Inhibitors of Complex I of the mitochondrial respiratory chain, such as rotenone, promote Parkinson disease-like symptoms and signs of oxidative stress. Dopamine (DA) oxidation products may be implicated in such a process. We show here that the o-quinone dopaminochrome (DACHR), a relatively stable DA oxidation product, promotes concentration (0.1–0.2 μM)- and respiration-dependent generation of H₂O₂ at Complex I in brain mitochondria, with further stimulation by low concentrations of rotenone (5–30 nM). The rotenone effect required that contaminating Ca²⁺ (8–10 μM) was not removed. DACHR apparently extracts an electron from the constitutively autoxidizable site in Complex I, producing a semiquinone, which then transfers an electron to O₂, generating O₂⁻ and then H₂O₂. Mitochondrial removal of H₂O₂, formed by either monoamine oxidase or DACHR, was performed largely by glutathione peroxidase and glutathione reductase, which were negatively regulated by low intramitochondrial Ca²⁺ levels. Thus, the H₂O₂ formed accumulated in the medium if contaminating Ca²⁺ was present; in the absence of Ca²⁺, H₂O₂ was completely removed if it originated from monoamine oxidase, but was less completely removed if it originated from DACHR. We propose that the primary action of rotenone is to promote extracellular O₂⁻ release via activation of NADPH oxidase in the microglia. In turn, O₂⁻ oxidizes DA to DACHR extracellularly. (The reaction is favored by the lack of GSH, which would otherwise preferably produce GSH adducts of dopaminoquinone.) Once formed, DACHR (which is resistant to GSH) enters neurons to activate the rotenone-stimulated redox cycle described.

Parkinson disease (PD) is one of the major human neurodegenerative disorders and is clinically characterized by resting tremors, rigidity, slowness of voluntary movement, and postural instability. The neuropathological hallmark of PD is the progressive loss of the nigrostriatal dopamine (DA)-containing neurons, the cell bodies of which are in the substantia nigra pars compacta and nerve terminals in the striatum (1, 2). Lewy bodies are characteristic aggregates that form in affected cells, and increasing evidence suggests that α-synuclein, ubiquitin, and iron constitute a major fraction of Lewy body aggregates (3). Defects in mitochondrial Complex I activity and DA deficiency have been detected in patients suffering from the disease (4–6). Mitochondrial Complex I inhibitors such as 1-methyl-4-phenylpyridinium (MPP⁺), a metabolic product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone have been shown to induce symptoms similar to those of PD in experimental animals and humans (7, 8). Several in vitro studies using MPP⁺ and rotenone have shown that these compounds can induce oxidative stress, apoptosis, and other biochemical changes similar to those observed in patients afflicted with idiopathic PD (9, 10). The conversion of MPTP to MPP⁺ is a complex process that is required for toxicity and that is catalyzed by monoamine oxidase (MAO) type B, localized in non-DA glial cells, followed by spontaneous oxidation to MPP⁺ (11, 12). The latter is taken up by DA neurons via the DA transporter and is concentrated by an active process in mitochondria (13). Rotenone needs no specific transport mechanism. It is the classical Complex I inhibitor that indiscriminately affects respiration in every cell type. However, its low specificity is accompanied by the striking specificity of the pathology following its slow infusion, which includes degeneration of the substantia nigra pars compacta neurons accompanied by the formation of inclusions similar to authentic Lewy bodies (14). It therefore appears that some link must exist between the presence of the neurotransmitter DA and mitochondrial Complex I inhibitors for the development of toxicity.

Oxidative stress appears to contribute to the neurodegeneration observed in PD (8, 15–17). Brains of PD patients have decreased levels of GSH and exhibit oxidative damage to DNA, lipids, and protein (18, 19). Reactive oxygen species responsible for damage are supposed to be produced during DA metabolism or oxidative phosphorylation (20). Normal O₂⁻-consuming metabolic processes (e.g. the mitochondrial electron transport chain and the oxidative deamination of DA by MAO) and non-enzymatic autoxidation of DA are believed to result in intraneuronal formation of O₂⁻ and H₂O₂ (21). Like all catecholamines, DA is easily oxidizable. O₂⁻ promptly accepts an electron from DA, which is transformed into the o-semiquinone radical; two such radicals disproportionate, generating the quinone dopaminoquinone (DAQ) plus DA. The unstable DAQ undergoes spontaneous 1,4-intramolecular cyclization and further oxidation, eventually forming the relatively stable dopaminochrome (DACHR) (see Scheme 1) (21–23). The similar
reaction sequence of the catecholamine adrenaline with O$_2$ is extremely fast, and the production of adrenochrome has been used as a method for measuring O$_2$ (24). Also, DA forms DACHR upon interaction with peroxynitrite or other oxidants. Of note, DA oxidation to DAQ and then to DACHR can be performed by the peroxidase component of cyclooxygenase, with the electron acceptor being prostaglandin H$_2$ or also H$_2$O$_2$ (25). When a thiol like GSH is present at the site of DAQ formation, a nucleophilic addition of the thiol at C-5 of DAQ takes place (see Scheme 1). This reaction prevails over the intramolecular cyclization (26). Neuromelanin, the dark pigment that accumulates with age in the substantia nigra, is the product of DA oxidation processes induced primarily by DAQ. Analysis of its composition indicates that it is formed in part from compounds originating from the interaction of DAQ with cellular GSH, leading to 5-S-thiol derivatives (mainly 5-S-cysteine, which is formed by degradation of the GSH adduct) (21). This is what is expected from the facile interaction of DAQ with GSH. However, ~50% of neuromelanin derives from cyclization products (i.e., from DACHR-like species) (27, 28). As noted, such compounds have to originate from a cellular location where GSH is not present at sufficiently high concentrations. This observation is a strong indication that DACHR is formed in vivo.

The mechanism by which DA metabolites may be involved in reactive oxygen species generation is, however, not fully elucidated, and the molecular basis for facilitation of reactive oxygen species production in DA-containing neurons is not established. In this study, we report that the interaction between DACHR and respiring brain mitochondria generates H$_2$O$_2$. We show that DACHR dose-dependently increases the production of H$_2$O$_2$ constitutively observed at Complex I of the mitochondrial respiratory chain and that the presence of very low, marginally inhibitory concentrations of the Complex I inhibitor rotenone further increase peroxide production. We also report on Ca$^{2+}$ control of H$_2$O$_2$ generation and removal. The relevance of these results in the pathogenesis of PD is discussed.
induced in the presence of 50 μM GSH. Under these conditions, the high reactivity of DAQ with the thiol led to the quantitative formation of the GSH adduct at C-5 (5-S-glutathionyl dopamine) as evidenced by the consumption of only 1 atom of oxygen/molecule of DA and the absence of an absorbance increase at 470 nm (Scheme 1). In our experiments, the formation of DACHR was conducted in the presence of bovine serum albumin, which decreased the yield of DACHR by 20–25%, probably due to interaction of the thiols in bovine serum albumin with DAQ. The concentration of DACHR under each experimental condition was determined by the absorbance at 470 nm.

Hydrogen Peroxide Measurements—5 μM Amplex Red and 15 μg/ml HRP (3.75 units) were included in the incubations. H2O2 was detected by the formation of the fluorescent Amplex Red oxidation product resorufin using excitation and emission wavelengths of 563 and 587 nm, respectively, on a Shimadzu RL-5000 spectrofluorometer in a stirred cuvette thermostatted at 30 °C (33). The H2O2 calibration scale is linear in the 0–6 μM range, and at the end of each assay, traces were calibrated by the addition of H2O2 (500 pmol).

Results

As shown in Fig. 1, H2O2 was generated by brain mitochondria respiring on the NAD-linked substrates glutamate and malate. The release rate was low and could be reliably detected only with the high sensitivity reagent Amplex Red. H2O2 production is to be ascribed to autoxidation of some redox component within Complex I (generating O2−, which is then transformed by superoxide dismutase into H2O2) located on the substrate side of the rotenone inhibition site as evidenced by the stimulation of peroxide release induced by fully inhibitory rotenone concentrations (data not shown) (34–36). DACHR (3.6 μM) induced a strong long lasting potentiation of H2O2 generation. The DACHR effect was almost completely eliminated in the absence of substrates (and with some malonate to minimize the oxidation of endogenous substrates). With substrates and DACHR, an additional large increase in H2O2 production was induced by a very low concentration of rotenone (15 nM), which only marginally inhibited respiration (see below) and was almost without effect on H2O2 release in the absence of DACHR. The DACHR potentiation of H2O2 release was visible at concentrations as low as 0.1–0.2 μM and increased essentially linearly with DACHR (Fig. 2A). Concentrations in excess of 10 μM were not tested as they were likely of no physiological significance. 15 nM rotenone was stimulatory at all DACHR concentrations tested, and the potentiation of H2O2 output was also linear. The effect of increasing rotenone concentrations (up to 30 nM) at a fixed DACHR concentration is shown in Fig. 2B. In this concentration range, rotenone only very slightly increased H2O2 production in the absence of DACHR. The rotenone potentiation of H2O2 release was visible at <5 nM. Fig. 2C shows the dose-response inhibition by rotenone of mitochondrial respiration. The H2O2 production rate at a fixed DACHR concentration as a function of substrate concentration was as described in the legend for Fig. 1. A, dependence of H2O2 output on the DACHR concentration; ○, glutamate/malate; ●, glutamate/malate and 15 nM rotenone. B, dependence of H2O2 output on the rotenone concentration: ○, glutamate/malate; ●, glutamate/malate and 3.6 μM DACHR; △, glutamate/malate plus DACHR and 300 μM EGTA. C, dose-response inhibition of mitochondrial respiration by rotenone in the absence (○) and presence (●) of EGTA. 1.5 mM ADP was added. D, H2O2 output as a function of substrate concentration: ○, no addition; ●, 15 nM rotenone; △, 3.6 μM DACHR; •, DACHR and 15 nM rotenone. When necessary, the pH was adjusted to compensate for the H+ released upon metal binding by EGTA. In A, B, and D, data are means ± S.E. from at least five independent experiments. In C, data points from a single experiment are representative of at least four independent experiments.
Dopaminochrome-dependent \( \text{H}_2\text{O}_2 \) Release in Brain Mitochondria

Table I

| Substrate | Trapped | Accumulated |
|-----------|---------|-------------|
| DA, malonate, ± EGTA | 6.6 ± 0.10 | 4.05 ± 0.10 |
| DA, glutamate/malate | 7.00 ± 0.15 | 2.19 ± 0.15 |
| DA, EGTA, glutamate/malate | 7.10 ± 0.10 | ≤0.05 |

In the experiments described above, the Ca\(^{2+}\)-dependent removal of the incubation mixture (8–10 \( \mu M \)) was not removed. When such contaminating Ca\(^{2+}\) was removed by EGTA, the constitutive \( \text{H}_2\text{O}_2 \) production and the stimulated release induced by DACHR were essentially unchanged. However, rotenone stimulation was less evident (Fig. 2B). The decreased rotenone effect in the presence of EGTA was not due to a variation of the inhibitory potency of rotenone on respiration (Fig. 2C) and appeared to depend solely on the removal of free Ca\(^{2+}\), which does not mimic the heavy metal chelator TPEN (15–30 \( \mu M \)) or the iron chelator deferoxamine (15 \( \mu M \)) (data not shown). Adding small amounts of Ca\(^{2+}\) to the incubation medium (with EGTA omitted) did not increase the rotenone effect further (data not shown).

DACHR- and MAO-dependent Production of Peroxide and Its Removal by Mitochondria: Effect of Ca\(^{2+}\) —The results presented above were obtained by monitoring \( \text{H}_2\text{O}_2 \) as soon as it formed with the high affinity trap Amplex Red/HRP and DA (75 \( \mu M \)). Alternatively, HRP was added at 5 min of incubation (30 s after Amplex Red addition) to monitor net \( \text{H}_2\text{O}_2 \) accumulation. Results were corrected for a small nonspecific deflection upon the addition of HRP. No fluorescent signal was detected upon the addition of Amplex Red. Other additions were 300 \( \mu M \) EGTA, 1 \( \mu M \) glutamate, 1 \( \mu M \) malate, and 0.5 \( \mu M \) malonate. Values are the means ± S.E. from at least four independent experiments.

Amplex Red/HRP detection system at a fixed time (5 min) of the reaction (Table II). As described above (Fig. 2), trap measurements with glutamate/malate showed a strong stimulation of peroxide production by DACHR, which was further increased by rotenone in the absence of EGTA. Residual \( \text{H}_2\text{O}_2 \) accumulation in the suspension medium was relatively high in the absence of EGTA and especially with rotenone, suggesting that the peroxidase activity was not sufficient to dispose of all the \( \text{H}_2\text{O}_2 \) produced. In the presence of EGTA, accumulated \( \text{H}_2\text{O}_2 \) was greatly reduced. However, a small measurable accumulation of residual \( \text{H}_2\text{O}_2 \) was consistently detectable, suggesting that the \( \text{H}_2\text{O}_2 \) removal was not as efficient as with MAO-derived \( \text{H}_2\text{O}_2 \) (Table I). In the presence of ADP, which decreases the mitochondrial membrane potential (ΔΨ) (State 3), the DACHR-induced authentic peroxide production (measured with the trap) was generally lower. This was particularly evident when EGTA was present: under these conditions, \( \text{H}_2\text{O}_2 \) production was the lowest, and no \( \text{H}_2\text{O}_2 \) accumulation was detected. Without EGTA, \( \text{H}_2\text{O}_2 \) production rates in State 3 were closer to those measured with no ADP (State 4) in both the absence and presence of rotenone. Under these conditions, \( \text{H}_2\text{O}_2 \) accumulation was evident as expected; it was lower than without ADP.

Succinate is a powerful generator of \( \text{O}_2\text{H}_2\text{O}_2 \) in mitochondria under some very specific metabolic conditions. \( \text{H}_2\text{O}_2 \) production with succinate, which depends on reverse electron transfer from Complex II (the electron carrier from succinate to coenzyme Q) back to Complex I, requires the ΔΨ to be high and is extremely sensitive to even small decreases in the potential (it is completely removed in State 3) (29). Rotenone at a high concentration acts as an inhibitor, an indication that the antioxidizable carrier is located uphill of the rotenone inhibition site (29, 35, 36). Furthermore, \( \text{H}_2\text{O}_2 \) release is inhibited by modestly increasing intramitochondrial Ca\(^{2+}\) (i.e. in the presence of contaminating Ca\(^{2+}\)) and is partially inhibited by the contemporary oxidation of NAD-linked substrates (29). In vivo, succinate is never oxidized alone by the mitochondria, and its concentration is dictated by the concentration of other tricarboxylic acid cycle substrates, which are NAD-dependent. Some of the experiments in Table II were performed with succinate together with glutamate and malate to try to reproduce a possible in vivo situation. In the absence of DACHR, the constitutive \( \text{H}_2\text{O}_2 \) release in State 4 with the three substrates was quite high when contaminating Ca\(^{2+}\) was removed by EGTA (this allowed a large contribution from succinate to the overall \( \text{H}_2\text{O}_2 \) output). Without EGTA, constitutive \( \text{H}_2\text{O}_2 \) generation was greatly decreased because contaminating Ca\(^{2+}\) depresses the succinate component of the \( \text{H}_2\text{O}_2 \) release (29). Despite the high production rate, no \( \text{H}_2\text{O}_2 \) accumulation in the medium was visible with EGTA because the glutathione reductase/glutathione peroxidase system was fully functional under these conditions. In the presence of ADP (State 3), the succinate-dependent contribution to the overall constitutive \( \text{H}_2\text{O}_2 \) production both without and with EGTA was eliminated because of the decreased membrane potential. When DACHR was included, \( \text{H}_2\text{O}_2 \) output was invariably increased. The highest values were obtained in State 4 and with EGTA (i.e. under conditions in which the succinate-dependent component of peroxide release was maximal); without EGTA, the release was greatly reduced. The accumulation of \( \text{H}_2\text{O}_2 \) was relatively high, as expected, without EGTA (i.e. when the glutathione reductase/glutathione peroxidase system was inhibited). However, relatively large amounts of \( \text{H}_2\text{O}_2 \) accumulated in the suspension medium also when contaminating Ca\(^{2+}\) was removed by EGTA. This is clearly at variance with the results of Table I, where a similar rate of \( \text{H}_2\text{O}_2 \) generation by MAO activity was
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Table II

DACHR-dependent production of \( \text{H}_2\text{O}_2 \) and its \( \text{Ca}^{2+} \)-controlled removal by glutathione reductase/glutathione peroxidase

| Condition | \( \text{H}_2\text{O}_2 \) (nmol released per 5 min/mg) |
|-----------|-----------------------------------------------|
|           | State 4 | State 3 |
|           | Trapped | Accumulated | Trapped | Accumulated |
| EGTA      |         |           |         |           |
| - Glutamate/malate | **0.44 ± 0.10** | **≤0.05** | **0.27 ± 0.05** | **≤0.05** |
| - Glutamate/malate, DACHR | **2.35 ± 0.15** | **0.55 ± 0.10** | **1.95 ± 0.10** | **0.50 ± 0.05** |
| - Glutamate/malate, DACHR, rotenone | **3.90 ± 0.15** | **1.33 ± 0.10** | **3.10 ± 0.10** | **0.90 ± 0.05** |
| + Glutamate/malate | **0.41 ± 0.15** | ND | **0.17 ± 0.05** | ND |
| + Glutamate/malate, DACHR | **2.40 ± 0.20** | **0.15 ± 0.05** | **0.80 ± 0.10** | **≤0.05** |
| + Glutamate/malate, DACHR, rotenone | **2.65 ± 0.20** | **0.23 ± 0.05** | **0.95 ± 0.10** | **≤0.05** |
| - Glutamate/malate, succinate, DACHR | **0.70 ± 0.10** | **0.10 ± 0.05** | **0.50 ± 0.05** | **≤0.05** |
| - Glutamate/malate, succinate, DACHR | **3.06 ± 0.15** | **0.90 ± 0.05** | **2.41 ± 0.10** | **0.50 ± 0.05** |
| - Glutamate/malate, succinate, DACHR, rotenone | **4.26 ± 0.10** | **1.70 ± 0.05** | **2.73 ± 0.10** | **0.70 ± 0.05** |
| + Glutamate/malate, succinate | **2.05 ± 0.05** | ND | **0.43 ± 0.05** | ND |
| + Glutamate/malate, succinate, DACHR | **6.96 ± 0.15** | **1.27 ± 0.10** | **0.79 ± 0.05** | **≤0.05** |
| + Glutamate/malate, succinate, DACHR, rotenone | **7.22 ± 0.15** | **1.36 ± 0.05** | **1.22 ± 0.05** | **≤0.05** |

accompanies no \( \text{H}_2\text{O}_2 \) accumulation when EGTA was present. Thus, the peroxide-removing activity appears to be compromised when a high \( \text{H}_2\text{O}_2 \) production rate originates from the interaction of DACHR with the mitochondria. In all cases, 15 nM rotenone resulted in the potentiation of \( \text{H}_2\text{O}_2 \) production and accumulation, most evident in the absence of EGTA. The presence of ADP (State 3) depressed \( \text{H}_2\text{O}_2 \) output, particularly with EGTA. A low level of rotenone promoted the increased production as well as the increased accumulation of \( \text{H}_2\text{O}_2 \) when EGTA was omitted.

In general, these results indicate that DACHR extracts electrons from Complex I, apparently from the same site that constitutively generates \( \text{O}_2^- \) and with a similar control system. A main difference is that, with DACHR, contaminating \( \text{Ca}^{2+} \) induces a substantial potentiation of the \( \text{H}_2\text{O}_2 \) release in the presence of low rotenone levels. The peroxide released following the interaction of DACHR with Complex I is removed by the mitochondrial glutathione reductase/glutathione peroxidase, with its normal control by \( \text{Ca}^{2+} \). However, with DACHR, the maximal peroxide-scavenging activity, as observed in the absence of contaminating \( \text{Ca}^{2+} \) (i.e. with EGTA), appears to be somewhat compromised.

Exogenous superoxide dismutase was reported to increase the mitochondrial production of \( \text{H}_2\text{O}_2 \) when it derives from \( \text{O}_2^- \) generated on the cytosolic face of the inner membrane (36), but not when it derives from internally produced \( \text{O}_2^- \). The former situation applies to non-physiological antimonycin-stimulated \( \text{H}_2\text{O}_2 \) production (37), which originates from \( \text{O}_2^- \) generated on the cytosolic face of Complex III, the latter to Complex I-derived \( \text{H}_2\text{O}_2 \), which is from intramitochondrially formed \( \text{O}_2^- \) (29). The DACHR-stimulated \( \text{H}_2\text{O}_2 \) release was not increased by exogenous superoxide dismutase (data not shown), additional proof that the site of electron leakage to DACHR is internal and likely the same that undergoes constitutive autoxidation.

The GSH Adduct of DAQ Does Not Promote \( \text{H}_2\text{O}_2 \) Generation, but DACHR-dependent Peroxide Production Is Not Prevented by GSH—When the tyrosinase-catalyzed oxidation of DA was performed in the presence of GSH, no DACHR formation took place, and 5-S-glutathionyl dopamine was formed instead (see “Experimental Procedures” and Scheme 1). This is explained by the high reactivity of tyrosinase-produced DAQ with thiols, taking precedence over cyclization of DAQ to leuco-DACHR. When tested during respiration-dependent \( \text{H}_2\text{O}_2 \) production, the GSH adduct exhibited no activity both without and with rotenone (Fig. 3). However, if DACHR was first produced (i.e. in the absence of GSH), and GSH was supplied later together with mitochondria, the respiration-promoted \( \text{H}_2\text{O}_2 \) release and stimulation by rotenone were unmodified. Accordingly, the DACHR absorbance at 470 nm was not altered by GSH (data not shown). Thus, once formed, DACHR is relatively stable and is capable of performing redox cycling also in the presence of GSH.

DISCUSSION

Reactive oxygen species originating from DA oxidation products have long been suspected to participate in the pathogenesis of PD. DA oxidation to DAQ depends on its interaction with \( \text{O}_2^- \) peroxynitrite, and other reactive species such as ‘OH originating via Fenton chemistry from \( \text{H}_2\text{O}_2 \) and metals such as iron, which is elevated in PD substantia nigra. DAQ is unstable and readily undergoes additional reactions with thiols such as GSH or cyclization to leuco-DACHR, followed by oxidation to DACHR. Furthermore, DA can be transformed into DAQ by

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The text contains scientific information about the effects of DACHR on mitochondrial \( \text{H}_2\text{O}_2 \) production and the role of GSH and rotenone in these processes. It discusses the mechanisms of electron leakage and the interactions with complex I and II, as well as the production of reactive oxygen species from DA.

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The table summarizes the conditions and results of experiments involving the production and removal of \( \text{H}_2\text{O}_2 \) in the presence of different conditions, including changes in the presence of GSH, EGTA, ADP, DACHR, and rotenone.
cyclooxygenase, being used as an electron donor in the peroxydase component of the cyclooxygenase reaction, where prostaglandin \( \text{G}_2 \) (but also \( \text{H}_2\text{O}_2 \)) is the electron acceptor (25). Although cyclooxygenase-1 is constitutively present in the microglia, cyclooxygenase-2 has recently been shown to be present in DAergic neurons of the substantia nigra of PD patients (and to be induced upon MPTP treatment), and this may represent a strong contribution to DA oxidation (38).

The involvement of Complex I of the respiratory chain in the pathogenesis of PD has long been suspected because Complex I inhibitors such as MPTP-derived MPP\(^+\) and, more recently, rotenone have been shown to promote PD-like symptoms (7, 8) and because the activity of Complex I has been reported to be decreased in affected individuals (4–6). Particularly impressive is the finding that a slow infusion of low concentrations of rotenone, a specific, highly hydrophobic inhibitor, induces typical PD-like lesions, including the formation of Lewy bodies (14).

In the absence of respiratory chain inhibitors, Complex I is the main site where \( \text{O}_2 \) and \( \text{H}_2\text{O}_2 \) are generated (29, 36). \( \text{H}_2\text{O}_2 \) is formed from both NAD-linked substrates and succinate (in this case, via reverse electron flow to Complex I). In the first case, the production is very slow and is increased by fully inhibitory concentrations of the Complex I inhibitor rotenone, which acts distal to the autoxidizable component. Succinate-dependent \( \text{H}_2\text{O}_2 \) production is much faster. It is inhibited by rotenone (which interrupts the backwards electron transfer), by decreasing \( \Delta \Psi \) (such as during ADP-stimulated respiration), and by small amounts of \( \text{Ca}^{2+} \) (such as present as a contaminant in saline medium) (29).

We have shown in this study that \( \text{H}_2\text{O}_2 \) is formed following the interaction of DACHR with respiring mitochondria, apparently exclusively at the level of Complex I, with properties that are complex and very similar to those of constitutive \( \text{H}_2\text{O}_2 \) generation, but at higher rates. Stimulation of \( \text{H}_2\text{O}_2 \) release above basal levels was detected at DACHR concentrations as low as 0.1–0.2 \( \mu \text{M} \), and the rate increased strongly and essentially linearly with increasing DACHR concentrations. In essence, it appears that the site within Complex I responsible for the nonelectronic reduction of \( \text{O}_2 \) to \( \text{O}_2^{-} \) is also capable of transferring an electron to the \( \alpha \)-quinone DACHR, with the likely generation of a highly reactive semiquinone radical, which in turn transfers an electron to \( \text{O}_2 \), forming \( \text{O}_2^{-} \) and regenerating DACHR, which operates redox cycling. Electron transfer from Complex I to DACHR takes precedence over transfer to \( \text{O}_2 \), given that its rate is much faster at much lower concentrations. A similar mode of interaction has previously been proposed for adrenochrome interaction with Complex I (39). Furthermore, a low concentration of rotenone, which marginally inhibits respiration and induces a negligible increase in the constitutive \( \text{H}_2\text{O}_2 \) output, induced a strong potentiation of peroxide release by DACHR. The rotenone-induced extra electron leakage to DACHR was largely prevented by removing contaminating \( \text{Ca}^{2+} \).

When some succinate is present together with glutamate/malate, which may mimic a more physiological situation, \( \text{H}_2\text{O}_2 \) release was generally higher than with glutamate/malate alone also when DACHR was omitted. In particular, the succinate potentiation of constitutive \( \text{H}_2\text{O}_2 \) release was highest with EGTA because the succinate component of the overall \( \text{H}_2\text{O}_2 \) production was \( \text{Ca}^{2+} \)-inhibited. In this situation of higher constitutive \( \text{H}_2\text{O}_2 \) production, the inclusion of DACHR promoted an additional large increase, as may be expected if the sites of \( \text{O}_2 \) and DACHR reduction are the same (and if the reaction with DACHR takes precedence over that with \( \text{O}_2 \)). The succinate-dependent increased constitutive \( \text{H}_2\text{O}_2 \) production was largely eliminated when \( \Delta \Psi \) was decreased by ADP. Accordingly, also the high rate of \( \text{H}_2\text{O}_2 \) output induced by DACHR with the three substrates decreased to values similar to the no-succinate situation in the presence of ADP. In summary, the high peroxide output obtained with succinate plus glutamate/malate in the presence of EGTA with (and, to a lesser extent, also without) DACHR was readily eliminated as soon as \( \Delta \Psi \) was decreased. It is likely that, in vivo, \( \Delta \Psi \) is not at its maximum and that these high levels of \( \text{H}_2\text{O}_2 \) released are not normally observed. When EGTA was omitted, the succinate-dependent component of \( \text{H}_2\text{O}_2 \) release was largely eliminated, and peroxide production was closer to that observed without succinate both without and with DACHR. The site of \( \text{O}_2^-/\text{H}_2\text{O}_2 \) production with DACHR is located on the inner face of the inner mitochondrial membrane.

An important point that has been addressed in this study is how the mitochondrial peroxidase(s) deals with mitochondrially produced \( \text{H}_2\text{O}_2 \). Allowing \( \text{H}_2\text{O}_2 \) formed at a known rate to accumulate in the suspension medium for some minutes before introducing the detection system provided information about how \( \text{H}_2\text{O}_2 \) is handled. We found recently that the glutathione reductase/glutathione peroxidase system, responsible for much of the peroxide removal in mitochondria, is inhibited by low intramitochondrial \( \text{Ca}^{2+} \) levels (29). At 75 \( \mu \text{M} \) \( \Delta \Psi \), a relatively high concentration that may, however, be of physiological significance, MAO activity monitored by immediately measuring \( \text{H}_2\text{O}_2 \) release was unaffected by mitochondrial respiration or by EGTA. In contrast, \( \text{H}_2\text{O}_2 \) that accumulated in the medium was highest without respiration; it was greatly decreased with substrates and no EGTA, but was totally absent if EGTA was also present. Also the DACHR-dependent accumulation of \( \text{H}_2\text{O}_2 \) was under the control of intramitochondrial \( \text{Ca}^{2+} \) and was at its lowest when contaminating \( \text{Ca}^{2+} \) was removed by EGTA. However, there are indications that the glutathione reductase/glutathione peroxidase system is partially inhibited in the presence of DACHR. In fact, when the DACHR-induced peroxide production was high, i.e. in State 4 with succinate and EGTA (a rate that was very similar to the MAO activity at 75 \( \mu \text{M} \) \( \Delta \Psi \)), accumulated peroxide was still relatively high, whereas it was undetectable when it originated from MAO (compare Tables I and II). Some \( \text{H}_2\text{O}_2 \) accumulated under similar conditions even in the absence of succinate. A possible explanation for the decreased \( \text{H}_2\text{O}_2 \)-removing activity with DACHR-derived peroxide is that, with DACHR, the primary product formed is the intramitochondrial \( \text{Ca}^{2+} \) semiquinone, followed by electron transfer to \( \text{O}_2 \), forming \( \text{O}_2^{-} \). It is likely that some intramitochondrial DACHR semiquinone accumulates, escaping reaction with \( \text{O}_2 \), and that such a species or its derivatives interact with mitochondrial proteins, altering their activity. Decreased glutathione reductase/glutathione peroxidase activity in the presence of intramitochondrial DACHR semiquinone would likely secondarily affect also the removal of MAO-derived \( \text{H}_2\text{O}_2 \). It is important to emphasize that the duration of the experiments in this study was rather short. It follows that this aspect may likely become of great importance in longer time frames.

The control of both MAO- and DACHR-induced \( \text{H}_2\text{O}_2 \) production by intramitochondrial \( \text{Ca}^{2+} \) (an increase in which prevents efficient peroxide removal, leading to a higher \( \text{H}_2\text{O}_2 \) steady state) may be an important new aspect of cell physiology. The \( \text{Ca}^{2+} \) increase required for the inhibition of \( \text{H}_2\text{O}_2 \) removal is small (semimaximal effect at 0.9 \( \mu \text{M} \) (29)) and readily achieved in vivo. It is the same order of increase required for the activation of pyruvate, isocitrate, and \( \alpha \)-oxoglutarate dehydrogenases (40). Increased intracellular \( \text{Ca}^{2+} \) levels in PD have been repeatedly suggested. Recently, it was shown that the inhibition of \( \text{Ca}^{2+} \)-activated proteases (calpains) attenuates MPTP toxicity, directly involving increased \( \text{Ca}^{2+} \) levels in the pathogenesis of PD (41).
An increase in cytosolic Ca\(^{2+}\) levels is readily followed by increased mitochondrial Ca\(^{2+}\) levels (42).

Both in vitro and in vivo studies have established that the primary lesions in rotenone-induced toxicity are not in DAergic neurons, but in the microglia (43–45). Extremely low rotenone concentrations promote the extracellular release of NADPH oxidase-derived O\(_2^-\) from the microglia (43). The importance of NADPH oxidase in the pathogenesis of PD has been well documented (44, 46). Such activation of glial derived O\(_2^-\) may indeed be the primary event in rotenone toxicity (and likely also in MPP\(^+\) toxicity because MPP\(^+\) is produced from MPTP in the microglia and is actively taken up first by the mitochondria in these cells). Although it seems unlikely that significant DA levels are present in the microglia, it is conceivable that microglial released O\(_2^-\) initiates DA oxidation by acting on extracellular DA in the synaptic cleft or its vicinity via monoelectronic DA oxidation to the DA semiquinone radical, followed by dismutation of the semiquinone to produce DAQ. The low concentration of GSH in the extracellular environment may favor the transformation into DACHR of the unstable DAQ, which is produced from MPTP in vitro (44, 55). Data on the efficacy of anti-inflammatory agents in PD are accumulating (55, 56).

In summary, we have shown that the DA oxidation product Dopaminochrome-dependent H\(_2\)O\(_2\) Release in Brain Mitochondria

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