Induction of Apoptosis in Catecholaminergic PC12 Cells by L-DOPA

Implications for the Treatment of Parkinson's Disease

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Abstract

The hypothesis that L-DOPA therapy in Parkinson's disease may augment neuronal damage and thus accelerate the progression of the disease remains controversial. In this study, we demonstrate that L-DOPA induces death of catecholaminergic cells in vitro via an active program of apoptosis. Treatment of PC12 cells with clinically applicable concentrations of L-DOPA (25–100 μM) induced cell death via a mechanism which exhibited morphological and biochemical characteristics of apoptosis, including chromatin condensation, membrane blebbing, and internucleosomal DNA fragmentation. L-DOPA–induced apoptosis was cell and drug-type specific. Toxicity is an intrinsic property of the drug molecule since it was not suppressed by inhibiting conversion of L-DOPA to dopamine. However, L-DOPA toxicity was inhibited by antioxidants, suggesting that activation of apoptosis is mediated by oxygen radicals. Our finding that L-DOPA–induced cell death in vitro occurs via apoptosis explains the lack of evidence supporting its toxicity in vivo, since apoptotic neurons are rapidly phagocytosed in vivo without causing damage to surrounding tissue. Furthermore, since apoptosis is an active cellular program which can be modulated, we suggest clinical approaches for decreasing L-DOPA toxicity, thus preventing acceleration of neuronal damage in Parkinson's disease. (*J. Clin. Invest.* 1995. 95:2458–2464.) Key words: levodopa • toxicity • neurodegeneration • programmed cell death • antioxidants

Introduction

Parkinson's disease is characterized by selective degeneration of dopaminergic neurons in the substantia nigra. The etiology of this disease remains unknown, but one hypothesis is that endogenous or exogenous neurotoxins may be responsible for the destruction of nigrostriatal neurons. Current clinical therapies for Parkinson's disease are limited and the few treatments that are available alleviate the neurological symptoms of the disease rather than the primary event of cell death. L-3,4-dihydroxyphenylalanine (levodopa or L-DOPA),[1] the natural precursor to dopamine, is the most effective and frequently prescribed therapy for controlling the symptoms of Parkinson's disease (1). It remains the drug of choice in the treatment of this disease despite the fact that there are limiting adverse side effects associated with chronic L-DOPA treatment. The major side effects are central and include the appearance of involuntary movements (dyskinesia), fluctuations in motor performance ("on-off" phenomenon), and psychiatric complications (1–4). In addition, the effects of L-DOPA therapy on the progression of Parkinson's disease are unknown and conflicting reports have suggested that L-DOPA may or may not accelerate deterioration of the patients (5–10). L-DOPA produces initial improvements in dopaminergic function, but therapy is only successful for a limited period of time after which patients become unresponsive and neurological disability reappears and worsens (2, 3). Despite this eventual decline in "effectiveness," L-DOPA remains the most successful drug used to treat Parkinson's disease and is often administered successfully for many years before treatment becomes unsatisfactory.

Several recent studies have demonstrated that L-DOPA is toxic to some neuronal cells in vitro (11–15). It has been suggested from these studies that long-term administration of L-DOPA to patients with Parkinson's disease may exacerbate neuronal damage and thus accelerate the progression of the disease. However, this hypothesis has remained unsubstantiated due to a lack of evidence supporting L-DOPA toxicity in vivo. It has been shown in both rats (16) and mice (17) that long-term administration of L-DOPA does not cause detectable damage to nigrostriatal dopaminergic neurons. Furthermore, no evidence of damage to nigral neurons was found in a normal individual consuming large amounts of L-DOPA over a prolonged period (18). However, recent in vivo studies, involving chronic administration of L-DOPA to animals in which dopaminergic neurons have been damaged previously by exposure to 6-hydroxydopamine, have provided some evidence that L-DOPA toxicity may also occur in vivo (19, 20).

Although toxicity of L-DOPA towards catecholaminergic neurons has been demonstrated in cell cultures (11–15), the mechanism by which L-DOPA, especially at clinically relevant concentrations, induces cell death has not been investigated. Cell death is proposed to occur by two distinct mechanisms, necrosis and apoptosis. Necrosis is a passive, uncontrolled form of cell death, typically observed in response to an acute toxic insult. Necrotic cells lose control of cellular homeostasis, swell, and undergo cell lysis, thus causing inflammation and damage to surrounding tissue. In contrast, apoptosis is an active form of cell death involving the participation of the cell in an internal

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1. *Abbreviations used in this paper:* AO, acridine orange; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; L-DOPA, levodopa; NMDA, *N*-methyl-D-aspartate; 6OHDA, 6-hydroxydopamine.

program of self-destruction. This physiological "cellular suicide" plays an important role in embryonic development, particularly development of the nervous system (21, 22). Apoptosis can be recognized by distinct morphological and biochemical features (23, 24). Apoptotic cells shrink in size, undergo membrane blebbing, and exhibit highly condensed chromatin. The biochemical hallmark of apoptosis is cleavage of DNA into regular sized fragments, typically large fragments of 50 kb and often into multiples of 180 bp internucleosomal fragments (25–27). These latter fragments give a characteristic "ladder" pattern when separated by agarose gel electrophoresis.

An important feature of apoptotic cell death in vivo is that dying cells maintain their membrane integrity and are rapidly phagocytosed by neighboring cells or patrolling macrophages, leaving no trace of their existence and causing no damage to surrounding tissue (23, 28). We have demonstrated recently that death of neuronal cells in response to a specific neurotoxin, 6-hydroxydopamine, can occur via an apoptotic mechanism (29). To explain the paradoxical reports that L-DOPA toxicity can be observed in vitro but not in vivo, we hypothesize that L-DOPA–induced cell death is mediated via an apoptotic mechanism rather than via a necrotic process. The rapid engulfment of apoptotic cells without causing damage to surrounding tissue would explain how the death of neurons after administration of L-DOPA might go undetected in in vivo studies. Furthermore, the possibility that L-DOPA toxicity is mediated via an apoptotic mechanism is not purely of academic interest, but also has several important clinical implications. Since apoptosis is an active form of cell death involving a specific cellular program, it has the attractive property of being amenable to modulation by, for example, addition of exogenous agents or small reductions in the dose administered. Thus, determining the exact mechanism of cell death induced by L-DOPA has important implications for the treatment of Parkinson's disease.

In this study we have investigated the effect of L-DOPA on rat pheochromocytoma PC12 cells. These cells are catecholaminergic cells, which can be induced to differentiate into sympathetic-like neuronal cells upon addition of nerve growth factor (30). PC12 cells have been used in many recent studies of neuronal cell death after trophic factor deprivation (31–33). We have demonstrated in this study that L-DOPA is toxic to catecholaminergic PC12 cells and have further investigated the mechanism of cell death induced by L-DOPA using morphological and biochemical criteria. The specificity of L-DOPA toxicity has been addressed and we have also examined possible mediators of L-DOPA toxicity.

Methods

Materials. RPMI 1640 medium and donor horse serum were obtained from Imperial Laboratories (Andover, Hampshire, United Kingdom). Fetal calf serum was from Advanced Protein Products, Ltd. (Brierley Mill, United Kingdom). Carbidopa was obtained from ICN Biomedicals, Inc. (Carson, CA). CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), NMDA (*N*-methyl-D-aspartate), and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid were bought from Tocris Cookson (Bristol, United Kingdom). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Cell culture. PC12 cells were maintained in RPMI 1640 medium supplemented with 10% vol/vol donor horse serum and 5% vol/vol fetal calf serum. FaO cells were grown in Hams nutrient F12 mixture supplemented with 10% vol/vol fetal calf serum. In addition, both culture media contained 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Drug treatment of cells. L-DOPA, dopamine, and carbidopa were added to cells from 2.5 mM stock solutions dissolved in PBS. Glutathione was added from a 100 mM stock solution dissolved in PBS. Vitamin E (α (+)-tocopherol acid succinate) and ascorbic acid were added from 10 mM stock solutions dissolved in DMSO and PBS, respectively. CNQX was added from a 2 mM stock solution dissolved in DMSO. Control flasks were treated with either DMSO or PBS to the same concentration.

Morphometric analysis of cells. The morphology of detached PC12 cells after treatment with L-DOPA was examined under fluorescence on a confocal microscope (MRC 600; Biorad, Hercules, CA). Cells were pelleted by centrifugation (250 *g*, 5 min), resuspended in 10 μl RPMI medium, and fixed with an equal volume of 4% wt/vol paraformaldehyde. 20 μl acridine orange (AO) solution (10 μg/ml in PBS) was added to the cells before mounting on slides. For trypan blue staining, cells were resuspended in 10 μl RPMI medium and added to an equal volume of trypan blue solution. Cells were then viewed under bright field.

Extraction and electrophoretic analysis of DNA. DNA was extracted from the detached cell population of treated cultures or the viable monolayer of untreated control cultures. 1×10^6 cells were used for the preparation of each sample. In cultures treated with L-DOPA, detached cells were collected by centrifugation (250 *g*, 5 min) every 2 h between 16 and 24 h after addition of the drug. The detached cells collected at these intervals were pooled to obtain 1×10^6 cells.

Cells were resuspended in 20 μl of buffer containing 10 mM EDTA, 50 mM Tris/HCl (pH 8), 0.5% wt/vol sodium lauryl sarkosinate, and 0.5 mg/ml proteinase K and incubated for 1 h at 50°C. 10 μl TE (10 mM Tris-HCl, 10 mM EDTA, pH 8) containing 0.5 mg/ml RNaseA was added, and the cells were incubated at 50°C for 1 h. 10 μl 1% wt/vol low melting point agarose (in 10 mM EDTA, pH 8) was added to each sample, and the samples were then loaded on a 2% agarose (FMC Corp. BioProducts, Rockland, ME) gel containing 0.1 μg/ml ethidium bromide. The samples were subjected to electrophoresis at 40 V in TPE running buffer containing 0.8 M Tris-phosphate, 20 mM EDTA (pH 8) for 3–4 h and then viewed under ultraviolet illumination.

Apoptotic cell counts. Detached cells from treated or untreated cultures were collected by centrifugation (250 *g*, 5 min), and the cell pellet was resuspended in 20 μl RPMI medium. Cell counts were carried out using a hemocytometer. The nuclear morphology of the cells was analyzed by staining with 10 μg/ml AO solution. A 10 μl sample of the cell suspension was mixed with an equal volume of AO solution and viewed under fluorescence. Apoptotic cells were counted as those exhibiting brightly fluorescing, highly condensed chromatin.

Statistical analysis. All data are expressed as the mean±SEM of at least three observations. Statistical analysis was performed using the Students *t* test.

Results

Characterization of L-DOPA–induced toxicity. Initial experiments were carried out to determine whether catecholaminergic PC12 cells were susceptible to the toxic effects of L-DOPA. Treatment of PC12 cells with 200 μM L-DOPA, a concentration which has been shown in previous studies to be toxic to other catecholaminergic cells (11–13), resulted in a decrease in viable cell number after 24 h (data not shown). This observation confirmed that PC12 cells were sensitive to L-DOPA toxicity and therefore could be used to examine the mechanism by which L-DOPA induces cell death.

To investigate the morphology of L-DOPA–induced cell death, a lower and more clinically applicable concentration was used. PC12 cells were treated with 50 μM L-DOPA for 24 h. After this time, cells which had detached from the viable monolayer were collected and their morphology was examined by staining with AO or trypan blue. AO staining revealed that treatment of PC12 cells with 50 μM L-DOPA induced cell death

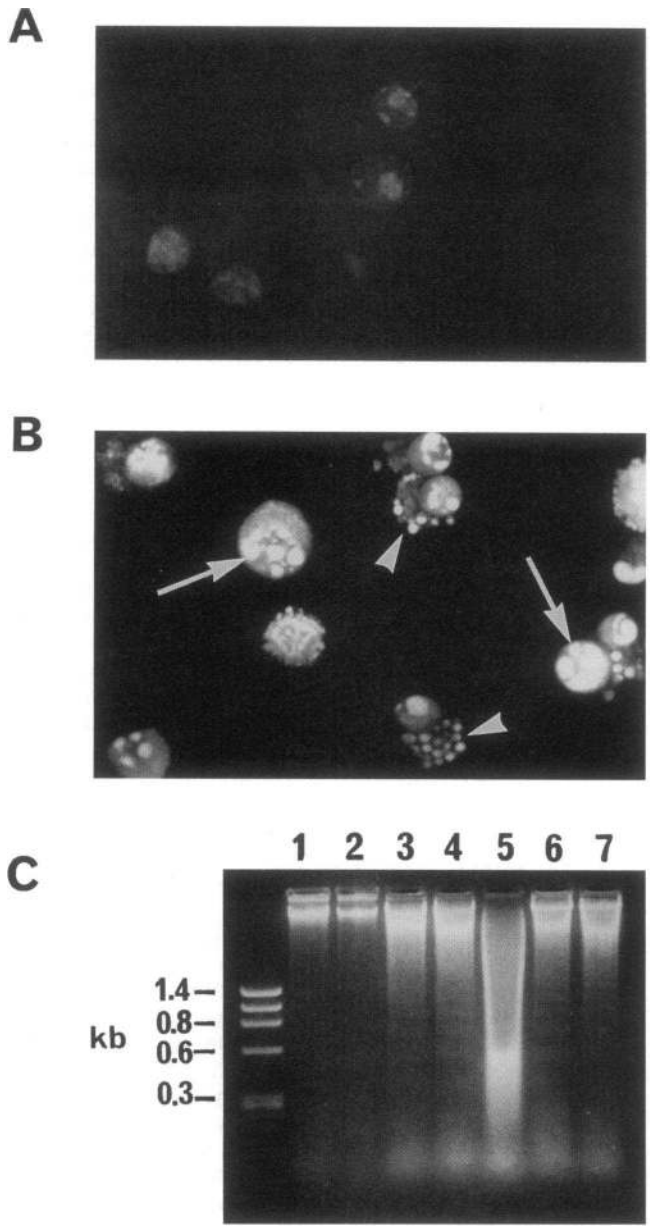


Figure 1. Morphological and biochemical characteristics of L-DOPA–induced cell death. (*A* and *B*) Photomicrographs of PC12 cells stained with 5 μg/ml AO and viewed under fluorescence with confocal microscopy. (*A*) Untreated control cells. (*B*) Detached cells from cultures treated with 50 μM L-DOPA for 24 h. These cells exhibited characteristic features of apoptosis including highly condensed chromatin (*arrows*) and extensive membrane blebbing (*arrowheads*). (*C*) Electrophoretic analysis of DNA extracted from PC12 cells after treatment with L-DOPA. Detached cells from cultures treated with 50 μM L-DOPA were collected every 2 h between 16 and 24 h after addition of the drug and pooled. DNA extracted from these pooled samples exhibited a laddering pattern indicative of internucleosomal fragmentation (lanes *3*, *4*, *6*, and *7* are samples from two independent experiments). No fragmentation was observed in DNA samples extracted from untreated control PC12 cells (lanes *1* and *2*). A smearing pattern indicative of random DNA degradation was seen in DNA samples from detached cells after treatment with 200 μM L-DOPA for 24 h (lane *5*).

via a mechanism which possessed characteristic morphological features of apoptotic cell death, including highly condensed chromatin and extensive membrane blebbing (Fig. 1 *B*). Staining of the cells with trypan blue indicated that, at this concentration, L-DOPA did not induce necrotic cell death. The only cells which stained with trypan blue were small, shrunken cells which had undergone "secondary necrosis." This is a term used to describe the fate of an apoptotic cell in vitro. Although an apoptotic cell maintains its membrane integrity initially, if it is not phagocytosed it eventually starts to disintegrate and becomes permeable to dyes such as trypan blue.

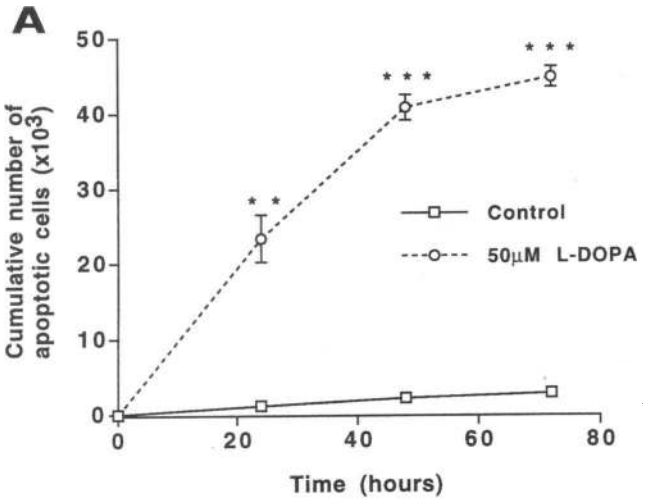
A biochemical hallmark of apoptosis observed in many cells is the characteristic cleavage of DNA at internucleosomal sites to produce a DNA ladder (25). The integrity of DNA from PC12 cells induced to die by treatment with L-DOPA was examined by agarose gel electrophoresis (Fig. 1 *C*). Cells detaching from the viable monolayer between 16 and 24 h after treatment with 50 μM L-DOPA were collected and pooled. Analysis of DNA extracted from these cells revealed the classical fragmentation pattern typical of apoptosis (Fig. 1 *C*, lanes *3*, *4*, *6*, and *7*). DNA fragmentation was not observed in untreated control cells (Fig. 1 *C*, lanes *1* and *2*) nor in viable monolayer cells of L-DOPA–treated cultures (not shown). Induction of necrotic cell death by higher concentrations of L-DOPA (200 μM) produced a DNA smear indicative of random digestion of the DNA (Fig. 1 *C*, lane *5*).

The induction of apoptosis by L-DOPA as a function of time is shown in Fig. 2 *A*. PC12 cell cultures were treated with 50 μM L-DOPA and the induction of apoptosis was measured over a 72-h period. The amount of apoptosis induced in treated cultures was measured after 24, 48, and 72 h and expressed as the cumulative number of apoptotic cells. Induction of apoptosis by L-DOPA reached a plateau after 48 h, but the largest number of apoptotic cells were generated within 24 h of exposure to the drug. A 24-h exposure to L-DOPA was therefore used in all subsequent experiments. Treatment of PC12 cells with concentrations of L-DOPA between 0 and 100 μM for 24 h revealed that the induction of apoptosis by L-DOPA was a dose-dependent effect (Fig. 2 *B*). At higher concentrations of L-DOPA (above 200 μM), necrosis was the predominant form of cell death (not shown).

Specificity of L-DOPA–induced apoptosis. To investigate whether the induction of apoptosis by L-DOPA was specific to catecholaminergic cells, or a general phenomenon observed in all cell types, the effect of L-DOPA on noncatecholaminergic cells was examined. The noncatecholaminergic cells chosen for this investigation were FaO rat hepatoma cells and rat-1 fibroblasts. Fig. 3 *A* shows the amount of apoptosis induced after treatment of PC12 cells or FaO cells with 50 and 100 μM L-DOPA for 24 h. No induction of apoptosis was observed in cultures of FaO cells treated with either concentration of L-DOPA. Similarly, no toxic effect was observed after addition of 50 μM L-DOPA to rat-1 fibroblasts for 24 h (data not shown).

To investigate if induction of apoptotic cell death was a specific effect of L-DOPA, PC12 cells were incubated with equimolar concentrations of various other amino acids for 24 h (Fig. 3 *B*). No apoptotic cell death was observed after treatment of PC12 cells with 50 μM phenylalanine (an aromatic amino acid), arginine (a basic amino acid), or serine (a neutral amino acid).

Investigation into the mechanism of L-DOPA–induced apoptosis. It has been demonstrated previously that dopamine is toxic to dopaminergic cells in vitro (14, 34). Thus, we considered the possibility that induction of apoptosis after exposure of PC12 cells to L-DOPA may be due to a toxic effect of dopamine or of dopamine formation. This possibility was inves-



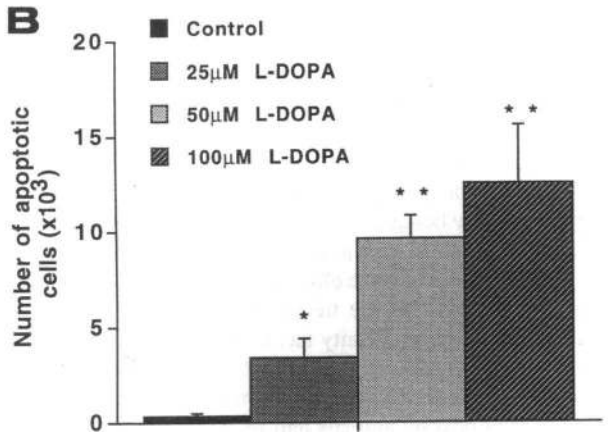
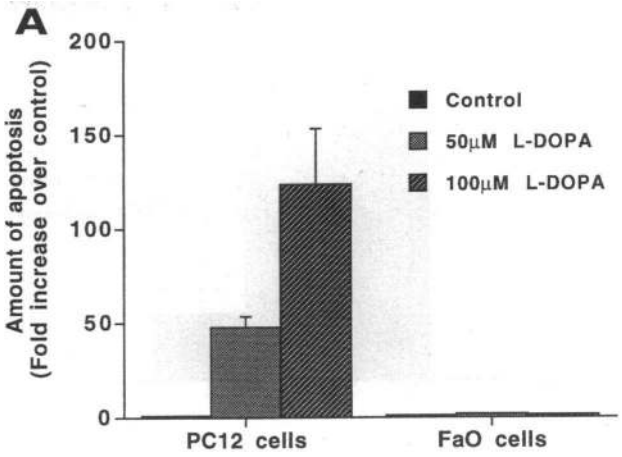


Figure 2. Time and concentration dependency of L-DOPA–induced apoptosis. (*A*) Amount of apoptosis induced in PC12 cell cultures treated with 50 μM L-DOPA over a 72-h period. The amount of apoptosis was measured after 24, 48, and 72 h and expressed as the cumulative number of apoptotic cells. (*B*) Amount of apoptosis measured in PC12 cell cultures after treatment with different concentrations of L-DOPA for 24 h. Data points represent the mean±SEM of three experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with untreated controls.



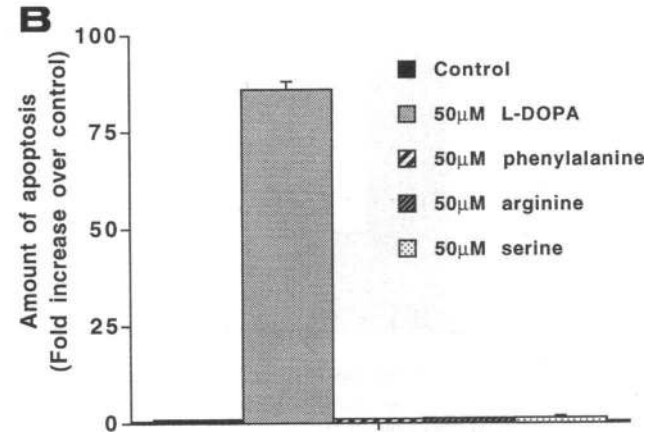


Figure 3. Cell-type and amino acid specificity of L-DOPA–induced apoptosis. (*A*) Amount of apoptosis measured in cultures of catecholaminergic PC12 cells or FaO hepatoma cells after treatment with 50 and 100 μM L-DOPA for 24 h. (*B*) Amount of apoptosis measured in PC12 cell cultures treated with 50 μM L-DOPA or equimolar concentrations of other amino acids for 24 h. The amount of apoptosis is expressed as fold increase over control. Data points represent the mean±SEM from three independent experiments.

tigated by preventing the conversion of L-DOPA to dopamine using carbidopa, an inhibitor of the decarboxylase which catalyzes this conversion. The amount of apoptosis observed in PC12 cell cultures treated for 24 h with 50 μM L-DOPA + 25 μM carbidopa was not significantly different from that observed in cultures treated with 50 μM L-DOPA alone (Fig. 4 *A*). PC12 cells were also treated with 50 μM dopamine to determine whether dopamine itself induced apoptosis in these cells. Although an induction of apoptosis was observed in cultures treated with 50 μM dopamine for 24 h, the amount of apoptosis was significantly less than that induced after treatment with 50 μM L-DOPA for 24 h (Fig. 4 *A*).

Reactive oxygen species such as hydrogen peroxide and associated oxy radicals can be generated during the autoxidation of L-DOPA. To investigate whether the induction of apoptosis by L-DOPA was mediated by generation of reactive oxygen species, the ability of antioxidants to inhibit L-DOPA–induced apoptosis was examined. PC12 cells were treated with 50 μM L-DOPA alone or in the presence of one of the following antioxidants: 100 μM vitamin E, 1 mM glutathione, or 100 μM ascorbic acid. Fig. 4 *B* shows the amount of apoptosis induced 24 h after addition of the drugs. Induction of apoptosis by L-DOPA was completely inhibited by addition of glutathione or ascorbic acid. Induction of apoptosis by L-DOPA was also inhibited, although to a lesser extent, by vitamin E.

Olney and colleagues (35) have reported that high concentrations of L-DOPA (1–2 mM) exhibit weak excitotoxic properties in the chick embryo retina. L-DOPA excitotoxicity was shown to be blocked by CNQX, an antagonist at non-NMDA subtypes of excitatory amino acid receptors (35). To investigate whether the induction of apoptosis by L-DOPA was mediated via activation of excitatory amino acid receptors, we examined the ability of CNQX to block L-DOPA–induced apoptosis in PC12 cells. Induction of apoptosis after treatment of PC12 cells with 50 μM L-DOPA for 24 h was not significantly inhibited

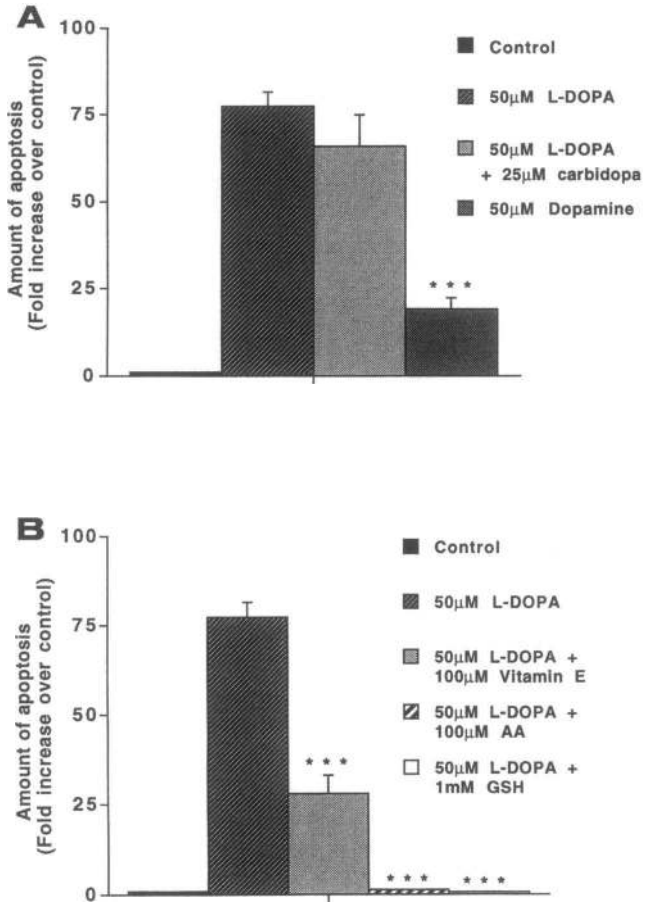


Figure 4. Analysis of possible mediators of L-DOPA–induced apoptosis. (*A*) Amount of apoptosis measured in PC12 cell cultures treated with 50 μM dopamine or 50 μM L-DOPA in the presence or absence of 25 μM carbidopa for 24 h. (*B*) Effect of 1 mM glutathione (*GSH*), 100 μM ascorbic acid (*AA*), or 100 μM vitamin E on the amount of apoptosis induced after treatment of PC12 cells with 50 μM L-DOPA for 24 h. The amount of apoptosis is expressed as fold increase over control. Data points represent the mean±SEM from three independent experiments. *** $P < 0.001$ compared with L-DOPA treatment alone.

by the presence of 20 μM CNQX (not shown). Furthermore, induction of apoptosis was not observed after treatment of PC12 cells with either 1 mM glutamate, 1 mM amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, or 1 mM NMDA for 24 h (not shown).

Discussion

L-DOPA is the most frequently prescribed drug for controlling the symptoms of Parkinson's disease. However, there are limitations to chronic treatment, including debilitating side effects and a progressive decrease in efficacy with time (1–4). In addition, there is disagreement as to whether L-DOPA treatment should be initiated early or late in the course of the disease. Conflicting evidence suggests that L-DOPA may, or may not, accelerate the progression of the disease (5–10). In this study, we have demonstrated that L-DOPA is toxic to catecholaminergic PC12 cells in vitro and that at clinically relevant concentrations this toxicity is mediated via an apoptotic mechanism rather than via a necrotic process. The concentration of L-DOPA reported in human plasma after administration of an average dose of 1,000 mg of L-DOPA (e.g., 1,000 mg/70 kg person = 14.3 mg/kg) is 1.7 μg/ml (8.6×10^{-6} M) (15). However, there is a lack of information available regarding the tissue levels of L-DOPA in parkinsonian patients treated with this drug. In animals treated with L-DOPA (100 mg/kg), striatal concentrations of the drug reached $15–30 \times 10^{-6}$ M (36). Although the average dose of L-DOPA administered to patients is severalfold lower than the dose used in the above experiment in animals, the concentrations of L-DOPA expected in the striatum of patients with Parkinson's disease are close to the range of concentrations used in our study. Therefore, although it is difficult to extrapolate the concentrations of L-DOPA used in our study to a clinical situation, where large swings in plasma levels are produced by standard L-DOPA treatments, we observe induction of apoptosis with concentrations of L-DOPA which could quite possibly be reached in patients. Necrotic cell death is observed with higher concentrations of drug, as has been described in previous studies of L-DOPA toxicity (11–15). Although the toxic effects of L-DOPA reported in this study are of a far lesser magnitude than the neurotoxic effects of a dopaminergic toxin such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine), the cumulative effect of a small, but highly significant, amount of apoptosis might be predicted to be catastrophic in a nucleus containing a small number of post-mitotic cells. Our finding that L-DOPA–induced cell death occurs via an active "suicide" pathway which is controlled by the cell, rather than simply being an uncontrolled loss of cellular homeostasis, provides an explanation for conflicting observations made in recent studies of the effect of L-DOPA in vivo, and has several implications for the treatment of Parkinson's disease.

Although L-DOPA toxicity has been reported previously in catecholaminergic cells in vitro (11–15), several studies have examined the effect of chronic administration of L-DOPA in vivo in normal healthy animals and found no evidence of neuronal damage, although extensive quantitation of the number of nigral cells remaining after drug treatment was not performed (16, 17). Damage to nigrostriatal dopaminergic neurons could not be detected after chronic treatment of rats (16) or mice (17) with L-DOPA for 4 or 18 mo, respectively. In addition, Quinn et al. (18) found no evidence of damaged nigral dopaminergic neurons in a normal individual receiving L-DOPA over a 4-yr period. Our finding that L-DOPA–induced cell death occurs by apoptosis may explain this apparent paradox between the toxic effects of L-DOPA in vitro and in vivo. An important feature of apoptotic cell death in vivo is that cells which have died by apoptosis maintain their membrane integrity and are rapidly phagocytosed before they can cause inflammation and damage to the surrounding tissue. This means that apoptotic cells have a very short half-life in vivo. Thus, the induction of small amounts of apoptosis by L-DOPA would not readily be detected and therefore could have gone unnoticed in in vivo studies aimed at looking for large scale neuronal degeneration. Of interest, Steece-Collier and co-workers (15) did observe increased infiltration of macrophages in their studies of L-DOPA toxicity to fetal grafts, which may reflect ongoing apoptosis in the tissue. Our own in vivo investigations have revealed an induction of apoptotic cell death in the substantia nigra after administration of L-DOPA to parkinsonian rats (Walkinshaw, G., and C. M. Waters, manuscript in preparation), suggesting that our in vitro findings can be extended to the in vivo situation.

An important difference between apoptosis and necrosis is

that after a toxic insult, necrotic cell death will occur irrespective of other factors, whereas because apoptosis is an active cellular process, the susceptibility of a cell to undergo apoptotic cell death in response to a certain stimuli can vary depending on intracellular and extracellular factors. In other words, the threshold for induction of apoptosis depends upon the status of the cell. Healthy cells may be completely resistant to low concentrations of a toxin but damaged cells, or cells which have been compromised in some way, may be more susceptible and may activate the apoptotic pathway in response to the same concentration. The limited numbers of dopaminergic neurons which remain in the substantia nigra of parkinsonian patients might be predicted to be damaged, and therefore have a lower threshold for activation of an active apoptotic death program than normal healthy neurons. Although plasma concentrations of L-DOPA may not be sufficiently elevated to activate an apoptotic program in healthy animals or individuals, equivalent concentrations may induce a significant amount of apoptosis of dopaminergic cells in patients with Parkinson's disease. The hypothesis that L-DOPA–induced apoptosis and hence toxicity may depend on the condition of the cell is supported by in vivo evidence (19, 20). Ogawa et al. (19) examined the effects of chronic L-DOPA treatment in mice in which catecholaminergic neurons had been damaged by pretreatment with 6-hydroxydopamine (6OHDA). Using lipid peroxidation in the brain as an indicator of neuronal damage, these investigators reported that L-DOPA treatment resulted in damage to neurons which had been injured previously by 6OHDA treatment, but not in normal neurons. Blunt et al. (20) have also reported that in rats with a unilateral 6OHDA lesion, chronic administration of L-DOPA decreased the number of dopaminergic cells in the ventral tegmental area ipsilateral to the lesion, but had no effect on the number of cells in the corresponding area from the contralateral, nonlesioned side.

It has been demonstrated that there are significantly lower levels of free radical scavengers and reducing agents in the substantia nigra from patients with Parkinson's disease (37), thus neurons in this area of the parkinsonian brain would be especially vulnerable to oxidative damage. The generation of free radicals has been implicated in the pathogenesis of Parkinson's disease and Perry et al. (37) have previously suggested that prophylactic antioxidants might slow the onset of the disease. However a recent major study in North America demonstrated that administration of tocopherol, a biologically active component of vitamin E, to patients with Parkinson's disease did not slow down the progression of neuronal degeneration (38). This clinical trial, however, did not investigate the effect of tocopherol in patients being treated with L-DOPA. The patients used in the study were untreated patients with Parkinson's disease, and the end point of the trial was the onset of disability prompting the clinical decision to withdraw tocopherol treatment and to begin administering L-DOPA. Thus, the effects of coadministering antioxidants with L-DOPA on the progression of Parkinson's disease were not investigated in the DATATOP study (38). Our data show that L-DOPA–induced apoptosis is mediated via generation of reactive oxygen species and can be completely prevented by certain antioxidants in vitro. A recent study has demonstrated that L-DOPA also increases production of hydroxyl radicals in the substantia nigra in vivo (39). We suggest that an approach to improving L-DOPA therapy would be to elevate the depleted levels of free radical scavenging agents in the substantia nigra of parkinsonian patients perhaps by coadministration of antioxidants. This adjuvant therapy might protect dopaminergic neurons from L-DOPA–induced apoptosis.

Our finding that L-DOPA induces cell death via an apoptotic mechanism may also have implications in patients with Parkinson's disease who are being considered for transplant therapy with grafts of fetal catecholaminergic cells. During embryonic development, the nervous system is shaped into its final form by a process that involves apoptosis of over 50% of the developing neurons (21). It is possible that during this developmental stage fetal neurons are primed to undergo apoptosis and are therefore more susceptible to activation of an apoptotic pathway by L-DOPA. It has been demonstrated that chronic administration of L-DOPA impairs both the development of grafts of fetal nigral neurons in 6OHDA lesioned animals (15), and subsequent recovery of rotational behavior after grafting (40). We suggest that fetal neurons will also be susceptible to the toxic effects of L-DOPA and may have an increased propensity to undergo apoptosis. This hypothesis supports the idea that withdrawal of post-implantation L-DOPA therapy in patients with Parkinson's disease who have received a fetal graft may increase the success of graft survival.

We were interested to determine whether the free radical damage which leads to apoptosis of PC12 cells is generated during the conversion of L-DOPA to dopamine, or even by dopamine itself. Direct application of dopamine to PC12 cells did induce apoptosis, but to a far lesser extent than did L-DOPA. The induction of apoptosis by L-DOPA could not be inhibited by preventing the conversion of L-DOPA to dopamine using carbidopa, an inhibitor of the enzyme which catalyzes this conversion. Thus L-DOPA appears to be an intrinsically toxic molecule, and therapeutic approaches could be made which might protect against the noxious side effect of L-DOPA, but retain the beneficial property of conversion to the active metabolite, dopamine. It can be envisaged that clinically, separation of toxicity and pharmacological activity might be achieved by the development of an L-DOPA analogue which could be converted to a dopaminergic agonist with efficacy on dopamine receptors, but which failed to exhibit formation of free radicals when metabolized. A more immediate clinical objective would be coadministration of antioxidants which remove the potential toxicity of L-DOPA but retain its conversion to dopamine.

This paper presents evidence that L-DOPA, the most successful and frequently prescribed drug for alleviating the symptoms of Parkinson's disease, is toxic to catecholaminergic cells and that this toxicity is mediated by a process of apoptosis. Apoptosis is a form of cell death with active kinetics, thus the toxicity of the drug may be considerably reduced by changes in the therapeutic regimen such as giving the minimum effective dose and avoiding fluctuations in serum levels by using slow release preparations (1). Since the induction of apoptosis by L-DOPA does not appear to be inextricably linked to its pharmacological activity, the toxicity may be viewed as a side effect which may be abrogated by, for example, the use of antioxidants, without loss of therapeutic benefit. Furthermore, we believe that L-DOPA–induced apoptosis of nigral neurons may underlie the decrease in efficacy observed with long-term administration of this anti-parkinsonian agent and may be responsible for accelerating the progression of neuronal degeneration in Parkinson's disease.

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