Research report

Differential neurotoxicity induced by L-DOPA and dopamine in cultured striatal neurons

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Abstract

The neurotoxicity of L-DOPA and dopamine (DA) on striatal neurons was examined by using primary cultures of rat striatum. Exposure to L-DOPA and DA at concentrations of 30–300 μM induced dose-dependent cell death in both younger cultures (3 days in culture, 3 DIC) and elder cultures (10 days in culture, 10 DIC). The cytotoxicity of L-DOPA and DA was also dependent on the exposure time (6–24 h). Ascorbic acid (200 μM) inhibited both L-DOPA- and DA-induced cytotoxicity in 3 DIC cultures, whereas it provided significant protection against DA- but not L-DOPA-induced cytotoxicity in 10 DIC cultures. The L-DOPA cytotoxicity in 10 DIC cultures was prevented by a non-NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and by an NMDA receptor antagonist, MK-801. Neither antagonist prevented DA cytotoxicity. D-DOPA did not affect the viability of 10 DIC cultures, though it elicited marked toxicity in 3 DIC cultures. These results suggest that there are two components in the mechanisms that mediate the L-DOPA neurotoxicity on striatal neurons: one is autoxidation-relevant and the other is autoxidation-irrelevant. With respect to the latter, glutamate receptor stimulation may be involved. In contrast, autoxidation plays an important role in DA neurotoxicity.

Keywords: Ascorbic acid; Autoxidation; Dopamine; Glutamate; L-DOPA; Neurotoxicity; Striatum

1. Introduction

The nigrostriatal dopamine (DA) system plays an important role in the regulation of striatal function [19,26], and a number of studies have been performed to elucidate the physiological role of DA in the striatum. L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor substance of DA in catecholaminergic neurons, is the most effective therapeutic agent for Parkinson’s disease [17]. On the other hand, DA and its related substances have been implicated in the cause of neuronal injury. The mechanism underlying neuronal toxicity has been considered to be oxidative damage by degradation products of catecholamines, including quinone derivatives [14] and oxygen free radicals [27]. In addition, it has been proposed that DA and related substances have glutamatergic properties. For the instance, the dopaminergic system has been suggested to play a role in the neuronal injury and death accompanying cerebral ischemia [12], a process mediated at least in part by glutamate receptor activation. In another line of investigation, it has been shown that a methamphetamine-induced loss of dopaminergic fiber in the striatum was blocked by an N-methyl-D-aspartate (NMDA) receptor antagonist, MK-801 [29] and that the toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to the substantia nigra was blocked by several NMDA antagonists [30]. In vivo microdialysis studies have revealed that the stimulation of dopamine receptors in rat striatum or substantia nigra increases the release of glutamate [1,23]. These results imply a link between dopaminergic systems and glutamate toxicity, and led us to consider the possibility that L-DOPA and DA, their metabolites, or substances derived from them by normal or abnormal degradation pathways might have glutamatergic properties. The credibility of this theory is further heightened by the finding that L-DOPA induces endogenous glutamate release from rat striatal slices [13].

We therefore examined the effects of L-DOPA and DA on the survival of cultured striatal neurons. We observed that an exposure of the cultures to L-DOPA or DA induced
cell death with different mechanisms: one is related to oxidation and the other involves glutamate receptor stimulation, probably due to the facilitated release of endogenous glutamate by L-DOPA.

2. Materials and methods

2.1. Cell culture

Primary striatal cell cultures were prepared from fetal Wistar rats on the 17–19th day of gestation, according to the method described previously [2,3]. Briefly, the anterior striatum was removed bilaterally and mechanically dissociated using scalpel blades, then filtered through a stainless steel mesh (150 mesh). The resultant single-cell suspension was plated on plastic coverslips that were placed in Falcon 60 mm dishes at a density of $4.5 \times 10^6$ cells/dish. The cultures were maintained in Eagle's MEM supplemented with 10% heat-inactivated FCS (1–7 days after plating) or 10% heat-inactivated horse serum (8–10 days after plating). Culture dishes were placed in a 37°C, 5% CO$_2$ humidified atmosphere in an incubator. After 8 days in culture, overgrowth of non-neuronal cells was prevented by adding 10 μM cytosine arabinoside in the media and incubating for 48 h. Thereafter, two-thirds of the medium volume in each dish was replaced with fresh medium every 2 days. The immunostaining for glial fibrillary acidic protein (GFAP) and microtubular associated protein (MAP-2) revealed that after 10 days of incubation under the above-mentioned conditions, neurons accounted for more than 93% of the cultured cells and glial cells for less than 7%.

2.2. Measurement of neurotoxicity

Neurotoxicity was quantified by a Trypan blue exclusion assay. The drug treatment was performed by transferring the cell-loaded coverslips into drug-containing media. After the desired incubation, the cultures were stained with 1.5% Trypan blue for 10 min at room temperature, then fixed with isotonic formalin for 2 min, rinsed with saline and photographed using a Hoffman modulation microscope (TMD 300, Nikon, Tokyo, Japan). Over 200 cells were counted to determine the viability of the cells on a coverslip, and five coverslips were used to obtain the means ± S.E.M. in each experiment.

2.3. Drugs

Drugs and their sources were as follows: L-DOPA, dopamine chloride, kainic acid (KA), L-(+)-ascorbic acid (AA) and D-DOPA were all from Nacalai Tesque (Kyoto, Japan); (+)-MK-801 hydrogen maleate and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Research Biochemicals International (MA, USA).

2.4. Statistics

Values are expressed as means ± S.E.M. Significant differences were determined by Dunnett’s two-tailed test. Significance was defined as $P < 0.05$.

Fig. 1. Effects of L-DOPA and dopamine (DA) on survival of cultured striatal neurons. Culture (10 DIC) fields were photographed after Trypan blue staining followed by formalin fixation. A indicates the sham-treated cells (sham). B and C show the cells treated for 24 h with 100 μM L-DOPA and 100 μM DA, respectively. Calibration bar = 30 μm.
3. Results

3.1. Effects of L-DOPA and DA on neuronal survival

The exposure of the striatal cultures in 10 days in culture (10 DIC) to either L-DOPA or DA elicited marked reduction of cell viability as revealed by Trypan blue exclusion (Fig. 1). Cells incubated for 24 h with Eagle’s MEM containing L-DOPA or DA had blue-stained cell bodies with disrupted neurites, whereas sham-treated cells had the bright cell bodies with well-developed neurites. As shown in Fig. 2, exposing cultures to L-DOPA or DA (30–300 μM) for 6–24 h reduced the viability of the striatal neurons in a concentration- and time-dependent manner.

3.2. Maturation-dependent alterations in susceptibility of neurons to the L-DOPA and DA cytotoxicity

To determine whether cell maturation influences the cytotoxic action of L-DOPA and DA, the neurotoxicity induced in 10 DIC cells and in 3 DIC cells was compared using sister cultures. Cultures were exposed to 100 μM of either L-DOPA or DA for 24 h. As shown in Fig. 3, the younger (3 DIC) cells were more vulnerable to the cytotoxic actions of L-DOPA and DA than were the elder (10 DIC) cells. It was interesting that the neurotoxic profile of kainic acid (KA) was markedly distinct from that of L-DOPA and DA: a 24-h exposure to 1 mM KA evoked a marked decrease in the viability of the elder cultures, while it elicited no cytotoxicity in the younger cultures. In contrast, 100 μM D-DOPA, an inactive enantiomer of L-DOPA, induced prominent toxicity in the younger cells but did not significantly affect the viability of the elder cultures (Fig. 4).

3.3. Protective effects of ascorbic acid on L-DOPA and DA neurotoxicity

It has been reported that oxidized products of catecholamines exert toxic actions on striatal neurons. Therefore, the effects of ascorbic acid (AA) on L-DOPA and DA neurotoxicity in striatal cultures were examined (Fig. 5). A 24-h exposure to 200 μM AA alone did not affect cell viability. In 3 DIC cultures, both L-DOPA- and DA-induced neurotoxicity were significantly ameliorated by simultaneous addition of AA to L-DOPA- or DA-containing medium. In 10 DIC cultures, AA did not affect L-DOPA-induced neurotoxicity but significantly reduced DA-induced neurotoxicity. We examined the autoxidation of L-DOPA and dopamine after 24-h incubation of these cultures using HPLC analysis. When 100 μM L-DOPA or dopamine was incubated for 24 h in the cell-free medium, the contents of L-DOPA and dopamine after 24-h incubation were 35.5 ± 3.1% and 15.2 ± 1.8% of that immedi-
Fig. 5. Effects of ascorbic acid (AA) on L-DOPA and DA cytotoxicity in striatal neurons. AA was applied simultaneously with L-DOPA or DA to striatal cultures. * \( P < 0.05 \), ** \( P < 0.01 \). NS, no significance.

ately after their addition, respectively \( (n = 5) \). The simultaneous addition of AA inhibited the decomposition of both L-DOPA and dopamine. When AA with L-DOPA or dopamine was incubated, the contents of L-DOPA and dopamine after 24-h incubation were 59.7 ± 5.8% and 25.7 ± 2.4% of that immediately after addition, respectively \( (n = 5) \).

3.4. Glutamatergic antagonists differentiate between L-DOPA and DA neurotoxicity

To elucidate whether L-DOPA- and DA-neurotoxicity involve glutamatergic properties, either CNQX, a non-NMDA receptor antagonist, or MK-801, an NMDA receptor antagonist, was administered simultaneously with L-DOPA or DA in 10 DIC cultures. The results are summarized in Fig. 6. L-DOPA neurotoxicity was exclusively inhibited by a simultaneous application of CNQX \( (10 \mu M) \) or MK-801 \( (10 \mu M) \). In contrast, these drugs did not affect DA neurotoxicity.

Fig. 6. Effects of glutamate receptor antagonists on L-DOPA and DA cytotoxicity in cultured striatal neurons. CNQX \( (10 \mu M) \) and MK-801 \( (10 \mu M) \) were applied simultaneously with L-DOPA or DA to striatal cultures. * \( P < 0.01 \) vs. sham. NS, no significance when compared with DA alone.

4. Discussion

The present findings demonstrate that L-DOPA and DA are cytotoxic to cultured striatal neurons in concentration- and time-dependent manners. The proposed mechanism of the neurotoxic actions of L-DOPA and DA is their autoxidation into reactive free radicals and quinones [4,5]. The generated radicals have potent toxicity to the respiratory chain in the mitochondria; the radicals cause oxidation of mitochondrial pyridine nucleotides and thereby stimulate \( Ca^{2+} \) release from intact mitochondria [24]. Thus, an energy crisis evoked by mitochondrial dysfunction results in cell death. However, in the present study, we observed that the susceptibility of striatal neurons to L-DOPA and DA toxicity varied with an increase in days in culture. The younger cells \( (3 \text{ DIC}) \) were more vulnerable to their toxicity than were their elder counterparts \( (10 \text{ DIC}) \). We have examined the effects of AA on L-DOPA and DA toxicity, because preceding papers employed AA to prevent the autoxidation of L-DOPA [18]. The younger cultures were also more protected by AA against the toxicity, implying that the autoxidation products of L-DOPA and DA contribute more to their toxicity on younger cultures than to that on elder cultures. This is further supported by the fact that D-DOPA, which lacks antiparkinsonism activity but undergoes autoxidation during incubation, also produced the cytotoxicity in the younger cells but not in the elder cultures. These observations may reflect a deficiency in the younger neurons of the defensive mechanism against the neurotoxicity of free radicals and quinones generated from L-DOPA and DA autoxidation [20]. Maturation dependency was particularly prominent in KA neurotoxicity in the present study. KA was neurotoxic to the elder cultures but inert to the younger ones. This is in agreement with the result of a Northern analysis of KA/AMPA receptor subunit mRNAs of the rat striatum; the quantity of the mRNAs were augmented with the development of the rat during postnatal days 1 to 14 [15]. The maturation-dependent toxicity of KA is possibly based on the difference between the number of KA receptors expressed in younger cultures and in elder ones.

Our results demonstrated that AA discriminated between L-DOPA and DA neurotoxicity on the elder cultures but not on the younger cultures. Moreover, D-DOPA was neurotoxic to the younger cultures but not to the elder cultures. These results lead us to the question of why the elder cultures were sensitive to DA autoxidants but not to L-DOPA or D-DOPA autoxidants. Catecholamine autoxidation yields various quinone derivatives [14], oxygen free radicals [27], and hydroxyl radicals [7]. Thus, many studies have already indicated that hydroxyl radicals play an important role in the cytotoxicity induced by dopamine and L-DOPA [28]. The products of autoxidized DA which are absent among those of L-DOPA or D-DOPA may exert the neurotoxicity on elder cultures, although these products have not yet been identified. There may also be some
additional autoxidation-independent mechanism contributing to the DA-induced neurotoxicity in the elder cultures. Indeed, several lines of evidence have indicated that AA has a DA antagonist property in addition to its reductive feature [16]. More evidence is needed to clarify the issue.

In the elder cultures, l-DOPA-induced neurotoxicity was not explicable in terms of its autoxidation, in view of the failure of AA to provide full protection. This suggests another autoxidation-independent mechanism underlying the neurotoxicity. It is noteworthy that a recent study has raised another possibility to explain l-DOPA toxicity, which is based on the finding that l-DOPA induces a transmitter-like release of endogenous glutamate [13]. This led us to examine the effects of glutamate receptor antagonists on l-DOPA and DA neurotoxicity. As shown by our results, both an NMDA antagonist and non-NMDA antagonist were capable of protecting the elder cultures against l-DOPA neurotoxicity. This suggests that l-DOPA-induced neuronal death is elicited by the following: exposure of the neurons to l-DOPA stimulates glutamate release from the neurons; the released glutamate acts on non-NMDA receptors to depolarize the neurons; depolarized neurons facilitate the release of NMDA receptor channel function from Mg²⁺-block; glutamate acts on NMDA receptors to induce Ca²⁺-influx to the neurons; Ca²⁺, forming the complex with calmodulin, activates the neuronal type of nitric oxide synthase (nNOS); nNOS with increased activity produces NO with a detrimental property to the cell and mitochondrial membrane, leading to neuronal death. This is supported by the present observation that d-DOPA did not induce neurotoxicity on the elder cultures, which could reasonably be associated with its inability to induce glutamate release [13]. On the other hand, neither non-NMDA nor NMDA receptor antagonist affected dopamine neurotoxicity in 10 DIC, contradicting some reports that dopamine has the ability to release glutamate [1,24]. We estimate that glutamate release is not involved in dopamine neurotoxicity for the following reasons: first, as shown in Fig. 5, an anti-oxidizing agent, ascorbate, blocked dopamine neurotoxicity in both the 3 and the 10 DIC, but glutamate receptor antagonists had no influence on the neurotoxicity; second, there is little direct evidence for glutamatergic properties of dopamine and there is disagreement among the lines of evidence. Moreover, there is a report that dopamine inhibited glutamate release induced by veratrine in striatal slices [8]. Thus, it is not likely that glutamate release plays a role in dopamine-induced neurotoxicity.

l-DOPA, a DA precursor, is well established as a medicine for symptomatic treatment of patients with Parkinson’s disease (PD), but its influence on the progression of the disease is debated [6,10]. The toxic concentration of l-DOPA used in the present study was about 10-times as high as the therapeutic plasma concentrations in PD patients [27]. However, neurotoxicity cannot be ruled in PD patients who receive long-term l-DOPA therapy, because they are chronically exposed to l-DOPA and may have particular susceptibility to neurotoxic agents; low levels of reducing agents and free radical scavengers have been found in their nigrostriatal tissues [9,22,25]. The present results favor the notion that long-term l-DOPA therapy has the potential to accelerate the progression of PD [11], and suggest that dose increasing, in an attempt to compensate the inevitable decline in the efficacy of l-DOPA therapy, should be limited. Olney et al. have proposed that the cytotoxicity of l-DOPA and its ortho-hydroxylated derivative, 6-OH-DOPA, might be implicated in the degeneration of striatal neurons in Huntington’s disease [21]. Our present results provide experimental support for this hypothesis.

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References


