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6-Hydroxydopamine toxicity to dopamine neurons in culture: potentiation by the addition of superoxide dismutase and *N*-acetylcysteine

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6-Hydroxydopamine (6OHDA) is a neurotoxin specific for catecholamine neurons of both the central and the peripheral nervous systems. There is strong evidence that 6OHDA neurotoxicity is correlated with its property to auto-oxidize rapidly at neutral pH and produce H_2O_2 and hydroxyl and superoxide radicals [1, 2]. The binding of quinones, formed during the auto-oxidation of 6OHDA, to proteins could also be contributing to the cytotoxicity of 6OHDA [3].

Cysteine and its derivative, *N*-acetylcysteine (NAC), are therapeutic agents that are widely used because of their antioxidant properties and ability to restore hepatic

glutathione levels depleted during metabolic stress [4, 5]. In the course of our studies on the mechanism of action of the specific neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridine (MPP^+) and 6OHDA, we examined whether NAC could modify their toxicity. We have shown that NAC does not modify the toxicity of MPTP or MPP^+ to dopamine (DA) neurons in culture, suggesting a lack of free radical involvement in their mechanism of action [6]. In the present communication, we report that the damage to DA neurons in culture produced by 6OHDA was potentiated by NAC.

Cysteine and NAC can be oxidized in the presence of metals to yield thiyl and hydroxyl radicals and H_2O_2, which result in lipid peroxidation [7, 8]. Superoxide (O_2^-) has also been suggested as a product of cysteine auto-oxidation [9]. To determine whether free radicals and H_2O_2, generated in the incubation medium by the auto-oxidation of NAC, contribute to the increased 6OHDA toxicity, we examined the effects of catalase and superoxide dismutase (SOD) on the potentiation of 6OHDA toxicity by NAC. We also examined the effect of NAC on the accumulation in the culture medium of 6OHDA-quinones, produced during 6OHDA auto-oxidation [10].

Materials and methods

Dissociated cell cultures were prepared from embryonic rat mesencephalon as described previously [11]. All experiments were performed during week 3 *in vitro*, when the growth of DA neurons has reached a plateau. The feeding medium consisted of Minimum Essential Medium (MEM; Gibco) containing 33 mM glucose, 2 mM glutamine and supplemented with 10% horse serum. NAC (1 mM; Sigma Chemical Co., St Louis, MO) was added to the cultures before and during exposure to 6OHDA. 6OHDA·Hbr (Regis Chemical Co.) was dissolved in ice-cold distilled deionized water containing 11.4 mM ascorbic acid and added to the feeding medium at a final concentration of 0.1 mM 6OHDA and 0.1 mM ascorbic acid. Toxicity to DA neurons was evaluated by measuring the uptake of [^{3}H]DA (New England Nuclear, Boston, MA; 29.5 Ci/mmol) 24 hr after removing 6OHDA, as described previously [11]. The quinone products generated from 6OHDA auto-oxidation were measured spectrophotometrically at 490 nm [12]. At time zero, 6OHDA dissolved in ascorbic acid was added to the feeding medium as described above for the toxicity studies. The medium was buffered with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid; Research Organics Inc.) and adjusted to pH 7.4 with sodium bicarbonate. The absorbance at 490 nm was followed every 0.5 min for the first 5 min and every 1 min thereafter. The addition of NAC or SOD did not affect the pH of the medium.

Results and discussion

Incubation of cultures with 0.1 mM 6OHDA for 1 hr resulted in a 22% inhibition of [^{3}H]DA uptake measured 24 hr later (Table 1). This effect was prevented completely in cultures pretreated with 10 μM mazindol, which inhibits the uptake pump of the DA neurons, suggesting that intraneuronal accumulation is needed for the toxic action of 6OHDA ([^{3}H]DA uptake, percent of control: 6OHDA, 72 ± 5; mazindol + 6OHDA, 103 ± 3). Exposure of cultures to 1 mM NAC alone did not result in toxic changes, as assessed by [^{3}H]DA uptake (Table 1) or by phase contrast microscopy of living cultures (results not shown). However, the toxicity of 6OHDA was potentiated by 1 mM NAC, the [^{3}H]DA uptake being further reduced from 78 to 53% of control (Table 1).

To determine whether the auto-oxidation of 6OHDA and NAC in the feeding medium contributed to the toxicity to DA neurons, we examined the effect of catalase and SOD. SOD, the enzyme that inactivates the superoxide anion (O_2^-) to form molecular oxygen and H_2O_2 [13], also potentiated the toxicity of 6OHDA to cultured DA neurons (Table 1). Addition of SOD (66 μg/ml) to the feeding medium potentiated the neurotoxicity of 6OHDA ([^{3}H]DA uptake reduced from 78 to 27% of control). The presence of both SOD and NAC had an additive effect on 6OHDA neurotoxicity ([^{3}H]DA uptake reduced from 27 to 13% of control). Addition of catalase (83 μg/ml) had no significant effect on 6OHDA neurotoxicity or on the potentiation of 6OHDA toxicity by NAC (results not shown). Since SOD probably does not enter the cells, the potentiation of 6OHDA toxicity by SOD was most likely due to the dismutation of O_2^- generated during oxidation of 6OHDA in the feeding medium. It is known that the auto-oxidation of 6OHDA is catalyzed by O_2^- and the presence of SOD retards it [12]. A delay in extracellular oxidation would increase 6OHDA levels in DA neurons, which should lead to greater toxicity.

The mechanism involved in the potentiation of 6OHDA neurotoxicity by NAC is not evident from these data. *In vitro* exposure to NAC can induce lipid peroxidation in rat liver hepatocytes without causing cell death [8]. In addition, cysteine, which can be formed from NAC *in vivo*, induces glutathione and ATP depletion in rat hepatocytes *in vitro* [7]. It is possible that sublethal changes caused by NAC could render the DA neurons more susceptible to 6OHDA toxicity. However, this seems unlikely in our experiments, because NAC must be present during exposure to 6OHDA to induce potentiation of neurotoxicity. Pretreatment with NAC for 90 min before addition of 6OHDA did not have any effect if NAC was not also added to the feeding medium during 6OHDA exposure ([^{3}H]DA uptake: control, 100 ± 4%; 6OHDA, 59 ± 3%; NAC pretreatment followed by 6OHDA, 65 ± 3%). It is unlikely that any sublethal damage to the neurons caused by NAC, such as membrane lipid peroxidation or glutathione depletion, could be reversed immediately upon removal of NAC from the medium.

We also examined the effects of SOD and NAC on the accumulation of 6OHDA-quinones in the feeding medium spectrophotometrically by measuring the absorbance at 490 nm [12]. The results of these experiments are shown in

Table 1. Effect of NAC and SOD on the 6OHDA induced toxicity to cultured DA neurons

Treatment	[^{3}H]DA uptake (% of control)
Control	100.0 ± 7.7
6OHDA	78.2 ± 6.7*
NAC	107.6 ± 6.1
NAC + 6OHDA	52.9 ± 4.1†
SOD	100.0 ± 3.7
SOD + 6OHDA	26.5 ± 1.9
SOD + NAC + 6OHDA	12.8 ± 0.9‡

Mesencephalic cultures were exposed to 0.1 mM 6OHDA for 1 hr. Treatments began 15 min before addition and during exposure to 6OHDA at the following concentrations: NAC, 1 mM; SOD, 66 μg/ml. All drugs were removed at the end of 6OHDA treatment. [^{3}H]DA uptake was measured 24 hr after the end of 6OHDA treatment by a 10-min exposure to 34 nM [^{3}H]DA (sp. act. 29.5 Ci/mmol). The uptake of the controls was 6.0 ± 0.5 pmol [^{3}H]DA/culture. Statistical significance was determined using ANOVA (one-way analysis of variance).

* Significantly different from control, $P < 0.01$.

† Significantly different from 6OHDA, $P < 0.001$.

‡ Significantly different from SOD + 6OHDA, $P < 0.001$.

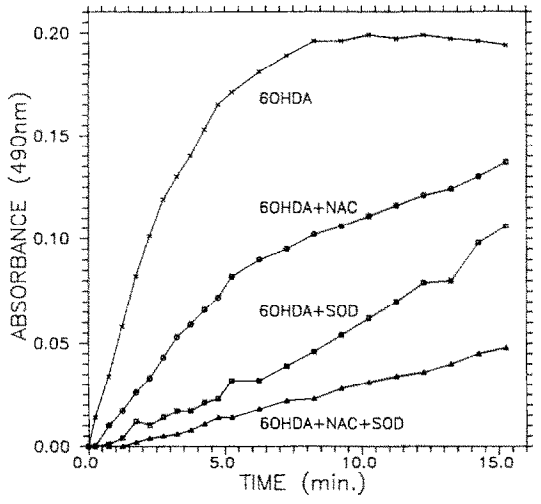

Fig. 1. Effect of SOD (66 μg/ml), NAC (1 mM), or the combination of SOD and NAC on the rate of 6OHDA-quionone accumulation in the tissue culture feeding medium after addition of 0.1 mM 6OHDA. The graph shows data from one representative experiment. The experiment was repeated three times with similar results. The rate of 6OHDA-quinone accumulation was followed by measuring the color formation spectrophotometrically at 490 nm as described in the text.

Fig. 1. The absorbance at 490 nm increased rapidly after addition of 6OHDA (0.1 mM), reaching a plateau at about 8 min. When NAC (1 mM) was present in the feeding medium, there was a significant delay in color formation, with approximately one-half of the 6OHDA oxidation product present by 8 min. SOD (66 μg/ml) caused an even greater delay in quinone accumulation, and after 8 min only about one-quarter of the quinone could be measured. Finally, the presence of both NAC and SOD had an additive effect on quinone accumulation. At 8 min just about 10% of color had been formed.

SOD is believed to retard 6OHDA auto-oxidation by removing O_2^-, which is a catalyst in 6OHDA oxidation [12]. Both a delay of quinone formation and a decrease in O_2 consumption are caused by the presence of SOD during auto-oxidation of 6OHDA [12]. NAC, which itself auto-oxidizes in the presence of metals to yield free radicals, H_2O_2, and possibly O_2^-, has not been shown to delay 6OHDA auto-oxidation. A delay in 6OHDA-quinone accumulation in the medium could result from the reduction of the quinone to 6OHDA by NAC or the binding of 6OHDA or its quinone to the thiol group of NAC. A difference in the effects of NAC and SOD on 6OHDA auto-oxidation in the feeding medium is supported by our observation that maximum absorbance, although delayed, was reached in solutions containing SOD and 6OHDA after approximately 30 min. However, in the presence of NAC (with or without SOD) maximum absorbance was not reached even after 1 hr, the longest time examined.

Our experiments show a negative correlation between the rate of accumulation of 6OHDA-quinones in the feeding medium and the toxicity of 6OHDA to DA neurons. The intraneuronal accumulation of 6OHDA is necessary for the expression of neurotoxicity, as shown by the complete protection from 6OHDA toxicity by mazindol. Therefore, a delay in 6OHDA auto-oxidation in the feeding medium by SOD could increase the amount of 6OHDA available for accumulation in the DA neurons and, consequently, potentiate toxicity. SOD is a high molecular weight protein, and its action on 6OHDA auto-oxidation would be expected to be restricted to the extracellular space (the feeding medium). However, NAC is a peptide, which has been used therapeutically because of its ease of penetration within cells [4]. Since binding of 6OHDA and its quinone to thiol compounds has been demonstrated both *in vitro* and *in vivo* [14, 15] and because NAC can also act as an antioxidant, one would expect that the presence of NAC within the DA neurons should be protective against 6OHDA neurotoxicity.

A possible explanation for the potentiation of 6OHDA toxicity by NAC could be the inhibition or delay of its auto-oxidation in the feeding medium, as we believe to be the case with SOD. However, in experiments where 6OHDA auto-oxidation was monitored by measuring the oxygen consumption in the solution by an oxygen electrode, no change in the rate of oxygen consumption was observed when 1 mM NAC was added to the 6OHDA solution (Danias and Mytilineou, manuscript in preparation). Furthermore, the presence in the feeding medium of 1 mM glutathione, a compound that does not easily pass through the cell membrane, did not modify 6OHDA neurotoxicity to DA neurons (data not shown). Thus, it appears that the potentiation of toxicity produced by NAC is probably an intracellular event. Recent studies demonstrating a potentiation of paraquat toxicity to cultured endothelial cells by NAC [16], and a potentiation of 6OHDA toxicity to hepatocytes *in vivo* by a thiol compound [17], suggest the need for a careful evaluation of the intracellular effects of these molecules. The mechanisms underlying the potentiation of 6OHDA toxicity by SOD and NAC are currently being investigated in our laboratory.

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