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PARTICIPATION OF ACTIVE OXYGEN SPECIES IN 6-HYDROXYDOPAMINE TOXICITY TO A HUMAN NEUROBLASTOMA CELL LINE

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Abstract—Catalase, superoxide dismutase, and dimethylsulfoxide were tested for their ability to prevent the cytotoxic effect of 6-hydroxydopamine (6-OHDA) on the human neuroblastoma line SY5Y. Viability was measured at two time points after 6-OHDA treatment: at 3 hr by means of amino acid incorporation and at 24 hr by trypan blue dye exclusion. Survival of cells treated concomitantly with catalase (50 μg/ml) and 6-OHDA was at least 90 per cent that of untreated controls. Cells receiving 6-OHDA alone showed less than 30 per cent survival relative to untreated controls. Superoxide dismutase (50 μg/ml) temporarily protected cells from a high concentration of 6-OHDA. Dimethylsulfoxide treatment increased survival from the control level 24 hr after treatment with 6-OHDA. Two other cell lines (A_1B_1 human glial cells and CHO fibroblasts) had intermediate and high resistance to the drug, respectively, compared to the low resistance of SY5Y cells. CHO and SY5Y cells had similar responses to 6-OHDA and to H_2O_2 when tested at twice the molarity of 6-OHDA. Specific activities of three enzymes known to detoxify H_2O_2 or H_2O_2-generated organic hydroperoxides (catalase, glutathione *S*-transferase, and glutathione peroxidase) were compared in the three cell lines. Catalase activity was 2.5 times as high in A_1B_1 and CHO cells as in SY5Y cells when expressed as units/mg protein and 7 times as high in units/culture dish. Other enzyme activities showed no correlation to 6-OHDA resistance.

The selective toxicity of 6-hydroxydopamine (6-OHDA) for catecholaminergic nerve cells *in vivo* [1, 2] and adrenergic-like neuroblastoma cells *in vitro* [3–6] is a well-studied phenomenon in neuropharmacology. However, the exact molecular mechanism of toxicity remains uncertain. Two hypothetical mechanisms are currently favored, both based on the generation of toxic molecular species by the autoxidation of 6-OHDA. At physiological pH (7.4), 6-OHDA spontaneously reacts with molecular oxygen to form a 1,4-*para*-quinone and its products [7], as well as oxidative radicals and their products, specifically hydrogen peroxide (H_2O_2), the superoxide radical ($O_2^{\cdot-}$), and the hydroxyl radical ($^{\cdot}OH$). The generation of these species is proposed to occur by the following reaction steps [8]:

$$\text{6-OHDA} + O_2 \rightarrow SQ^{\cdot} + O_2^{\cdot-} + H^+ \quad \text{(I)}$$

$$H^+ + \text{6-OHDA} + O_2^{\cdot-} \rightarrow SQ^{\cdot} + H_2O_2 \quad \text{(II)}$$

$$SQ^{\cdot} + O_2 \rightarrow Q + O_2^{\cdot-} + H^+ \quad \text{(III)}$$

$$SQ^{\cdot} + O_2^{\cdot-} + H^+ \rightarrow Q + H_2O_2 \quad \text{(IV)}$$

where $SQ^{\cdot}$ is a semiquinone free radical of 6-OHDA and Q is a quinone. The formation of hydroxyl radicals can be catalyzed from the breakdown of H_2O_2 by trace metals in a Fenton-type reaction [9],

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \quad \text{(V)}$$

or by the Haber–Weiss reaction [9],

$$H_2O_2 + O_2^{\cdot-} \rightarrow {}^{\cdot}OH + OH^- + O_2 \quad \text{(VI)}$$

Hydrogen peroxide may be formed additionally by the reduction of the superoxide radical:

$$2H^+ + 2O_2^{\cdot-} \rightarrow H_2O_2 + O_2 \quad \text{(VII)}$$

According to one hypothesis of 6-OHDA toxicity, the quinones generated by 6-OHDA oxidation bind covalently with sulfhydryl or other nucleophilic groups, leading to the inactivation of essential macromolecules [7, 10, 11]. The demonstration of quinone binding to the cytoplasmic and axonal membrane proteins of neurons damaged by 6-OHDA supports this view [12, 13]. Also, a *p*-quinone reaction product of 6-OHDA, 2-*S*-(glutathionyl)-6-OHDA quinone, resulting from the interaction of injected 6-OHDA with brain tissue of animals, has been isolated [14]. Neither demonstration, however, proves that quinones are the cause of cell death or axonal degeneration.

An alternative hypothesis states that the active oxygen species formed by autoxidation of 6-OHDA initiates cell destruction. The spontaneous formation of H_2O_2 [15], $^{\cdot}OH$ [16], and $O_2^{\cdot-}$ [8] from 6-OHDA is documented at physiological pH. Furthermore, the cytotoxicity of 6-OHDA correlates with its rate of autoxidation, but not with the sulfhydryl reactivity of its quinoid oxidation products [17]. H_2O_2 and $O_2^{\cdot-}$ may be toxic because they are strong oxidizing agents. $O_2^{\cdot-}$ takes part in the generation of $^{\cdot}OH$ via the Haber–Weiss reaction. The hydroxyl radical is

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implicated in the damaging effects of ionizing radiation [18] and alloxan-induced diabetes [19].

If the second hypothesis is correct, superoxide dismutase (SOD), which catalyzes the reaction of superoxide radicals with hydrogen ions to yield H_2O_2 and O_2 [20], or catalase, which speeds the decomposition of H_2O_2 to H_2O and O_2 [21], should modify the cytotoxic effects of 6-OHDA. Both enzymes also should prevent ·OH formation by a Haber–Weiss or Fenton-type reaction by removing H_2O_2 and O_2^- from the system.

Recently, in a study using the human neuroblastoma cell line SK-N-SH-SY5Y (SY5Y), we found that cell death caused by 6-OHDA was completely prevented by the simultaneous administration of catalase during drug treatment. SOD conferred partial, transient protection. These results, presented elsewhere in preliminary form [22], were compared in the present study with the protective potential of dimethylsulfoxide (DMSO), a presumptive ·OH scavenger [23]. We also compared the toxic effects of 6-OHDA and H_2O_2 upon SY5Y cells and two cell lines relatively resistant to 6-OHDA, A_1B_1 human glial tumor cells and CHO Chinese hamster ovary fibroblasts. Lastly, we compared the specific activities of catalase, glutathione *S*-transferase (GSH S-Tr), and glutathione peroxidase (GSH-Px) in the three cell lines. GSH S-Tr protects cells from oxidative damage [24]. GSH-Px is postulated to protect brain tissue from glycolysis-inhibiting dihydro phenols or trihydro benzenes and quinones [25].

We demonstrated that (a) catalase and SOD did not inhibit the accumulation in the medium of quinones generated by 6-OHDA autoxidation; (b) DMSO (0.12 mM) approximately doubled the percentage of SY5Y cells surviving treatment with 0.12 mM 6-OHDA; (c) H_2O_2 (0.12 to 0.8 mM) was not as selectively toxic to neuroblastoma cells as 6-OHDA (0.12 to 0.4 mM), and (d) the specific activity of catalase was lower in SY5Y cells than in A_1B_1 or CHO cells.

MATERIALS AND METHODS

Cell culture. Monolayer stock cultures of the human neuroblastoma clonal cell line SK-N-SH-SY5Y (SY5Y), the Chinese hamster ovary (CHO) cell line and the human glioma clonal cell line A_1B_1 were maintained at 37° in tissue culture flasks (Corning Glass Works, Corning, CA) with complete medium consisting of Ham's F-12 nutrient mixture (Grand Island Biological Co., Grand Island, NY) supplemented with 15% fetal calf serum (Irvine Scientific Sales Co., Irvine, CA). Subcultures were prepared for drug tests from cells in exponential growth phase. The cells were dislodged by incubation (5 min, 37°) in 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)-buffered saline, pH 7.4, containing 1.0 mM EDTA. They were diluted in complete medium, counted in a hemacytometer, and seeded 1×10^5 cells per dish in 35 mm Corning tissue culture dishes. Trypsin was not used. We received the SY5Y cell line from Dr. June Biedler, Memorial Sloan–Kettering Cancer Center, New York, NY, and the CHO cell line from Dr. Sam Barranco, The University of Texas Medical Branch, Galveston, TX. The A_1B_1 line was cloned from human glioma line U-251-MG, which was a gift from Drs. J. Pontén and B. Westermark, University of Uppsala, Sweden.

6-Hydroxydopamine and enzyme treatment. Twenty-four hours after seeding, the medium was removed from the culture dishes and replaced with 1 ml of drug solution consisting of bicarbonate-buffered serum-free F-12 medium, pH 7.4, plus 1% penicillin–streptomycin–neomycin antibiotic mixture (GIBCO) and a fixed dose of 6-OHDA (0, 0.12 or 0.4 mM). After 1 hr of incubation at 37°, the drug was removed and dishes were either replenished with complete medium or assayed for amino acid incorporation. Some samples were treated concomitantly with 6-OHDA and enzyme: either 50 μg/ml superoxide dismutase (ontosein, beef liver; Diagnostic Data, Mountain View, CA), 50 μg/ml catalase (beef liver, 63,863 units/mg; CalBiochem, San Diego, CA), or both at 50 μg/ml each. Solutions were prepared immediately before use. Other samples were treated simultaneously with 6-OHDA and DMSO. A 1.2×10^{-4} solution of DMSO was prepared by diluting Cryoprotective medium (Micro-biological Associates, Bethesda, MD) containing 15% DMSO 3:60 in serumless F-12.

Hydrogen peroxide treatment. SY5Y, CHO and A_1B_1 cultures were treated as described for 6-OHDA with H_2O_2 at concentrations of 0.12, 0.4 or 0.8 mM. However, enzymes were not used. The molarity of the H_2O_2 stock solution was determined by titration of H_2O_2 with thiosulfate, according to the procedure of Kokatnur and Jelling [26].

Viability assays. Two assays performed at different times after drug treatment permitted a temporal evaluation of cell viability. We measured the level of ^{3}H-amino acid incorporation into acid-precipitable proteins during the period 0–3 hr after treatment with 6-OHDA and trypan blue exclusion 24 hr after treatment. At these time points, in the absence of protective agents, the percentage (relative to controls) of amino acid incorporation by a drug-treated cell population is similar to the percentage of cells excluding dye [6]. For the incorporation assay, during the period 0–3 hr (in one case 21–24 hr) after 6-OHDA treatment, half the samples were incubated in amino acid-free F-12 (GIBCO) containing 5 μCi/ml ^{3}H-amino acid mixture (New England Nuclear Corp., Boston, MA). One set of control samples was incubated for <10 sec to determine background counts (zero-time). The amino acid solution was removed, and the cell monolayers were digested with 1 ml Lowry solution A per sample for 30 min. The digested samples were incubated overnight at 4° in 0.5 ml of 50 per cent trichloroacetic acid (TCA, Sigma Chemical Co., St. Louis, MO), and then washed through GF/C filters (Whatman, Inc., Clifton, NJ) with 30 ml of ice-cold 5% TCA [27]. The filters were dried, placed in scintillation fluid (Scintiverse, Fisher Scientific Co., Fair Lawn, NJ), and counted for radioactivity.

Samples assayed by the trypan blue technique were incubated in complete medium for 24 hr after drug treatment, and then harvested from the dishes at room temperature with saline + EDTA. The cells were pelleted and resuspended in 0.4% trypan blue (GIBCO) diluted 1:6 in saline. We counted stained

and unstained cells in a hemacytometer with a phase microscope 2–10 min after their suspension in the dye.

Spectrophotometric analysis. The formation of quinoidal products by the autoxidation of 6-OHDA was monitored by absorbance at 490 nm with a Beckman model 25 spectrophotometer. A concentrated stock solution of 6-OHDA (8.0 mM) was prepared in 0.01 N HCl. It was diluted to 0.12 or 0.4 mM in sodium bicarbonate-buffered F-12 medium, pH 7.4, and assayed immediately at room temperature. Catalase (50 μg/ml) or superoxide dismutase (50 μg/ml) was included in half the samples.

Enzyme assays. CHO, A_1B_1, and SY5Y cells in exponential growth phase were harvested with saline + EDTA and centrifuged. The pellets were lysed in hypotonic phosphate buffer. Enzyme activities were measured in the cell lysates, including membrane fragments. Catalase (EC 1.11.1.6) activity was determined by the method of Beers and Sizer [28]. The L-dopa peroxidase activity of catalase was measured as described by Awasti *et al.* [29]. Glutathione *S*-transferase (GSH S-Tr, EC 2.5.1.18) activity was measured with 1-chloro-2-4-dinitrobenzene as the substrate, according to the procedure of Habig *et al.* [30]. Glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity was determined using t-butyl hydroperoxide or H_2O_2 as substrates [31]. Protein concentrations were estimated by the Bradford [32] technique.

Statistical analysis. Mean values of treatment groups within each experiment were compared using one-way analysis of variance with *a priori* comparisons of means. F-tests were used to test significance, with an accepted significance level of $P < 0.05$.

RESULTS

Selective toxicity of 6-OHDA. Cell lines A_1B_1 (human glioma), SY5Y (human neuroblastoma) and CHO (Chinese hamster ovary fibroblasts) were tested for sensitivity to the cytolytic effects of 6-OHDA. Figure 1 shows a comparison of their responses as measured by the trypan blue viability test 1 day after a 1-hr incubation with 6-OHDA. Over the dose range 0–4.0×10^{-4} M (0–100 μg/ml), CHO cells were resistant to the drug. On the other hand, over 90 per cent of the SY5Y cells were killed at doses greater than or equal to 1.2×10^{-4} M. A_1B_1 cells showed intermediate sensitivity: cell survival was unaffected at 1.2×10^{-4} M, but approached that of SY5Y cells at 4×10^{-4} M.

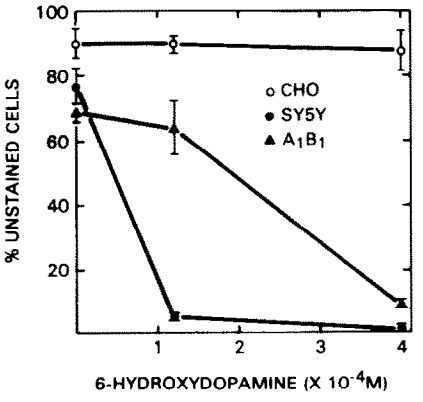

Fig. 1. Dose-response of CHO, SY5Y and A_1B_1 cells to 6-OHDA. Cells were seeded at a density of 10^5 cells/35 mm culture dish in complete medium. After 24 hr the medium was replaced with serum-free F-12 medium containing 6-OHDA for 1 hr. Drug was then removed and complete medium was re-added. The percentage of cells excluding trypan blue was assayed 24 hr after drug treatment. Vertical range bars represent standard deviations. For each point N = 3.

Protection of SY5Y cells by catalase and SOD. SY5Y cells were treated for 1 hr with intermediate

Table 1. Effects of catalase and superoxide dismutase on inhibition of ^{3}H-amino acid incorporation by 6-OHDA 3 hr after treatment*

	1.2×10^{-4} M 6-OHDA		4×10^{-4} M 6-OHDA	
	Mean cpm/plate (N = 3)	Coefficient of variation (%)	Mean cpm/plate (N = 3)	Coefficient of variation (%)
Zero time	204	53	113	19
Control	110,731	16	79,872 (N = 2)	0
SOD	123,899	9	89,268	19
Catalase	124,852	6	93,173 (N = 2)	4
6-OHDA	14,624†	19	3,769†	16
6-OHDA + SOD	18,652†	17	57,139†	18
6-OHDA + catalase	102,826	11	82,629	5
6-OHDA + both	100,495	10	90,636	6
Mean no. cells/plate	56,000 ± 15,000 (S.D.)		53,000 ± 6,000 (S.D.)	

* SY5Y human neuroblastoma cells were seeded at a density of 10^5 cells/35 mm culture dish in complete medium. After 24 hr the medium was replaced for 1 hr with serum-free medium containing 6-OHDA ± enzyme. The drug-containing solution was removed, and the cells were incubated with 1 ml F-12 containing 5 μCi ^{3}H-amino acid mixture for 3 hr. Zero time points (background) were obtained by incubation with the radiolabeled substance for less than 10 sec. Incorporation of radiolabel was measured by scintillation counting of material precipitated by trichloroacetic acid. Significance levels were determined by *a priori* comparisons of means between treatment and control groups.

† Differs significantly from control, $P < 0.001$.

Table 2. Viability indices of human neuroblastoma cells after treatment with 6-hydroxydopamine ± enzyme*

Enzyme	No 6-OHDA		1.2×10^{-4} M 6-OHDA		4×10^{-4} M 6-OHDA		
	Amino acid incorporation (3 hr)	Dye exclusion (24 hr)	Amino acid incorporation (3 hr)	Dye exclusion (24 hr)	Amino acid incorporation (3 hr)	(24 hr)	Dye exclusion (24 hr)
No enzyme (control)	100	100	13	28	5	< 1	10
SOD	112	99	17	21	72†	< 1	5
Catalase	113	106	93†	106†	103†	ND‡	100†
Both	129†	102	91†	110†	113†	ND	95†

* Results of amino acid incorporation viability assays 3 and 24 hr after drug treatment and trypan blue dye exclusion 24 hr after treatment are expressed as percentages of non-drug-treated control values (underlined). Significance levels were determined by analysis of variation with *a priori* comparisons of means between enzyme-treated and control samples within each drug dose group (top value of each column). For each point, N = 3.

† Differs significantly from control (no enzyme), $P < 0.001$.

‡ Not determined.

(1.2×10^{-4} M) and high (4×10^{-4} M) concentrations of 6-OHDA in the presence of catalase (50 μg/ml), superoxide dismutase (50 μg/ml), or both. Controls lacked the drug or either enzyme. Cell culture viability was measured by the level of ^{3}H-amino acid incorporation into proteins during the period 0–3 hr after drug treatment (Table 1) and by trypan blue dye exclusion 24 hr after drug treatment. The results of both assays are expressed in Table 2 as percentages of the values obtained from controls receiving neither enzymes nor 6-OHDA.

According to the amino acid assay, catalase fully protected cells from both intermediate and high concentrations of 6-OHDA. Similar results were obtained with the trypan blue test. The relative survival values from both assays were always greater than 90 per cent compared to 30 per cent or less in drug-treated controls which received no enzyme.

On the other hand, SOD afforded only temporary protection at the higher dose of 6-OHDA and no significant protection at the lower dose. At 1.2×10^{-4} M 6-OHDA, the survival of cultures treated with 60 HDA alone as well as SOD plus 6-OHDA was below 30 per cent as judged by either viability assay. However, 72 per cent protection by SOD was observed in groups treated with the high dose of 6-OHDA, according to amino acid incorporation 3 hr after drug treatment. No such protection was detected by either the trypan blue test or the amino acid incorporation assay 24 hr after treatment (Table 2). At 24 hr both assays showed less than 10 per cent survival in controls given 4×10^{-4} M 6-OHDA and less than 5 per cent in cells receiving SOD + 6-OHDA.

Toxicity of H_2O_2. The results of the above experiment implied a major contribution of H_2O_2 to the mechanism of 6-OHDA toxicity. We therefore tested the effects of H_2O_2 over a dose range of $0–8 \times 10^{-4}$ M in the three cell lines previously used: SY5Y, A_1B_1, and CHO. Table 3 contains the relative values of the trypan blue assay (determined at 24 hr) and amino acid incorporation measured from 0 to 3 hr after treatment. At twice the molarity, H_2O_2 evoked a response from SY5Y and CHO cells similar to that caused by 6-OHDA, in terms of dye exclusion 24 hr after treatment. However, dye exclusion by cell line A_1B_1 dropped markedly from its 6-OHDA values, the response closely approximating that of cell line SY5Y. Amino acid incorporation values, like dye exclusion, were similar for cell line SY5Y treated with H_2O_2 or 6-OHDA.

Protection of SY5Y cells by DMSO. Since 6-OHDA generates hydroxyl radicals (·OH) during autoxidation in cell-free systems [16], we tested the ability of a putative ·OH scavenger [23], DMSO to protect SY5Y cells from 6-OHDA toxicity. The results in Table 4 show that the proportion of cells surviving (6-OHDA-treated survival ÷ untreated

Table 3. Toxic effects of hydrogen peroxide on cultured cell lines*

H_2O ($\times 10^{-4}$ M)	SY5Y		Cell lines A_1B_1		CHO	
	Amino acid incorporation (3 hr)	Dye exclusion (24 hr)	Amino acid incorporation (3 hr)	Dye exclusion (24 hr)	Amino acid incorporation (3 hr)	Dye exclusion (24 hr)
0 (control)	100	100	100	100	100	100
1.2	89	64†				
4.0	43†	41†	23†	43	44†	94
8.0	9†	17†	18†	19	17†	83

* Viability of three cell lines treated for 1 hr with H_2O_2 was measured by ^{3}H-amino acid incorporation 3 hr after treatment and trypan blue dye exclusion 24 hr after treatment. Values expressed are percentages of control values. Significance levels were determined by analysis of variance. For each value, N = 3.

† Differs significantly from control, $P < 0.001$.

Table 4. Effect of DMSO on 6-OHDA toxicity in SY5Y cells*

	(A) No 6-OHDA		(B) 6-OHDA (1.2×10^{-4} M)			
	% Cells unstained (mean ± S.D.)	S.L. vs control	% Cells unstained (mean ± S.D.)	S.L. vs control	% Cells protected (B ÷ A)	S.L. A vs B
Control	71.5 ± 1.3†		19.9 ± 6.1		28	$P < 0.001$
DMSO (1.2×10^{-4} M)	83.6 ± 4.2	$P < 0.01$	41.6 ± 3.6	$P < 0.001$	50	$P < 0.001$

* Cells were treated concomitantly with dimethylsulfoxide (DMSO) and 6-OHDA at an equal molar ratio, for 1 hr. The percentage of cells excluding trypan blue dye was assayed 1 day later. Significance levels were determined by analysis of variance. For each point N = 3, except as noted.
† N = 2.

survival × 100) was 50 per cent in the presence of DMSO, compared to 28 per cent in controls. Furthermore, the survival rate of cells treated with DMSO alone (83.6 per cent) significantly exceeded that of controls (71.5 per cent $P < 0.01$).

Effects of enzymes on quinone formation. The formation of quinones by the autoxidation of 6-OHDA is easily followed spectrophotometrically at 490 nm absorbance [33]. In Fig. 2, quinone formation is traced for 6-OHDA dissolved in F-12 medium, pH 7.4 at 22°, in the presence and absence of enzymes. Drug and enzyme concentrations were identical to those used in the cell culture tests. At 1.2×10^{-4} M 6-OHDA, absorption of control and catalase-containing samples was the same, reaching maximum levels at 1 min. Thus, catalase did not affect quinone formation. Maximum absorbance of the SOD samples was not reached until 4 min, and thereafter did not differ significantly from the control. Therefore, SOD inhibited quinone formation for 2–4 min, after which inhibition disappeared. At 4×10^{-4} M 6-OHDA, SOD again inhibited quinone formation for 3.5 to 4 min, thereafter allowing absorbance to reach that of the control. Absorbance in the presence of catalase was about 16 per cent higher than the maximum control value, indicating increased quinone formation. One set of samples was incubated at room temperature for 1 hr, the length of drug incubation used in the 6-OHDA toxicity assays. Absorbance values at 1 hr did not change markedly from those at 5 min, as shown in Table 5.

Catalase, GSH S-transferase, and GSH-peroxidase activities in SY5Y cells. The specific activities of three enzymes which detoxify H_2O_2 or H_2O_2-generated organic hydroperoxides were compared in cell lines SY5Y, A_1B_1, and CHO (Table 6). Earlier we found that A_1B_1 human glial cells and CHO fibroblasts were both less sensitive to 6-OHDA than SY5Y cells. Catalase activity (units/mg protein) in A_1B_1 and CHO cells was about 2.5 times higher than in SY5Y cells. No L-dopa activity of catalase was detectable in any of the cell lines. GSH-Tr activity was 29 per cent lower in A_1B_1 cells than in SY5Y cells, but in CHO cells it was more than double the SY5Y level. Activity of GSH-Px against an organic hydroperoxide (tertiary butyl hydroperoxide) was 72 per cent higher in A_1B_1 cells and 30 per cent higher in CHO cells than in SY5Y cells. The H_2O_2-reducing activity of GSH-Px was the same in A_1B_1 and SY5Y cells, both of which were twice that of CHO cells.

DISCUSSION

Using SY5Y human neuroblastoma cells as a target for selective 6-OHDA cytotoxicity, we found evidence that three active oxygen species participated

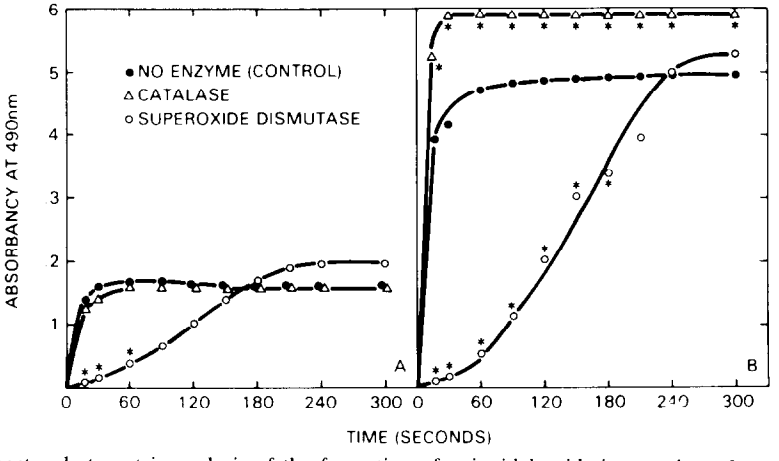

Fig. 2. Spectrophotometric analysis of the formation of quinoidal oxidation products from 6-OHDA at 1.2×10^{-4} M at 22° (A) and 4.0×10^{-4} M (B). Absorbance at 490 nm is shown for 6-OHDA dissolved in F-12 medium, pH 7.4, in the presence and absence of catalase (50 μg/ml) or superoxide dismutase (50 μg/ml). Values plotted are the means of two or three samples. Asterisks indicate means that differ significantly ($P < 0.05$) from the control.

Table 5. Absorbance 490 of 6-OHDA oxidation products*

Incubation time (min)	No enzyme		Catalase		Superoxide dismutase	
	Concentration 6-OHDA × 10^{-4} M					
	1.2	4.0	1.2	4.0	1.2	4.0
5	0.110	0.502	0.168	0.555	0.211	0.547
60	0.112	0.424	0.202	0.512	0.154	0.436

* One sample from each of the assays in Fig. 3 was allowed to stand at room temperature for 1 hr. Absorbance was read at 490 nm.

in the process of cell killing: H_2O_2, which is decomposed in a reaction enhanced by catalase; ·OH, which is trapped by DMSO; and O_2^-, whose reduction is catalyzed by SOD. Catalase, DMSO, and SOD all inhibited cytotoxicity to various degrees. Differences in the power of each agent to delay or prevent cell death implied unequal contributions of the three molecules to the mechanism of 6-OHDA toxicity.

Since catalase completely prevented cell killing at the time points and 6-OHDA concentrations tested, we conclude that H_2O_2 was a major cause of 6-OHDA toxicity, either as a primary agent or as a necessary intermediate for the generation of other toxic species. In an experimental system comprised of a different toxin and target where catalase prevents toxicity, Rose and György [34] discuss the likelihood that the actual toxic agent is not H_2O_2 itself, but a compound formed by reactions requiring H_2O_2. They show that diaburic acid, which induces hemolysis of vitamin E-deficient rat erythrocytes, is prevented from doing so by catalase. Moreover, H_2O_2 alone at comparable doses does not hemolyze cells, though it does at higher concentrations. With SY5Y cells we found similarly that twice the molar concentration of H_2O_2 as 6-OHDA was required to achieve comparable cell killing.

As shown in reactions II, IV, V, and VI, H_2O_2 may take part in several reactions during which potential toxins are generated. Our results provide information on two of them, quinones and hydroxyl radicals. The formation of quinones by the autoxidation of 6-OHDA is easily followed spectrophotometrically at 490 nm absorbance. In Fig. 2, quinone formation is traced for 6-OHDA dissolved in F-12 medium, pH 7.4, in the presence and absence of enzymes. Drug and enzyme concentrations were identical to those used in the cell culture tests. At 1.2×10^{-4} M 6-OHDA, absorbance of control and catalase-containing samples was the same, reaching maximum levels at 1 min. Thus, catalase did not affect quinone formation. Maximum absorbance of the SOD-containing samples was not reached until 4 min, but it did not differ significantly from the control. Thus, SOD inhibited quinone formation for 2–4 min, after which inhibition disappeared. At 4×10^{-4} M 6-OHDA, SOD again inhibited quinone formation for 3.5 to 4 min, thereafter allowing the absorbancy level to reach that of the control. Absorbance in the presence of catalase was about 16 per cent higher than the maximum control level, indicating increased quinone formation. One set of samples was allowed to proceed at room temperature for 1 hr, the length of drug incubation in cell culture tests. Absorbance values at 1 hr did not change markedly from those at 5 min, as shown in Table 5. Observed spectrophotometrically, quinone formation was not inhibited by catalase, as would be expected by examination of reaction IV. On the contrary, it was actually enhanced at 0.4 mM 6-OHDA, i.e. the equilibrium of reaction IV shifted to the right as H_2O_2 was removed. In addition, SOD inhibited quinone formation for less than 4 min, after which it reached control levels. Therefore, quinones were not directly toxic to the neuroblastoma cells.

Hydrogen peroxide may form hydroxyl radicals by Fenton (V) or Haber–Weiss (VI) reactions. If ·OH is the toxic species, both catalase and DMSO should prevent 6-OHDA toxicity. Furthermore, SOD should also inhibit ·OH formation via the Haber–Weiss reaction. We found only partial protection by DMSO and SOD. The reduction of 6-OHDA toxicity by DMSO is in agreement with an *in vivo* study reporting the prevention of 6-OHDA-induced damage to mouse sympathetic

Table 6. Catalase, GSH *S*-transferase and GSH peroxidase activities of cultured cell lines*

Cell line	(μg Protein/culture dish)	Catalase		GSH S-Tr	GSH-Px	
		H_2O_2	L-Dopa activity		tBHP	H_2O_2
		U/mg protein	U/mg protein	U/mg protein	U/mg protein	U/mg protein
SY5Y	12	1.38	0	0.17	0.32	0.13
A_1B_1	24	3.53†	0	0.12†	0.55†	0.13
CHO	24	3.64†	0	0.37†	0.44†	0.055†

* Enzyme activities were determined for untreated cells in exponential growth phase. Two substrates were used for GSH-Px assays, tertiary butyl hydroperoxide (tBHP) and H_2O_2. All determinations were made in triplicate and the standard deviations were < 1 per cent.

† Differs significantly from SY5Y value, $P < 0.001$.

nerves by 1-phenyl-e-(2-thiazoyl)-2-thiourea, which traps ·OH and semiquinone radicals [35]. Failure of DMSO to preserve viability completely in our experiments may have resulted either because we used too low a concentration (equimolar concentrations of 6-OHDA and DMSO were used) or because ·OH is not the major toxic species generated by 0.12 mM 6-OHDA. The question cannot be resolved from these experiments. Since SOD temporarily inhibited but did not ultimately prevent cell death, either the Haber–Weiss reaction was not a significant source of ·OH radicals or SOD was ineffective for other reasons.

The finding that SOD alone did not prevent cell death, while catalase alone did, implies that O_2^- was not deleterious to the cells. However, the delay of cell death by SOD at 0.4 mM 6-OHDA demonstrated some involvement of O_2^- in toxicity. The transient protection at 0.4 but not 0.12 mM is difficult to explain since several factors are involved. For example, H_2O_2 inhibits SOD [36]. Since SOD reduces O_2^- to H_2O_2 and O_2, a buildup of H_2O_2 may have reduced the protective action of SOD, and H_2O_2 itself would have caused cell death. However, SOD appeared to be able to inhibit quinone formation equally at 0.12 and 0.4 mM 6-OHDA via the removal of O_2^-.

A hypothetical explanation of the events described which depends on the Haber–Weiss generation of ·OH is as follows: ·OH generated from O_2^- and H_2O_2 may be the predominant toxic species at 0.4 mM 6-OHDA, but not at 0.12 mM. At the lower 6-OHDA concentration, H_2O_2 would have greater toxicity than ·OH. The ability of SOD to affect toxicity would be seen only at the higher concentration by its inhibition of the Haber–Weiss reaction. To account for the disappearance of SOD protection with time, we may consider the explanation of Cohen and Heikkila [16] that SOD, by removing O_2^-, causes an accumulation of hydroxyl radicals that would otherwise be decomposed by the following reaction:

$$\cdot OH + O_2^- \rightarrow OH^- + O_2 \qquad (VIII)$$

The reaction cannot occur until after the system has generated a sufficient concentration of ·OH. Thus, at 0.4 mM 6-OHDA, SOD would enhance ·OH toxicity after initially displaying protective action against superoxide radicals.

The probable inability of catalase or SOD to enter cells would suggest that any effects they produced were extracellular. Thus, catalase presumably protected cells by removal of H_2O_2 from the surrounding medium, indicating that 6-OHDA need not generate its toxic products within the cell. If the damage caused by O_2^- and H_2O_2 is a cell membrane effect, it may involve simple oxidative attack or more specific interaction. For example, active oxygen species can act as messengers in such processes as inflammation [37] and control of nucelotide cyclases [38, 39].

Extracellular protection by catalase implies that 6-OHDA or its quinones need not be transported into the cell for destruction to occur. However, extracellular toxicity confutes the prevalent hypothesis that the selective toxicity of 6-OHDA derives from its uptake by susceptible cells. In mature neurons *in vivo*, studies with [^{3}H]-6-OHDA and catecholamine uptake blockers suggest that 6-OHDA accumulation by the axonal terminals is required for their destruction [12, 40, 41].

Two studies in mouse neuroblastoma cell lines link high uptake of radiolabeled dopamine and 6-OHDA to high drug sensitivity and low uptake to low sensitivity, as in cell lines CHO-K1, C-6, and L929 [4, 42]. A third study demonstrates a 2 to 6-fold higher accumulation of labeled 6-OHDA in N1E-115 mouse neuroblastoma cells than in L cells over a period of 1 hr [13]. No evidence exists, however, to show that in neuroblastoma cells, or even in immature sympathetic neurons, catecholamine uptake is a prerequisite for the expression of 6-OHDA toxicity. Thus, the phenomenon of selective cell death caused by 6-OHDA may be different from the process of axonal destruction in mature adrenergic neurons.

The present study suggests that a factor in the selective destruction of neuroblastoma cells *in vitro* is their selective sensitivity to H_2O_2 or ·OH. However, the toxicity of 6-OHDA is not the same as that delivered by a "lump sum" of H_2O_2, as was seen by the response of CHO and A_1B_1 cells to H_2O_2. Though CHO cells survived H_2O_2 treatment, their vital signs differed markedly from those following 6-OHDA treatment. Neither amino acid incorporation (3 hr) nor dye exclusion (24 hr) was decreased in CHO cells by treatment with 6-OHDA (see Table 1 and Ref. 6).

Experiments were therefore conducted to measure the activity in the three cell lines of various enzymes which defend cells against H_2O_2 and organic hydroperoxides by the oxidation of lipids. Three such enzymes are known: catalase, glutathione peroxidase, and glutathione *S*-transferase. Catalase is proposed to have a dual function dependent upon the steady-state concentration of H_2O_2 [21]. At high concentrations ($> 10^{-6}$ M) of H_2O_2, catalase accelerates the destruction of H_2O_2. At low concentrations of H_2O_2 and in the presence of a suitable hydrogen donor, such as L-dopa [29], it expresses a peroxidative activity. GSH-Px catalyzes the reduction of both lipid peroxides and H_2O_2 in some systems [43]. GSH S-Tr expresses GSH-Px activity with organic hydroperoxides, but has little activity with H_2O_2 [24].

Comparison of the enzyme activities of control SY5Y cells with those of two 6-OHDA-resistant cell lines (A_1B_1 and CHO) yielded the following correlations to drug resistance (Table 6). Catalase activity was 2.5 times as high and GHS-Px at least 1.2 times as high in A_1B_1 and CHO cells as in SY5Y cells. However, GSH-Px activity was 10 times lower than catalase activity in SY5Y cells, and thus was not the primary protective agent, as Cohen and Hochstein [25] proposed it to be in brain against benzenoid neurotoxins. GSH S-Tr specific activity was relatively high in all three cell lines compared to human red blood cells (data not shown), but the values revealed no correlation between drug refractoriness and enzyme activity.

We analyzed the catalase values for each cell line in terms of specific activity per culture dish to determine the present effective concentration. One culture dish of SY5Y controls (5.0×10^4 cells when 6-

OHDA was added) contained 12 μg protein and 17 mU catalase activity. Similarly, CHO cells (24 μg protein/dish) possessed 89 mU/dish and A_1B_1 (24 μg protein/dish) 88 mU/dish. One unit of catalase is defined as that which catalyzes the conversion of 1 μg substrate per minute at 37°. Values are reported by Heikkila and Cohen [15], as well as others [44], for the rate of H_2O_2 generation by 6-OHDA. At pH 7.4, 37°, H_2O_2 generation (oxygen consumption) by 0.1 mM 6-OHDA is 28.6 nM/min, or 1 ng/min from 1 ml solution. For 0.5 mM 6-OHDA, the rate in 1 ml solution is 90 ng/min. The values are higher in the presence of ascorbic acid, and may be higher in a cell system which contains other reducing agents. Since SY5Y, CHO and A_1B_1 cells can detoxify up to 17, 89 and 88 ng H_2O_2 per min respectively, the rate of H_2O_2 generation at 0.1 mM 6-OHDA should be well within the defense capabilities of all three cell lines. This concentration is close to the lower dose (0.12 mM) used in the present study. However, as shown in the dose–response curve, (Fig. 1), only A_1B_1 and CHO cells were resistant to 0.12 mM 6-OHDA. SY5Y cells were sensitive presumably because H_2O_2 generation was actually higher than the value assumed above and because of the cytotoxicity of the free radicals O_2^- and ·OH. At the higher concentration of 6-OHDA, which approximates the higher dose (0.4 mM) used in this study, the limit of effective catalase activity was reached in cell lines CHO and A_1B_1, and exceeded in cell line SY5Y. According to Fig. 1, SY5Y and A_1B_1 cell survival is severely reduced by 0.4 mM 6-OHDA, while CHO survival is unaffected. The resistance of cell line CHO may have resulted from its GSH S-Tr activity, which was at least three times as high as that of the other cell lines in terms of units per dish: 9 mU/dish for CHO cells compared to 3 mU for A_1B_1 cells and 2 mU for SY5Y cells. According to this analysis, CHO resistance to 6-OHDA toxicity relied in part on a defense mechanism against organic hydroperoxides, which are probably formed by the interaction of H_2O_2 with cell constituents such as lipids.

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