease in the intracellular GSH concentration (Kane et al., 1993). Interestingly, buthionine sulfoximine-induced neuronal death was detectable only 24 h after buthionine sulfoximine removal due to the increased oxygen/free radical formation during the reoxygenation period (Nutter et al., 1991; Myers et al., 1995).

Conversely, nicergoline did not prevent the massive neuronal death induced by menadione in GT1-7 cells. This lack of effect is not surprising, because menadione acts by a wide variety of pharmacological actions (increased consumption of molecular oxygen and generation of superoxide, GSH depletion, oxidation of protein sulfhydryl groups in cytoskeletal proteins, decreased mitochondrial and cellular ATP pools, effects on calcium homeostasis, and single-strand DNA breaks) that may all contribute to cell death (Nutter et al., 1991). Our results are in line with those of earlier experiments, in which neither IGF-I nor GSH itself was able to modify the toxic effect of menadione (Sortino and Canonico, 1996).

Interestingly, the protective effect exerted by nicergoline against GSH depletion-caused neuronal damage was similar to that produced by the potent radical scavengers idebenone or vitamin E, which prevent neuronal death in different neuronal systems (Miyamoto et al., 1989; Bruno et al., 1993). The lack of additivity between nicergoline and idebenone or vitamin E suggests that these compounds interfere with the same intracellular events to produce a protective effect. In support of this possibility, nicergoline has been reported to inhibit lipid peroxidation in brain homogenates of hypoxic rats (Shintomi et al., 1986), and to antagonize in vitro the cyanate-induced inhibition of cytochrome oxidase activity in mouse brain homogenate (Shintomi et al., 1986).

The ability of nicergoline to prevent diethylmaleate-induced GT1-7 cell death does not seem to be mediated by interference with protein kinase C. Nicergoline has been reported to induce the translocation of the Ca^{2+} dependent protein kinase C isoenzymes in vitro and in vivo in rat hippocampus and striatum (Caputi et al., 1998). However, in GT1-7 cells the protein kinase C activator PMA was unable to modify cell viability either under basal conditions or in the presence of diethylmaleate and/or nicergoline. In addition, the protein kinase C inhibitor staurosporine did not affect the protective effect of nicergoline against diethylmaleate-induced GT1-7 cell death.

In conclusion, our data indicate a neuroprotective action of nicergoline in immortalized neuronal cells exposed to compounds that induce cell death by depleting endogenous GSH levels. This protective activity against free radical-mediated damage (probably due to the inhibitory effect of nicergoline on lipid peroxidation and/or to its scavenging action), which occurs at clinically relevant concentrations, gives novel insight into the understanding of the possible mechanisms involved in the therapeutic efficacy of nicergoline in neurodegenerative diseases, including brain aging and dementia.

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