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Dopamine- and L-β-3,4-Dihydroxyphenylalanine Hydrochloride (L-Dopa)-Induced Cytotoxicity Towards Catecholaminergic Neuroblastoma SH-SY5Y Cells

EFFECTS OF OXIDATIVE STRESS AND ANTIOXIDATIVE FACTORS

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ABSTRACT. Enhanced oxidative stress has been suggested to be involved in the degeneration of nigrostriatal dopaminergic neurons in Parkinson's disease. The high turnover rate of dopamine and/or unsequestered dopamine may cause an increase of formation of hydrogen peroxide via either oxidative deamination of dopamine by monoamine oxidase or autoxidation. Hydrogen peroxide would be converted to more toxic hydroxyl free radicals. L-β-3,4-Dihydroxyphenylalanine hydrochloride (L-DOPA), the most useful drug in the symptomatic treatment of Parkinson's disease, has been considered to possess deteriorating degenerative side-effects. The catecholaminergic neuroblastoma SH-SY5Y cells were chosen to investigate the cytotoxic effect of dopamine and L-DOPA. Both dopamine and L-DOPA were found to be cytotoxic towards SH-SY5Y cells. Such toxic effects were accompanied by an increase of oxidative stress in the cell cultures and could be reversed effectively by catalase and to a lesser extent by superoxide dismutase. The non-enzymatic antioxidants L-ascorbic acid, glutathione, *N*-acetyl-L-cysteine, but not (+)-α-tocopherol, also completely protected SH-SY5Y cells against the cytotoxic effects induced by dopamine and L-DOPA. Antioxidative factors, namely free radical scavengers (including *N-tert*-butyl-α-phenylnitrone, salicylic acid, and D-mannitol) and a strong iron chelator, deferoxamine, however, did not protect the SH-SY5Y cells against dopamine and L-DOPA. The generation of reactive oxygen species and the resulting enhanced oxidative stress was clearly involved in the dopamine- and L-DOPA-induced cytotoxic effects. Hydrogen peroxide played the most important role related to cytotoxicity of dopamine and L-DOPA. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:363–372, 1997.

KEY WORDS. SY-SY5Y cells; oxidative stress; cytotoxicity; dopamine; L-DOPA; antioxidants

Several lines of evidence have suggested that enhanced oxidative stress may be an important pathogenetic mediator in Parkinson's disease. The levels of antioxidant enzyme activities of catalase [1] and glutathione peroxidase [2], and of glutathione [3–6] were found to be reduced in the substantia nigra of the parkinsonian brain. The levels of copper, zinc-dependent, and/or manganese-dependent superoxide dismutase are increased in the substantia nigra of the parkinsonian brain. This may be an adaptive response to increased free radical formation reflecting an oxidative stress condition [7, 8]. The iron level is also increased in the post-mortem substantia nigra of Parkinson's patients [9–12]. Iron is involved in the generation of highly reactive hydroxyl radical from hydrogen peroxide via Fenton reaction or Haber–Weiss reaction. Hydroxyl radical can initiate lipid peroxidation chain reaction. It has been observed that the levels of basal lipid peroxidation [13] and lipid hydroperoxide [14] are increased in the substantia nigra of the parkinsonian brain. DNA could also be damaged by hydroxyl radical. 8-Hydroxy-deoxyguanosine, a product of hydroxyl radical attack on guanine in DNA, was also found to be increased in Parkinson's post-mortem brain tissue [15]. Dopamine is a neurotransmitter released during dopaminergic function, and the principal means to inactivate the released dopamine are active re-uptake and monoamine oxidase-catalyzed oxidative deamination. Hydrogen peroxide is generated as an end-product of the oxidative deamination by monoamine oxidase and can induce cellular damage directly or also indirectly by forming oxygen free radicals such as superoxide and hydroxyl radical.

Nigrostriatal dopamine deficiency is a consequence of the degeneration of nigrostriatal dopaminergic neurons in the substantia nigra of the parkinsonian brain [16–19]. To restore dopaminergic neurotransmission and function, the remaining dopaminergic neurons become hyperactive, and thus the dopamine turnover is increased. In turn, an in-

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creased generation of hydrogen peroxide is associated with an increased turnover of dopamine. In addition to oxidative deamination of dopamine by monoamine oxidase, unsequestered dopamine may be subjected to autoxidation [20–22]. Autoxidation of dopamine leads to the formation of reactive oxygen species. It has been speculated, therefore, that increased release, re-uptake, and presynaptic turnover of dopamine may accelerate the progressive degeneration of nigrostriatal dopaminergic neurons as observed in Parkinson's disease [23–27]. Cytotoxicity of catecholamines towards cultured neurons has been demonstrated [28, 29].

DOPA, which can replenish the striatal dopamine, is the most important therapeutic agent in alleviating the symptoms of Parkinson's disease. However, the effectiveness of L-DOPA‡ therapy declines with the length of L-DOPA treatment [30, 31]. It was even considered that L-DOPA treatment may exacerbate the progress of degeneration of dopaminergic neurons in Parkinson's patients treated with L-DOPA [24–33]. Indeed, reactive oxygen species can potentially be generated by the metabolism and/or the autoxidation of L-DOPA [22, 34, 35]. Therefore, it is possible that L-DOPA treatment may increase oxidative stress towards the nigrostriatal dopaminergic neurons, which have already suffered from enhanced oxidative stress.

In this study, we try to substantiate whether or not the toxicity of dopamine and L-DOPA towards dopaminergic neurons is via oxidative stress and whether such stress can be reversed by antioxidants. Catecholaminergic neuroblastoma SH-SY5Y cells were employed to assess the effects induced by dopamine and L-DOPA, because these cells are quite sensitive to oxidative stress, i.e. towards 6-hydroxydopamine [36]. The effects of various antioxidant enzymes, reducing agents, and free radical scavengers were assessed.

MATERIALS AND METHODS

Materials

Dopamine (3,4-dihydroxyphenylethylamine hydrochloride), L-DOPA, catalase (from bovine liver), superoxide dismutase (from bovine erythrocytes), L-ascorbic acid, (+)-α-tocopherol, glutathione (reduced form), *N*-acetyl-L-cysteine, deferoxamine mesylate, *N*-*tert*-butyl-α-phenylnitrone, salicylic acid (sodium salt), D-mannitol, DMEM, FBS (heat-inactivated), HBSS, MTT, BSA, NAD^+, sodium dodecyl sulfate 1,1,3,3-tetraethoxypropane and Triton X-100 were purchased from the Sigma Chemical Co. (St. Louis, Mo, U.S.A.). DMSO was obtained from the BDH Co. (Toronto, Ontario, Canada). Treated disposable sterile multiple well plates (96 wells w/lid, flat bottom) were ordered from the Corning Laboratory Sciences Co. (Corning, NY, U.S.A.). Sterile/gamma-irradiated tissue culture dishes (100 × 20 mm style), B–D® 22G1 needles, and B–D® 10-mL syringes were purchased from Becton Dickinson & Co. (Lincoln Park, NJ, U.S.A.). Syringe filters (0.2 μm) were from Gelman Sciences (Ann Arbor, MI, U.S.A.). All other chemicals were obtained from the BDH Co. and were of analytical grade or of the highest purity available.

Preparation of Drugs

All the test compounds except (+)-α-tocopherol were dissolved in DMEM. The pH of the DMEM solution containing test compounds was adjusted to pH 7.4. These solutions were sterilized by passing through 0.2 μm syringe filters and then were diluted to the required concentrations with DMEM containing 10% FBS. (+)-α-Tocopherol was dissolved in DMSO and diluted with DMEM containing 10% FBS to the desired concentrations (final concentration of DMSO less than 0.01%) [37]. DMSO at concentrations of 0.1% or lower has no effect on the viability of SH-SY5Y cells (unpublished data).

Catecholaminergic Neuroblastoma SH-SY5Y Cell Cultures

Catecholaminergic neuroblastoma SH-SY5Y cells, which were obtained from Dr. C. Haymen (Regeneron Pharmaceuticals, Tarrytown, NY, U.S.A.) [36], were grown in DMEM supplemented with 10% FBS in a water-jacketed incubator (Forma Scientific, Marietta, OH, U.S.A.) with a humidified atmosphere of 5% CO_2 in air at 37°. The cells were maintained in the proliferative growth phase on Falcon® 100 × 20 mm polystyrene tissue culture dishes. Passages were done at 3- to 5-day intervals under aseptic conditions in a biological safety cabinet (Forma Scientific) at room temperature. As the cells approached the subconfluent stage, they were harvested from tissue culture dishes mechanically by pipetting the medium up and down to detach the cells from the bottom of each of the tissue culture dishes. Single cells were dissociated by gently passing the cell suspension five times through a B–D® 22G1 needle with a B–D® 10-mL syringe. The cell density of this cell suspension was counted using a La Fontaine® hemacytometer counting chamber (Fisher, Toronto, Ontario, Canada) and was diluted to a cell density of 2×10^4 cells/well for seeding. Cells were then transferred into 96-well plates for the subsequent cytotoxic studies.

The optimal cell density, i.e. 2×10^4 cells/well, for seeding was chosen for all studies. Twenty-four hours after seeding, 25 μL of medium containing the test compounds at different concentrations was added into each well. Then, 25 μL of medium containing dopamine or L-DOPA at different concentrations was added. After 48 hr, cell viability was determined by the MTT assay, and melanin formation was also measured.

MTT Assay

The MTT assay was based on the conversion of the yellow tetrazolium salt MTT by mitochondrial dehydrogenases of

‡ *Abbreviations:* L-DOPA, L-β-3,4-dihydroxyphenylalanine hydrochloride; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HBSS, Hanks' balanced salts (modified); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; and LDH, lactate dehydrogenase.

live cells to the purple MTT formazan, which can be measured at 570 nm spectrophotometrically [38, 39]. MTT stock solution was prepared by dissolving MTT at 5 mg/mL in HBSS and sterilized by passing through 0.2-μm syringe filters. MTT reagent was prepared freshly from MTT stock solution (i.e. 1/10 dilution, 1 part stock MTT solution diluted in 9 parts DMEM containing 1% FBS), and 50 μL of MTT reagent was added to each well. The plates were incubated in the same CO_2 incubator for 4 hr. Acid-isopropanol (100 μL of 0.04 N HCl in isopropanol) containing 10% Triton X-100 was then added to each well and mixed thoroughly on a Titer plate shaker (Lab-Line Instruments Inc., Melrose Park, IL, U.S.A.) to dissolve the purple crystals. The plates were read with a Macintosh® Microcomputer-operated UVmax kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA, U.S.A.) at a wavelength of 570 nm. Wells without cells were used as blanks.

Data obtained from the software SOFTmax® (Molecular Devices Corp.) were transferred directly to Microsoft® Excel (Microsoft® Corp. Redmond, WA, U.S.A.) within the Macintosh® Microcomputer for the calculation of means, standard error, and percent viability.

LDH Assay

The LDH assay is based on the production of NADH from NAD^+ during the conversion of lactate to pyruvate by LDH. NADH has a maximum absorbance at 340 nm. The assay was performed using the 96-well plates. Aliquots (50 μL) of cell culture medium were incubated in the presence of NAD^+ (1 mg/mL in 0.05 M sodium pyrophosphate buffer, pH 9.0) and 100 μL lactate (1% in 0.05 M sodium pyrophosphate buffer, pH 9.0). The rates of NADH formation (milli-optical density per minute [mOD/min]) were monitored using the Macintosh® Microcomputer-operated UVmax kinetic microplate reader at 340 nm.

Estimation of Lipid Peroxidation

Malondialdehyde, the major end product of lipid peroxidation, was measured by a spectrophotometric method [40]. The samples were heated with thiobarbituric acid at low pH, and the pink chromogen products were measured at 532 nm spectrophotometrically. SH-SY5Y cells were harvested and washed with ice-cold HBSS three times. The cells were then homogenized with a Polytron® homogenizer (Brinkmann, Toronto, Ontario, Canada) in a 1.15% KCl solution (1:10, w/v) and 0.075% of sodium dodecyl sulfate was added to solubilize the tissues and to extract malondialdehyde from them. Then acetic acid (7.5%) and thiobarbituric acid (0.335%) were added. The solutions were mixed thoroughly and heated in boiling water for 60 min. The reaction was stopped by cooling with tap water. The pink chromogen was extracted with *n*-butanol and pyridine (15:1, v/v) and centrifuged. The organic layer was assessed at 532 nm using a Beckman DU-600 spectrophotometer (Beckman, Fullerton, CA, U.S.A.). The malondialdehyde concentrations in the sample were assessed according to a standard curve of malondialdehyde, which was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane as previously described [41]. 1,1,3,3-Tetraethoxypropane (220 mg) was dissolved in 100 mL of 1% (v/v) sulfuric acid. One milliliter of this solution was diluted to 100 mL with 1% (w/v) sulfuric acid, and the concentration was estimated by measuring the UV absorbance in a 1-cm cuvette at 245 nm (ϵ = 13,700).

Measurement of the Melanin Formed from Dopamine and L-DOPA

Melanin, the end product of the autoxidation of dopamine and L-DOPA, was monitored at 405 nm spectrophotometrically with a Macintosh® Microcomputer-operated UVmax kinetic microplate reader.

Protein Determination

The Bio-Rad dye-binding method (Bio-Rad, Richmond, CA, U.S.A.) was adapted according to Bradford [42]. Samples (800 μL; 1/40 dilution) were mixed with Bio-Rad Dye Reagent Concentration (200 μL) and then were incubated at 37° for 5 min. The absorbance of the solutions was measured at 595 nm using a Zeiss model PM2DL spectrophotometer (Carl Zeiss, Oberkochen, Germany). Bovine serum albumin was used as the standard.

Statistical Analyses

Statistical analyses were performed using one-way and/or two-way ANOVA with Newman–Keuls pairwise comparisons (CLR ANOVA software, Clearlake Research Inc., Houston, TX, U.S.A.). The results are expressed as means ± SEM.

RESULTS

Cytotoxicity Induced by Dopamine and L-DOPA

As can be seen from Fig. 1 (A and C), both dopamine and L-DOPA, at concentrations as low as 50 μM, were found to be significantly cytotoxic to SH-SY5Y cells. The percentages of cell viability were approximately 60 and 30% in the presence of 100 μM dopamine or L-DOPA, respectively. This concentration was chosen for both dopamine and L-DOPA in the subsequent experiments to assess the effects of various antioxidants and antioxidative factors. The cytotoxic effects induced by dopamine and L-DOPA were correlated with the formation of melanin in the cultures (Fig. 1, B and D), which is the end product of autoxidation of dopamine and L-DOPA. The cytotoxic effects of both dopamine and L-DOPA at 100 μM were time dependent, with the maximal toxicity reached after 48 hr of incubation. We also found that over 90% of dopamine or L-DOPA remained detectable after 1 hr of incubation in DMEM and 30–40% of these catecholamines still presented in the DMEM after 24 hr of incubation.

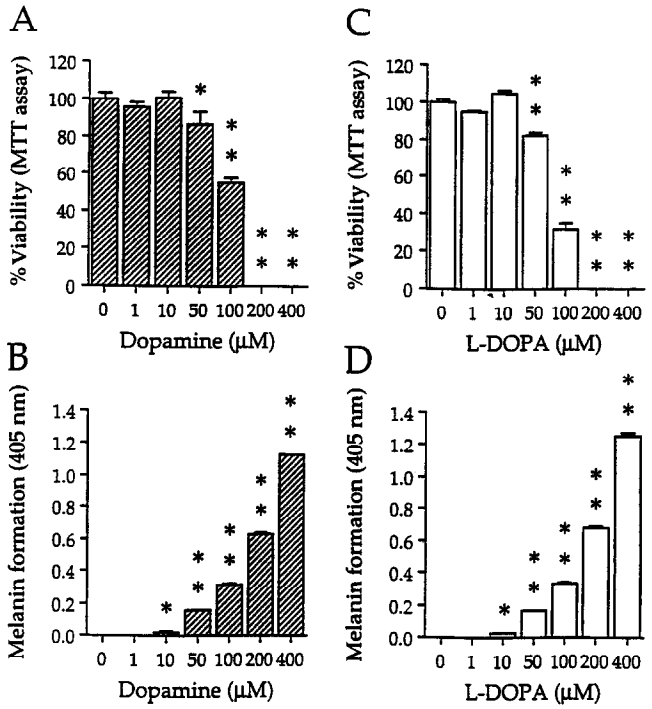

FIG. 1. Cytotoxicity of dopamine (A and B) and L-DOPA (C and D) towards SH-SY5Y cells, and the formation of melanin from dopamine and L-DOPA. Data are means ± SEM, N = 8. Statistical analyses were by one-way ANOVA with Newman–Keuls pairwise comparisons. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with dopamine = 0 μM or L-DOPA = 0 μM.

Effect of Dopamine on Lipid Peroxidation

The SH-SY5Y cells treated with a series of concentrations of dopamine for 48 hr and the levels of malondialdehye, which is an end product of lipid peroxidation, were measured. As can be seen in Fig. 2, dopamine induced lipid peroxidation of the neuroblastoma cells in a concentration-dependent manner. It reached a statistically significant level of 10 μM (Fig. 2A). However, dopamine at higher concentrations (i.e. above 100 μM) apparently inhibits the formation of malondialdehyde. This is due to the fact that dopamine can chemically react with malondialdehyde [43], but does not reflect a real reduction in malondialdehyde formation.

The cytotoxicity induced by dopamine was also confirmed by an LDH assay (Fig. 2B). Dopamine at higher concentrations (ie 100 and 1000 μM) caused substantial damage to the integrity of the cell membrane, which caused leakage of cytosol LDH. Lipid peroxidation was detected following treatment with 10 μM dopamine, namely, before the disintegration of the cell membranes.

Effects of the Antioxidant Enzymes Catalase and Superoxide Dismutase

Catalase (100 U/mL) completely prevented the cytotoxic effects of both dopamine- and L-DOPA-induced toxicity towards SH-SY5Y cells (Fig. 3, A and C). These results

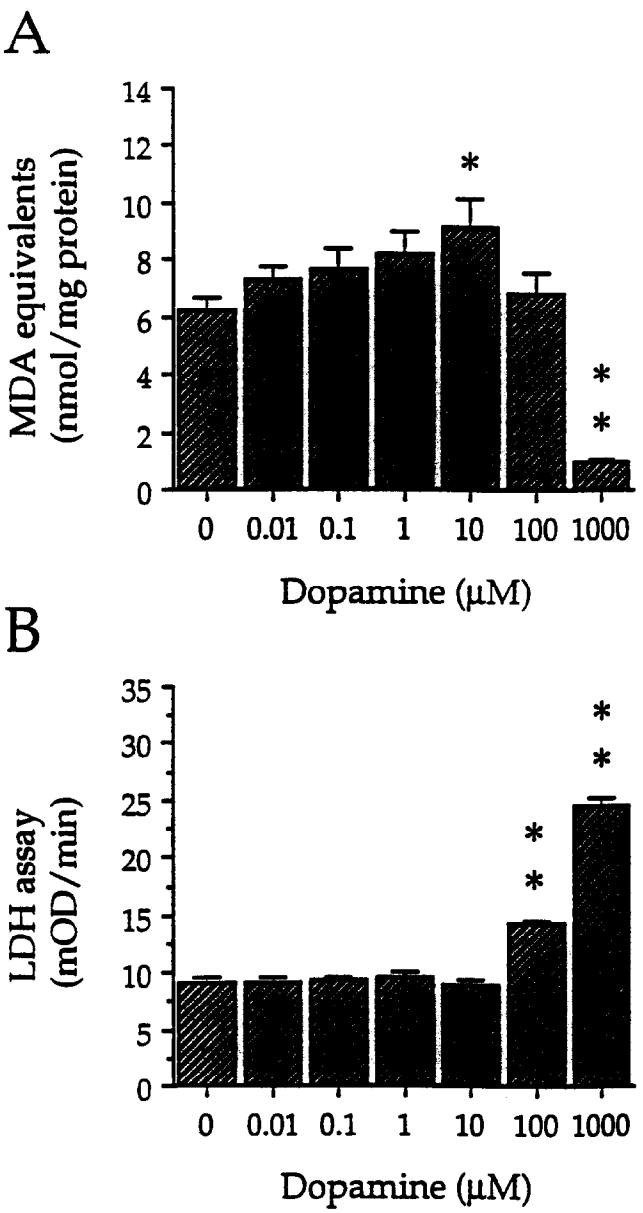

FIG. 2. Effect of dopamine on lipid peroxidation (A) and cell membrane integrity (B) of SH-SY5Y cells. Data are means ± SEM, N = 8. Statistical analyses were by one-way NOVA with Newman–Keuls pairwise comparisons. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with dopamine = 0 μM.

indicate that hydrogen peroxide is a byproduct during the autoxidation of dopamine and L-DOPA. It was interesting to note that catalase did not block the oxidative polymerization of dopamine or L-DOPA into melanin (Fig. 3, B and D).

Superoxide dismutase (from bovine erythrocytes) at a concentration of 500 U/mL produced partial protection of the SH-SY5Y cells against the cytotoxicity of 100 μM L-DOPA (Fig. 4B), but did not protect SH-SY5Y cells from the damage induced by 100 μM dopamine (Fig. 4A). The results suggested that superoxide might play a minor role in the cytotoxicity as induced by dopamine. The results seem to suggest that the mechanisms of oxidative damage of do-

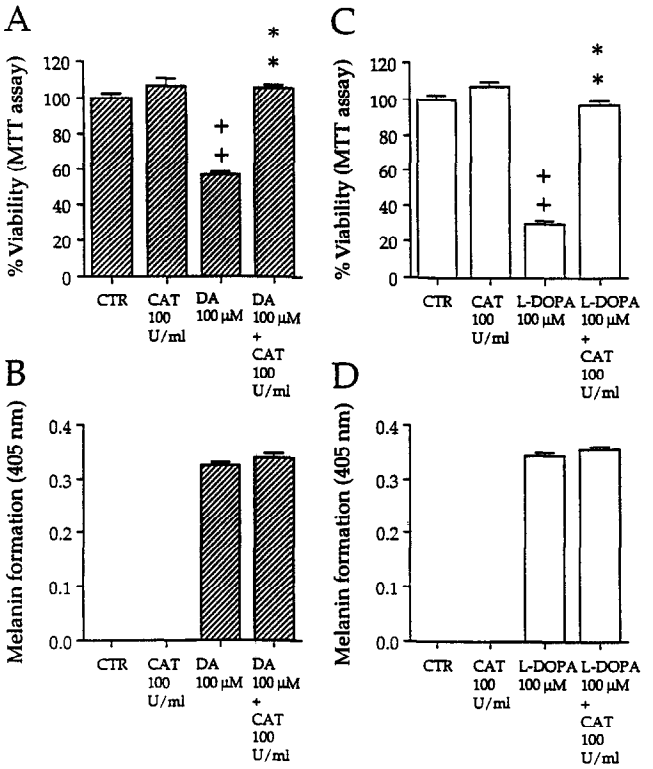

FIG. 3. Effect of catalase (CAT) on the cytotoxicity of dopamine (DA) and L-DOPA towards SH-SY5Y cells and the formation of melanin from DA and L-DOPA. Data are means ± SEM, N = 8. Statistical analyses were by two-way ANOVA with Newman–Keuls pairwise comparisons. Key: (++) $P < 0.01$ compared with control; and (**) $P < 0.01$ compared with DA = 100 μM or L-DOPA = 100 μM.

pamine and L-DOPA could be slightly different, because superoxide dismutase was capable of protecting the cells against L-DOPA.

Effects of Non-Enzymatic Antioxidants— L-Ascorbic Acid, (+)-α-Tocopherol, Glutathione, and N-Acetyl-L-Cysteine

Dopamine- or L-DOPA-induced cytotoxicity towards SY-SY5Y cells could be significantly prevented by L-ascorbic acid at concentrations of 100 and 200 μM (Fig. 5, A and C). L-Ascorbic acid by itself, however, at a higher concentration (i.e. 400 μM) was cytotoxic to both dopamine- or L-DOPA-treated or untreated SH-SY5Y cells. The protective effect could be related to the reducing ability of L-ascorbic acid, which inhibited the formation of melanin (Fig. 5, B and D).

(+)-α-Tocopherol up to 10 μM failed to exhibit any protective effects against dopamine or L-DOPA (Fig. 6, A and C). This agent also did not prevent the autoxidation of dopamine and L-DOPA, as measured by the formation of melanin (Fig. 6, B and D).

It has been proposed that (+)-α-tocopherol and L-ascorbic acid may act synergistically, wherein L-ascorbic acid can assist in recycling the antioxidative ability of (+)-α-tocopherol [44–46]. The effects of a combination of (+)-α-tocopherol (10 μM) and L-ascorbic acid (100 μM) against the dopamine- and L-DOPA-induced cytotoxicity were therefore assessed. No synergistic protective effect between (+)-α-tocopherol and L-ascorbic acid was observed under our experimental conditions (results not shown).

Both glutathione and *N*-acetyl-L-cysteine at concentrations of 1 and 10 mM significantly protected SH-SY5Y cells from the cytotoxicity induced by 100 μM dopamine or L-DOPA (Figs. 7 and 8). *N*-Acetyl-L-cysteine (10 mM) completely prevented the cytotoxic damage induced by either dopamine or L-DOPA (Fig. 8, A and C). The potency of glutathione (10 mM) was similar to that of *N*-acetyl-L-cysteine against dopamine (Fig. 7A), but only partially effective against L-DOPA-induced toxicity (Fig. 7C). Both thiol compounds were capable of reducing the formation of melanin (Figs. 7 and 8, B and D). These results are consistent with the idea that preventing autoxidation of dopamine and L-DOPA by depletion of reactive oxygen species by glutathione and *N*-acetyl-L-cysteine might account for their protective effects.

Effect of the Iron Chelator Deferoxamine

Deferoxamine at a concentration of 10 μM showed a statistically significant protective effect against dopamine-induced cytotoxic damage (Fig. 9A) but not against L-DOPA-induced damage (Fig. 9C). This iron chelator was shown to be cytotoxic towards SH-SY5Y cells, and it did not inhibit the formation of melanin (Fig. 9, B and D). These results indicate that iron contributed to the cytotoxic actions of dopamine, but was not involved in the oxidative synthesis of melanin in the present system.

Effects of the Free Radical Scavengers N-tert-Butyl-α-Phenylnitrone, Salicylic Acid, and D-Mannitol

The protective effects of three free radical scavengers, i.e. *N-tert*-butyl-α-phenylnitrone, salicylic acid, and D-

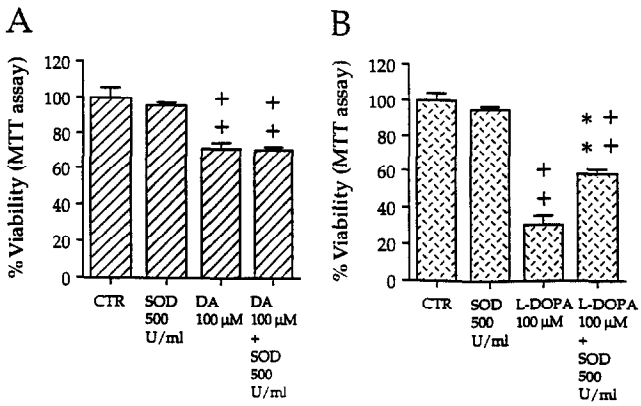

FIG. 4. Effect of superoxide dismutase (SOD) on the cytotoxicity of dopamine (DA) and L-DOPA. Data are means ± SEM, N = 8. Statistical analyses were by two-way ANOVA with Newman–Keuls pairwise comparisons. Key: (++) $P < 0.01$ compared with control (CTR); and (**) $P < 0.01$ compared with DA = 100 μM or L-DOPA = 100 μM.

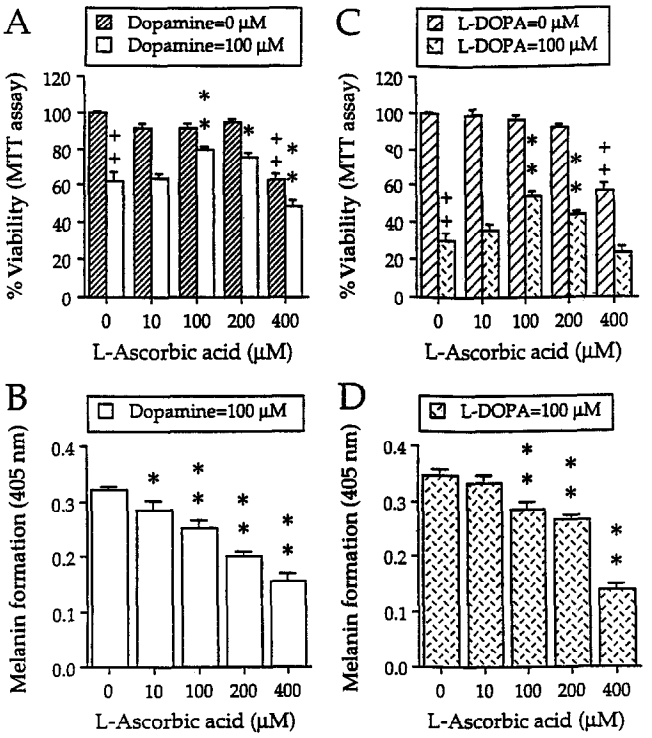

FIG. 5. Effect of L-ascorbic acid on the cytotoxicity of dopamine and L-DOPA towards SH-SY5Y cells and on the formation of melanin from dopamine and L-DOPA. Data are means ± SEM, N = 8. Statistical analyses were by two-way ANOVA with Newman–Keuls pairwise comparisons for A and C and by one-way ANOVA with Newman–Keuls pairwise comparisons for B and D. Key: (++) $P < 0.01$ compared with L-ascorbic acid = 0 μM at dopamine = 0 μM or L-DOPA = 0 μM; (*) $P < 0.05$; and (**) $P < 0.01$ compared with L-ascorbic acid = 0 μM at dopamine = 100 μM or L-DOPA = 100 μM.

mannitol, were also assessed. Up to 10 μM did not protect SH-SY5Y cells against the damage induced by either dopamine or L-DOPA (results not shown). Also these three free radical scavengers also did not affect the autoxidation of dopamine and L-DOPA (results not shown).

DISCUSSION

Catecholamines, especially dopamine, have long been suspected of being potential, endogenous neurotoxic agents towards dopaminergic neurons in the substantia nigra [23–27, 33]. It has been estimated that the average concentration of dopamine in a dopaminergic nerve terminal is approximately 50 mM [47, 48]. It is quite possible that dopamine at such a high concentration, if its action is not well controlled, can be harmful. It has also been considered that the nigral dopaminergic neurons may be damaged by long-term administration of L-DOPA [24, 25, 32]. Indeed, chronic treatment with L-DOPA has been shown to suppress the remaining dopaminergic neurons in the ventral tegmental area of rats previously treated with 6-hydroxydopamine [49]. Reactive oxygen species could be generated by both the monoamine oxidase-catalyzed oxidative deamination of dopamine and by the autoxidation of dopamine or L-DOPA. This could be the underlying mechanism of the cytotoxic effects of dopamine and L-DOPA.

We observed that the viability of catecholaminergic neuroblastoma SH-SY5Y cells declined following treatment with either dopamine or L-DOPA in a concentration-dependent manner (Fig. 1) and was inversely proportional to the formation of melanin. These results are consistent with the idea that autoxidation of dopamine and L-DOPA is a major factor of cytotoxicity. Increased lipid peroxidation and leakage of the cytosolic LDH following dopamine and L-DOPA treatments were detected (Fig. 2). Such an oxidative stress clearly disrupts the integrity and function of the cell membranes. L-DOPA was found to be slightly more toxic than dopamine. This is probably due to the fact that L-DOPA can be transported into the SH-SY5Y cells and autoxidation of L-DOPA could therefore occur both extracellularly and intracellularly, whereas the cells lack a dopamine transport system (Lai C-T and Yu PH, unpublished observation) and therefore dopamine could probably only cause extracellular damage. The present study suggests that dopamine and L-DOPA are quite potent toxic agents. This seems to imply that unsequestered dopamine at the synaptic gap may become harmful to the nerve terminals and sub-

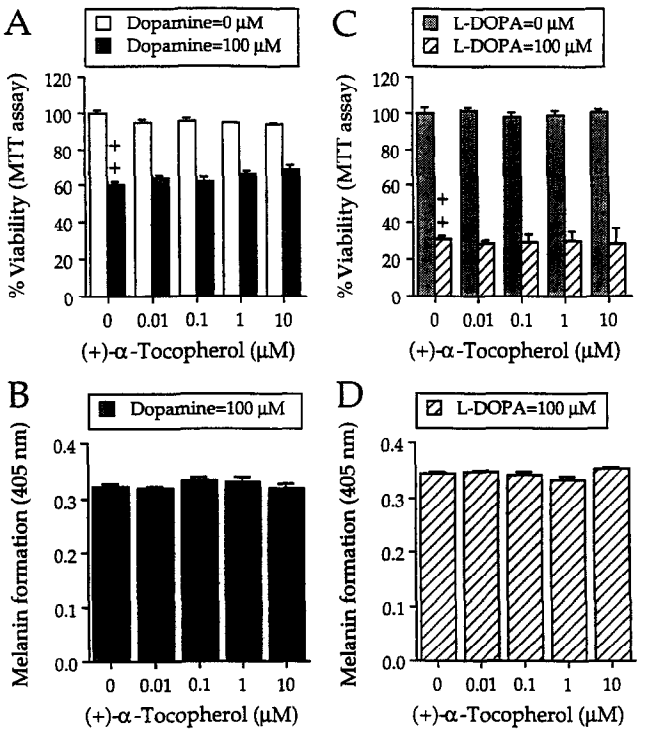

FIG. 6. Effect of (+)-α-tocopherol on the cytotoxicity of dopamine and L-DOPA towards SH-SY5Y cells and on the formation of melanin from dopamine and L-DOPA. Data are means ± SEM, N = 8. Statistical analyses were by two-way ANOVA with Newman–Keuls pairwise comparisons for A and C and by one-way ANOVA with Newman–Keuls pairwise comparisons for B and D. Key: (++) $P < 0.01$ compared with (+)-α-tocopherol = 0 μM at dopamine = 0 μM or L-DOPA = 0 μM.

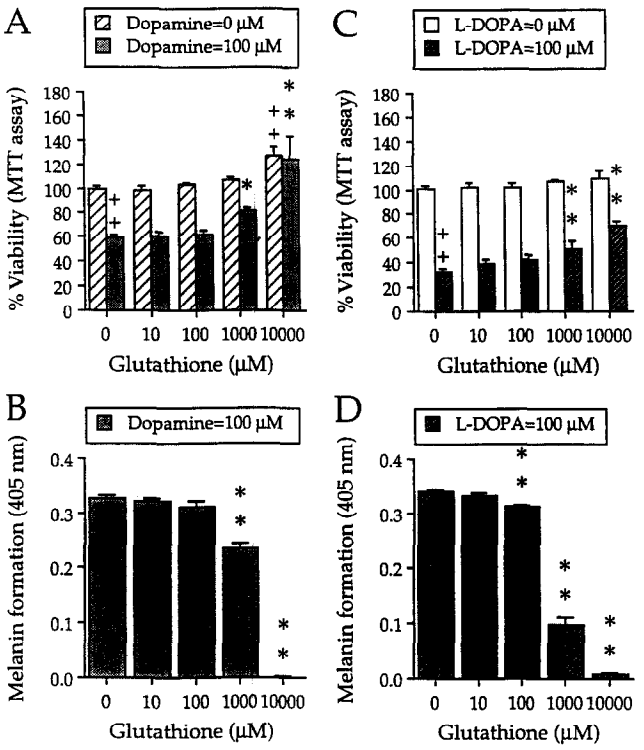

FIG. 7. Effect of glutathione on the cytotoxicity of dopamine and L-DOPA towards SH-SY5Y cells and on the formation of melanin from dopamine and L-DOPA. Data are means ± SEM, N = 8. Statistical analyses were by two-way ANOVA with Newman–Keuls pairwise comparisons for A and C and by one-way ANOVA with Newman–Keuls pairwise comparisons for B and D. (++) $P < 0.01$ compared with glutathione = 0 μM at dopamine = 0 μM or L-DOPA = 0 μM; and (*) $P < 0.05$, and (**) $P < 0.01$ compared with glutathione = 0 μM at dopamine = 100 μM or L-DOPA = 100 μM.

sequently result in retrograde neuronal death. It has been proposed that neuronal damage, i.e. by *d*-amphetamine, could be a result of increased synaptic dopamine due to increased dopamine release and inhibition of uptake [50].

When catalase was co-administered with either dopamine or L-DOPA to SH-SY5Y cells, it almost completely protected these cells from the cytotoxicity of dopamine or L-DOPA (Fig. 3). These results indicate that during autoxidation of dopamine or L-DOPA, hydrogen peroxide is formed. SH-SY5Y cells have been shown to be very sensitive to the cytotoxic effects of hydrogen peroxide (Zuo DM and Yu PH, unpublished observation).

Superoxide dismutase catalyzes the dismutation of the cytotoxic free radical superoxide. We observed, however, that superoxide dismutase exhibits a markedly less protective effect than catalase towards SH-SY5Y cells, even though superoxide is generated during the autoxidation of dopamine or L-DOPA. Hydrogen peroxide is formed in the superoxide dismutase-catalyzed reaction. It is known that both superoxide dismutase and glutathione peroxidase are localized in the same subcellular compartments, i.e. in the cytosol or in the mitochondrial matrix [51–53]. The superoxide dismutase action is coupled with the glutathione peroxidase activity under normal physiological conditions, namely, hydrogen peroxide generated from the superoxide dismutase-catalyzed reaction would be detoxified immediately by glutathione peroxidase. In our cell culture study, there was no such cooperative mechanism to remove the hydrogen peroxide. Therefore superoxide dismutase alone seems to be insufficient to protect the SH-SY5Y cells.

L-Ascorbic acid, a water-soluble antioxidant, also exhibited protection for SH-SY5Y cells against the cytotoxicity induced by dopamine and L-DOPA (Fig. 5). At higher concentrations (pH neuralized), however, L-ascorbic acid is toxic. It has been shown that L-ascorbic acid possesses both an antioxidant effect and a pro-oxidant action [54, 55]. Despite the fact that L-ascorbic acid is capable of scavenging superoxides and hydroxyl radicals [56–58] at higher concentrations, it is also capable of reducing transition metals from a less active stage to a highly active stage, e.g. ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), thereby facilitating the Fenton reaction and the subsequent hydroxyl radical formation. It has been shown that a combination of L-ascorbic acid and ferric ions can induce lipid peroxidation in rat cortical homogenate [59] and in rat neostriatum synaptosomes [60].

(+)-α-Tocopherol, a lipid-soluble, chain-breaking anti-

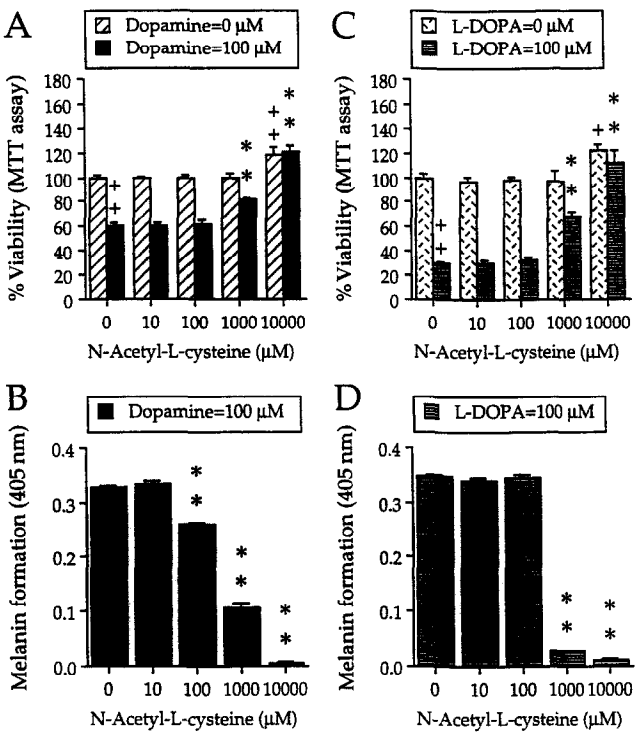

FIG. 8. Effect of *N*-acetyl-L-cysteine on the cytotoxicity of dopamine and L-DOPA towards SH-SY5Y cells and on the formation of melanin from dopamine and L-DOPA. Data are means ± SEM, N = 8. Statistical analyses were by two-way ANOVA with Newman–Keuls pairwise comparisons for A and C and by one-way ANOVA with Newman–Keuls pairwise comparisons for B and D. Key: (++) $P < 0.01$, and (+) $P < 0.05$ compared with *N*-acetyl-L-cysteine = 0 with *N*-acetyl-L-cysteine = 0 μM at dopamine = 100 μM or L-DOPA = 100 μM.

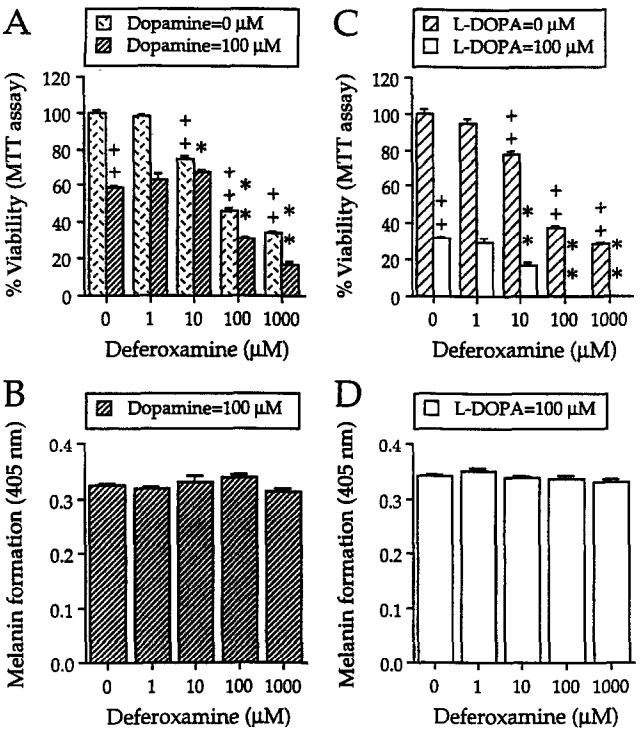

FIG. 9. Effect of deferoxamine on the cytotoxicity of dopamine and L-DOPA towards SH-SY5Y cells and on the formation of melanin from dopamine and L-DOPA. Data are means ± SEM, N = 8. Statistical analyses were by two-way ANOVA with Newman–Keuls pairwise comparisons for A and C and by one-way ANOVA with Newman–Keuls pairwise comparisons for B and D. Key: (++) $P < 0.01$ compared with deferoxamine = 0 μM at dopamine = 0 μM or L-DOPA = 0 μM; and (*) $P < 0.05$, and (**) $P < 0.01$ compared with deferoxamine = 0 μM at dopamine = 100 μM or L-DOPA = 100 μM.

oxidant, did not protect the cells against the dopamine- or L-DOPA-induced cytotoxicity (Fig. 6). These results suggested that (+)-α-tocopherol did not terminate the propagation of lipid peroxidation chain reaction in this SH-SY5Y model. (+)-α-Tocopherol is known to be capable of suppressing membrane lipid peroxidation [61, 62]. In the present experiment, (+)-α-tocopherol was administered to the SH-SY5Y cells at the same time as either dopamine or L-DOPA. Perhaps a prolonged pretreatment of (+)-α-tocopherol or increasing its concentration is required in order for it to be fully incorporated into the cell membrane before initiation of lipid peroxidation chain reactions. It is interesting to note that (+)-α-tocopherol (2000 IU/day) was found to be ineffective in slowing down the progress of Parkinson's disease in the DATATOP clinical trial [63].

Glutathione, a tripeptide (L-γ-glutamyl-L-cysteinylglycine), is the most abundant intracellular antioxidant. Both glutathione and *N*-acetyl-L-cysteine, a synthetic compound of cysteine, completely protected the SH-SY5Y cells from the dopamine- and L-DOPA-induced cytotoxicity (Figs. 7 and 8). It was also demonstrated that both compounds could suppress the autoxidation of dopamine or L-DOPA. The protective effects of these reducing agents are clearly attributed to the suppression of the autoxidation of dopamine or L-DOPA.

The iron chelator deferoxamine and hydroxyl free radical scavengers (*N-tert*-butyl-α-phenylnitrone, salicylic acid, and D-mannitol) did not protect the SH-SY5Y cells against the cytotoxicity induced by dopamine or L-DOPA in the present study. Hydroxyl free radicals are known to be generated during autoxidation of catechols. It is puzzling why these potent free radical scavengers fail to prevent the toxicity induced by the catechols. It is probably related to the selective sites of action. It has been shown, for example, that membrane-bound acetylcholine esterase was subjected to the oxidative inactivation by ascorbate and copper *in vitro* [64]. Mannitol at concentrations up to 100 mM did not protect the enzyme from inactivation. It was explained that the hydroxyl radicals are generated by the metal-mediated Fenton and/or the Haber–Weiss reactions at the sites where the metals are located. Interaction of the enzyme and the hydroxyl free radicals occurs immediately at the sites, i.e. copper binding sites, where they are formed. It seems to be possible that trace amounts of metals, such as ferrous ions, are located in the cell membranes of the SH-SY5Y cells, and hydrogen peroxide, i.e. generated via autoxidation of dopamine or L-DOPA, might be converted to hydroxyl free radicals at the metal sites and then cause *in situ* cellular damage. Catalase removes hydrogen peroxide and therefore protects the cells from damage. The free radical scavengers, however, are too remote from the selective "metal" sites, and do not have an opportunity to react with the radicals. The half-life of the hydroxyl radical is extremely short (10^{-9} sec) [65, 66]. The cellular damage may occur before the interaction of these radical scavengers with the hydroxyl free radicals.

In conclusion, our findings support the hypothesis that excessive dopamine and L-DOPA can be potentially neurotoxic towards dopaminergic neurons. The mechanism of such cytotoxic effects is due to increased oxidative stress via autoxidation. Hydrogen peroxide plays the most crucial role in the cascade of oxidative events induced by dopamine or L-DOPA.

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