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European Journal of Pharmacology 430 (2001) 235–241

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(−)-D-Deprenyl attenuates apoptosis in experimental brain ischaemia

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Received 19 April 2001; received in revised form 28 August 2001; accepted 30 August 2001

Abstract

(−)-D-Deprenyl protects neurons from oxidative damage and helps to maintain the mitochondrial membrane potential by influencing intracellular anti-apoptotic oncoproteins, such as Bcl-2. The cellular rescue in the penumbra region by (−)-D-deprenyl administration was examined after permanent middle cerebral artery occlusion in rats. (−)-D-Deprenyl was given continuously following permanent middle cerebral artery occlusion. Two days later, the rats were killed and their infarct volumes were determined. Coronal brain sections were stained with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate deoxyribonucleic acid (DNA) nick-end labelling (TUNEL) and caspase-3, TUNEL and anti-neuronal nuclei (NeuN) double labelling. Neural plasticity was characterized by growth-associated protein-43 (GAP-43) immunohistochemistry. A 1000 × 1000-μm region was sampled at both cortical margins of the TUNEL-positive area at its borders. The numbers of TUNEL-labelled and TUNEL–caspase-3-labelled cells decreased significantly. (−)-D-Deprenyl treatment increased the number of GAP-43-positive cells. We conclude that (−)-D-deprenyl reduced the number of affected cells and induced neuronal plasticity.

Keywords: (−)-D-Deprenyl; Middle cerebral artery occlusion; Apoptosis; TUNEL

1. Introduction

Ischaemic insult in the brain results in neuronal loss and cell necrosis and/or apoptosis (reviewed by Lipton, 1999; Sharp et al., 2000; Graham and Chen, 2001). Therefore, the primary aim of any therapeutic intervention is to reduce the volume of brain damage and thus to minimize neurological impairment. A further therapeutic goal might be the activation of neural plasticity in the peri-infarct rim, which may promote rehabilitation (Cramer and Chopp, 2000; Hortobágyi et al., 1998).

(−)-D-Deprenyl, the *N*-propargyl analogue of (−) methamphetamine, is a widely known monoamine oxidase-B inhibitor (Knoll, 1998; Magyar et al., 1998; Mahmood, 1997) with well-documented antiparkinson effects. Clinical benefits of (−)-D-deprenyl via different mechanisms in neurodegenerative diseases have been demonstrated as reviewed by Tatton et al. (1996). It might protect neurons from oxidative damage and death by reducing the production of H_2O_2 (Cohen and Spina, 1989). It stereospecifically reduces the number of CA1 hippocampal neurons that die after ischaemia and hypoxia (Barber et al., 1993), and it can influence the growth of some glia and neuronal populations (Sensenbrenner et al., 1997; Shankaranarayana et al., 1999; Lakshmana et al., 1998; Holm et al., 2001; Semkova et al., 1996).

(−)-D-Deprenyl has well-characterized antiapoptotic properties (Tatton et al., 1996; Paterson and Tatton, 1998). (−)-D-Deprenyl ameliorates ischaemic stroke states at multiple levels (Semkova et al., 1996; Tang et al., 1998; Kitani et al., 1996, 1999; Thomas et al., 1997; Thiffault et al., 1997; Wadia et al., 1998). In a permanent middle cerebral artery occlusion model of stroke, following a 7-day treatment, (−)-D-deprenyl significantly reduced the lesion size (Ekblom et al., 1998; Semkova et al., 1996); however, the mechanism and the exact extent of the protective action of (−)-D-deprenyl have been evaluated only in in vitro models.

It has been demonstrated in a PC12 cell culture system that (−)-D-deprenyl upregulates Bcl-2 and reduces the number of apoptotic cells (Tatton et al., 1994). Bcl-2 expression helps to keep mitochondria functionally intact (Kluck, 1997). Bcl-2 is an antiapoptotic protein that could interfere with the proapoptotic proteins of the same protein family (e.g. Bax, Bad, Bid), blocking cytochrome *c* release from mitochondria, and thus preventing the ensuing activation of the apoptotic execution machinery (Reed, 1997; Eskes et al., 1998).

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01375-9

Upregulation of Bcl-2 is characteristic of axonal growth cones and may regulate synaptic plasticity (Holm and Isacson, 1999; Holm et al., 2001). With Bcl-2 and BclXL gene therapy, overexpression of growth-associated protein-43 (GAP-43) has been observed in a permanent middle cerebral artery occlusion model in the rat (our unpublished data). It has been suggested that this upregulation of GAP-43 may signify enhanced synaptic plasticity and is characteristic for neuronal plasticity following middle cerebral artery occlusion (Li et al., 1998). GAP-43 has been used as an index of axonal sprouting and reflects enhancement of neuronal plasticity (Buffo et al., 1997; Skene, 1989).

In this paper, we focus on the mechanisms by which (−)-D-deprenyl reduces infarct volume. Apoptotic neurons and non-neuronal cells were visualized by fluorescence immunohistochemistry and counted in the region of peri-infarct penumbra. Furthermore, GAP-43-positive cells in the peri-infarct region were visualized and considered as a sign of neural plasticity and survival (Chopp et al., 1999).

2. Materials and methods

2.1. The animals

The animals used were male Wistar rats weighing between 320 and 460 g ($n = 20$). The treatment and care of animals complied with EU standards. The experimental procedure was reviewed and approved by the local Ethics Committee.

2.2. Lesion induction

Anaesthesia was induced with 4% and maintained with 2–2.5% of halothane in 70% N_2O and 30% O_2, using a face mask. A standardised technique was used to perform the permanent middle cerebral artery occlusions. Ischaemic lesions were induced by electrocoagulating the left middle cerebral artery on the surface of the brain (Coyle, 1982). The method was slightly modified. Briefly, a craniectomy hole about 3 mm in diameter was made just above the left middle cerebral artery occlusion. The dura mater was removed. Subsequently, the middle cerebral artery occlusion was identified and a bipolar coagulator was applied to it to obstruct the distal branches above the lenticulostriatal branches.

2.3. Treatment

Treatment was started immediately following the permanent middle cerebral artery occlusion. In Group I ($n = 8$), the animals were infused with 0.2 mg/kg/day of (−)-D-deprenyl in a vehicle of 0.9% physiological saline, delivered via osmotic minipumps (Alzet, ALZA, Palo Alto, CA) intraperitoneally for 2 days. In Group II ($n = 8$), the control rats with permanent middle cerebral artery occlusion were infused with the vehicle only. The rats were killed after 2 days of treatment. In Group III animals ($n = 2$), the control rats without a permanent middle cerebral artery occlusion were infused with the vehicle only. The control rats without a permanent middle cerebral artery occlusion (Group IV, $n = 2$) were infused with 0.2 mg/kg/day (−)-D-deprenyl in 0.9% physiological saline.

2.4. Measurement of infarct volume

Rat brains were removed, sliced and the 2-mm-thick fresh brain slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC) for delineation of the tissue damage (Bederson et al., 1986). The TTC reaction stains functional mitochondrial dehydrogenases red. The unstained area was identified as an infarct area. The TTC-stained brain slices were then photographed, digitised and the territory of the lesion was defined using computer-aided morphometry (using UTHSCA ImageTool) in each slice. The infarct volumes in cubic millimeters were calculated by summing the products of lesioned areas and slice thickness in the lesioned slices.

2.5. Immunohistochemistry

After fixation in 10% formaldehyde and embedding into paraffin, 20-μm-thick sections were cut and stained with haematoxylin and eosin and Cresyl violet. For confocal laser scanning microscopic (CLSM) studies, 4-μm-thick sections were cut and mounted on precleaned, 3-aminopropyltriethoxy-silane-coated glass slides. For the identification of apoptotic cells in the peri-infarct region, broken deoxyribonucleic acid (DNA)-ends were visualized using an in situ fluorescent terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (conjugated to the green fluorophore fluorescein isothiocyanate) DNA nick-end labelling (TUNEL) kit (Boehringer-Mannheim), and caspase-3 (Immunotech, Mouse anti-caspase-3, prediluted) fluorescence immunohistochemistry was also performed. A high-temperature antigene unmasking procedure for paraffin sections was not used so that the baseline expression of caspase-3 could not be detected. The enhanced expression of caspase-3 after middle cerebral artery occlusion could be localized (Guegan and Sola, 2000). For the identification of neurons, NeuN (Chemicon) mouse anti-neuronal nuclei (1:100) primary antibodies were used. TUNEL–NeuN and TUNEL–caspase-3 double labelling was performed. For indirect immunofluorescence, goat anti-mouse antibody conjugated to Alexa 568 (Molecular Probes) was used as a secondary antibody (1:100). GAP-43 was also visualized using a mouse anti-GAP-43 primary antibody (1:100) (Zymed), and an ABC Elite kit (Vector Laboratories) to characterize neuronal plasticity.

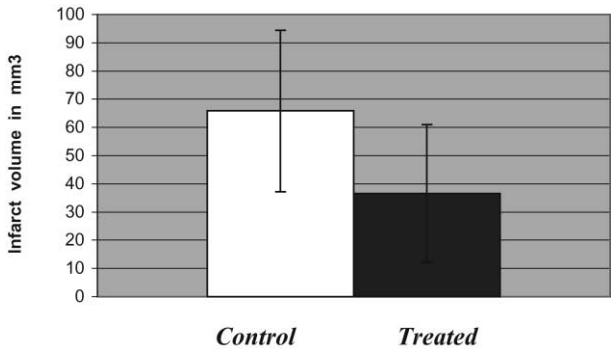

Fig. 1. The lesion size decreased after 48 h of continuous treatment with (−)-D-deprenyl. The average lesion size in control animals was 65.8 ± 28.6 mm^3, whereas in treated animals, it was 36.5 ± 24.4 mm^3 ($P < 0.05$). Results are presented as means ± S.D.

2.6. Fluorescence detection

A BIO-RAD MRC 1024 confocal system was used, installed on a Nikon DIAPHOT inverted microscope (Donsanto). For excitation, 488- and 568-nm lines of a Krypton–Argon laser were applied sequentially. Detection was performed with a standard filter set.

2.7. Sampling

The lesion was defined on the basis of Cresyl violet- and haematoxylin and eosin-stained sections. There is a rim of eosinophilic cells just outside the infarct, which apparently represents an area of selective neuronal cell death (Nedergaard, 1987; Nedergaard et al., 1987). These cells are characterized by intense TUNEL staining (Li et al., 1995a,b; States et al., 1996) and caspase-3 overexpression (Sasaki et al., 2000; Guegan and Sola, 2000).

A 1000 × 1000-μm region was sampled at both cortical ends of the TUNEL-positive area at its borders. Each area was sampled by zooming three times into it, thereby gaining six samples per section, 270 × 270 μm each. The samples were nonoverlapping and randomly selected. The data were analysed blind to exclude operator bias. In each group, we took 60 samples from TUNEL-labelled cortical tissue, 30 samples from TUNEL and NeuN double-labelled cortical tissue and 30 samples from TUNEL and caspase-3 double-labelled cortical tissue.

2.8. Reverse transcriptase-polymerase chain reaction analysis of mRNA products

Control rats treated with 0.9% physiological saline (Group III) and control rats treated with 0.2 mg/kg/day of (−)-D-deprenyl in a vehicle of 0.9% physiological saline (Group IV) (all of them without permanent middle cerebral artery occlusion) were killed after a 2-day treatment. A tissue sample was then harvested from the parietal cortex and subcortical structures, homogenized and processed for reverse transcriptase-polymerase chain reaction analysis of Bcl-2 and GAP-43 mRNA.

2.9. Statistical analysis of data

Volumetric data were statistically analysed using a Mann–Whitney "*U*" test. The data gained by counting the TUNEL-labelled, caspase-3-labelled and NeuN-labelled cells in the samples were analysed using a two-tailed Student's *t*-test. Values were considered significant if $P < 0.05$.

3. Results

The lesion size decreased after 48 h of continuous treatment with (−)-D-deprenyl by 50% on average ($P < 0.05$). The average lesion size in treated animals was 36.5 mm^3, whereas it was 65.8 mm^3 in control rats (Fig. 1).

Two days after the permanent middle cerebral artery occlusion, a well-defined ischaemic lesion could be characterized on Cresyl violet-stained sections. In the infarcted area, no neural elements could be identified; however,

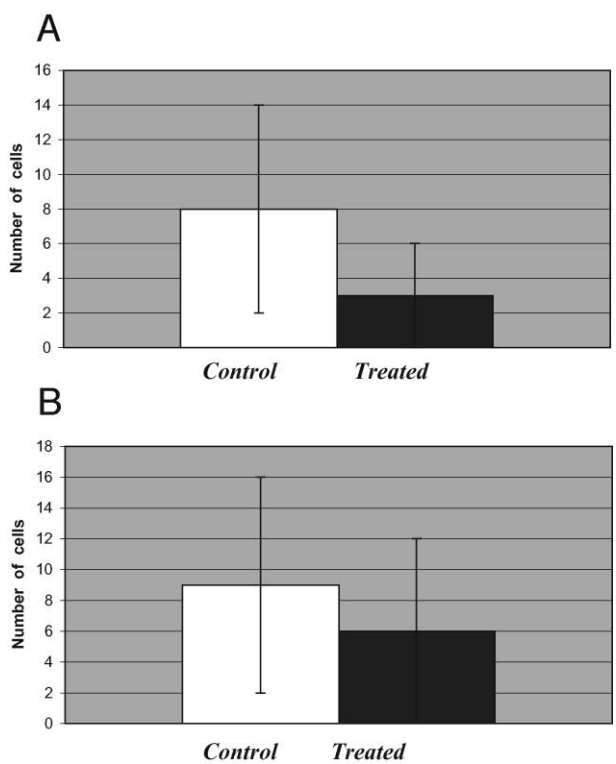

Fig. 2. (A) The number of TUNEL and caspase-3 double-labelled cells averaged over 30 samples was 8 ± 6 in control rats and 3 ± 3 in treated rats ($P = 0.0003$). Results are presented as means ± S.D. (B) The number of TUNEL-labelled neurons averaged over 30 samples was 9 ± 7 in control rats and it was 6 ± 6 in treated rats ($P = 0.1460$). Results are presented as means ± S.D.

endothelial cells of capillaries and microvessels and glia cells were morphologically well preserved. In some of the sections, polymorphonuclear leucocytes were detected in various numbers, mostly in the periphery of the infarcted brain tissue.

The number of TUNEL-labelled cells averaged over 60 samples was 17 ± 12 in treated rats, whereas it was 28 ± 25 in control rats ($P = 0.002$). The number of TUNEL–caspase-3 double-labelled cells averaged over 30 samples was 3 ± 3 in treated rats, whereas it was 8 ± 6 in control rats ($P = 0.0003$) (Fig. 2A). The number of TUNEL-labelled neurons averaged over 30 samples was 6 ± 6 in treated rats, whereas it was 9 ± 7 in control rats ($P =$ 0.1460) (Fig. 2B). The number of caspase-3-labelled cells averaged over 30 samples was 18 ± 13 in treated rats, whereas it was 26 ± 20 in control rats ($P = 0.0654$). Samples stained with GAP-43 showed a slight upregulation of GAP-43 in (−)-D-deprenyl-treated rat brains after permanent middle cerebral artery occlusion as compared to that in control rat brains after middle cerebral artery occlusion. GAP-43-positive cells were strictly localized to a thin ring-like area of healthy looking cortex surrounding the lesion (Fig. 3A,B). Reverse transcriptase-polymerase chain reaction analysis showed that in (−)-D-deprenyl-treated control rat (Group IV), temporal cortices Bcl-2 mRNA expression was increased by about 100% as compared to that in physiological saline-treated control rat (Group III), and in (−)-D-deprenyl-treated control rat (Group IV), temporal cortices GAP-43 mRNA expression increased only slightly as compared to that in physiological saline-treated control rat temporal cortices (Group III) (Fig. 4).

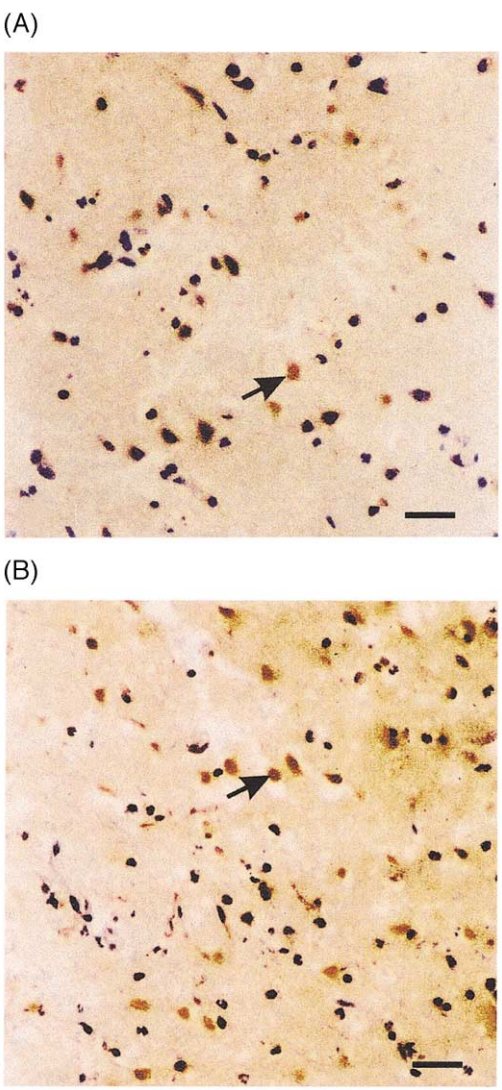

Fig. 3. (A) A light microscopy picture of the peri-infarct rim of a rat following 2-day permanent middle cerebral artery occlusion. GAP-43-positive cells can be seen in red. Red cells are strictly localized to a thin ring-like area of the healthy looking cortex surrounding the infarct. (B) A photomicrograph from an identical area. An increased number of GAP-43-positive cells were detected following (−)-D-deprenyl treatment. (The arrows indicate GAP-43-positive cells.) (Bar = 10 μm).

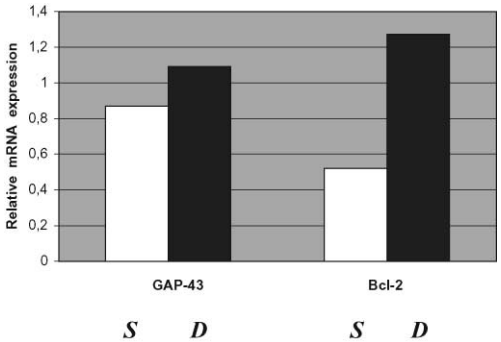

Fig. 4. In (−)-D-deprenyl-treated naive control rat temporal cortex (D) (Group III), Bcl-2 mRNA expression increased by about 100% as compared to that in physiological saline-treated control rat temporal cortex (S) (Group IV), whereas GAP-43 mRNA only slightly changed in (−)-D-deprenyl-treated naive control rat temporal cortex (D) (Group III) as compared to physiological saline-treated control rat temporal cortex (S) (Group IV).

4. Discussion

In this paper, (−)-D-deprenyl, a compound with well-characterized antiapoptotic properties, was found to reduce the number of TUNEL-positive cells and the number of TUNEL-positive and caspase-3-overexpressing cells in a lesioned rat cortex after permanent middle cerebral artery occlusion, leading to a decrease in lesion size and enhanced post-stroke neuronal plasticity. In these experiments, the developing ischaemic lesion was studied 48 h after permanent middle cerebral artery occlusion in rats. If cellular death and damage characterized by TUNEL positivity and increased caspase-3 positivity are expressed as percentages relative to saline-treated controls, then in (−)-D-deprenyl-treated animals, the number of TUNEL-positive cells decreased by 40%, the number of TUNEL-positive and caspase-3-overexpressing cells decreased by 60%,

the number of TUNEL-positive neurons decreased by 30%, and the number of caspase-3-overexpressing cells decreased by 32%. Furthermore, it is important to note that the comparatively small number of TUNEL-positive neurons present in the samples indicates that not only neurons are at risk in the region examined.

(−)-D-Deprenyl treatment produced an increase in the number of surviving cells compared to saline treatment following permanent middle cerebral artery occlusion. The comparatively high number of TUNEL-positive cells in both treated and control animals indicates that necrotic processes were going on in the sampled areas (Didenko and Hornsby, 1996; Gold et al., 1994). Cell necrosis following Ca^{2+} influx and calpain activation and apoptosis with caspase cascade activation are closely related and interconnected at several levels (Wang, 2000).

However, since oxidative stress can damage DNA and may result in both single- and double-stranded DNA nicks (Sun, 1990; Du et al., 1996), it may well be that the significant decrease in the number of TUNEL-positive cells is partly due to the immediate scavenging activity of (−)-D-deprenyl. Immediate radical trapping by (−)-D-deprenyl may involve the trapping of secondary peroxyl radicals (Thomas et al., 1997). Furthermore, the expression of the scavenger proteins Cu^{2+}/Zn^{2+} superoxide dismutase and Mn^{2+} superoxide dismutase is also altered by (−)-D-deprenyl in the long run, thus making preconditioning against focal cerebral ischaemia possible (Tatton et a1., 1996). It could be suggested that the protective activity of (−)-D-deprenyl at least partly is due to its binding to the enzyme glyceraldehyde-3-phosphate dehydrogenase (Kragten et al., 1998) and to changes in gene expression, especially the overexpression of Bcl-2. This altered gene expression results in the maintenance of mitochondrial integrity and thus in the prevention of caspase-3 activation (Reed, 1997).

GAP-43 is heavily expressed during neuronal development and regeneration. It is primarily associated with axonal outgrowth. GAP-43 expression is used as an index of axonal sprouting and reflects the activation of neuronal plasticity (Buffo et al., 1997). GAP-43 has been detected in the adult rat after focal ischaemic injury (Li et al., 1998). The slight upregulation of GAP-43 expression in the peri-infarct rim may signify enhanced neuronal plasticity. This may be an additional feature of the drug besides its promotion of dendritic arborization (Shankaranarayana et al., 1999; Lakshmana et al., 1998). It has been shown that the upregulation of Bcl-2 and Bcl-X expression in surviving neurons close to the penumbra might reflect an active survival mechanism that protects these neurons from cell death following a sublethal insult (Isenmann et al., 1998), and adenovirus-mediated Bcl-2 overexpression results in increased GAP-43 expression in the peri-infarct rim (our unpublished observation). Moreover, (−)-D-deprenyl is said to have a 'trophic-like' nature, which may also contribute to its protective effect. (−)-D-Deprenyl induces nerve growth factor (NGF) expression in cultured rat astrocytes and in rat cortex in vivo, which contributes to a reduction in lesion size following middle cerebral artery occlusion (Semkova et al., 1996).

Our results support the observation that the cellular sparing effect of (−)-D-deprenyl is related to its anti-apoptotic nature. We conclude that (−)-D-deprenyl significantly attenuates selective cell death and probably to some extent necrotic events induced by permanent middle cerebral artery occlusion in the peri-infarct rim and in the peripheral region of the lesion. It also downregulates the ischaemia-induced overexpression of caspase-3. Furthermore, it may cause a slight increase in neuronal plasticity in the peri-infarct rim.

(−)-D-Deprenyl has had a long career since it was synthesized in 1965 (Knoll et al., 1965). Its use in the therapy of Parkinson's disease is well established. Besides its other pharmacological actions, the neuroprotective effect of (−)-D-deprenyl is becoming a focus of interest. In this study, the anti-apoptotic effect and a modest pro-plasticity effect of (−)-D-deprenyl were demonstrated in an ischaemic stroke model. These features of the drug could have clinical implications.

Acknowledgements

We are grateful for the assistance, outstanding expertise and patience of Volner Lászlóné and Druskó Miklósné.

All experiments were conducted in accordance with the appropriate ethical guidelines. (This work was supported by EU Grant contract nos. BMH4-CT-98-3277 and IC20-CT-98-0206.)

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