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DETERIORATION OF AXONAL MEMBRANES INDUCED BY PHENOLIC PRO-OXIDANTS

ROLES OF SUPEROXIDE RADICALS AND HYDROGEN PEROXIDE

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Abstract—The susceptibility of axons to oxidative free radicals generated by pro-oxidant neurotoxins and related compounds was tested by applying the reagents to the disheathed ventral nerve trunk of the crayfish. Electrophysiological characteristics of the axons, including spike amplitude and rise time, were recorded, using intracellular glass microelectrodes. L-Dopa, or L-dopa in the presence of copper-(bis)-histidine (Cu-his), did not change significantly the electrophysiological characteristics of the axon. A 20 mM concentration of 6-hydroxydopamine (6-OHDA), 20 mM 6-OHDA in an anaerobic environment, and 20 mM 6-OHDA with inactivated catalase–SOD accelerated the rate of decline of the spike amplitude with time to 5–8 times the control rate. Simultaneously, parallel increases in rise time and spike duration were observed, consistent with partial depolarization of the resting membrane presumably resulting from increased permeability. Catalase, superoxide dismutase (SOD), or a mixture of catalase and SOD all afforded partial protection, catalase having the least protective effect, and catalase + SOD the greatest. In contrast, 20 mM H_2O_2, 2 mM H_2O_2, or Cu-his alone did not significantly accelerate deterioration of the axon. Most of the damage results from the interaction of H_2O_2 with O_2^-, rather than from the direct action of either species. *p*-Hydroxyphenylpyruvate (pHPP) in the presence of Cu-his induced a similar accelerated deterioration of the axon to 4.2 times the control rate. Catalase plus SOD partially protected against this effect, but either enzyme alone was not significantly protective.

The central nervous system is susceptible to damage by pro-oxidant drugs and neurotoxins. Some catecholamines and catecholamine derivatives, e.g. 6-hydroxydopamine (6-OHDA), generate a mixture of superoxide radicals and hydrogen peroxide by transferring electrons to oxygen [1]. However, little is known regarding the mediators of their action, nor have the primary targets of the damage been identified [2–9]. The relative contributions of some of the reactive species can be determined by the use of superoxide dismutase (SOD) (which converts $2O_2^-$ to H_2O_2 and oxygen [10]) and catalase (which converts $2H_2O_2$ to $H_2O + O_2$ [6, 11]).

6-OHDA selectively destroys catecholamine-secreting neurons, into which it is transported by the catecholamine uptake mechanisms. It has been estimated that, when the intra-axonal concentration of 6-OHDA reaches a threshold of about 30 mM, destruction of the axon occurs [1]. Intracerebral injections of 6-OHDA cause "unspecific" damage to neurons near the tip of the cannula [12, 13]. Many different neurons may be susceptible to its toxic effects, but only at the site of its injection and in the catecholamine-secreting neurons does its concentration reach toxic levels [1].

Several potential targets for 6-OHDA toxicity have been studied *in vitro*. Heikkila and Cohen [6] showed that both SOD and catalase protect against 6-OHDA-induced inhibition of the uptake of tritiated biogenic amines. They concluded that O_2^- plays a role in the degeneration of the nerve terminal caused by 6-OHDA, largely by inducing formation of hydroxyl radicals. However, Tiffany-Castiglioni *et al.* [14] found that SOD alone does not protect the neuroblastoma cell culture from 6-OHDA toxicity, but merely delays the destruction. Since catalase alone *did* afford protection, it was concluded that H_2O_2 was the primary toxic agent despite the fact that H_2O_2 added alone could not account for all the damage.

Another avenue of toxicity is that which results from covalent binding of the quinone of 6-OHDA. This compound binds *in vitro* to proteins such as albumin [15]. These authors conclude that nerve degeneration potentially results from a similar reaction with nucleophilic groups on neuronal proteins. These possible roles have been assessed by Sachs and Jonsson [1] and, clearly, several or all reactive species generated by 6-OHDA and the reactive semiquinone can contribute to cellular toxicity.

In the presence of oxygen, *p*-hydroxyphenylpyruvate (pHPP) undergoes autoxidation with the production of *p*-hydroxyphenylacetic acid, H_2O_2 and CO_2, [16]. Jaynes *et al.* [17] state that pHPP undergoes autoxidation in the presence of high concentrations of Mn^{2+} but do not identify the products. Catalase protects pHPP hydroxylase or tyrosine hydroxylase from substrate-induced destruction *in vitro*, thus implicating H_2O_2 in its toxicity [5, 18].

As part of a series of studies of the susceptibility of various possible targets to free radical damage, we report the ability of some pro-oxidant compounds (including 6-OHDA) to alter some of the elec-

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trophysiological properties of axons. In systems where damage was observed, protective roles of superoxide dismutase and catalase were investigated.

MATERIALS AND METHODS

Southern crayfish (*Pacifastacus*) were supplied by College Biological Suppliers, Seattle, WA. The ventral nerve cord was dissected out using the method outlined by Bures *et al.* [19], the nerve trunk being mechanically desheathed over a distance of about 5 mm. The preparation was repeatedly flushed with crayfish electrolyte solution [20] adjusted to pH 7.5 [21] to ensure that potassium release from any damaged cells did not appreciably affect the extracellular fluid composition.

Following dissection, the nerve was positioned over a pair of AG–AgCl stimulating electrodes 2 mm apart, and the bath was drained to the desired level. A Sobotka micromanipulator was used to insert a glass electrode into an axon of the dissected nerve trunk. The apparatus was magnetically mounted on a 70lb steel plate resting on a 200lb concrete slab separated from the bench top by a number of pneumatic buffers which served as vibration dampers.

Following the stabilization period, 0.25 ml of reagent was applied every 20 min to the desheathed region of the nerve trunk. Solutions used were made up by dissolving the required quantity of chemical in deoxygenated crayfish electrolyte solution, sufficient NaOH being added to give a pH of 7.5 at 17°, and were again deoxygenated using a Virtis gas manifold with high purity nitrogen (Liquid Carbonic Gas Co., B.C.: O_2 content less than 60 ppm). We verified that pH did not change during incubation, and that changes in the ionic strength equivalent to the presence of 20 mM reagent had no measurable effect on the measurements made.

For the treatments in low O_2 environment, the crayfish solution was bubbled with N_2 gas for a period of 20 min prior to placing the nerve trunk in the solution. During the experiment, N_2 (see above) was continuously passed over and into the covered treatment trough to replace O_2 in the manifold which enclosed the preparation.

The Cu II-(bis)–histidine complex was prepared by mixing stoichiometric quantities of cupric sulfate and histidine, followed by neutralization to pH 7.5 using NaOH. Superoxide dismutase [22] was used at a final concentration of 10 nM, it having been confirmed by a series of direct assays that this was five times more than the amount necessary to scavenge 90% of the superoxide radicals produced under comparable conditions. Catalase (when present) was at a final concentration of 10 nM. Inactivated enzyme solutions for use as controls were produced by incubation at 100° for 30 min.

The nerve cord was stimulated five times per second, and the average of a dozen spikes was recorded at 5-min intervals for an initial 20-min stabilization period and, then, for a further 20-min experimental period. Axons which had spike amplitudes less than 80 mV or rise times greater than 5 mVsec after the initial period were not tested further.

Twenty superimposed spikes were recorded at the end of the 20-min pretreatment period, for use as the stabilized normal characteristic for that axonal preparation. Subsequent readings on that axon were converted to a percentage of that initial reading. Each treatment was repeated on five independently cannulated axonal preparations. The significances of the differences between treatment and control and between treatments, were evaluated by analysis of variance. Two separate control experiments (axons

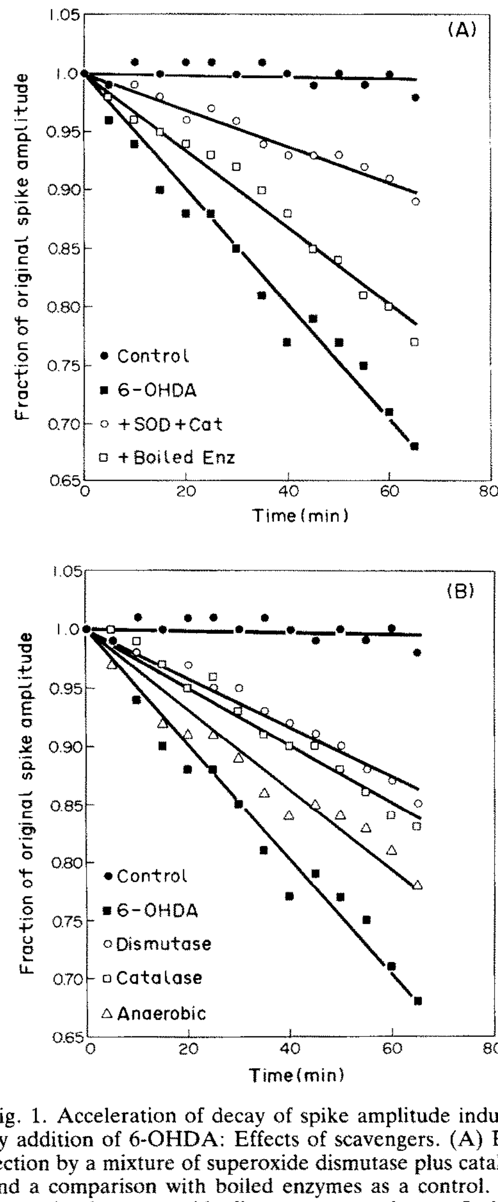

Fig. 1. Acceleration of decay of spike amplitude induced by addition of 6-OHDA: Effects of scavengers. (A) Protection by a mixture of superoxide dismutase plus catalase and a comparison with boiled enzymes as a control. (B) Protection by superoxide dismutase or catalase or flushing with nitrogen. Spike amplitude (fraction of original value) is plotted as a function of time in minutes under the stated experimental conditions. Each point represents the average value at that time, obtained from twenty summed spikes measured on each of five independently cannulated axons. Incubation medium was Crayfish Ringer's solution stabilized at pH 7.5 with 0.01 M Tris buffer at 17°. Where present, 6-OHDA was at 20 mM, and catalase or dismutase at 10 nM.

treated with buffer alone) were carried out, one before and one after all of the other experimental groups, to ensure that procedures remained constant during the project. Values for these two control groups did not differ significantly and were pooled to obtain the control values.

RESULTS

Effects of pro-oxidants on electrophysiological characteristics. The typical patterns of loss of electrophysiological characteristics of axons in the presence or absence of test reagents are shown in Fig. 1, A and B. The gradients and standard errors of the gradients for the linear relationships between electrophysiological characteristics and treatment time are tabulated in Figs. 2 and 3.

6-OHDA (20 mM) induced rapid deterioration of the axon membrane, sufficient to destroy much of its physiological electrical activity in 65 min of exposure (Figs. 1 and 2). Changes included a significant decrease in spike amplitude and significant increases in both rise time and duration (Fig. 4). In the case of pHPP + Cu-his, the only statistically significant change was decreased spike amplitude.

The following reagents had little or no effect on the axon membrane and are not shown: 5 mM L-dopa, 5 mM pHPP, 5 mM 6-OHDA, 5 mM H_2O_2 and 20 mM H_2O_2. Cu-his by itself had no detectable effect. Similarly, Cu-his produced no significant change in the effects of 5 mM L-dopa, but it did enhance damage in the presence of 5 mM pHPP to approximately ten times that of the controls (Fig. 3).

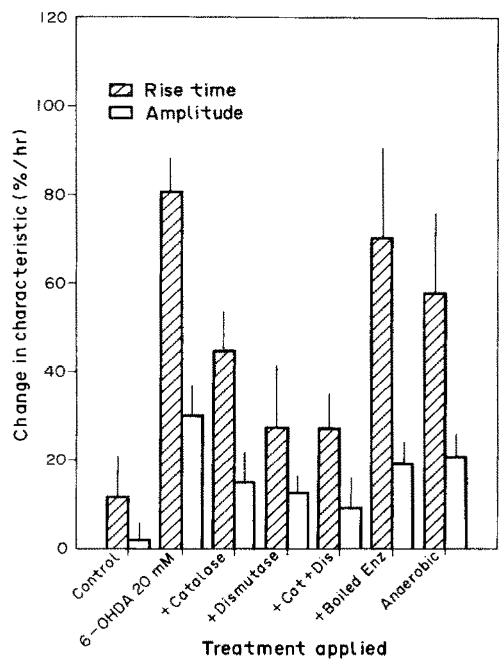

Fig. 2. Acceleration of changes in spike amplitude and rise time induced by addition of 6-OHDA: Effects of catalase, superoxide dismutase, catalase plus dismutase, boiled enzyme, or decreased pO_2 ("anaerobic"). Incubation conditions were as stated in the legend of Fig. 1. Values plotted represent average percent change per hour of the measured value during the 90-min observation period. Values are the positive or negative slopes of the regression line through the stated spike characteristic (as a percentage of the value during 20 min preceding treatment) plotted as a function of time, i.e. 100 × 60 × slopes of curves plotted in Fig. 1A and 1B, etc. Each value is the mean of the slope obtained for five independently cannulated axons. Vertical lines on bars represent standard errors of means.

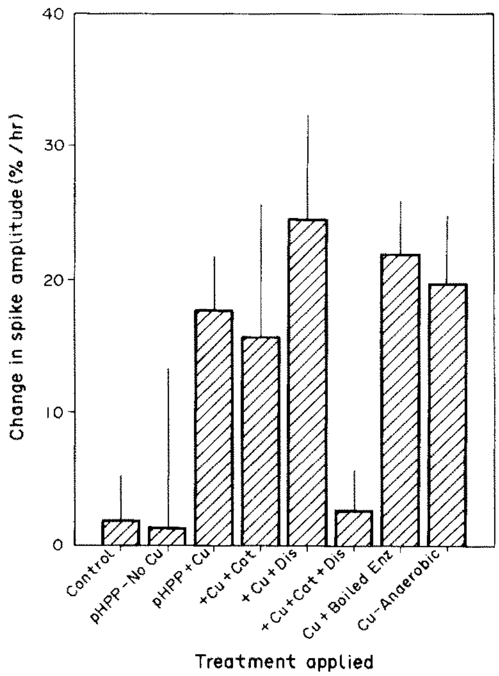

Fig. 3. Acceleration of decrease in spike amplitude induced by addition of copper plus *p*-hydroxyphenylpyruvate: Comparison with pHPP alone, effects of catalase, superoxide dismutase (separately and together), boiled enzyme mixture as a control, and effect of decreased pO_2 ("anaerobic"). Explanation of the values plotted is as stated in the legend of Fig. 2. Incubation conditions were as stated in the legend of Fig. 1, except that pHPP at 5 mM replaced 6-OHDA, and copper-(bis)-histidine (where present) was at 1 mM.

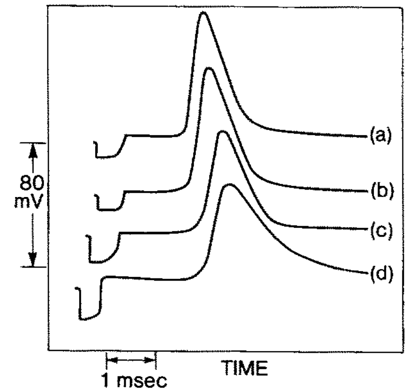

Fig. 4. Progressive alteration of electrophysiological characteristics of a single axon over 60 min of treatment with 6-OHDA (20 mM). Conditions were as stated in the legend of Fig. 1. Curves a–d represent 0, 20, 40 and 60 min of treatment time respectively.

Effect of "anaerobic" conditions. Flushing the system with high purity nitrogen produced no significant protection against damage by either 6-OHDA or Cu-his (Figs. 2 and 3).

Effects of catalase and/or superoxide dismutase. The roles of O_2^- or H_2O_2 in altering the electrophysiological properties may be inferred from the actions of catalase, superoxide dismutase, or a combination of the two.

In treatments with 6-OHDA, it was found that SOD decreased the rate of loss of spike amplitude by 62.5%, catalase produced 53.4% protection, while both together provided 73.5% protection (Fig. 1). These changes were statistically significant at the predetermined 95% level of confidence, although the effects of catalase and SOD were not significantly different from each other.

In the treatments with pHPP + Cu-his (Fig. 3), the presence of catalase + SOD produced a statistically significant protection, while the presence of heat-inactivated catalase–SOD mixture, or catalase or SOD alone, or anaerobic conditions did not significantly decrease the damage.

DISCUSSION

Nature of the axonal damage. Since the primary purpose of this study was to apportion the relative contributions of intermediates in the neurotoxic action of 6-hydroxydopamine, we will draw only the following limited conclusions as to the nature of the damage to the axon. In general, the electrophysiological changes observed—decreased spike amplitude, increased rise time, and increased spike duration (Fig. 4)—are consistent with partial depolarization of the resting membrane, presumably resulting from increased permeability. In the case of either 6-OHDA or pHPP + Cu-his, amplitude always declined as a linear function of time, whereas duration or rise time increased in an autocatalytic manner. This implies either an independent action of 6-OHDA at the different sites of damage reflected in the amplitude measurements and measures of spike duration (including rise time), or that a non-linear relationship between changes in these two measures results from action at a single site.

Direct and indirect damaging effects of O_2^- and H_2O_2. Destructive roles for intermediates in the reduction of oxygen in the action of both 6-OHDA and pHPP + Cu-his are inferred from the observed protection by the combination of catalase and superoxide dismutase in both cases. The relative contributions of O_2^- and H_2O_2 to the total damage caused by 6-OHDA could not be simply apportioned. As with some other systems involving these substances, the amount of protection produced by either superoxide dismutase or catalase alone (over 50%) seems high in comparison with the protection afforded when they are both present together (73.5%). Moreover, the fact that catalase protects contrasts dramatically with the failure of H_2O_2 (when added externally) to produce comparable damage.

The axonal membrane must, therefore, be added to a growing list of biological targets toward which the combination of H_2O_2 and O_2^- is much more damaging than the sum of the damage produced by both acting individually. In some of these other systems [14, 23], it has been established that, in the presence of reducing agents such as superoxide, H_2O_2 reacts (in a "Haber–Weiss"-like process, or more generally a metal catalyzed Fenton-type reaction [24] to yield hydroxyl radicals which are, in contrast, extremely destructive. The list of such biological targets now includes mitochondria [25] erythrocyte membranes [23, 26] and cultured neuroblastoma cells [14]. Further experiments with hydroxyl radical scavengers will be necessary to verify any role of hydroxyl radicals in the present system.

Direct and indirect protective actions of the scavengers. Interpretation of the protective actions of superoxide dismutase and catalase must take into consideration that either of these scavengers can play dual roles. On the one hand, each has a specific action in scavenging its substrate, and thus in preventing (by removal) the *action* of superoxide or hydrogen peroxide formed in the autoxidation of 6-OHDA. On the other hand, each plays a more general role in decreasing the rate of *formation* of all of the free radical intermediates of the reaction by slowing the rate of initial electron transfer from 6-OHDA to oxygen [27, 28]. Thus, the 50–75% inhibition produced by these reagents is quite consistent with a major role for the semiquinone intermediate.

Effects of diminished concentrations of oxygen. Although truly anaerobic conditions are not attainable in practice, it is of interest that very substantial decreases in the concentration of oxygen failed to retard the actions of either of the damaging pro-oxidants. In other systems, an enhancement of catechol-mediated damage by decreased concentrations of oxygen has been explained on the basis that a major fraction of the damage is due to semiquinone radicals produced in the initial transfer of an electron from the catechol. This explanation is based on the presumption that oxygen reacts rapidly with semiquinones to form the relatively inert *p*-quinone. On this basis, the increased steady-state levels of the semiquinone which occur under conditions of lowered oxygen tension compensate for the decreased contribution of free radical forms of oxygen [29].

Although there are no selective scavengers for semiquinone radicals which would allow direct assessment of their role in the current system, a damaging role for the semiquinone intermediates is consistent with the failure of a mixture of superoxide dismutase and catalase to protect completely. If either superoxide or hydrogen peroxide, or hydroxyl radicals resulting from Fenton-type or Haber–Weiss-like reactions were together responsible for all of the damage, then the destructive actions of 6-OHDA should be inhibited completely by the presence of SOD and catalase. Clearly this is not the case. Moreover, direct actions of 6-OHDA acting as a reducing agent cannot account for more than half of the damage at decreased oxygen concentrations. This follows from the aerobic studies, in which the maximum amount of damage attributable to the direct action of 6-OHDA cannot exceed the small amount of residual damage in the presence of superoxide dismutase and catalase. Future studies of the effects of other reducing agents (e.g. dithiothreitol), or of nonspecific scavengers of semiquinones (e.g. cytochrome *c*), will help to clarify these questions.

Significant participation of the *p*-quinone product is excluded by the observed failure of the products of oxidation of 6-OHDA to produce detectable damage when added alone. pHPP alone is only marginally damaging to axons but in the presence of cupric ions it is significantly toxic. L-Dopa which is a rather more powerful reductant of oxygen than pHPP was not damaging even in the presence of cupric ions. The role of copper in promoting free radical damage is at least 2-fold: transition metal ions accelerate production of free radical intermediates by accelerating the overall reactivity of 6-OHDA toward oxygen [27, 28]. Second, transition metal ions accelerate production of hydroxyl radicals by catalyzing the transfer of electrons to hydrogen peroxide, with the resultant formation of hydroxyl radicals in a Fenton-type reaction [24].

In conclusion, we have described a preparation of nervous tissue in which damage by 6-OHDA can be assessed quantitatively. The method should be useful for further investigation of the mechanism of action of pro-oxidant neurotoxins, and for testing the efficacy of potential protective agents. Addition of more selective electrophysiological measurements could lead to identification of the targets of the damage within the membrane. The axonal membrane, although not as sensitive to damage as microtubules [30] must be included among potential targets for the neurotoxic actions of oxygen radicals in living cells.

To sum up therefore, taking the current results together with those of other workers, we conclude that, among the pro-oxidants and mixtures studied, 20 mM 6-OHDA, or 5 mM pHPP in the presence of copper, has deleterious effects on the measured electrophysiological properties of the axonal membrane. Contributing to the damage is a multiplicity of intermediates among which superoxide and hydrogen peroxide play important roles, both in accelerating the reaction of 6-OHDA with oxygen [27] and in increasing the steady-state yield of reactive intermediates. Neither hydrogen peroxide alone nor the products of oxidation of 6-OHDA have significant direct contributions, while 6-OHDA itself has little, if any, direct damaging effect.

Oxygen has ambiguous effects, increasing the yield of oxygen free radical intermediates, but decreasing the yield of semiquinone. Copper accelerates damage in the case of pHPP (but not L-dopa) by accelerating both production of potentially harmful radicals and synergistic interactions between them.

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