Generation of the Neurotoxin 6-Hydroxydopamine by Peroxidase/H_2O_2 Oxidation of Dopamine

Alessandra Napolitano, Orlando Crescenzi, Alessandro Pezzella, and Giuseppe Prota*

Department of Organic and Biological Chemistry, University of Naples "Federico II", Via Mezzocannone 16, 80134-Naples, Italy

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At physiological pH values, oxidation of the neurotransmitter dopamine (DA) by the peroxidase/H_2O_2 system leads to, besides dopaminochrome and 5,6-dihydroxyindole resulting from oxidative cyclization of dopaminequinone (DQ), significant amounts of the neurotoxin 6-hydroxydopamine (6-OHDA) in the oxidized quinonoid form (topaminequinone, TQ). Formation of TQ was shown to depend critically on the presence of hydrogen peroxide in the reaction medium and was not observed when DA oxidation was carried out using the tyrosinase/O_2 system or chemical agents such as periodate or ferricyanide. These and other data suggest that, under the conditions adopted, nucleophilic attack of the hydrogen peroxide anion on DQ leading to TQ significantly competes with the intramolecular cyclization path. In line with this mechanism, the reaction course was not affected by the presence of hydroxyl radical scavengers. Peroxidase/H_2O_2 oxidation of the model *N*-acetyldopamine (**1**) gave, as expected, the 2-hydroxy-1,4-benzoquinone **3** in yields up to 55%, depending on the catecholamine/H_2O_2 mole ratio. Likewise, reaction of 4-methyl-1,2-benzoquinone (**4**) with hydrogen peroxide afforded 2-hydroxy-5-methyl-1,4-benzoquinone (**5**) in good yields. Collectively, these results would point to the possibility that intraneuronal formation of 6-OHDA is associated with an increased production of hydrogen peroxide under oxidative stress conditions.

Introduction

Since the late 1960's 6-hydroxydopamine (6-OHDA) has represented an important pharmacological tool in neurobiology for chemical lesioning of catecholaminergic neurons.[1] It is generally agreed that the selectivity associated with this catecholamine analogue stems from its high-affinity uptake by the neuron membrane pump. Such a specific transport mechanism results in an efficient intraneuronal accumulation allowing the degenerative action to be selectively restricted to the catecholamine neurons.[2]

There are also several observations indicating the involvement of 6-OHDA, as generated in brain by aberrant oxidations of catecholamines, in some severe mental disorders.[3–5] However this view has not reached general consensus.[6] On the basis of marked and long-lasting depletion of brain norepinephrine and DA following administration of 6-OHDA,[7] and on the results of the tracer studies by Senoh *et al.*,[8] Stein and Wise[3] proposed that the endogenous generation of even small amounts of the neurotoxin might be responsible for the progressive damage to the noradrenergic reward mechanism associated with schizophrenia. More recently, the observed ability of 6-OHDA to induce the symptomatology of Parkinson's disease in humans and animals[9] has been taken to mean that this or structurally related neurotoxins may be implicated[5] in the etiology of the neurodegenerative processes which selectively affect the nigrostriatal dopamine neurons.[10] 6-OHDA was also reported to be generated from endogenous DA in rat brain following administration of methylamphetamine in large doses.[11]

Most of the chemical and biochemical work carried out on 6-OHDA in the last decades have addressed the molecular mechanisms underlying its neurotoxic effects. As a result of these studies, two main theories have been put forward, both revolving around the marked ease of the catecholamine to undergo oxidation with intracellular formation of electrophilic quinonoid intermediates and/or highly reactive oxygen species capable of targeting critical functionalities within vital biomolecules, including enzymes and lipids.[12] However, how the neurotoxin may be generated intraneuronally from DA has so far escaped definition.

We report now evidence that the quinone derived from 6-OHDA is generated *in vitro* by peroxidase/H_2O_2 oxidation of DA *via* addition of the hydrogen peroxide anion to the transiently formed dopaminoquinone (DQ). By contrast, no detectable amounts of the neurotoxin were obtained by Mn^{2+}- or tyrosinase-catalyzed oxidation of DA at physiological pH, as previously reported.[13,14]

Results and Discussion

There are some reports in the literature dealing with the *in vitro* formation of 6-OHDA by oxidation of DA under conditions of physiological relevance.[8,13–15] In an initial series of experiments, we found that oxidation of DA by the Udenfriend or Fenton system leads to 6-OHDA, as reported,[8,15] whereas the tyrosinase-[14] or Mn^{2+}-catalyzed[13] DA oxidation failed to give the toxin. These latter oxidation conditions were therefore carefully reexamined using improved techniques for product analysis and identification.

1. Manganese(II)-Catalyzed Autooxidation. Manganese, an essential trace element which upon excessive exposure produces a neurological syndrome similar to chronic Parkinson's disease in animals and humans,[16] was reported to catalyze the autooxidation of DA with formation of 6-OHDA in aqueous buffer at neutral pH.[13] By HPLC analysis with electrochemical detection of the components of the oxidation mixture, evidence was

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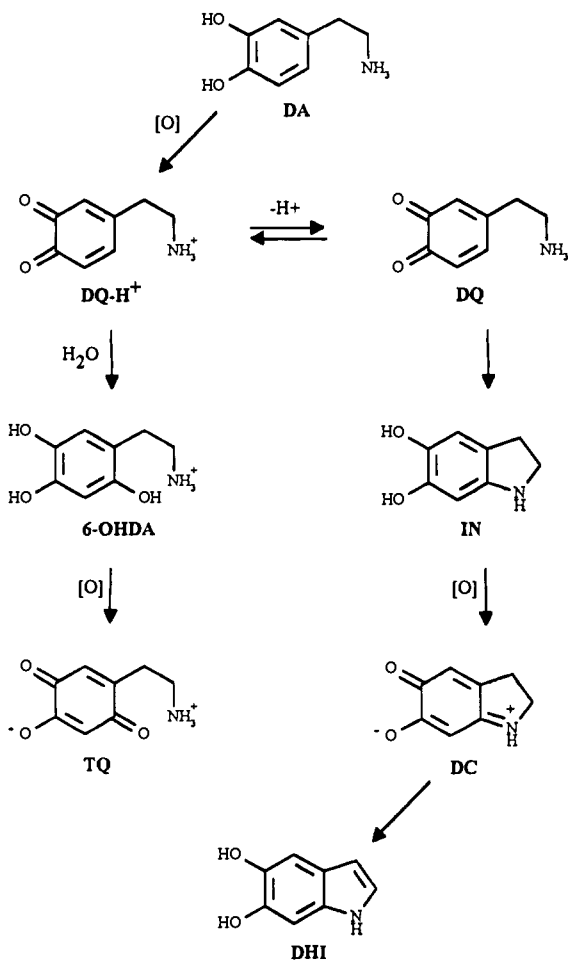
Scheme 1



provided for a 78% consumption of DA, at 1 min reaction time, with formation of 6-OHDA at about a 1:1 mole ratio with respect to unchanged DA. In repeating the experiment, using different analytical conditions, we found that the metal-catalyzed oxidation proceeds with the rapid depletion of the substrate, as described, with concomitant formation of dopaminochrome (DC)[17] along with minor amounts of other unidentified species which rapidly disappeared in the subsequent phases of the reaction and could not be further characterized. However, 6-OHDA or the corresponding quinone, topaminequinone (TQ), was not present in yields higher than 0.2% (estimated lower detection limit), as evidenced by analysis of the mixture either as such or after $NaBH_4$ reduction. Such a discrepancy in the results may probably be ascribed to the inadequacy of the chromatographic analytical conditions adopted in the work by Garner *et al.*[13] for identification of the reaction product. In any event, the unexpected outcome of our experiment is of interest in that it casts doubts on the possibility envisaged by the authors that generation of the neurotoxicant 6-OHDA may account for the pathophysiology of Mn exposure.

2. Tyrosinase-Catalyzed Oxidation. Accumulation of 6-OHDA and TQ in the tyrosinase-promoted oxidation of DA as the result of addition of water to transiently generated DQ (Scheme 1) has been postulated by Garcia-Moreno *et al.*[14] on the basis of kinetic evidence. Support for the proposed mechanism has also been provided in a recent paper by Ito *et al.*[18] Such a reaction would represent a side branch of the main oxidation pathway of DA, which involves in the early stages oxidative cyclization of DQ to DC, *via* the leuco intermediate 5,6-dihydroxyindoline (IN), followed by rearrangement to 5,6-dihydroxyindole (DHI).[12d,19] The relative operation of the hydroxylation path with respect to the cyclization route was reported to be dictated by the pH of the medium, becoming significant at pH values <6. Surprisingly, on HPLC analysis under our experimental conditions, the product pattern of the tyrosinase-catalyzed oxidation of DA at pH 5 was not significantly different from that observed at pH 7. No appreciable amounts of 6-OHDA or its quinone (<0.2% yield) could be detected at either pH.

It should be noted, however, that the only direct evidence for the formation of 6-OHDA and its quinone from DA followed from voltammetric experiments performed at pH 4.2.[14] It is possible that under such more acidic conditions the formation of 6-OHDA prevails over cyclization. In fact, a similar shift from the cyclization to the hydroxylation route was observed to occur in the case of the oxidation of dopa at low pH,[20] due to the increased extent of protonation of the amino group. It is clear however that the relevance of these observations to the oxidative metabolism of DA *in vivo* should be carefully considered.

3. Peroxidase/H_2O_2 Oxidation. Given the results of the above experiments, as a further step in our investigation, we wanted to explore other conditions of potential physiological interest under which 6-OHDA might be generated.

In a previous study[21] on the structure of dopamine melanin, a polymeric product of DA oxidation, we obtained evidence that the peroxidase/H_2O_2 system is able to effect a rapid and efficient conversion of the catecholamine to melanin. We therefore chose the peroxidase/H_2O_2 couple as a biomimetic oxidizing agent to examine the early stages of DA oxidation. It should be noted however that the postulated involvement of this enzymic system in the oxidative metabolism of DA in brain, particularly in neuromelanin biosynthesis,[22,23] has not definitively been assessed.[5]

HPLC analysis of the reaction mixture in the early stages of the peroxidase/H_2O_2 oxidation of DA showed a rather complex pattern of products (Figure 1, elutogram a). Peaks C and D were identified as the aminochrome DC and its rearrangement product, DHI, by analysis of the UV spectra and comparison of the chromatographic behavior with those of authentic samples. Like C, the other two major components of the mixture, compounds A and B, were characterized by a red chromophore in the visible region suggestive of an aminochrome structure.[17] Of these, peak A, which proved highly unstable, could not be identified by coinjection with a number of standard samples, including noradrenochrome. On the other hand, peak B, which could be obtained from the reaction mixture by preparative HPLC fractionation, exhibited under different pH conditions absorption maxima identical to those described for 2-hydroxy-5-(2-aminoethyl)-1,4-benzoquinone (topaminequinone, TQ).[24] Consistent with this assignment, $NaBH_4$ reduction of the compound gave a colorless species with the spectral features and the chromatographic properties of 6-OHDA. On standing under a nitrogen atmosphere, the chromophore of compound B was smoothly converted to that of DC, in agreement with the reported formation of the ami-

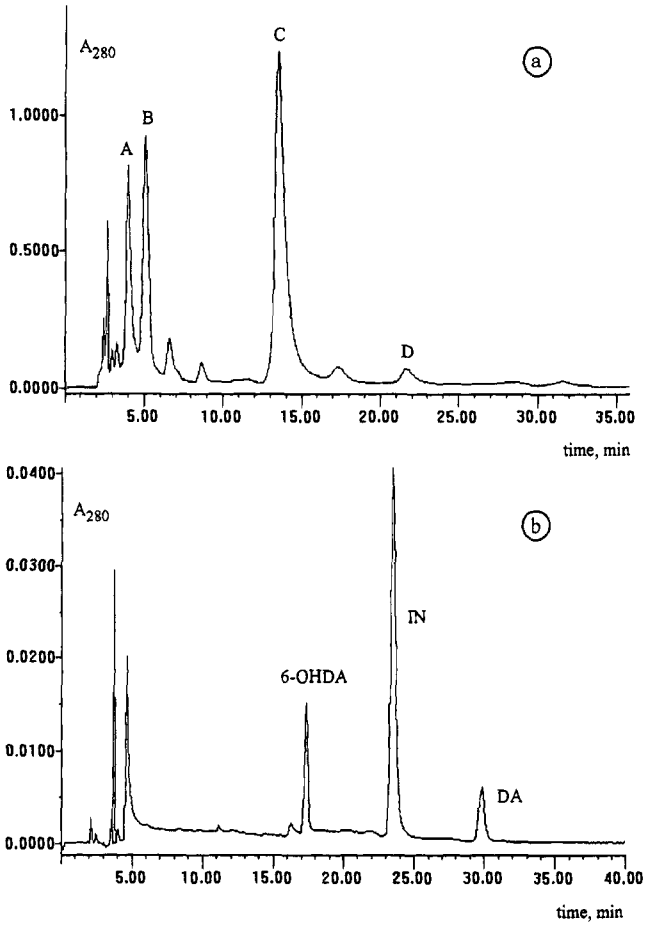


Figure 1. HPLC elution profile of the products formed by oxidation of DA (5 mM) promoted by peroxidase (2.8 U/mL)/H_2O_2 (10 mM) in 0.1 M phosphate buffer, pH 7.0 at 2 min reaction time, with (b) or without (a) reduction with $NaBH_4$. Eluants A and B were used for analysis of the nonreduced and reduced mixtures respectively; all other conditions were as described in the Experimental Section.

nochrome by intramolecular cyclization of TQ in the oxidation pathway of 6-OHDA.[25,26]

Additional support for the formation of TQ in the course of the peroxidase/H_2O_2-promoted oxidation of DA was provided by analysis of the reaction mixture after $NaBH_4$ reduction (Figure 1b). When the oxidation mixture was reduced after a 2 min reaction time, HPLC analysis revealed the presence of the reduction product of DC, 5,6-dihydroxyindoline (IN), identified by coinjection with a synthetic sample, along with significant amounts of 6-OHDA and the starting amine DA.

Monitoring of TQ formation with time showed that it is transiently generated and accumulates in the very first minutes of the oxidation course. In the subsequent stages of the process, the concentration decreases with concomitant formation of DC. Thus, at 5 mM DA concentration, in the presence of 2 equiv of H_2O_2, the yield of 6-OHDA with respect to the starting catecholamine was 7% at 2 min reaction time. The yield halved after 10 min.

In further experiments, the course of DA oxidation, as effected by a number of chemical and enzymic agents including periodate, ferricyanide, and the tyrosinase/O_2 system, was examined in comparison with that observed in the case of the peroxidase-promoted reaction. Under all the conditions examined, however, DA was converted to the melanin pigment through DC and

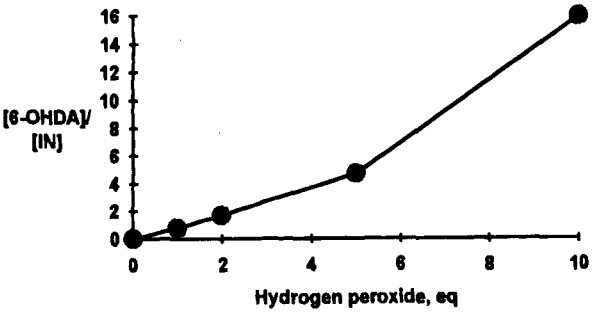


Figure 2. Effect of H_2O_2 concentration on the initial ratio of formation of 6-OHDA to IN in the peroxidase-promoted oxidation of DA (5 mM) by peroxidase. The values of the initial formation rates were obtained from plots of product concentration vs time by extrapolation at zero time.

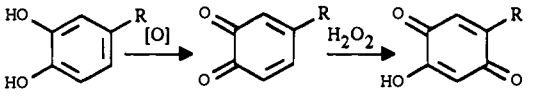
DHI intermediates. No detectable amounts of 6-OHDA or the corresponding quinone were formed.

Incubation of DA with hydrogen peroxide in the absence of peroxidase for up to 20 min did not produce a significant consumption of the catecholamine (<2%), and in addition 6-OHDA, as such or in the oxidized quinonoid form, was not obtained. Conversely, incubation of DA with tyrosinase/O_2 in the presence of H_2O_2 gave rise to TQ in significant yields along with the usual pattern of cyclization product. It should be noted, however, that under these conditions the rate of the tyrosinase-catalyzed oxidation of DA appreciably decreases with respect to a control reaction carried out in the absence of H_2O_2, probably because of a partial inhibition of the enzymatic activity by hydrogen peroxide.[27]

4. Mechanistic Studies. In the light of the above results, it appears that the presence of hydrogen peroxide together with an agent capable of effecting DA oxidation is a requisite for the formation of 6-OHDA (or TQ) from DQ to take place. In support of this view is the observed formation of TQ from 6-OHDA following the addition of DQ, generated in acid solution by cerium ammonium nitrate oxidation of DA, to a buffered solution of H_2O_2 at pH 7. On the other hand, no trace of TQ could be detected in the pattern of reaction products in control experiments when DQ, generated as above, was poured in the neutral buffer, but in the absence of H_2O_2. Attempts to confirm these observations using other chemical agents for *in situ* generation of DQ at neutral pHs in the presence of H_2O_2 did not provide reliable results. This may be ascribed to the ability of H_2O_2 to undergo redox exchange with most of the common oxidants, e.g., sodium peroidate or potassium ferricyanide.[28]

The dependence of the yield of 6-OHDA in the peroxidase/H_2O_2-promoted oxidation of DA (5 mM) on the concentration of H_2O_2 in solution was shown by HPLC analysis of reaction mixtures with different DA/H_2O_2 mole ratios, after treatment with $NaBH_4$. In Figure 2 the initial ratio of 6-OHDA to IN is plotted vs H_2O_2 concentration. This takes into account the slowing down of the oxidation rate observed as the concentration of H_2O_2 in solution increases, due probably to a partial inhibition to the enzyme activity.[29] The increase of the relative yield of 6-OHDA with increasing concentration of hydrogen peroxide points to a direct competition of this latter species with the side chain amino group of DA (or possibly of its oxidation product DQ).

Scheme 2



1: R = CH_2CH_2NHAc 2: R = CH_2CH_2NHAc 3: R = CH_2CH_2NHAc
4: R = CH_3 5: R = CH_3

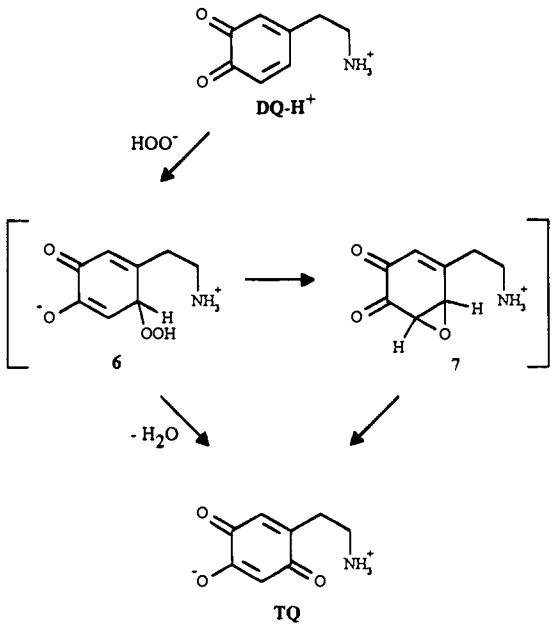
Further information on the mechanism of the reaction was gained by investigation of the behavior of the model compound *N*-acetyldopamine (1) in which blocking of the amino group of the ethylamine side chain prevents the cyclization route to operate. The peroxidase/H_2O_2-promoted oxidation of **1** leads to the initial development of an absorption maximum at 390 nm, attributable to the *o*-quinone **2**, which accumulated in solution but was not sufficiently stable to be isolated even under carefully controlled conditions. This was subsequently replaced by a purple compound identified as 5-(2-acetamidoethyl)-2-hydroxy-1,4-benzoquinone (**3**) on the basis of the spectral features which were closely reminiscent of those of TQ (Scheme 2). Using the value of the extinction coefficient reported for the quinone under similar conditions,[24] a 55% yield of **3** could be estimated when the oxidation was carried out with a 20-fold excess of H_2O_2. In close agreement with this observation, reaction of 4-methyl-1,2-benzoquinone (**4**) with hydrogen peroxide under the same conditions adopted for DA oxidation afforded 2-hydroxy-5-methyl-1,4-benzoquinone (**5**), identified by comparison with an authentic sample prepared by an alternative synthetic route,[30] in yields of up to 47%, depending on the quinone/hydrogen peroxide mole ratio (Scheme 2). By contrast, on standing in the buffer system, but in the absence of H_2O_2, 4-methyl-1,2-benzoquinone was rapidly converted into a brownish mixture exhibiting an almost featureless absorption spectrum.

From consideration of all of these data, it may be reasonable to suggest that, under the conditions examined, the nucleophilic attack of the hydrogen peroxide anion on the quinone DQ, transiently generated in the early stages of DA oxidation, significantly competes with the intramolecular cyclization path leading to the aminochrome DC. It should be emphasized that the proposed mechanism of formation of 6-OHDA is linked to the intrinsic reactivity of DQ toward H_2O_2 and does not depend upon the specific oxidizing system used to generate DQ.

As depicted in Scheme 3, various mechanistic options may be considered for the conversion of the hydroperoxide adduct **6**, initially formed by H_2O_2 attack on TQ, i.e., formation of the epoxide **7**, or the direct decomposition with 1,2 elimination of a molecule of water. Reactions of *p*- and *o*-quinones toward H_2O_2 leading to 2-hydroxy-1,4-benzoquinones *via* 2,3-epoxides are well documented in the literature.[31] An alkaline medium is usually employed in order to generate the hydroperoxide anion, the actual reactive species. In our case, however, the failure to detect any reaction intermediate is not surprising considering that isolation or detection of quinone epoxides has been reported in the case of some less reactive 1,4-quinones, *e.g.*, 1,4-naphthoquinones, but only rarely for compounds of the 1,2-series, unless in the presence of a proper substitution pattern.[32]

An alternative mechanism of formation of 6-OHDA involving attack of the hydroxyl radical on DA has been

Scheme 3



reported to occur in Fenton-type or iron–EDTA/ascorbate reactions.[15] All other products which would be expected to result from a random hydroxylation process were also obtained. By contrast, the regioselective course of the peroxidase/H_2O_2 oxidation of DA and the observed increase of the 2-hydroxy-1,4-benzoquinone **3** obtained from the model compound 1 support the proposed ionic type mechanism for 6-OHDA formation. The possible intervention of the hydroxyl radical under the conditions of our study was definitively ruled out by the results of separate experiments showing that the pattern of reaction products and the 6-OHDA/IN formation ratio at fixed reaction times were unaffected by addition of OH• scavengers (DMSO, mannitol).

Conclusion

A growing body of information supports the role of oxidative stress in the pathogenesis and progression of Parkinson's disease and other neurodegenerative processes.[5,33] An increased turnover of DA by monoamine oxidase has been observed[10] in the surviving neurons of the nigrostriatal tract in Parkinsonian brain, resulting in an enhanced production of hydrogen peroxide, an oxidant species capable of inducing tissue damage and triggering cellular destruction. Extensive studies by Cohen and associates[34] pointed to a significant loss of GSH resulting from H_2O_2 scavenging by GSH peroxidase as the major effect of the increased DA catabolsim in pigmented neurons.

The results of the present investigation provide evidence that, under biomimetic conditions, DQ is able to react with hydrogen peroxide to give TQ, the quinone of 6-OHDA. If these model studies have some bearing on the *in vivo* situation, one could expect that the endogenous formation of the highly electrophilic TQ capable of alkylating key protein nucleophiles is a significant event underlying oxidative stress conditions and the associated neuronal damages. There are however some observations which would cast doubts on this interpretation. In a study on the formation of 6-OHDA in brain following administration of 6-hydroxydopa, Evans and Cohen reported that striatal dopaminergic nerve terminals are resistant to exposure to high levels

of 6-OHDA.[6] However, the effect produced by a persistent endogenous production of 6-OHDA over extended time periods was not considered and should be further investigated.

One of the reviewers has also suggested that formation of 6-OHDA may not be critical in the neurodegenerative processes associated with oxidative stress conditions, as both DQ and $O_2^{\bullet-}/H_2O_2/OH^{\bullet}$ formed in the initial oxidation of DA are cytotoxic. Of course, the possible involvement of these species can not be ruled out and remains to be assessed. However, from consideration of the values of the cyclization rates,[12d,19,25,26,35] it could be argued that an efficient self-deactivation pathway is available to DQ but not to TQ. Thus, any TQ formed is expected to undergo deactivation by interaction with cellular nucleophilic components and may therefore represent a major cytotoxic agent produced under oxidative stress conditions.

Experimental Section

UV spectra were recorded on a Perkin Elmer Lambda 7 spectrophotometer having the cell compartment controlled at 25 ± 0.1 °C with circulating water. HPLC analyses were performed with a Gilson instrument equipped with model 305 pumps and a model 317 UV detector. EI MS mass spectra were determined with a Kratos MS 50 spectrometer. ^1H NMR (270 MHz) and ^{13}C NMR (67.9 MHz) spectra were recorded on a Bruker AC 270 spectrometer.

Materials. Dopamine hydrochloride, 6-hydroxydopamine hydrobromide, *N*-acetyldopamine, mushroom tyrosinase (EC 1.14.18.1, o-diphenol:O_2 oxidoreductase; 6300 U/mg), and horseradish peroxidase (donor H_2O_2 oxidoreductase, EC 1.11.1.7) type II (200 purpurogallin U/mg, $E_{430}/E_{275} = 2.0$) were purchased from Sigma Chemicals (St. Louis, MO). Methyl-1,4-benzoquinone, 4-methylcatechol, and 5,6-dimethoxyindole were from Aldrich Chemie (Steinheim, Germany). Nonstabilized hydrogen peroxide (35% solution in water) was from Fluka. Hydrogen peroxide concentration was determined spectrophotometrically prior to each experiment.[27] 5,6-Dihydroxyindole was synthesized according to Benigni and Minnis;[36] 4-methyl-1,2-benzoquinone was prepared by sodium periodate oxidation of 4-methylcatechol as reported.[37] 2-Hydroxy-5-methyl-1,4-benzoquinone was obtained from methyl-1,4-benzoquinone by a three-step synthesis as previously described.[30]

Analytical Conditions. HPLC was performed using a Spherisorb S5 ODS 2 column (1 mL/min) and a 10 × 250 mm Alltech Econosil C_{18} column (6 mL/min) for analytical and preparative runs, respectively. Detection was carried out at 280 nm. Different elution conditions were employed as follows: 0.05 M citrate buffer, pH 4–methanol, 95:5 (eluant A); 0.05 M phosphate buffer, pH 3.0, containing 10 mM sodium 1-octanesulfonate–acetonitrile, 90:10 (eluant B); mixed 0.04 M phosphate–0.04 M citrate buffer, pH 3.0, containing 7.5 mM sodium 1-octanesulfonate, 0.08 mM ethylenediaminetetraacetic acid, 9.5% methanol, and 4.5% acetonitrile (eluant C).

Synthesis of 5,6-Dihydroxyindoline Hydrobromide (IN). The title compound was prepared from 5,6-dimethoxyindole by a modification of the method reported by Chavdarian *et al.*[38] In brief, 2.0 g (11.3 mmol) of 5,6-dimethoxyindole in 200 mL of 50% AcOH was treated with 2.8 g (44.4 mmol) of sodium cyanoborohydride and allowed to stand at room temperature under stirring. After 75 min, the mixture was poured into 500 mL of 4 M NaOH and extracted repeatedly with Et_2O. The combined organic layers were washed with saturated aqueous NaCl, dried over Na_2SO_4, and taken to dryness to give 1.62 g of crude 5,6-dimethoxyindoline (80% yield) which was used as such in the next step. Crystallization from benzene–petroleum ether gave an analytical sample exhibiting physical and spectral properties in good agreement with those reported.[38] A solution of 1.2 g (6.70 mmol) of the crude 5,6-dimethoxyindoline in 65 mL of 48% HBr was heated for 3 h at 140 °C. After cooling, the solution was evaporated to a reddish brown residue which by crystallization from EtOH–Et_2O afforded 0.96 g (62% yield) of 5,6-dihydroxyindoline hydrobromide: mp 222–224 °C dec; EI MS *m/z* 151 (M^+), 150, 149, 132, 122; ^1H NMR (DMSO-d_6) δ 3.08 (2H, t, $J = 7.6$ Hz, H-3, H-3'[Hl]), 3.72 (2H, t, $J = 7.6$ Hz, H-2, H-2'[Hl]), 6.84 (1H, s, H-7), 6.88 (1H, s, H-4), 9.46 (2H, bs, NH_2^+), 10.45 (2H, bs, OH); ^{13}C NMR (DMSO-d_6) δ 28.52, 45.77, 106.24, 111.64, 125.35, 126.76, 145.19, 146.72.

Oxidation of Dopamine (DA). (a) By Peroxidase/H_2O_2. A solution of dopamine hydrochloride (5 mM) in 0.1 M phosphate buffer, pH 7.0, was treated with horseradish peroxidase (2.8 U/mL) and a freshly prepared solution of H_2O_2 (10 mM). Aliquots of the reaction mixture were periodically withdrawn and analyzed by HPLC, using eluant A as the mobile phase. Alternatively, aliquots of the oxidation mixture were treated with an excess of solid $NaBH_4$ (ca. 100 mM final concentration) and, after 30 s, acidified to pH 1 with 6 M HCl prior to analysis (eluant B or C). Identification of 6-OHDA and other reaction products was achieved by comparison of their chromatographic behavior with those of authentic samples. In the preparative runs, the mixture was filtered through a 0.45 μm Millipore filter after 6 min reaction time and fractionated by HPLC, using eluant A as the mobile phase. UV spectra of the fractions corresponding to the major peaks were recorded either soon after collection or after storing under nitrogen atmosphere. Some of these were reduced by treatment with $NaBH_4$ and further analyzed by HPLC. Other experiments were performed with varying concentrations of H_2O_2 (10–100 mM) while the concentration of the substrate was held constant (5 mM). Estimation of the yield of the oxidation products at different reaction times in the mixtures, after $NaBH_4$ treatment, was achieved by measurement of peak areas in the HPLC elutograms (eluant C) and comparison with external calibration curves. In some experiments, 100-fold mole excess of dimethyl sulfoxide or mannitol was added to DA solution (5 mM) before the addition of the enzyme and H_2O_2. At given time periods, aliquots were removed, reduced as above, and analyzed by HPLC (eluant C). The ratio 6-OHDA/IN was determined by HPLC analysis, as above, in comparison with that of control runs.

(b) By Mn(II)/O_2. Mn^{2+}-catalyzed autooxidation of DA was performed as previously described.[13] The reaction course was monitored by HPLC analysis (eluant A) of aliquots of the oxidation mixture, periodically withdrawn, acidified to pH 3 with 1 M HCl and stored on ice. In other experiments, analysis of the mixture (eluant C) was carried out after reduction with $NaBH_4$ and subsequent acidification, as described in part a.

(c) By Tyrosinase/O_2. Dopamine hydrochloride (1 mM) in 0.05 M phosphate buffer at either pH 5.0 or 7.0 was incubated with tyrosinase (130 U/mL) under a stream of oxygen and vigorous stirring, using conditions similar to those described by Garcia-Moreno *et al.*[14] HPLC analysis (eluant B) of the mixtures was performed after $NaBH_4$ reduction as in part a. In another series of experiments, dopamine hydrochloride (5 mM) was oxidized with tyrosinase (310 U/mL) in 0.1 M phosphate buffer, pH 7.0, in the presence or absence of hydrogen peroxide (10 mM). Periodical analyses of the reaction mixture in either case were carried out after reduction treatment.

(d) By $NaIO_4$, $K_3Fe(CN)_6$, or $Ce(NH_4)_2(NO_3)_6$. Dopamine hydrochloride (5 mM) in 0.1 M phosphate buffer was treated with $NaIO_4$ (10 mM) or $K_3Fe(CN)_6$ (20 mM) in the same buffer. After 2 min, the reaction mixture was filtered through a 0.45 μm filter, reduced with $NaBH_4$ as in part a, and analyzed by HPLC. Oxidation of DA (40 mM) by $Ce(NH_4)_2(NO_3)_6$ (80 mM) was performed in 1 M sulfuric acid. Thirty seconds after addition of the oxidant, the resultant yellow orange mixture was neutralized by dilution with 80 volumes of 0.1 M phosphate buffer, pH 7.0, containing, when required, H_2O_2 (1 mM) and, after an additional 1 min, reduced with $NaBH_4$ and analyzed by HPLC (eluant B or C).

Oxidation of *N*-Acetyldopamine (1) by Peroxidase/H_2O_2. *N*-Acetyldopamine (0.1 mM) in 0.1 M phosphate buffer, pH 7.0, was treated with horseradish peroxidase (0.17 U/mL) and hydrogen peroxide at varying concentrations (0.2–2 mM). The yield of 5-(2-acetomidoethyl)-2-hydroxy-1,4-benzoquinone (**3**) was estimated spectrophotometrically at 5 min reaction time, taking the molar extinction coefficient at 495 and 268

nm as 1800 and 10 400, respectively.[24] By this method, a maximum 55% yield of formation of **3** was estimated in the experiment with a 20:1 H_2O_2 to substrate mole ratio.

Reaction of 4-Methyl-1,2-benzoquinone (4) with H_2O_2. The quinone (90% pure as determined spectrophotometrically) freshly predissolved in acetonitrile was diluted up to a 5 mM final concentration with 0.1 M phosphate buffer, pH 7.0, containing H_2O_2 at varying concentrations (50–200 mM). The purple chromophoric species which accumulated within the first 10 min of the reaction was identified as 2-hydroxy-5-methyl-1,4-benzoquinone (**5**) by comparison of the spectral features with those of an authentic sample.[30] The yield of formation of quinone **5** was estimated spectrophotometrically using the extinction coefficient reported by Wehrli *et al.*[24] and by comparison with external calibration curves. A maximum 47% yield of **5** was obtained using a 40:1 H_2O_2 to quinone mole ratio.

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