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SHORT COMMUNICATION

Protective Effect of L-Deprenyl against Apoptosis Induced by Okadaic Acid in Cultured Neuronal Cells

Tiina Suuronen, Petri Kolehmainen* and Antero Salminen*†‡*

*Department of Neuroscience and Neurology, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, and †Department of Neurology, Kuopio University Hospital, Kuopio, Finland

ABSTRACT. L-Deprenyl, an irreversible MAO-B (monoamine oxidase B, EC 1.4.3.4) inhibitor, is used for the treatment of Parkinson's disease and to delay the progression of Alzheimer's disease. L-Deprenyl also exhibits protective effects against neuronal apoptosis which are independent of its ability to inhibit MAO-B. The purpose of this study was to compare the antiapoptotic efficacy of L-deprenyl against different types of apoptotic inducers in three neuronal cell culture models. The level of apoptosis was quantified by measuring the activation of caspase-3 enzyme, which is the main apoptotic executioner in neuronal cells. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and LDH (lactate dehydrogenase, EC 1.1.1.27) assays were used to demonstrate the cytotoxic response of apoptotic treatments. Our results showed that okadaic acid, an inhibitor of protein phosphatase 1 and 2A, induced a prominent increase in caspase-3 activity both in cultured hippocampal and cerebellar granule neurons as well as in Neuro-2a neuroblastoma cells. Interestingly, L-deprenyl offered a significant protection against the apoptotic response induced by okadaic acid in all three neuronal models. The best protection appeared at the concentration level of 10^{-9} M. L-Deprenyl also provided a protection against apoptosis after AraC (cytosine β-D-arabinoside) treatment in hippocampal neurons and Neuro-2a cells and after etoposide treatment in Neuro-2a cells. However, L-deprenyl did not offer any protection against apoptosis caused by serum withdrawal or potassium deprivation. Okadaic acid treatment *in vivo* is known to induce an Alzheimer's type of hyperphosphorylation of tau protein, formation of β-amyloid plaques, and a severe memory impairment. Our results show that the okadaic acid model provides a promising tool to study the molecular basis of Alzheimer's disease and to screen the neuroprotective capacity of L-deprenyl derivatives. BIOCHEM PHARMACOL **59**;12:1589–1595, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. selegiline; caspase-3; etoposide; serum deprivation; cytosine β-D-arabinoside; Alzheimer's disease

L-Deprenyl (selegiline), an irreversible inhibitor of MAO-B,§ has been used in the treatment of Parkinson's disease [1, 2]. L-Deprenyl treatment also delays the progression of Alzheimer's disease [3]. Recent studies have shown that L-deprenyl can protect against neuronal apoptosis, both in cultured neurons and in animal models [for reviews see 4–6]. For instance, L-deprenyl provides neuroprotection against growth factor withdrawal in PC12 cells [7], oxidative stress in mesencephalic neurons [8], and the genotoxic compound, AraC, in cerebellar granule neurons [9], and against axotomy-induced motoneuronal degeneration [10] and delayed neuronal death in hippocampus after global ischemia [11]. The molecular basis of the neuroprotection induced by L-deprenyl is still unknown. However, it seems that the neuroprotective effect of L-deprenyl does not require MAO-B inhibition, but rather is dependent on gene expression [4, 12]. Tatton *et al.* [12] have shown that L-deprenyl induces selective changes in gene expression and protein synthesis that are accompanied by a decrease in responsiveness to apoptosis. L-Deprenyl, for instance, up-regulates the synthesis of the antiapoptotic proteins Bcl-2 and the superoxide dismutases SOD1 and SOD2.

The purpose of this study was to determine whether the antiapoptotic property of L-deprenyl is selective with respect to different kinds of apoptotic inducers and different types of cultured neuronal cells. We show here that L-deprenyl induced a general protective effect against okadaic acid -induced apoptosis, this being seen in cultured hippocampal and cerebellar granule neurons, as well as in Neuro-2a neuroblastoma cells.

MATERIALS AND METHODS

Chemicals

L-Deprenyl (selegiline) was provided by Orion-Farmos. Okadaic acid and etoposide were purchased from Calbiochem and AraC from Sigma Chemical Co. The fluorogenic substrate of caspase-3, Ac-DEVD-AMC, was obtained from

‡ Corresponding author: Dr. Antero Salminen, Department of Neuroscience and Neurology, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland. Tel. +358 17162015; FAX +358 17162048; E-mail: antero.salminen@uku.fi

§ *Abbreviations:* LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Ac-DEVD-AMC, *N*-acetyl-aspartate-glutamate-valine-aspartate-7-amino-4-methyl coumarin; MAO-B, monoamine oxidase B; AraC, cytosine β-D-arabinoside.

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Pharmingen. MTT and Hoechst 33258 were from Sigma Chemical Co. The LDH cytotoxicity kit was purchased from Promega. Dulbecco's modified Eagle's medium and Neurobasal cell culture media, fetal bovine serum, and B27 supplement were obtained from GIBCO Life Technologies.

Cell Culture

Mouse neuroblastoma Neuro-2a cells were obtained from American Type Culture Collection (ATCC) and cultured as described earlier in detail [13]. Hippocampal neurons were isolated from 17-day-old Wistar rat embryos and cultured as described by Brewer *et al.* [14]. Cerebellar granule cells were isolated from the cerebella of 7-day-old Wistar rats and cultured essentially as described by Schousboe *et al.* [15]. Cultured primary neurons were exposed to apoptotic inducers after the differentiation phase of 7–10 days and Neuro-2a cells 24 hr after plating. The percent of astrocytes (stained with antibody against glial fibrillary acidic protein) was 8.5% in hippocampal cultures and 1.7% in cerebellar granule cells (data not shown).

Induction of Apoptosis

Several different apoptotic inducers were studied to compare the antiapoptotic effects of L-deprenyl both in rat primary neurons and Neuro-2a neuroblastoma cultures. The final concentration of okadaic acid used to induce a prominent apoptosis varied between 10 and 50 nM in different cell cultures (see Figs. 1–3). Etoposide was used at the final concentration of either 5 or 10 μM. The AraC concentrations studied varied between 1 and 100 μM. Serum withdrawal experiments were performed only on Neuro-2a cells. In cerebellar granule cells, apoptosis was also induced by potassium deprivation. All these apoptosis models have been described earlier in detail either by us [13, 16] or by others [7, 9, 17–20].

Caspase-3 Activity Assays

The level of caspase-3 activity is a good quantitative parameter for neuronal apoptosis [13, 17, 21]. Accordingly, we used this enzyme in our recent apoptotic studies with both primary neurons and Neuro-2a neuroblastoma cells [13, 16]. The activities of caspase-3 were assayed from the cytosolic extracts using a fluorogenic substrate Ac-DEVD-AMC from Pharmingen. Assays were performed according to the protocol of the manufacturer. Hoechst 33258 nuclear staining was used in some experiments to calculate apoptotic cells containing nucleoid bodies. Staining was performed as described earlier [13]. The protein concentration in cytosol was assayed using the kit from Bio-Rad and their protocol for microassays.

MTT and LDH Cytotoxicity Assays

MTT viability [22, 23] and LDH cytotoxicity [23] assays were used to calculate the protective effects of L-deprenyl against cytotoxicity induced by apoptotic treatments. The MTT assay with improved sensitivity [22] was used to measure changes in mitochondrial oxidative capacity in neuronal cultures after drug treatments. LDH leakage to the medium was assayed with the cytotoxicity kit obtained from Promega.

Statistical Methods

All values were expressed as means ± SD. The difference between control and deprenyl-treated rats was analyzed using Student's *t*-test for unpaired values. Equality of variance was analyzed by Levene's test.

RESULTS

Cytotoxicity of L-Deprenyl to Cultured Neuronal Cells

Figure 1 shows the cytotoxicity data of L-deprenyl treatments concerning LDH release (Fig. 1, A and D), MTT staining (Fig. 1, B and D), and caspase-3 activities (Fig. 1, C and D) in hippocampal cultures and Neuro-2a cells. L-Deprenyl treatment itself did not cause any cytotoxic effects up to the highest concentration used (10^{-5} M).

L-Deprenyl-Induced Protection in Cultured Hippocampal Neurons

The antiapoptotic effects of L-deprenyl were studied in cultured rat hippocampal neurons using okadaic acid, etoposide, and AraC as apoptotic inducers. Earlier studies have shown that all these treatments induce apoptosis in cultured primary neurons [9, 19, 20, 24]. Figure 2A shows that okadaic acid treatment induced a prominent increase in the activity of caspase-3, much higher than that induced by AraC (Fig. 2E) or etoposide (Fig. 2F) treatments. Interestingly, L-deprenyl treatment resulted in a significant protection against okadaic acid -induced apoptotic response at final concentration levels from 10^{-11} to 10^{-7} M, but not at the concentration of 10^{-5} M. The apoptotic response of okadaic acid was also verified by calculating apoptotic cells containing nucleoid bodies by Hoechst staining (Fig. 2B). The effect of L-deprenyl was statistically significant at the level of 10^{-11} M. However, the percent increase in Hoechst-positive cells (Fig. 2B) was small after 24 hr, which shows that apoptosis had not yet proceeded to the nuclear fragmentation level, although caspase-3 was already at the high level possibly because caspase-3 may play a key role in nuclear fragmentation [e.g.21]. In the case of AraC-induced apoptosis, an antiapoptotic response of L-deprenyl appeared only at the lowest concentration used, 10^{-11} M, but not at the higher concentrations (Fig. 2E). L-Deprenyl had no ability to prevent apoptosis after etoposide treatment (Fig. 2F).

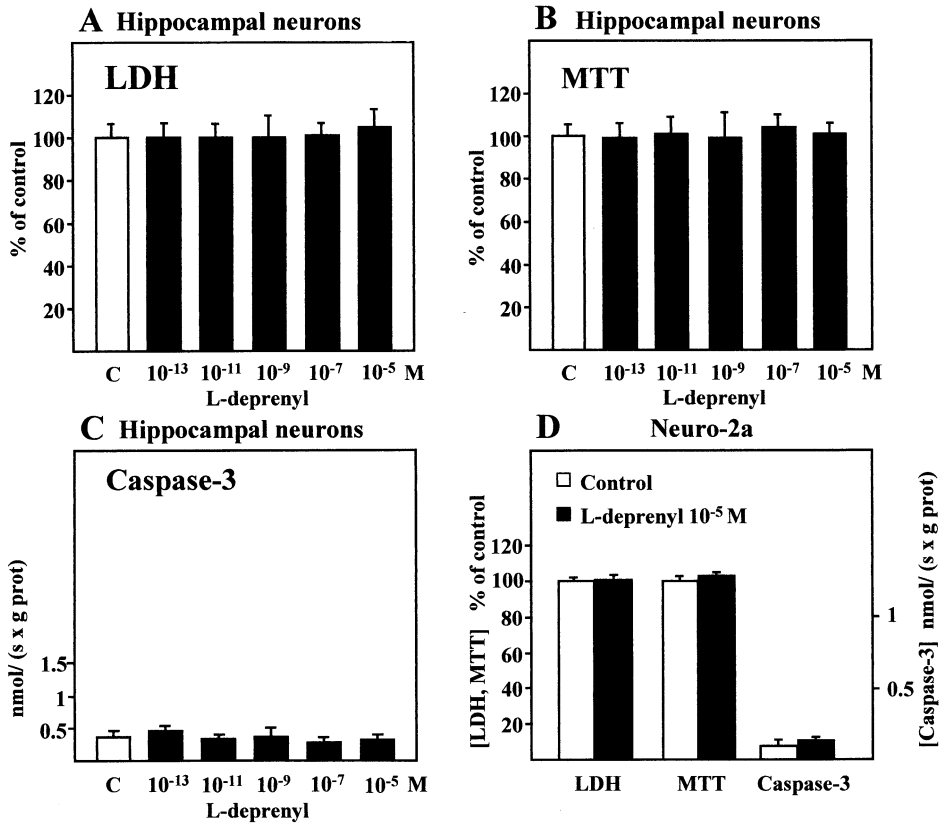

FIG. 1. Cytotoxicity of L-deprenyl in cultured neuronal cells. LDH release (A), MTT staining (B), and caspase-3 activities (C) in cultured hippocampal neurons (7-day-old culture) after 24 hr exposure to different concentrations of L-deprenyl. Panel D shows LDH release, MTT staining, and caspase-3 activities in Neuro-2a cells after 48 -hr exposure to 10^{-5} M L-deprenyl. Values for LDH and MTT assays are expressed as % control value and for caspase-3 as nmol AMC per second and gram of cytoplasmic protein. Values are mean ± SD (N = 4–6). There were no statistically significant differences between "L-deprenyl -treated" and "control" groups ($P > 0.05$).

The cytotoxic effects of okadaic acid were studied using the MTT viability and LDH cytotoxicity assays. Okadaic acid induced a significant decrease in MTT activity which was prevented by 10^{-9} M L-deprenyl treatment (Fig. 2C). The final concentration of 10^{-9} M also induced the best protection with the caspase-3 assay (Fig. 2A). L-Deprenyl treatment did not reduce the LDH leakage induced by okadaic acid (Fig. 2D). On the contrary, the highest concentrations of L-deprenyl enhanced LDH leakage (Fig. 2D).

L-Deprenyl-Induced Protection in Cultured Cerebellar Granule Cells

The effect of L-deprenyl against apoptosis was also studied in cultured cerebellar granule cells. Our preliminary experiments showed that in cultured cerebellar granule cells, the optimum concentration of okadaic acid was found to be higher than in hippocampal neurons. A final concentration of 50 nM was used in the apoptosis assays. Figure 3A shows that L-deprenyl treatment significantly decreased the okadaic acid-induced response as assessed by caspase-3 activities. LDH leakage was also slightly reduced in L-deprenyl-treated cultures (Fig. 3C). Potassium deficiency induces a well-characterized apoptosis in cerebellar granule cells [16, 21]. Figure 3B shows a prominent increase in the activity of caspase-3. Interestingly, L-deprenyl did not protect against apoptosis induced by potassium deficiency in cerebellar granule cells.

L-Deprenyl-Induced Protection in Neuro-2a Neuroblastoma Cells

We recently characterized several apoptosis models using Neuro-2a cells [13, 16]. In this study, we used four different models to induce apoptosis (Fig. 4). All the apoptotic agents used (okadaic acid, AraC, etoposide, and serum deprivation) induced a prominent increase in the activity of caspase-3. Interestingly, L-deprenyl provided protection against okadaic acid- and AraC-induced apoptotic responses (Fig. 4, A and B) but not against apoptosis induced by 5 μM etoposide (Fig. 4C) or serum deprivation (Fig. 4D). As in the case of hippocampal neurons (Fig. 2, A and E), the highest concentrations of L-deprenyl did not provide any protection.

Antiapoptotic Effect of L-Deprenyl at Different Concentrations of Okadaic Acid and Etoposide

Next, we studied whether the antiapoptotic effect of L-deprenyl is dependent on the concentration of apoptotic inducers okadaic acid and etoposide. Figure 5 shows that the level of caspase-3 response, a parameter of apoptosis level, was dependent on the concentration of okadaic acid and etoposide both in hippocampal neurons and Neuro-2a cells. Figure 5 shows that the deprenyl-induced (conc. 10^{-9} M) protection appeared only at the highest concentration of okadaic acid used, but not at the lower level where the apoptotic response in caspase-3 activity was milder. Inter-

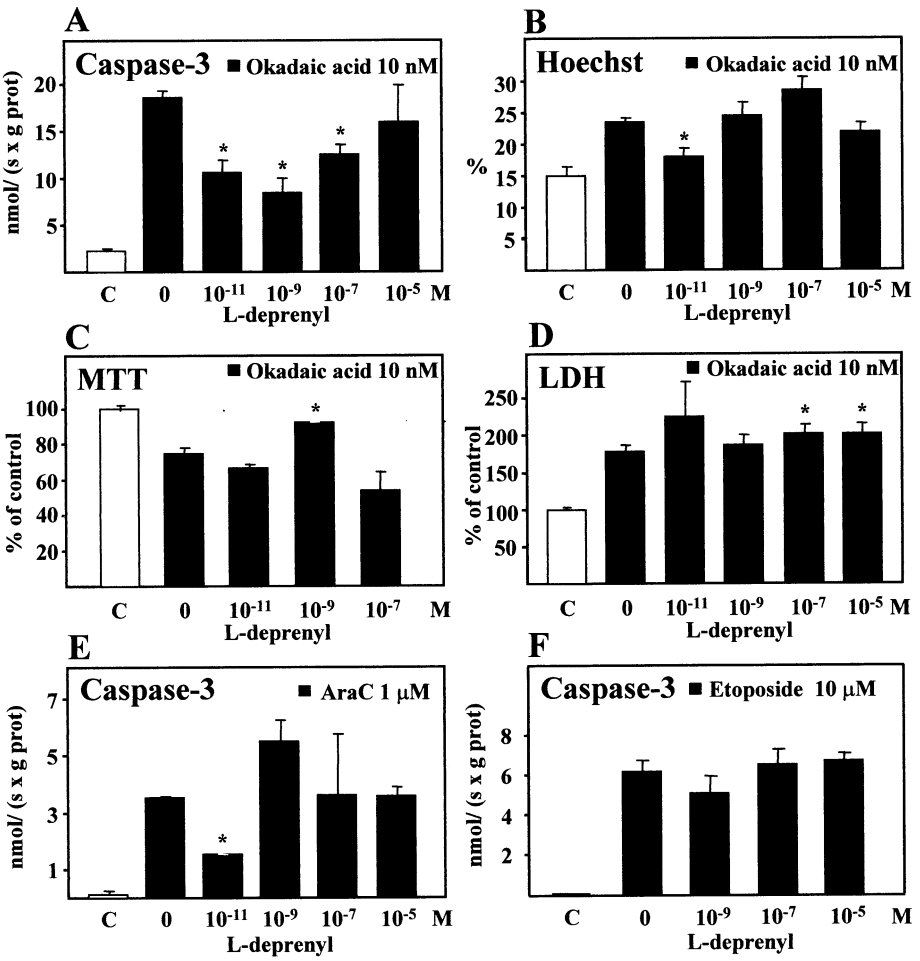

FIG. 2. L-Deprenyl protects against neuronal apoptosis induced by okadaic acid in cultured hipppocampal neurons. Apoptosis was induced in hippocampal neurons by treatment for 24 hr with 10 nM okadaic acid (A–D) and 1 μM AraC (E) or for 12 hr with 10 μM etoposide (F). Values of caspase-3 activity are expressed as nmol AMC per second and gram of cytoplasmic protein. Values of MTT and LDH release are percent of the control. Values of Hoechst–staining show the number of apoptotic cells (%). All values are means ± SD (N = 3–4). Statistical significance between "okadaic acid plus L-deprenyl -treated" groups is compared to "okadaic acid alone" group: *$P < 0.05$. Statistical comparison between "okadaic acid plus L-deprenyl" and "control with L-deprenyl" (C) showed a statistically significant difference ($P < 0.01$) for all comparisons.

estingly, in Neuro-2a cells, L-deprenyl (conc. 10^{-9} M) caused a protection against etoposide-induced apoptosis at the 2-μM level, but not at the 5-μM level (Fig. 5B).

DISCUSSION

Recent studies have shown that L-deprenyl can protect against neuronal apoptosis, both in cultured neurons and in animal models [for reviews see 4–6]. The mechanism of neuroprotection induced by L-deprenyl still remains to be clarified. However, several approaches have shown that neuroprotection is independent of MAO-B inhibition [4]. In the present study, we exposed both primary neurons and neuroblastoma cells to several different kinds of apoptotic inducers to determine whether L-deprenyl can offer a selective or ubiquitous antiapoptotic response. Interestingly, L-deprenyl was found to be most effective against the neuronal apoptosis induced by okadaic acid treatment. Furthermore, the response was very similar in primary hippocampal and cerebellar granule neurons and in Neuro-2a neuroblastoma cells. However, L-deprenyl did not provide any protection against serum withdrawal, a typical apoptosis inducer.

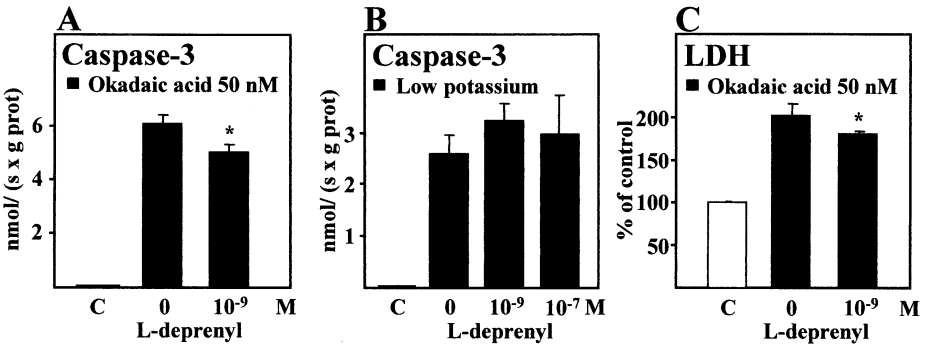

FIG. 3. L-Deprenyl protects against neuronal apoptosis induced by okadaic acid in cultured cerebellar granule neurons. Apoptosis was induced in cerebellar granule neurons by treatment for 26 hr with 50 nM okadaic acid (A and C) and for 48 hr with a low potassium (5 mM) medium (B). Values of caspase-3 activity and LDH release are expressed as in Fig. 2. All values are means ± SD (N = 3–4). Statistical significance (see Fig. 2): *$P < 0.05$.

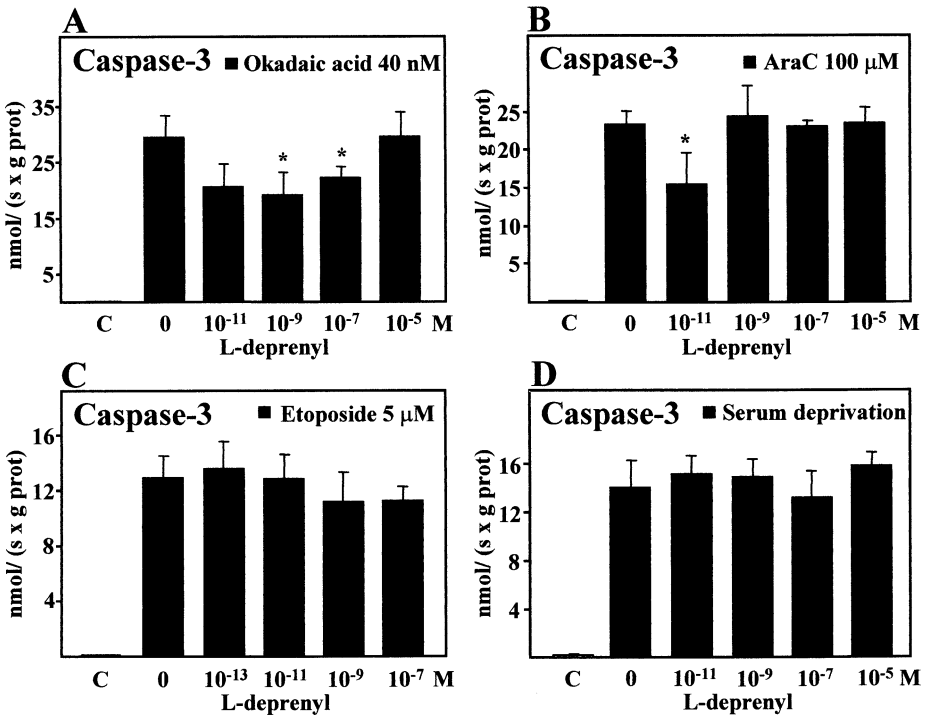

FIG. 4. L-Deprenyl protects against neuronal apoptosis induced by okadaic acid in cultured Neuro-2a neuroblastoma cells. Apoptosis was induced in Neuro-2a cells by treatment for 48 hr with 40 nM okadaic acid (A), 100 μM AraC (B), 5 μM etoposide (C), or serum-deficient medium (D). Values of caspase-3 activity are expressed as in Fig. 2. All values are means ± SD (N = 3–4). Statistical significance (see Fig. 2): *P < 0.05.

Okadaic acid is a well-known inhibitor of protein phosphatases 1 and 2A [25]. Inhibition of these important phosphatases induces hyperphosphorylation of several cellular proteins which affect, for instance, the regulation of gene expression and cellular metabolism [25, 26]. Interestingly, okadaic acid infusion to rat brain ventricles induces the formation of β-amyloid plaques and an Alzheimer's type hyperphosphorylation of tau protein [27]. Furthermore, okadaic acid treatment induces an apoptotic cell death in cultured neuronal cells [e.g. 20, 24]. We have also observed that okadaic acid treatment induces an activation of caspase-3 enzyme, the main executing caspase in neurons, and a characteristic apoptotic neuronal death in cultured neuronal cells [16]. From this viewpoint, it is possible that L-deprenyl treatment could delay the progression of Alzheimer's disease [3].

Apoptosis can be induced by multiple mechanisms [28, 29]. Our observation that L-deprenyl protects against apoptosis induced by okadaic acid provides an interesting tool to dissect the mechanism of the L-deprenyl-induced antiapoptotic response. Haldar *et al.* [30] have shown that okadaic acid treatment induces the phosphorylation of Bcl-2 protein, which leads to the inactivation of this critical antiapoptotic protein. Riordan *et al.* [31] showed that okadaic acid treatment can destabilize Bcl-2 mRNA and hence down-regulate the expression of Bcl-2. Furthermore, okadaic acid -induced apoptosis can be inhibited by overexpression of Bcl-2 and Bcl-X_L proteins [32]. These observations are interesting with respect to the observation of Wadia *et al.* [7], who showed that L-deprenyl protects against nerve growth factor withdrawal -induced apoptosis in PC12 cells by preventing the decrease in mitochondrial membrane potential ($\Delta\Psi_M$). One of the functions of Bcl-2 is to regulate mitochondrial permeability transition [33]

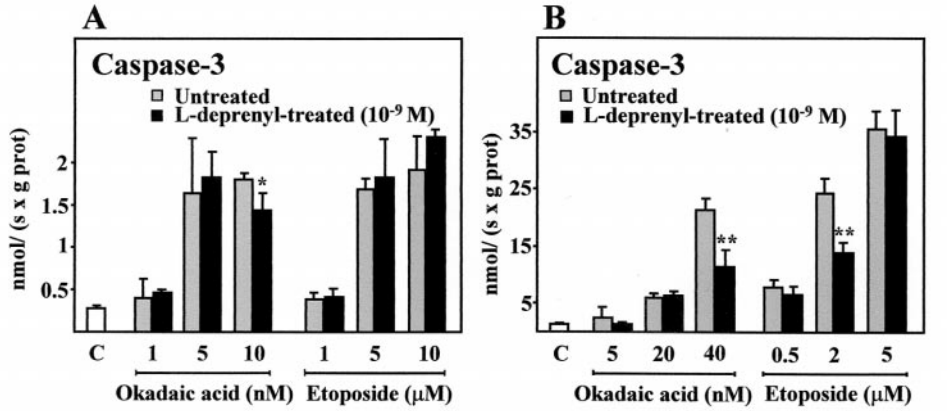

FIG. 5. Antiapoptotic effect of L-deprenyl at different concentrations of okadaic acid and etoposide. Panels A and B show the data in cultured hippocampal neurons and Neuro-2a cells, respectively. Values are means ± SD (N = 3–4). Statistical signficance (see Fig. 2): *P < 0.05, ** P < 0.01.

and the release of mitochondrial proapoptotic molecules [28, 33].

Interestingly, antiapoptotic capacity induced by L-deprenyl after okadaic acid treatment showed a bell-shaped profile both in hippocampal and Neuro-2a neuroblastoma cells. L-Deprenyl provided the strongest protection at 10^{-9} M concentration, whereas no protection was present at 10^{-5} M concentration. Tatton *et al.* [34] reported a very similar bell-shaped profile of PC12 cells after nerve growth factor withdrawal. This effect was not due to the toxicity of L-deprenyl or its metabolites, because the 10^{-5} M concentration of L-deprenyl did not affect any of the cytotoxic parameters. A bell-shaped protection profile could appear if L-deprenyl affected the function of gene family members which have either antiapoptotic or proapoptotic properties but are differently sensitive to L-deprenyl. Bcl-2 family of proteins forms such a family consisting as it does of anti- and proapoptotic members [35].

The okadaic acid model provides an interesting tool for studying the antiapoptotic mechanism of L-deprenyl and for screening the antiapoptotic capacity of L-deprenyl derivatives. However, it seems that the intensity level of apoptotic insults should be carefully tested, since the protective capacity of L-deprenyl varies with the intensity of apoptotic insult. This may be due to the mechanisms behind the bell-shaped protection profile.

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