

SELECTIVE DOPAMINERGIC VULNERABILITY: 3,4-DIHYDROXYPHENYLACETALDEHYDE TARGETS MITOCHONDRIA

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Abstract—Parkinson's disease (PD) is a major cause of age-related morbidity and mortality, present in nearly 1% of individuals at ages 70–79 and ~2.5% of individuals at age 85. L-DOPA (L-dihydroxyphenylalanine), which is metabolized to dopamine by dopa decarboxylase, is the primary therapy for PD, but may also contribute to disease progression. Association between mitochondrial dysfunction, monoamine oxidase (MAO) activity, and dopaminergic neurotoxicity has been repeatedly observed, but the mechanisms underlying selective dopaminergic neuron depletion in aging and neurodegenerative disorders remain unclear. We now report that 3,4-dihydroxyphenylacetaldehyde (DOPAL), the MAO metabolite of dopamine, is more cytotoxic in neuronally differentiated PC12 cells than dopamine and several of its metabolites. In isolated, energetically compromised mitochondria, physiological concentrations of DOPAL induced the permeability transition (PT), a trigger for cell death. Dopamine was > 1000-fold less potent. PT inhibitors protected both mitochondria and cells against DOPAL. Sensitivity to DOPAL was reduced \geq 30-fold in fully energized mitochondria, suggesting that mitochondrial respiration may increase resistance to PT induction by the endogenous DOPAL in the *substantia nigra*. These data provide a potential mechanism of action for L-DOPA-mediated neurotoxicity and suggest two potentially interactive mechanisms for the selective vulnerability of neurons exposed to dopamine. © 2001 Elsevier Science Inc.

Keywords—Free radicals, Parkinson's disease, Reactive species, Dopamine, Mitochondria, Permeability transition

INTRODUCTION

Selective vulnerability of dopaminergic neurons is manifest in the progressive loss of these neurons in both aging and PD. The mechanism(s) underlying selective vulnerability remain(s) unclear, but evidence from a variety of experimental approaches connects dopamine toxicity, MAO activity, and mitochondrial dysfunction. For example, inhibition of mitochondrial respiration by monoamine-derived H₂O₂ [1], L-dopa mediated inhibition of respiration [2], and dopamine auto-oxidation [3] have all been proposed to contribute to the oxidative

stress [4] and mitochondrial dysfunction [5–8] associated with the dopaminergic deficits in PD. The dopamine auto-oxidation product, 6-OH dopamine, can also stimulate induction of the Ca²⁺-mediated mitochondrial permeability transition (PT) [3], an event implicated in cell death under some circumstances [9–13]. The concentrations of both 6-OH-dopamine and Ca²⁺ required, however, are well beyond those known to occur in vivo [3].

DOPAL is the initial product of MAO activity on dopamine, and it is the precursor of 3,4-dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxyphenylethanol (DOPET). Recent work shows that severe metabolic inhibition (rotenone coupled with glucose deprivation) increases the concentration of DOPAL in neuronally differentiated PC12 cells [14]. The potential toxicity of monoamine-derived aldehydes [15] suggests that the re-

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active aldehyde DOPAL may be a physiological mediator of neurotoxicity. This hypothesis is supported by recent evidence that DOPAL can exacerbate toxicity induced by severe metabolic inhibition and by evidence that doses of 100 μM and 500 μM DOPAL can increase lactate dehydrogenase release from neuronally differentiated pheochromocytoma (PC12) cells and the neuroblastoma line SK-N-SH [14]. Involvement of a toxic monoamine metabolite in neurotoxicity is consistent with evidence that the aldehyde metabolites of catecholamines generate free radicals [16] and induce apoptotic neuronal death in catecholaminergic neurons [17, 18]. The catecholamines themselves or their other metabolites do not. Other reactive aldehydes, including the lipid peroxidation byproduct 4-hydroxyhexenal and DOPEGAL [3,4-dihydroxyphenylglycolaldehyde], the monoamine metabolite of epinephrine and norepinephrine, are also potent co-stimulators of the mitochondrial PT [16,19].

A potential role for dopamine metabolites in neuronal toxicity is supported by evidence that millimolar dopamine can kill undifferentiated PC12 cells in a manner that may, based on cyclosporin A studies, involve the PT [20]. Involvement of MAO is equivocal because the nonspecific MAO inhibitor pargyline protects, but so does deprenyl, which selectively inhibits the MAO-B isoform not present in PC12 cells. As noted by the authors of ref. [20], however, deprenyl's protection may be due to its antioxidant capacity. Deprenyl may also have other non-MAO-dependent effects on the PT (B.S.K./W.J.B., manuscript in preparation). Dopamine-induced cytotoxicity is inhibited by *bcl-2* [21]. The protective effects of *bcl-2* are consistent with possible mitochondrial involvement in dopamine toxicity, but give no insight into the active metabolite. We now report that DOPAL, at physiological levels, is a highly potent mediator of cell death that is active at the mitochondrial level.

MATERIALS AND METHODS

Animal husbandry

Male Fischer 344 rats at ~ 90 d of age were obtained from Charles River laboratories. Rats were housed in Thorin micro-isolator units in a conventional, sentinel-monitored, IACUC-approved colony for at least 3 weeks prior to sacrifice. Rats were maintained on Purina Rodent Chow 5001 and water ad libitum.

Chemicals

Ultrapure sucrose was from ICN Biomedicals (Costa Mesa, CA, USA). All other compounds were from Sigma

(St. Louis, MO, USA) and of the highest purity available. Substrate solutions were brought to neutral pH prior to addition to the reaction mixtures.

DOPAL stock solutions

DOPAL was chemically synthesized [22]. Stocks were characterized, and were 99.5% pure as judged by HPLC.

Toxicity experiments

Toxicity experiments were essentially as described previously [18]. Briefly, PC12 cultures were initially expanded in T-75 flasks with 20 ml Dulbecco's Modified Eagle's Medium supplemented with 4.5 g/l glucose, 4 mM L-glutamine, 5% fetal calf serum, 10% horse serum, 1000 units/l penicillin, and 100 mg/l streptomycin. Cells were transferred to six well culture plates with 2 ml media and grown to 1.5×10^5 cells/well, treated with 50 ng/ml nerve growth factor (NGF), and maintained in NGF-containing media for 7 d prior to experimentation. The medium was changed every 2–3 d. NGF treatment caused the cells to differentiate, as judged by neurite outgrowth. During the experiments, the medium was replaced once a day. Cells were detached with buffered saline containing EDTA, centrifuged at $200 \times g$ and resuspended in trypan blue for counting of viable cells. To prevent extreme values from exerting undue influence on mean values, counts > 4 standard deviations away from the mean of identically treated wells (3/59, $\sim 5\%$) were excluded from the data in Fig. 4, as described previously [23,24].

Mitochondria were isolated as previously described [25]. Briefly, livers were rapidly homogenized in ice-cold buffer [250 mM mannitol, 75 mM sucrose, 100 μM K-EDTA, 10 mM K-HEPES (pH 7.4)] supplemented with 500 μM K-EGTA (pH 7.4). Homogenates were centrifuged at $1000 \times g$ for 10 min. Supernatants were removed and centrifuged at $10,000 \times g$ for 15 min. Pellets were washed three times in the above buffer supplemented with 0.5% fatty acid-free bovine serum albumin (BSA, Sigma A-6003). The first wash was also supplemented with 500 μM EGTA. The final mitochondrial pellet was resuspended in the above buffer with 5 μM EGTA and no BSA.

PT induction

PT induction was assessed spectrophotometrically (loss of absorbance at 540 nm) essentially as previously described [25] by suspending ~ 0.1 – 0.2 mg mitochondrial protein at room temperature in 200 μl of 215 mM

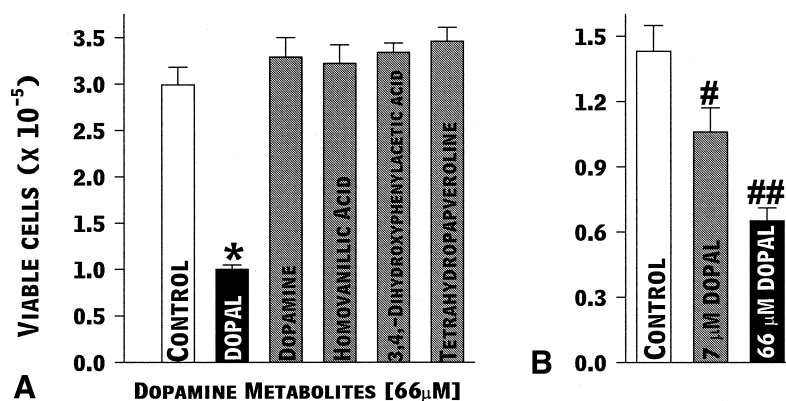


Fig. 1. DOPAL is more cytotoxic than other naturally occurring dopamine metabolites. (A) Differentiated PC-12 cells were cultured for 72 h in the presence or absence of 66 μM of the indicated dopamine metabolites. Each mean \pm SEM is derived from five wells. (B) Differentiated PC-12 cells were cultured for 48 h with or without DOPAL. Each mean \pm SEM is derived from three or four wells. * $p < .001$ vs. controls; # $p < .05$ vs. controls and vs. 66 μM DOPAL; ## $p < .001$ vs. controls and $p < .05$ vs. 6.6 μM DOPAL.

mannitol, 71 mM sucrose, 5 mM K-HEPES (pH 7.4). The total divalent cation concentration present in the buffer was estimated by a spectrophotometric sequestration assay to be $\sim 5 \mu\text{M}$ ($\sim 3 \mu\text{M Ca}^{2+}$) using 2.5 μM of the indicators Mag-Fura-2 and Fura Red and the cation chelators EDTA, EGTA, and TPEN [26]. ICP Mass spectroscopy and flame photometry confirmed that even the highest concentrations of DOPAL used did not contain significant levels of cations. Preliminary studies indicate essentially equivalent results occur in a KCl/phosphate-based assay system, which contained $< 0.3 \mu\text{M}$ divalent ions.

Statistical analysis

Unless noted, data were analyzed by ANOVA followed by the post hoc tests described in the figure legends.

RESULTS

DOPAL's toxicity at the cellular level was examined using neuronally differentiated PC12 cells as a model for dopaminergic neurons. PC12 cells are a rat pheochromocytoma-derived cell line that responds to nerve growth factor by extending neurites [27]. These cells are dopaminergic and sensitive to toxicants that kill dopaminergic neurons, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its metabolite, the 1-methyl-4-phenylpyridinium (MPP⁺) ion. The concentrations required for toxicity against PC12 cells are, however, higher than the concentrations required to kill primary neurons in culture [20,28].

Cytotoxicity studies revealed that DOPAL was the most toxic to PC12 cells of a series of related naturally

occurring metabolites of dopamine (Fig. 1). Incubation with 66 μM DOPAL killed $\sim 67\%$ of neuronally differentiated PC12 cells in 72 h, whereas cultures incubated with equivalent concentrations of dopamine, homovanillic acid, 3,4,-dihydroxyphenylacetic acid, and tetrahydropapveroline were indistinguishable from controls (Fig. 1A). This finding is consistent with past evidence that PC12 cells are resistant to dopamine doses below 1 mM [20], suggesting that DOPAL is ~ 100 -fold more toxic to these cells than dopamine. Further studies showed that DOPAL-induced cytotoxicity at near physiological levels (6.6 μM , Fig. 1B). Although PC12 cells are relatively resistant to dopamine toxicity, sensitivity to DOPAL was observed at concentrations close to those identified in normal human autopsy specimens of the substantia nigra ($354 \pm 23 \text{ pg/mg wet wt}$ [$N = 3$, mean \pm SEM] or $\sim 2.3 \mu\text{M}$, ref. [29] and unpublished data [W.J.B./S.W.L.]).

Biochemical studies of the effects of DOPAL on PT induction were conducted using isolated liver mitochondria because the properties of the PT in liver mitochondria are better documented and less controversial than the PT in brain mitochondria ([30,31] and see discussion). The ability of DOPAL to stimulate PT induction in liver mitochondria isolated from male Fischer 344 rats was measured by monitoring swelling spectrophotometrically by standard methods [19,25]. Assays were run in the presence of $\sim 2\text{--}3 \text{ nmol Ca}^{2+}/\text{mg}$ mitochondrial protein (see Fig. 2 legend); this Ca^{2+} exposure is comparable to physiological levels for mitochondrial populations that directly respond to cellular Ca^{2+} -signaling pathways [32–34]. In the absence of exogenous respiratory substrates, concentrations of DOPAL $\geq 125 \text{ nM}$ increased the rate of PT induction (Fig. 2A). Note that some doses that accelerate PT are actually *below* the

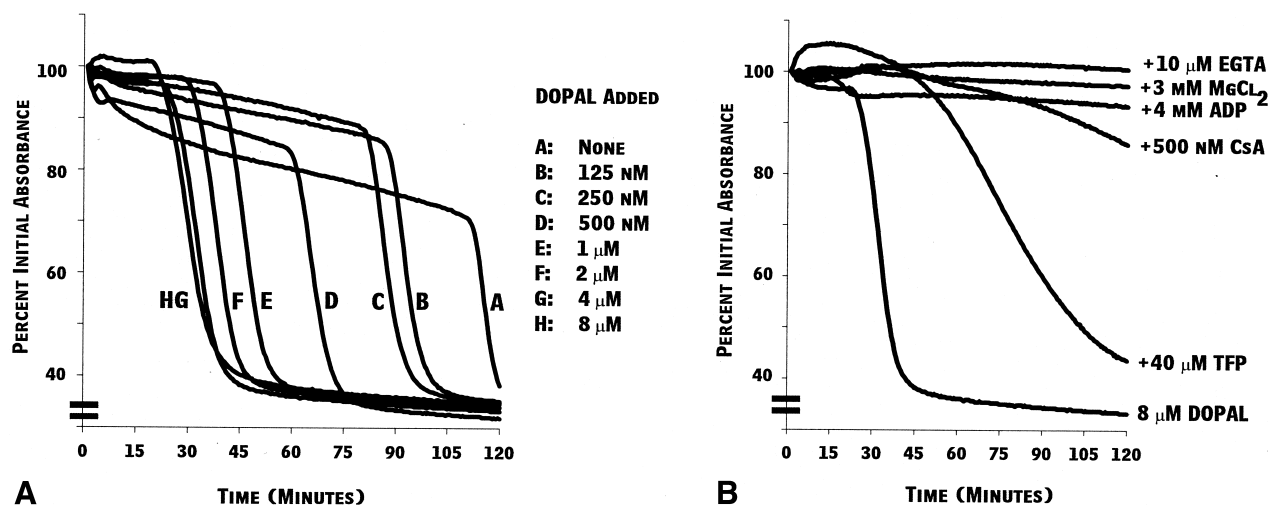


Fig. 2. DOPAL stimulates PT induction. (A) Representative traces showing the effects of 0–8 μM DOPAL on PT induction. Data for all traces were collected simultaneously using the same mitochondrial preparation. (B) Representative traces showing effects of well-characterized PT inhibitors on the loss of absorbance induced by 8 μM DOPAL. Data are representative of studies on three to five independent mitochondrial preparations.

observed level of DOPAL in tissue samples. In contrast to DOPAL, dopamine had no consistent effects on PT induction at concentrations $< 500 \mu\text{M}$ (not shown).

To confirm that the DOPAL-mediated loss of absorbance represented PT, and not a nonspecific loss of absorbance, we tested the efficacy of five well-characterized inhibitors of the PT—cyclosporin A (CsA), MgCl₂, ADP, EGTA, and trifluoperazine [25,35–38] in preventing DOPAL-mediated effects. As shown in Fig. 2B, all five inhibitors delayed induction by DOPAL, confirming that DOPAL-mediated loss of absorbance reflects PT induction.

The low resistance of mitochondria to DOPAL ob-

served in the experiment described above was somewhat surprising, since these concentrations of DOPAL approach those observed in human autopsy specimens. One possible explanation lies in the prior observation that respiration can protect mitochondria from the PT [25, 39]. Further studies revealed that actively respiring mitochondria are highly resistant to DOPAL-mediated PT induction. Addition of 10 mM exogenous succinate decreases mitochondrial sensitivity to DOPAL-mediated PT induction by ≥ 30 -fold (Figs. 2 and 3 and data not shown).

Data from cytotoxicity experiments conducted in the presence of rotenone, a mitochondrial complex I

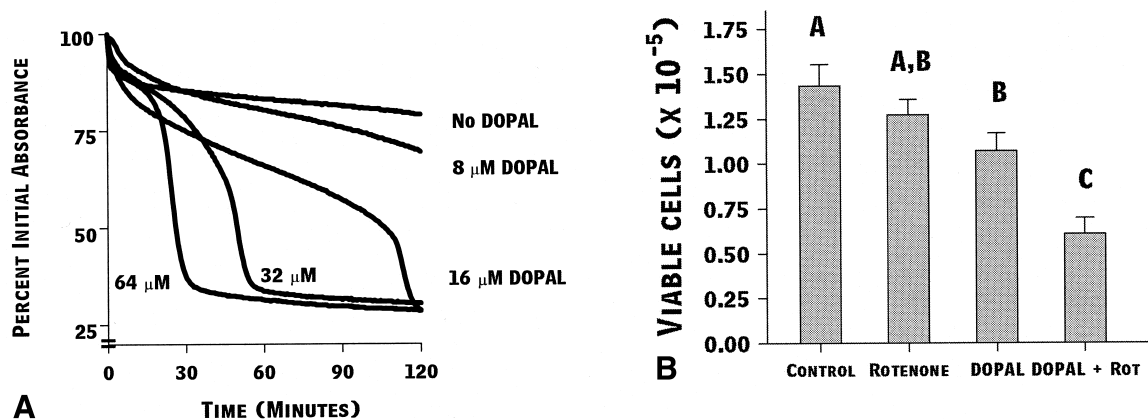


Fig. 3. Respiration protects against DOPAL. (A) Representative traces showing the effects of DOPAL on PT induction in the presence of 10 mM succinate. (B) Differentiated PC-12 cells were cultured for 48 h with or without DOPAL and/or rotenone. In the experiment shown, mean \pm SEM is derived from four wells. Groups not sharing a letter are statistically different by ANOVA followed by Fishers Protected Least Significant Difference post hoc test. DOPAL plus rotenone— $p < .01$ vs. all other groups; Control vs. DOPAL alone, $p < .05$.

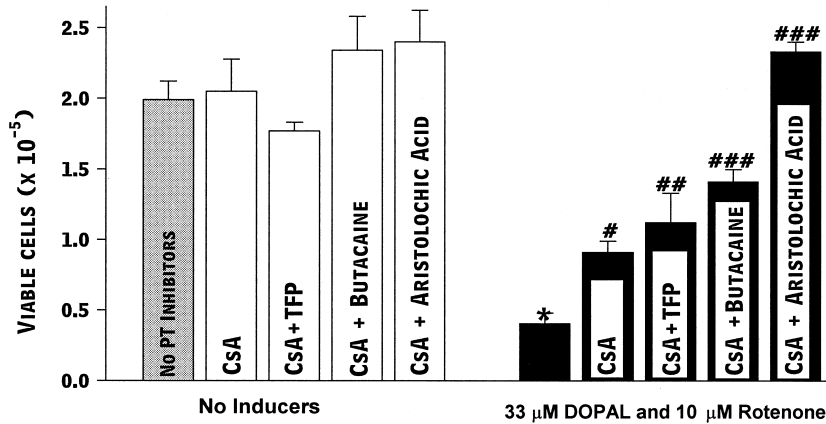


Fig. 4. DOPAL-mediated cytotoxicity in PC12 cells is prevented by PT inhibitors. Differentiated PC-12 cells were cultured for 72 h untreated or in the presence of 33 μ M DOPAL and 10 μ M rotenone with or without 1 μ M CsA either alone or in combination with 5 μ M trifluoperazine (TFP), 30 μ M butacaine, or 50 μ M aristolochic acid. Each mean \pm SEM is derived from five wells. Data were analyzed by ANOVA followed by the statistically conservative Tukey/Kramer post hoc test ($p < .05$ and $p < .01$); values significant at $p < .01$ were further evaluated by Fishers Protected Least Significant Difference Test. * $p < .0001$ vs. samples without DOPAL; # $p < .05$ vs. DOPAL alone; ## $p < .001$ vs. DOPAL alone; ### $p < .0001$ vs. DOPAL alone.

inhibitor that was used to decrease flux through complex I to mimic the complex I deficiency commonly observed in PD [5], are consistent with the hypothesis that respiration can protect mitochondria from the PT [6–8]. Concentrations of rotenone that were minimally toxic to differentiated PC12 cells nevertheless increased the cytotoxicity of 33 μ M DOPAL (Fig. 3B shows results from a single experiment used to establish experimental conditions for the study below). These data, and the resultant model used below, are consistent with recent evidence that DOPAL can exacerbate toxicity induced by severe metabolic inhibition deprivation [40]. This model, the combination of rotenone and DOPAL, was used to evaluate the role of PT in DOPAL-mediated cytotoxicity.

A role for the PT as a causative factor in DOPAL-mediated cytotoxicity in differentiated PC12 cells is supported by the finding that PT inhibitors decrease DOPAL-induced cell death. The PT inhibitor cyclosporin A (CsA), either alone [41–43] or in combination with trifluoperazine [44], butacaine [43], or aristolochic acid [45], had no effect on the viability of differentiated PC12 cells in the absence of DOPAL (Fig. 4). All PT inhibitors tested attenuated the toxicity of DOPAL and rotenone—CsA plus aristolochic acid gave complete protection. The synergistic effects of aristolochic acid, butacaine, or trifluoperazine with CsA suggest that the primary protective effect is due to inhibition of PT induction rather than by action of CsA on calcineurin [31]. Addition of 20 mM fructose to the CsA plus trifluoperazine combination, a combination used by others to delay PT [46,47], did not further increase protection (not shown).

DISCUSSION

Our studies using isolated liver mitochondria and “neuronally differentiated” PC12 cells identify DOPAL as a PT inducer and cytotoxin. The “neuronally differentiated” dopaminergic PC12 cell line is a well-standardized and widely used tissue culture model system for studying insults that might affect the *substantia nigra*, including DOPAL [14,40]. The current studies complement our initial in vivo studies, in which we have shown that DOPAL is neurotoxic to dopamine neurons (defined histochemically by tyrosine hydroxylase immunoreactivity) when injected into the ventro tegmental area [48]. The cell culture model enables us to study these phenomena under a more controlled condition. Isolated mitochondria further complement our investigation by providing a model capable of addressing mechanism.

The existence of a PT or PT-like phenomena in brain mitochondria is supported by studies in isolated mitochondria [3,30,49–52], in cell culture [42,53,54], and in vivo [55]. However, no widely accepted, well-defined model for the PT in isolated brain mitochondria currently exists [56–58]. Specifically, although we, and now others, have demonstrated the existence of a Ca^{2+} -mediated swelling in isolated brain mitochondria [30], the in vitro manifestations and pharmacology of the PT in brain mitochondria are not well defined. Second, the isolation of brain mitochondria yields a mixed population from several cell types that may have very diverse behavior, whereas liver mitochondria, although derived from several cell types, appear to behave homogeneously. For these reasons, basic biochemical studies on the PT were carried out in liver mitochondria [49]. The choice of the

young adult rat is based on practical considerations (availability, cost), and evidence from our laboratory that there are some relatively small quantitative differences, but few if any qualitative differences between the PT in livers from animals of different ages ([59] and B.S.K., unpublished data). No data on the PT in the brains of older animals currently exists.

Our data indicate that DOPAL, the MAO metabolite of dopamine, is a potent cytotoxin and PT inducer that may contribute to dopaminergic neuron depletion. Involvement of the PT in neurotoxicity is controversial in many systems [31], but the cellular protection experiments shown in Fig. 4 clearly implicate the PT in DOPAL-induced toxicity in our experiments. The lowest cytotoxic dose of DOPAL in PC12 cells (6.6 μ M) approaches the DOPAL concentrations observed in autopsy samples of the *substantia nigra* from individuals without PD.¹ Cytotoxic doses in true neurons may well to be even lower, well within the physiological range. Basal endogenous production of DOPAL may therefore approach the threshold of toxicity, implying that even modest increases in DOPAL levels may lead to DOPAL-mediated cytotoxicity. Such increases could result from age-related decreases in mitochondrial aldehyde dehydrogenase [60] or age-related increases in MAO activity [61], from increases in *locus coeruleus* neuronal cell body levels of catecholamine synthesizing enzymes that can occur during defective axonal transport in neurodegenerative disorders [62,63], or from other alterations in dopamine metabolism.

Our data also suggest that, in addition, mitochondrial dysfunction may lower the threshold for DOPAL-mediated toxicity. Thus, even basal DOPAL concentrations may serve as a lethal insult to cells already having compromised mitochondria. Compromised mitochondrial function may result from age-related effects, exposure to environmental toxins (e.g., pesticides such as rotenone), and/or genetic predisposition to decreased complex I activity. Note that some studies have identified a respiratory complex I defect in platelet mitochondria isolated from Parkinson's disease, and that this defect is maintained in cybrids formed from these cells [64]. These findings suggest just such a genetic predisposition in some cases of PD. Increased DOPAL and/or decreased

mitochondrial function may also act synergistically with each other and with other previously proposed mechanisms for dopaminergic neurotoxicity. For example, monoamine- [1] or L-dopa-mediated respiratory inhibition [2] may compromise respiration, subsequently rendering normally harmless levels of DOPAL cytotoxic, and inhibition of respiratory function may increase DOPAL concentrations.

The formation of DOPAL may also contribute to the toxicity that has been proposed to accompany long-term treatment with L-DOPA. L-DOPA, combined with a DOPA decarboxylase inhibitor, is the primary therapy for PD, and is currently given to over 500,000 people per day in the US alone. Because of the lack of effective alternatives, the use of this drug persists despite evidence from over 50 studies documenting the potential toxicity of this agent and its potential for contributing to the progression of PD [65]. Although multiple papers have documented toxic effects of L-DOPA, the nature of the harmful derivative remains unclear. Our results suggest that DOPAL may mediate some of the toxicity that has been observed.

The shared ability of DOPAL and DOPEGAL [16] to both stimulate Ca^{2+} -mediated PT induction invites comparisons and suggests a possible common mechanism of PT induction by aldehydes, specifically MAO metabolites. Our initial study on DOPEGAL showed that DOPEGAL stimulated Ca^{2+} -mediated PT induction in liver mitochondria at concentrations $\geq 6 \mu$ M. The current studies on DOPAL extend this observation in several ways. First, PT appears respiration sensitive, which was not previously observed. Second, DOPAL appears to be nearly 50-fold more potent when mitochondria are respiring on endogenous substrate. Third, DOPAL is demonstrated to be a cytotoxin whose toxicity is attenuated or prevented by PT inhibitors and appears exacerbated by conditions that impair mitochondrial respiration. In support of a role for aldehydes in PT induction, we have previously shown that the reactive lipid peroxidation byproduct 4-hydroxyhexenal is one of the most potent inducers described to date (active in the femtomolar range [19]). Currently, we are further exploring whether MAO activity and other MAO substrates stimulate PT induction. Current evidence suggests substantial structural specificity in the capacity of these compounds to induce PT (B.S.K./W.J.B., manuscript in preparation). These data are consistent with our previous work showing that the activity of 4-hydroxyhexenal and 4-hydroxynonenal differ by ~ 8 orders of magnitude [19]. We therefore currently hypothesize that aldehydes may play a general role in PT induction, and that the efficacy of a given aldehyde is strongly structure-specific.

In conclusion, the observed toxicity of DOPAL provides a plausible mechanism linking MAO activity, do-

¹Because MAO is localized to the mitochondrial outer membrane, mitochondrial DOPAL concentrations *in vivo* may be even higher than the bulk concentrations reported. This issue, however, is complicated by at least two opposing considerations. First, the existence of aldehyde dehydrogenase in the mitochondrial matrix, which might keep the local steady-state concentration of a given aldehyde below that of the bulk phase. It must be noted, however, that any DOPAL formed would have to pass through the inner membrane, where the pore is localized, in order to reach and be acted on by the dehydrogenase. Thus, the most critical parameter may be flux rather than concentration. These issues will require further consideration and clarification.

pamine, mitochondrial abnormalities, and the selective vulnerability of neurons exposed to dopamine. DOPAL-medical toxicity may contribute to both the onset and progression of PD as well as to toxicity associated with L-DOPA therapy.

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ABBREVIATIONS

DOPAL—3,4-dihydroxyphenylacetaldehyde

L-DOPA—L-dihydroxyphenylalanine

MAO—monoamine oxidase

PT—permeability transition

CsA—cyclosporin A

PD—Parkinson's disease