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# Characterization of reduced and oxidized dopamine and 3,4-dihydrophenylacetic acid, on brain mitochondrial electron transport chain activities

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ABSTRACT

Loss of dopamine (DA) homeostasis may be a contributing factor to cell damage in Parkinson's disease (PD). Past studies showing deleterious effects of DA on mitochondrial function, however, have been inconsistent raising questions about mitochondria as a downstream target for DA. Issues such as the dopamine species i.e., reduced or oxidized, time of exposure and the effect of major metabolites such as 3,4-dihydrophenylacetic acid (DOPAC) may contribute to the disparate findings. The present study used isolated, lysed rat brain mitochondria to characterize the effects of oxidized or reduced DA and DOPAC on complex activities of the electron transport chain (ETC). Time of exposure and quantitation of reduced or oxidized catachols for DA and DOPAC were monitored for all experiments. Reduced DA and DOPAC with or without a 30 min preincubation had no affect on NADH oxidase activity which monitors the activities of complexes I, III and IV. Complex II activity was inhibited by reduced DA ($\geq$500 μM), but not by reduced DOPAC and was significantly attenuated by SOD suggesting reactive oxygen species involvement. In contrast, fully oxidized DA and DOPAC dose dependently inhibited NADH oxidase, complex I and complex III activities with $IC_{50s}$ in the 50–200 μM range. No preincubation was required for inhibition with the catechols when they were fully oxidized. Oxidized DA inhibited complex I only when exposure occurred during stimulated electron flow, suggesting covalent binding of quinones to proteins within active sites of the complex. In intact, well coupled mitochondria, extramitochondrial DA was shown to access the mitochondrial matrix in a dose, time and energy-dependent fashion. The findings suggest that many of the reported inconsistencies with regards to the effects of DA and DOPAC on ETC function can be attributed to the oxidized state of the catechol at the time of exposure. In addition, the findings provide possible downstream targets for DA that could contribute to the vulnerability of dopaminergic neurons in PD.



## 1. Introduction

Parkinson's disease (PD) is a late-onset neurodegenerative disease in which the major feature is the loss of dopamine (DA) neurons in the substantia nigra. DA has been postulated to contribute to the cellular damage and degeneration that occurs in PD [1–3] through loss of DA homeostasis resulting in excess levels of DA and its metabolites. DA metabolism by monoamine oxidase (MAO) results in the formation of the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), ammonia, and hydrogen peroxide ($H_2O_2$), which can cause oxidative stress damage and subsequent cell death [4]. Alternatively, DA can undergo oxidation, to form a quinone and other downstream-oxidized products [5–7]. DA can also oxidize to form hydroquinones and semiquinone radical intermediates [8,9]. DA derived o-quinones in general are highly reactive electrophiles that may readily bind covalently to cellular nucleophiles such as DNA, glutathione (GSH), cysteine and reduced sulfhydryl groups on protein cysteinyl residues. Such protein adducts can result in protein dysfunction [10]. Elevation in 5-S-cysteinly-dopamine (5-S-Cys-DA) has been found in human substantia nigra [11], while in rodents exposed to exogenous DA, an increase in DA-protein adducts has been observed in vivo [12].

Mitochondrial dysfunction is also thought to contribute to the etiology of PD [13]. In PD brain as well as in blood platelets and muscle biopsy from PD patients, studies have shown decreases in the activity of the mitochondrial electron transport chain (ETC) complex I [14,15], and decreases in combined complex II/III and in the Kreb's cycle enzyme α-ketoglutarate-dehydrogenase [16,17]. In animal studies, we and others have shown that DA can have direct effects on intact

*Abbreviations:* DA, dopamine; DOPAC, 3,4-dihydrophenylacetic acid; DAH, reduced dopamine; DCPIP, 2,6-dichlorophenolindophenol; DOPAC-H, reduced DOPAC; DAQ, oxidized dopamine; DOPAC-Q, oxidized DOPAC; ETC, electron transport chain; GSH, reduced glutathione; H2O2, hydrogen peroxide; KCN, potassium cyanide; BSA, bovine serum albumin; MAO, monoamine oxidase; PD, Parkinson's disease; ROS, reactive oxygen species; 5′-S cysteinyldopamine, 5-S-Cys-DA; RCR, respiratory control ratio

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mitochondrial function; reducing $O_2$ consumption, causing mitochondrial permeability pore transition and uncoupling [3,18,19], raising the possibility that loss of DA homeostasis can contribute to and exacerbate mitochondrial dysfunction. Studies to pinpoint the site of action for DA toxicity in mitochondrial function, however, have been incomplete and report contradictory results that range from no effect of DA on mitochondrial function [20] to low $IC_{50s}$ of 8–12 μM for inhibition of complex I [21,22]. Inconsistencies among these studies have raised questions regarding DA and mitochondrial function with respect to the DA species i.e. reduced or oxidized (DAH, DAQ respectively) that may cause inhibition, the sites within the ETC that are affected and if the major DA metabolite, DOPAC, reduced or oxidized (DOPAC-H, DOPAC-Q), may also affect mitochondrial function.

The present study was, therefore carried out to characterize the effects of DA and DOPAC on individual ETC complexes paying attention to 2 variables likely contributing to the inconsistencies in prior studies, i.e., the extent of quinone formation and the time of exposure to the catechols. Findings from the study help to reconcile disparate past reports and provide insight into ongoing cellular sequelae that contributes to dopamine cell loss in PD.

## 2. Materials and methods

### 2.1. Materials

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

### 2.2. Animals

All experiments were conducted in Sprague Dawley rats from Charles River Laboratories (Wilmington, MA, USA) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The local Animal Care Committee approved all procedures. Rats were housed in pairs at 20–22 °C on a 12 h light–dark cycle with food and water available ad libitum.

### 2.3. Mitochondrial isolation

Mitochondrial isolation followed the procedure of Clark and Nicklas, [23] as we have reported in detail previously [18,19]. For studies using intact mitochondria, $O_2$ consumption was monitored and the respiratory control ratio (RCR), calculated as we have previously reported [18]. The mitochondrial RCR for studies using intact mitochondria was >8 indicating well coupled mitochondria at the time of assay. For studies using lysed mitochondria, the mitochondria were aliquoted and stored at −80 °C. Five freeze thaw cycles were conducted to lyse mitochondria prior to assay of ETC activities. All uptake and activity assay results were from 3 separate experiments run in duplicate.

### 2.4. DAH/DAQ and DOPAC-H/DOPAC-Q solutions

DAH and DOPAC-H solutions were made in ice cold HPLC water and kept on ice. The solutions were monitored at 480 nm and 400 nm, respectively, to verify any quinone formation [8,24]. DAQ and DOPAC-Q solutions were made in phosphate buffer pH 8.0 and kept at room temperature. A standard curve generated from different concentrations of DA oxidized by [25U] tyrosinase was used to quantify the desired DAQ concentrations. A standard curve for DOPAC-Q was generated from different concentrations of DOPAC oxidized by [250U] tyrosinase and used to quantify DOPAC-Q. DA used in the ETC assays was air oxidized rather than oxidized by tyrosinase due to interactions between tyrosinase mediated DAQ formation and the oxidation of NADH. The DAQ concentration was monitored at 480 nm until the desired absorbance for corresponding DAQ concentration on the standard curve was reached. For example, 50 μM DAQ had an absorbance of 0.16, whereas 100 μm DAQ had an absorbance of 0.31. Oxidized DOPAC used in the ETC assays was oxidized by tyrosinase due to the low auto-oxidation of DOPAC. Moreover, tyrosinase-mediated-DOPAC-Q formation did not interfere with NADH oxidation. The DOPAC-Q concentration was monitored at 400 nm until the desired absorbance for the corresponding DOPAC-Q concentration on the standard curve was reached.

### 2.5. NADH-oxidase

The oxidation of NADH was measured spectrophotometrically by following the decrease in absorbance at 340 nm. Activity was expressed as nmol of NADH oxidation/min/mg protein ($\varepsilon = 6.81\ mM^{-1}\cdot cm^{-1}$). The activity assay was modified from Ragan et al. [25] Briefly, the assay medium contained $K_2HPO_4$ (20 mM) pH 7.4, and EDTA (0.5 mM). To verify that NADH oxidation was associated with ETC activity, a parallel control with rotenone (10 μM) was run. Activity was inhibited by ≥90% with rotenone and results reported as the rotenone sensitive activity. DAH/DAQ or DOPAC-H/DOPAC-Q was monitored at 480 nm and 400 nm, respectively, to verify quinone content just prior to use. NADH (0.13 mM) was then added and the absorbance change was recorded for 2 min. Following this, mitochondria (50 μg/ml) were added, and enzyme-catalyzed NADH oxidation was measured for 3–5 min. Activity was measured in mitochondria either with or without a 30 min preincubation with DAH/DOPAC-H or DAQ. In control studies, DAH and DOPAC-H did not promote $NAD^+$ reduction and, therefore, did not produce artifacts in either the NADH-oxidase or complex I assay.

### 2.6. Complex I: NADH: ubiquinone oxidoreductase

The complex I assay was modified from Birch-Machin et al. [26] and measured spectrophotometrically the decrease in absorbance due to the oxidation of NADH at 340 nm. Activity was expressed as nmol of NADH oxidation/min/mg protein. Briefly, the assay medium contained $K_2HPO_4$(20 mM) pH 7.4, EDTA(0.5 mM), coenzyme Q1 (0.065 mM), antimycin A (0.002 mg/ml) to inhibit complex III and potassium cyanide (KCN) (2 mM) to inhibit complex IV. The reaction was initiated by the addition of NADH (0.13 mM) and the absorbance change was recorded for 2 min. Mitochondria (50 μg/ml) were then added and enzyme-catalyzed NADH oxidation was measured for 3–5 min. A parallel control with rotenone (10 μM) was run to define activity specific to complex I. Rotenone inhibited complex I activity by ≥95% and was reported as the rotenone sensitive activity. DAH/DAQ or DOPAC-H/DOPAC-Q was monitored for quinone content as stated above just prior to use. Complex I activity was measured in mitochondria either with or without a 30 min preincubation with DAH/DOPAC-H or DAQ.

### 2.7. Complex II: succinate: ubiquinone oxidoreductase

Complex II specific activity was measured by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm. Activity was expressed as nmol of DCPIP reduction/min/mg protein ($\varepsilon = 19.1\ mM^{-1}\cdot cm^{-1}$). Complex II assay was modified from Birch-Machin et al. [26]. Briefly, the assay medium contained $K_2HPO_4$ (25 mM) pH 7.4, EDTA(0.1 mM), KCN (2 mM), rotenone (10 μM), antimycin A(0.002 mg/ml), succinate (25 mM) and mitochondria (50 μg/ml) and incubated for 15 min at room temperature. DCPIP (0.050 mM) was added and absorbance change was recorded for 2 min. This was the non-enzymatic reduction rate of DCPIP and was subtracted from the enzymatic rate. Coenzyme Q1 (0.065 mM) was then added to initiate the reaction and the enzyme-catalyzed reduction of DCPIP was measured for 3–5 min. A parallel control with the complex II inhibitor malonate (10 mM) and mitochondria was also

run. Malonate inhibited complex II activity by ≥90–95%. In control studies, without mitochondria, DAH and DOPAC-H were found to reduce DCPIP. Since the complex II assay measures the reduction of DCPIP this added to the forward reaction and masked inhibition. To correct for the artifact, the non-enzymatic rate determined in the presence of DAH or DOPAC-H plus DCPIP and malonate but lacking mitochondria was subtracted from samples with mitochondria. DAQ and DOPAC-Q did not create an artifactual interference however, in order to maintain consistency of assay conditions parallel controls with inhibitors and DAQ/DOPAC-Q were also run. Complex II activity was the malonate sensitive activity.

### 2.8. Complex III: ubiquinol: ferricytochrome C oxidoreductase

Complex III specific activity was measured by monitoring at 550 nm the reduction of oxidized cytochrome C (III) to reduced cytochrome C (II). Activity was expressed as nmol of cytochrome C reduction/min/mg protein ($\varepsilon = 19\ mM^{-1}\cdot cm^{-1}$). The complex III assay was modified from Krahenbuhl et al. [27]. Briefly, cytochrome C was first oxidized by exposure to $O_2$ gas. The reaction medium contained $K_2HPO_4$ (25 mM) pH 7.4, $MgCl_2$(5.0 mM), KCN(2.0 mM), bovine serum albumin (BSA, 2.5 mg/ml), dodecyl maltoside(0.44 mg/ml), rotenone(10 μM), decylubiquinol (0.15 μM/ml) (see below), and oxidized cytochrome C (III) (80 μM). Mitochondria (10 μg/ml) were preincubated with catechols for 15 min at room temperature, then added to the assay at 10 μg/ml to initiate the reaction and the enzymatic reduction of cytochrome C (III) was measured for 3–5 min. A parallel control with complex III inhibitors myxothiazol (60 μM) and antimycin A (0.002 mg/ml) inhibited the activity by ≥95%. Complex III activity was the inhibitor sensitive rate. DAH/DAQ or DOPAC-H/DOPAC-Q was monitored for quinone formation just prior to use. In control studies, in the absence of mitochondria DAH or DOPAC-H but not their oxidized counterparts was found to pass electrons to oxidized cytochrome C (III) to produce reduced cytochrome C (II). This added to the forward reaction and created an artifact that masked inhibition. Corrections to the rate made by subtraction of the nonenzymatic rate could not be made as reaction rates were variable and inconsistent (see Results). DAQ and DOPAC-Q did not react with cytochrome C, however, for consistency and to control for any reduced catechols present when DAQ or DOPAC-Q was added to mitochondria, rates were run in the presence or absence of inhibitors. Decylubiquinol was synthesized according to Krahenbuhl et al. [27] by reduction of decylubiquinone (10 μmol) with sodium borohydride ($NaBH_4$) and the solution stored at −20 °C under light protection.

### 2.9. Complex IV: ferrocytochrome C oxidase: oxygen oxidoreductase

Complex IV specific activity was measured by following the oxidation of reduced cytochrome C (II) to oxidized cytochrome C (III) at 550 nm. Activity was expressed as nmol of cytochrome C oxidized/min/mg protein ($\varepsilon = 19\ mM^{-1}\cdot cm^{-1}$). The complex IV assay was modified from Birch-Machin et al. [26]. Briefly, the assay medium contained $K_2HPO_4$ (25 mM) pH 7.4, reduced cytochrome C (II) (80 μM) (see below) and dodecyl maltoside (0.44 mg/ml). Mitochondria (50 μg/ml) were added to initiate the reaction and the enzymatic oxidation of cytochrome C (II) was measured for 3–5 min. In some preparations, mitochondria (50 μg/ml) were pre-incubated with DAQ or DOPAC-Q for 15 min at room temperature prior to assay in the complex IV reaction. A parallel control with KCN (2 mM) inhibited complex IV activity ≥95–100%. Complex IV activity was the KCN sensitive activity. In the absence of mitochondria, DAH or DOPAC-H reduced oxidized cytochrome C back to reduced cytochrome C. This can create a competing back reaction since the complex IV assay measures the oxidation of reduced cytochrome C to oxidized cytochrome C producing an effect that mimics inhibition (see Results). Reduced cytochrome C (4.0 mM) was prepared in 20 mM potassium phosphate buffer, pH 7.0, and reduced using 7.8 mM L-ascorbic acid. This mixture was dialyzed for 16 h at 4 °C using 20 mM potassium phosphate, pH 7.0, as dialysis buffer, aliquoted and stored frozen at −80 °C.

### 2.10. Complex I/III: NADH: cytochrome C–oxidoreductase

Complex I/III specific activity was measured by monitoring at 550 nm the reduction of cytochrome C. Activity was expressed as nmol of cytochrome C reduction/min/mg protein ($\varepsilon = 19\ mM^{-1}\cdot cm^{-1}$). The assay was modified from Birch-Machin et al. [26]. Briefly, the assay medium contained $K_2HPO_4$ (20 mM) pH 8.0, EDTA (0.5 mM), KCN (2 mM), NADH (25 μM), and cytochrome C (50 μM). Mitochondria (50 μg of protein) were added to initiate the reaction and the enzymatic reduction of cytochrome C was measured for 3–5 min. Complex I/III activity was inhibited by ≥95% by the complex I inhibitor rotenone. Complex I/III activity was the rotenone (10 μM) sensitive activity.

### 2.11. Complex II/III: succinate: cytochrome C–oxidoreductase

Complex II/III specific activity was measured by monitoring at 550 nm the reduction of commercially available cytochrome C which contains a mixture of reduced and oxidized forms (Sigma Chem. Co, St. Louis MO.). Activity was expressed as nmol of cytochrome C reduction/min/mg protein. DAH/DAQ or DOPAC-H/DOPAC-Q was monitored for quinone formation just prior to use. Complex II/III assay was modified from Ragan et al. [25]. Briefly, the assay medium contained $K_2HPO_4$ (20 mM) pH 7.4, EDTA (0.5 mM), KCN (2 mM), succinate (pH 7.4) (20 mM), and cytochrome C (50 μM). Mitochondria (50 μg/ml) were preincubated with succinate for 15 min at room temperature. Cytochrome C was added to initiate the reaction and the rate was measured for 3–5 min. The complex III inhibitors myxothiazol (60 μM) and antimycin A (.002 mg/ml) or complex II inhibitor malonate (10 mM) inhibited activity by ≥90–95%. Complex II/III activity was the inhibitor sensitive rate. In the absence of mitochondria, DAH and DOPAC-H reduced oxidized cytochrome C. This artifact added to the forward reaction. To adjust for this, a parallel control with DAH/DOPAC-H plus inhibitors and mitochondria was also monitored and the rate subtracted from samples with DAH/ DOPAC-H. The mean non-enzymatic rate was 30.5 ± 0.09 (mean percentage ± 0.09 S.D., n from 3 separate experiments). In order to maintain consistency and control for any reduced catechols present when DAQ or DOPAC-Q was added to mitochondria, the assay was run in the presence or absence of inhibitors and the non-enzymatic rate was subtracted from the overall rate to obtain the corrected complex II/III inhibited rate.

### 2.12. DA uptake in intact mitochondria

Isolated, intact rat brain mitochondria (approximately 0.4 mg protein) were pre-incubated at either 0 °C or 30 °C for 5 min in 0.9 ml of mitochondrial incubation buffer (see above), containing 4 μM each clorgyline and pargyline (MAO A and B inhibitors, respectively), 100 μM mazindol (dopamine transport inhibitor), 10 μM Ro4-1284 (vesicular dopamine transporter inhibitor; kind gift of Hoffman-La Roche, Nutley, N.J., U.S.A.), and 200 μM ascorbate (to prevent dopamine oxidation). $^3$H-labeled dopamine (specific activity 28 Ci/mmol) supplemented with cold dopamine to final concentrations of 1–15 mM was added and incubations continued for another 10 min. At the end of incubation, uptake was stopped by the addition of 5 ml of ice cold incubation buffer. Mitochondria were washed extensively with incubation buffer using a Brandel Cell Harvester Model M24R (Gaitherburg, MD). Mitochondria collected on GF/F Whatman filters presoaked with 0.5% polyethylamine, were extracted with 0.2 N perchloric acid. Radioactivity from the acid extracted matrix was

quantified by scintillation counting. Nonspecific radioactive binding was eliminated by subtracting counts obtained at 0 °C from those at 30 °C. Results were reported as nmol dopamine per mg protein.

### 2.13. Statistical analysis

Statistical significance was calculated using ANOVA one-way test with the Tukey post hoc test for comparison or by an unpaired student *t* test using Graph Pad Instant version 3.0.

## 3. Results

### 3.1. DAQ and DOPAC-Q formation were monitored spectrophotometrically at 480 nm and 400 nm respectively and quantified using a standard curve

Prior studies of the effects of DA on mitochondrial function have not clearly differentiated reduced DA from oxidized DA and have reported conflicting results [20–22,28,29]. In the current study, DA was monitored at 480 nm [8] and DOPAC at 400 nm [24] and standard curves made from tyrosinase-mediated fully oxidized DA and DOPAC solutions were used to quantify the desired quinone concentration at the time of exposure (Insets of Fig. 1A and B). When a desired quinone concentration was reached, it was immediately used in the reactions. A lack of absorbance at the respective wavelength was interpreted as fully reduced DA or DOPAC. Past literature has also been inconsistent regarding the amount of exposure time of mitochondria to DA. In our study, NADH-oxidase and complex I activities were measured with and without 30 min preincubation periods at room temperature. No differences were found with pre-incubation versus no pre-incubation when care was taken to ensure that DA and DOPAC were fully oxidized or reduced at the time of the assay. All remaining studies were measured with a 15 min pre-incubation at room temperature.

### 3.2. Effects of reduced catechols DA and DOPAC on mitochondrial ETC complex activities

#### 3.2.1. NADH-oxidase activity was not attenuated by DAH or DOPAC-H

NADH-oxidase measures complex I, III and IV activities. NADH-oxidase was not inhibited by DAH or DOPAC-H at 1 mM with a 30 min pre-incubation (Fig. 1A and B and Table 1) or without a pre-incubation, (data not shown). The results demonstrate that complex I, III and IV are not inhibited by reduced DA or DOPAC and that time of exposure to the catechols was not a factor when the catechols remained fully reduced.

### 3.3. Complex I activity was not altered by DAH or DOPAC-H

To further validate the lack of effect of DAH and DOPAC-H on complex I, a spectrophotometric assay was used that isolates electron flow through complex I. Rotenone sensitivity (>95%) confirmed the bulk of electron flow through complex I. Consistent with the NADH-oxidase assay, complex I activity was not affected by DAH or by DOPAC-H with a 30 min pre-incubation (Table 1) or with no pre-incubation period (data not shown). The results demonstrate that exposure to the reduced catechols did not affect complex I activity.

### 3.4. Complex II activity was inhibited by DAH but not DOPAC-H

Complex II activity was corrected for the non-enzymatic reaction between DAH or DOPAC-H and DCPIP which masked inhibition as described in Materials and methods (2.7). The corrected rates show that complex II activity was inhibited 32.7 ± 2.0 and 41.9 ± 5.5% (± SEM) by 500 μM and 1 mM DAH, respectively, with a 15 min pre-incubation (Fig. 2A and Table 1). In contrast, complex II activity was not significantly affected by DOPAC-H (Fig. 2B and Table 1), although there was a trend towards decreased activity. Reduced DA can undergo oxidation to form ROS such as superoxide and hydrogen peroxide [28]. When studies were carried out in the presence of superoxide dismutase (SOD) (1U) and catalase (1U) (Cat), it was found that SOD protected from DAH mediated inhibition by 52.6% indicating that ROS generation was contributing to the inhibition of complex II by DAH.

### 3.5. DAH and DOPAC-H did not attenuate complex III activity

Complex III activity could not be independently assessed because in control studies in the absence of mitochondria, DAH and DOPAC-H reduced oxidized cytochrome C and added to the forward reaction masking inhibition. This artifactual interference resulted in inconsistent data that could not be corrected by subtraction in the presence of inhibitors. Although the complex II/III assay also measures the reduction of cytochrome C, unlike the complex III assay that requires oxidized cytochrome C (Section 2.8), the complex II/III assay uses a mixture of reduced and oxidized cytochrome C (Section 2.11). This mixture did not react with DAH and DOPAC-H to the same extent as oxidized cytochrome C and inhibitor corrected rates could be used to determine and correct for the artifactual interference. DAH inhibited complex II/III activity to a similar extent as was observed with complex II alone (Fig. 2C). As with complex II, DOPAC-H did not significantly inhibit complex II/III (Fig. 2D). These findings suggest that inhibition of complex II/III in the presence of DAH was due to

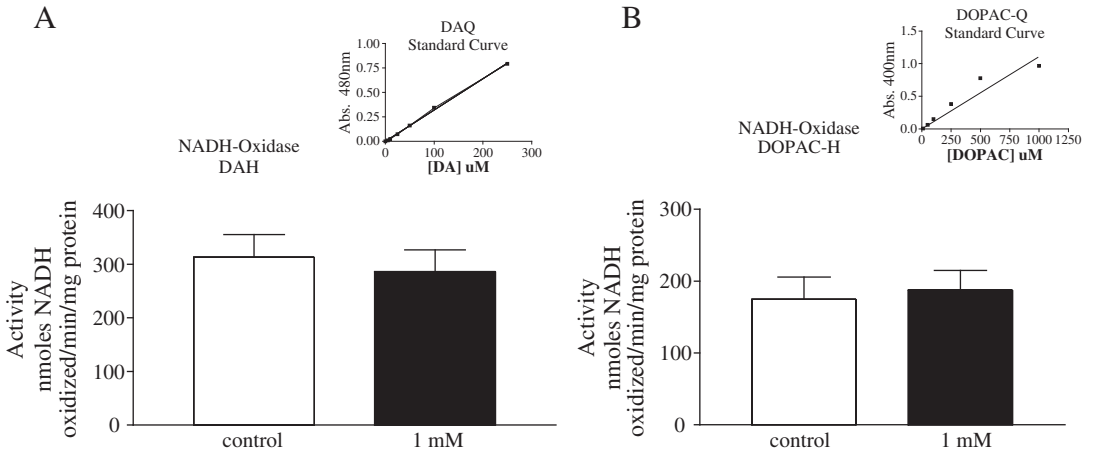


**Fig. 1.** The effect of reduced dopamine and DOPAC on NADH oxidase activity. NADH oxidation was measured spectrophotometrically as described in Materials and methods. Lysed rat brain mitochondria were exposed to (A) 1 mM reduced dopamine (DAH) or (B) 1 mM reduced DOPAC (DOPAC-H) for 30 min at room temperature prior to assay. Values for DAH and DOPAC-H were expressed as nmol of NADH oxidized/min/mg protein ± SEM. Inset: Standard curves for oxidized dopamine (DAQ) and oxidized DOPAC (DOPAC-Q) were generated as described in Materials and methods and were used to quantitate quinone formation prior to each individual assay.

**Table 1**
Effects of reduced and oxidized DA and DOPAC on ETC complex activities.

| Mitochondrial ETC complex | DAH | DOPAC-H | $IC_{50}$ Values for DAQ ± SEM | $IC_{50}$ Values for DOPAC-Q ± SEM |
|---|---|---|---|---|
| NADH-Oxidase (I, III, IV) | No effect at 1 mM | No effect at 1 mM | 158.5 μM ± 0.1 | 177.8 μM ± 0.1 |
| Complex I | No effect at 1 mM | No effect at 1 mM | 50.1 μM ± 0.036 | 181.9 μM ± 0.017 |
| Complex II | Significantly inhibited at 500 μM and 1 mM | No effect at 1 mM | No effect up to 400 μM | No effect up to 400 μM |
| Complex III | No effect at 1 mM | No effect at 500 μM | 151.4 μM ± 0.086 | 109.6 μM ± 0.065 |
| Complex IV | Inhibited at 1 mM due to artifactual competing back reaction between oxidized cytochrome C and reduced dopamine | No effect at 1 mM | No effect up to 250 μM | No effect up to 400 μM |

Summary of results for inhibition of mitochondrial electron transport chain (ETC) activities by reduced dopamine (DAH) and DOPAC ( DOPAC-H) and oxidized dopamine (DAQ) and DOPAC (DOPAC-Q). $IC_{50}$ values represent the means ± SEM of three separate experiments run in duplicate for each assay.

complex II inhibition. Additionally, NADH-oxidase activity, which measures complex I, III and IV activities, was not affected by 1 mM DAH or DOPAC-H (Fig. 1A and B) further supporting that complex III was not inhibited by DAH or DOPAC-H.

### 3.6. The effects of reduced DA and DOPAC on complex IV activity

Complex IV activity was not affected by 1 mM DOPAC-H, however; it was significantly inhibited by 1 mM DAH (activity 61% of control with 1 mM DAH) (Table 1 and Supplemental material Fig. 1). The inhibition observed in the presence of DAH was not prevented by: (2U) SOD plus (2U) Cat, MAO A or B inhibitors (10 μM) pargyline and (10 μM) clorgyline, respectively or dithioreitol (DTT) (50 μM) (data not shown). Control studies, in the absence of mitochondria, showed that DAH and DOPAC-H reduced oxidized cytochrome C. Since complex IV activity monitors the oxidation of reduced cytochrome C, the presence of DAH in the assay creates a competing back reaction that could mimic inhibition. This coupled with the finding that NADH oxidase activity, which includes complex IV, was not inhibited by DAH or DOPAC-H (Fig. 1A and B), suggested that DAH did not inhibit complex IV. Instead, the inhibition observed by DAH in the complex IV activity assay was likely an artifact due to an interaction between DAH and the product of the complex IV reaction, i.e., oxidized cytochrome C.

### 3.7. Effects of oxidized catechols DA and DOPAC on mitochondrial ETC complex activities

Oxidized DA and DOPAC did not produce internal artifacts with any of the components of the ETC assays. However, to maintain consistencies in assay methodology, for the reduced and oxidized catechols, mitochondrial ETC activities were measured in the presence or absence of the appropriate inhibitors.

### 3.8. NADH-oxidase was dose dependently inhibited by DAQ and DOPAC-Q

Immediate reaction of DAQ or DOPAC-Q with mitochondria, dose dependently inhibited NADH oxidase activity with $IC_{50s}$ of 158.5 ± 0.01 and 177.8 ± 0.10 (mean μM ± SEM) , respectively (Fig. 3A and B and Table 1). With a 30 min preincubation at room temperature with DAQ, the $IC_{50}$ was 150 ± 3.7 μM (data not shown) indicating that once DAQ was formed, time was not a factor. The results demonstrate the potential involvement of complex I, III or IV in the inhibition associated with DAQ and DOPAC-Q.

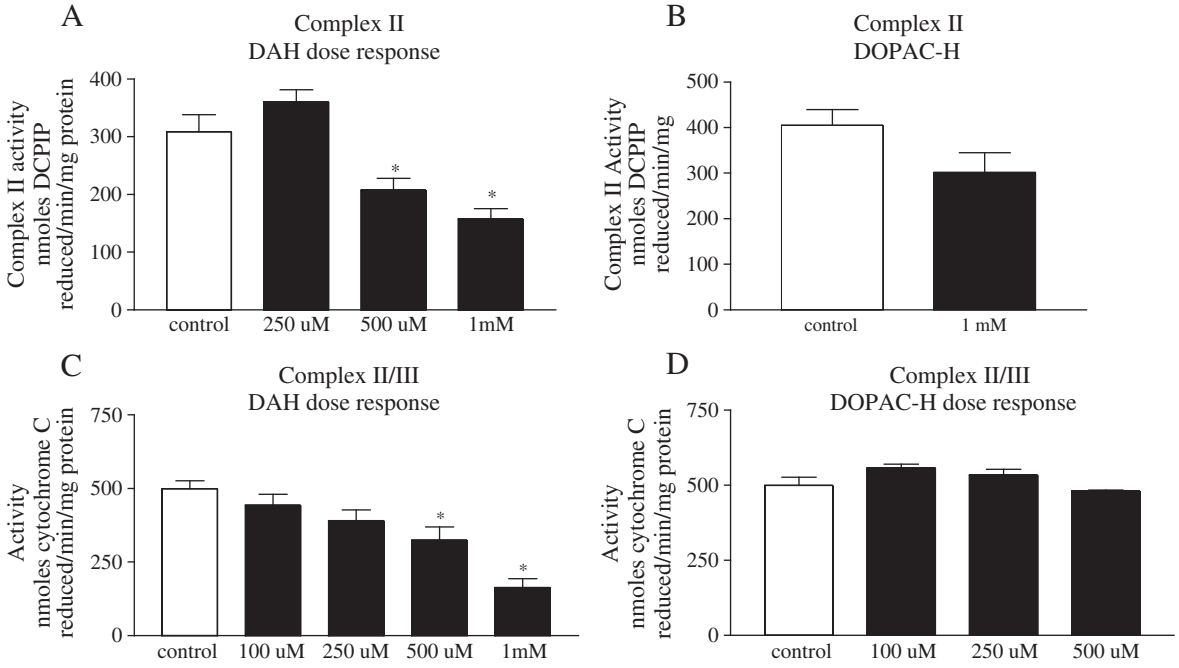


**Fig. 2.** Complex II and II/III activity in the presence of reduced dopamine and DOPAC. Complex II activity was measured spectrophotometrically as described in Materials and methods. Lysed rat brain mitochondria were exposed for 15 min at room temperature to (A) various concentrations of reduced dopamine (DAH) or to (B) 1 mM reduced DOPAC (DOPAC-H) prior to assay. Each value is the mean malonate sensitive activity ± SEM and is expressed as nmol of DCPIP reduction/min/mg protein. Complex II activity was not attenuated by DOPAC-H but was inhibited 32.7 ± 2.0 and 41.9 ± 5.5% by 500 μM and 1 mM DAH, respectively. Complex II/III activity was measured spectrophotometrically following 15 min exposure at room temperature to (C) various concentrations of DAH or (D) DOPAC-H. Values are the mean ± SEM and are expressed as nmol of cytochrome C reduction/min/mg protein. (*p, 0.05, different from control).

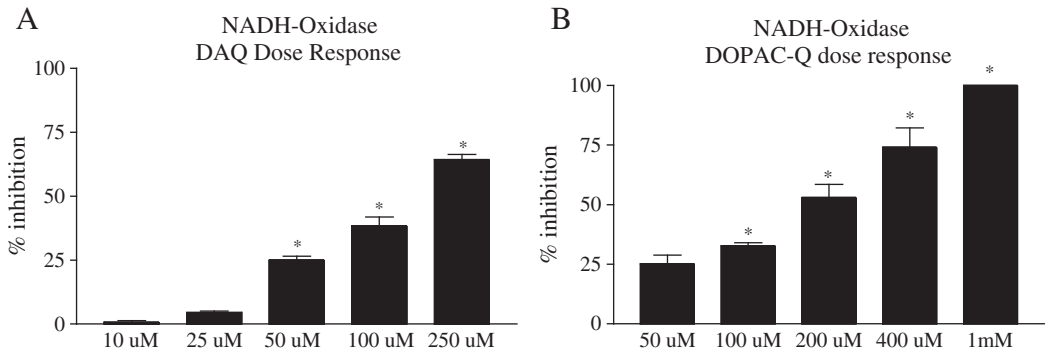


**Fig. 3.** The effect of oxidized dopamine and DOPAC on NADH oxidase activity. NADH oxidation was measured in lysed rat brain mitochondria immediately following exposure to (A) various concentrations of oxidized dopamine (DAQ) or (B) oxidized DOPAC (DOPAC-Q). Values are the mean ± SEM and were expressed as % inhibition as compared with control (*$p<0.05$, different from control). Activity was dose dependently inhibited by DAQ and DOPAC-Q.

### *3.9. Complex I activity was inhibited by DAQ and DOPAC-Q in a dose dependent manner*

In order to determine the site of DAQ, DOPAC-Q inhibitory action in the ETC, the activities of the individual complexes were studied. It was found that complex I activity was inhibited in a dose dependent manner by DAQ and DOPAC-Q when there was no pre-incubation with $IC_{50s}$ of 50.1 ± 0.036 and 181.9 ± 0.01 (mean μM ± SEM), respectively (Fig. 4A and B and Table 1). The $IC_{50s}$ for DAQ with a 30 min pre-incubation was 50.0 ± 1.4 μM. The results suggest that inhibition of complex I was quinone mediated and as with NADH-oxidase, once the quinone was formed time was not a factor.

### *3.10. Complex I inhibition by DAQ was not attenuated by the antioxidants SOD and catalase and required activation for inhibition by DAQ*

SOD and catalase failed to prevent the inhibition of complex I by DAQ (Fig. 4C). This supports that complex I activity was inhibited

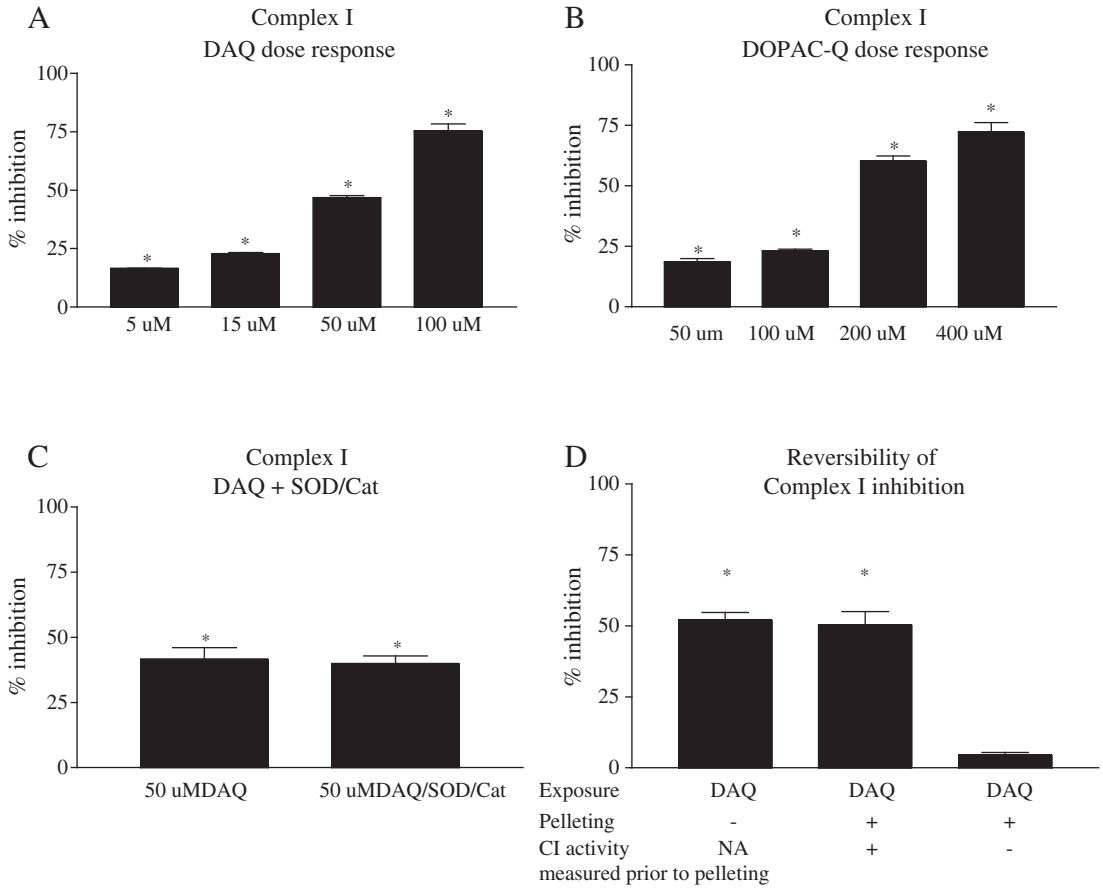


**Fig. 4.** Oxidized dopamine and DOPAC and their effect on complex I activity and reversibility. Complex I specific activity was measured in lysed rat brain mitochondria immediately following exposure to (A) various concentrations of DAQ or (B) DOPAC-Q. DAQ and DOPAC-Q effects were expressed as mean % inhibition of control are the mean ± SEM. (*$p<0.05$ different from control). Complex I was dose dependently inhibited by DAQ and DOPAC-Q. (C) Lysed mitochondria were exposed to 50 μM DAQ or 50 μM DAQ plus superoxide dismutase (SOD) [2U] and catalase (Cat) [1U]. Activity is expressed as the mean % inhibition of control ± SEM. SOD and catalase did not prevent DAQ mediated inhibition. (D) To examine the reversibility of inhibition, mitochondria were pre-incubated with 50 μM DAQ at room temperature for 30 min, thereafter the enzyme was activated by measuring activity (first bar on graph). The mitochondria from this reaction were then pelleted and the supernatant was removed. Mitochondria were resuspended and complex I activity was re-measured (second bar on graph). As a control, non-activated mitochondria were pre-incubated with DAQ for 30 min at room temperature, pelleted, and then complex I activity was measured. In this preparation complex I activity was not measured prior to pelleting, hence the enzyme was not activated (third bar on graph). Values are the mean ± SEM. (*$p<0.05$ different from control). Only activated complex I was irreversibly inhibited by DAQ.

by oxidized DA rather than ROS products formed during quinone formation. To examine if inhibition was reversible and if activation of complex I was necessary for DAQ mediated inhibition, mitochondria were pre-incubated with DAQ at room temperature for 30 min in the absence of substrate (Fig. 4D). Thereafter, in some incubations, the enzyme was activated by addition of NADH and activity was measured. Mitochondria were then pelleted, the supernatant was removed and the pellet resuspended. Complex I activity was then re-measured in the absence of any added DAQ. Non-activated complex I mitochondria were pre-incubated with DAQ for 30 min at room temperature, pelleted, resuspended and then complex I activity was measured. In this preparation, complex I activity was not measured prior to pelleting; hence the enzyme was not activated during exposure to DAQ. Complex I activity was only inhibited when DAQ exposure occurred during the active oxidation of NADH by complex I, suggesting that there was unmasking of a site sensitive to either DAQ or alternatively the semiquinone.

### 3.11. Complex II activity was not affected by oxidized DA or DOPAC

In contrast with the reduced catechols, the results show that oxidized DA (Fig. 5A and Table 1) or DOPAC (Fig. 5B) did not inhibit complex II activity. The lack of effect of DAQ and DOPAC-Q on complex II activity further supports that ROS generation during redox chemistry with DAH contributed to the inhibition of complex II by the reduced catechol as observed in Fig. 2.

### 3.12. Complex III activity was inhibited by both DAQ and DOPAC-Q in a dose dependent manner

DAQ and DOPAC-Q inhibited complex III activity in a dose dependent manner with $IC_{50s}$ of 151.4 ± 0.086 μM and 109.6 ± 0.065 μM, respectively (Fig. 5C and D and Table 1). Attempts to determine the reversibility of complex III inhibition by DAQ were carried out similar to what was done with complex I, however the results were unreliable due to the sensitivity of complex III activity to pelleting and resuspension resulting in loss of activity in both control and DAQ treated samples.

### 3.13. Oxidized DA and DOPAC do not attenuate complex IV activity

DAQ (up to 250 μM) and DOPAC-Q (400 μM) did not inhibit complex IV activity (Table 1). Higher concentrations were not run since the $IC_{50}$ for overall inhibition of NADH-oxidase activity, which includes complex IV was 158.5 ± 0.01 μM for DAQ, and 177.8 ± 0.16 μM for DOPAC-Q (Fig. 3A and B and Table 1).

### 3.14. GSH protects against complex I/III inhibition by DAQ

Since DAQ and DOPAC-Q inhibited complexes I and III activities, we studied the effects of antioxidants and a metal chelator on DAQ mediated inhibition of combined complex I/III. We assayed complex I/III activity with (200 μM) DA plus tyrosinase (25U), in the presence or absence of the hydrogen peroxide remover catalase (1U) (Cat), superoxide remover superoxide dismutase (SOD) (1U) plus (1U) Cat,

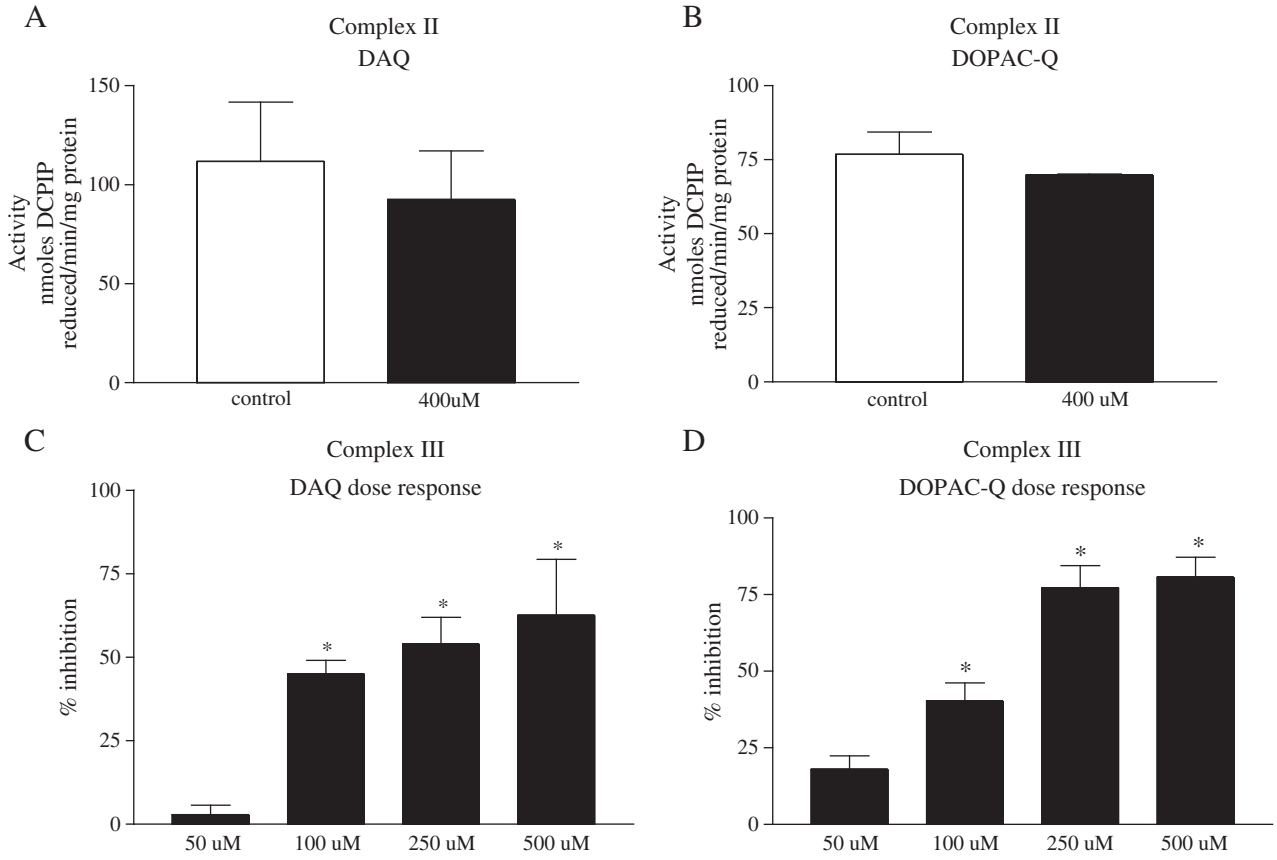


**Fig. 5.** The effect of oxidized dopamine and DOPAC on complexes II and III activities. Complex II activity was measured in freeze thawed rat brain mitochondria following exposure to (A) 400 μM of oxidized dopamine (DAQ) or (B) 400 μM oxidized DOPAC (DOPAC-Q) for 15 min at room prior to assay. Values are the mean malonate sensitive activity ± SEM and are expressed as nmol of DCPIP reduction/min/mg protein. Oxidized DA or DOPAC did not attenuate complex II activity. (C, D) Complex III activity was measured as described in Materials and methods. Lysed rat brain mitochondria were exposed to (C) different concentrations of DAQ or (D) DOPAC-Q in the reaction medium with a 15 min pre-incubation period at room temperature. Activity is expressed as nmol of cytochrome C reduction/min/mg protein and are the mean ± SEM. (*$p<0.05$different from control). Complex III activity was dose dependently inhibited by DAQ and DOPAC-Q.

the metal chelator desferoxamine (1 mM), antioxidant trolox (1 mM), or the quinone scavenger reduced glutathione (1 mM) (GSH). Only GSH provided significant and nearly complete protection from DAQ mediated inhibition (86 ± 0.06 mean % of control ± SEM for DA in the presence of tyrosinase plus GSH versus 7% ± 0.06 for DA and tyrosinase alone), while catalase, catalase/SOD, desferoxamine and trolox were without effect (Supplemental material Fig. 2). When added in the absence of DAQ, the antioxidants and metal chelator did not affect complex I/III activity. The results suggest a role for catechol quinones and/or oxidized DA products in mitochondrial ETC inhibition rather than ROS since of the agents tested, only GSH can prevent and/or sequester quinone formation.

### 3.15. DA uptake by intact mitochondria

In previous work we showed that exposure of intact mitochondria to DA significantly inhibited $O_2$ consumption [18,19] with a $K_m$ of ~7 mM. In the present study we found that DA can inhibit mitochondrial function in lysed mitochondria by interaction with ETC components with a $K_m$ in the low to moderate μM range. We next addressed the question of whether DA can gain access to the mitochondrial matrix. Well coupled, intact mitochondria (RCR >8) were exposed to various concentration of $^3$H-labeled DA as described in Materials and methods. The experimental paradigm used reduced DA since previous work by the laboratory showed that exposure of intact mitochondria to reduced DA resulted in inhibition of respiration [19]. Secondly, in the case of loss of DA homeostasis, vesicular release of DA into the cytosol, at least in terms of immediate exposure and prior to its metabolism, would likely be to DA in its reduced form. Following 10 min of incubation, mitochondrial matrix content of DA increased in a dose-dependent fashion (Fig. 6). DA uptake was energy dependent as only minimal counts were observed at 0 °C and final counts were corrected for this non-specific binding. Uptake was also time dependent. Percentage of total DA uptake was measured at various time points. At 1.25 min % of total uptake was 2.10 ± 0.60, at 2.5 min it was 12.80 ± 2.60, at 5.0 min it was 55.0 ± 4.0, and uptake was saturated by 10.0 min at 100.00 ± 5.5, (mean percentage ± SEM, n is from 3 separate determinations). Reduced DA uptake was independent of DA vesicular uptake, DA transporter activity, metabolism by MAO A or B or DA oxidation since uptake was determined in the presence of the VMAT inhibitor Ro4-1284, DAT inhibitor mazindol, MAO A and B inhibitors, clorgyline and pargyline, respectively and the antioxidant ascorbate. These findings demonstrate that extramitochondrial DA can access the mitochondrial matrix.

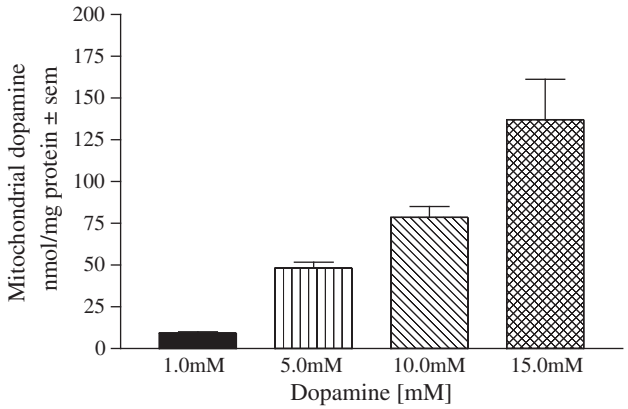


**Fig. 6.** Dopamine uptake by intact mitochondria. Dopamine uptake in isolated intact rat brain mitochondria was determined by incubation for 10 min at 30 °C with $^3$H-labeled dopamine as described in Materials and methods. Ascorbate (200 μM), Ro4-1284 (10 μM), mazindol (100 μM), clorgyline (4 μM) and pargyline (4 μM) were present to block autoxidation, dopamine vesicular uptake, uptake by the dopamine transporter, and metabolism of dopamine by MAO A or B, respectively. Radiolabeled dopamine extracted from the mitochondrial matrix was quantified by scintillation counting and corrected for non-specific binding by subtraction of counts from 0 °C incubations. Uptake was expressed as concentration of mitochondrial dopamine in nmol/mg of protein.

## 4. Discussion

Three major general conclusions arise from this study which shed light on the inconsistencies in the literature with regard to DA and its effects on mitochondrial function. Firstly, data show that knowledge of the redox state of the catechol is critical when determining the inhibitory effect on mitochondrial function and the sites at which interaction occurs. Secondly, time of exposure to the catechol is a factor when there is a changing redox state. In the presence of a fully reduced or oxidized catechol, the factor of time is removed. This further exemplifies the need to know the oxidation/reduction state of the catechol at the time of exposure. Thirdly, the oxidized catechols of DA and DOPAC are more deleterious to ETC activities than are the reduced forms.

The study is the first to differentiate between reduced and oxidized DA and DOPAC and measure their effects on each individual ETC complex activity. Characterization of effects can be summarized as follows: 1) reduced DA (DAH) inhibited only complex II activity of the ETC, while reduced DOPAC (DOPAC-H) was without affect on any ETC activities 2) oxidized DA and DOPAC (DAQ and DOPAC-Q respectively) inhibited complexes I and III of the ETC with low to moderate μM potencies and 3) DAQ irreversibly inhibited complex I only when activated suggesting unmasking of a site sensitive to DAQ or the semiquinone during active respiration.

The oxidation status of DA and DOPAC at the time of assay, as well as, the time of exposure was carefully assessed as both may contribute to differing effects on activities. The results showed that when kept reduced, DA and DOPAC did not inhibit NADH-oxidase activity with or without a pre-incubation period of up to 30 min. In contrast, no pre-incubation was necessary to fully inhibit NADH-oxidase when exposure was carried out with the completely oxidized catechols. Thus, time of exposure is a factor as DA becomes oxidized. At its extremes, no inhibition of NADH-linked electron flow occurs in the presence of reduced DA or DOPAC, while robust and rapid inhibition is observed with the oxidized catechols. Since the NADH-oxidase assay measures the combined activities of complexes I, III and IV the effects of the catechols were determined on individual ETC complex activities. Complex I was not inhibited by either DAH or DOPAC-H up to 1 mM regardless of a pre-incubation. Consistent with this, Morikawa et. al., [20] reported no inhibition of complex I in mouse brain mitochondria by 10 mM DA or 5 mM DOPAC, however, time of exposure was not indicated. Khan et al. [29] also found no inhibition of complex I with DA in rat brain mitochondria with a 15 min pre-incubation. In contrast Ben-Sachaar et al., reported inhibition of complex I in rat brain mitochondria by DA and DOPAC with $IC_{50s}$ of 8.12 ± 0 .75 μM [21] and 61.2 ± 8.08 μM [22] respectively, with a 1 min pre-incubation. In these latter studies mitochondria were pretreated with 2% digitonin. Digitonin is a detergent and may have altered the mitochondrial membranes exposing sites sensitive to inhibition that may not have been available in their normal state. None of the studies mentioned above quantified for oxidized DA, leaving open the possibility that the degree to which DA was oxidized in the different preparations contributed to the disparate results.

In the current study, NADH-oxidase activity was dose dependently inhibited by DAQ and DOPAC-Q with $IC_{50s}$ of 158.5 μM and 177.8 μM, respectively, and complex I inhibited by DAQ with an $IC_{50}$ of 50.1 μM. Ben-Sachaar and colleagues [22] reported that desferoxamine partially attenuated the DA mediated inhibition of complex I activity consistent with a quinone mediated inhibition. Our results of quinone mediated inhibition of complex I is also consistent with the studies of Khan et al. [29] and Jana et al. [30] who reported inhibition of complex I by 400 μM DA after an incubation period of 2 h. Khan et al. [29]

measured quinone production after the 2 h incubation and found an increase. Moreover, similar to our findings, Khan et al. [29] found that 5 mM GSH prevented DA mediated inhibition. GSH can keep DA in its reduced form and prevent quinone formation or chelate quinones already formed, thus further supporting a toxic role for quinones. Although Khan et al. [29] monitored for quinone production, it was not quantified making the concentration of quinone required for inhibition uncertain.

Complex I was also dose dependently inhibited by DOPAC-Q with an $IC_{50}$ of 181.9 μM. In contrast, Jana et al. [30] found a marginal but statistically significant inhibition of complex I activity with 400 μM DOPAC after 2 h incubation. DOPAC quinone formation was not measured, however quinoprotein adduct formation was monitored and none observed. It was concluded that DOPAC was far less toxic to ETC activities than DA. It should be noted that in their study they air oxidized DA and DOPAC. In the current study, DOPAC had to be oxidized via tyrosinase because it did not air oxidize as did DA. Taking into account the lack of DOPAC mediated quinoprotein formation and method of oxidation in the Jana et al. [30] study; it is likely that DOPAC had not oxidized under their assay conditions. Our findings would indicate that in the oxidized state, DOPAC is similar in potency to DA in inhibiting complex I.

The present study is the only study to investigate the effects of reduced and oxidized DA and DOPAC on the individual activities of complexes II and III. Malonate sensitive complex II activity was significantly inhibited by 1 mM DAH but not by DOPAC-H or oxidized DA and DOPAC. Monitoring of the malonate sensitive rate is important as DAH can pass electrons to DCPIP and mask inhibition. The superoxide removing enzyme SOD significantly protected from DAH mediated inhibition, suggesting that ROS generation contributed to the inhibition. In the current study the effects of the reduced catechols on complex III activity could not be measured due to an internal artifact created by an interaction between DAH, DOPAC-H and cytochrome C that was not amenable to correction (see Materials and methods and Results for details). Therefore, the effects of DAH and DOPAC-H were assayed on complex II/III activity. Similar to results with complex II, complex II/III activity was inhibited by DAH with comparable potency, but not by DOPAC-H. This finding coupled with the lack of effect of the reduced catechols on NADH oxidase activity which includes complex III, would suggest that complex III activity was not affected by DAH or DOPAC-H.

The inhibition of complex IV by 1 mM DAH in the present work was concluded to be an internal artifact created by the interaction between DAH and reduced cytochrome C mimicking inhibition (see Results). NADH-oxidase activity, which includes complex IV activity, was not attenuated by DAH. This finding supports that complex IV activity was not affected by DAH. Ben-Sachaar et al. [22] reported no inhibition of complex IV by DA up to 1 mM with a 1 min incubation. In the present study, oxidized DA up to 250 μM did not inhibit complex IV activity. In contrast, Khan et al. [29] and Jana et al. [30] reported 62.8% and 57% inhibition of complex IV by 400 μM DA following 2 h of incubation. Since the $IC_{50}$ for DAQ inhibition in the NADH-oxidase assay in our study was 158.5 μM, concentrations of DAQ greater than 250 μM were not tried. Higher concentrations of DAQ may have inhibited complex IV. On the other hand, DAQ concentration was not quantified in the Khan et al. [29] or Jana et al. [30] studies. Consequently, it is possible that there was residual DAH present in the assay medium. As we have observed, DAH can interact with the product of the complex IV assay, i.e. oxidized cytochrome C producing a competing back reaction that would mimic inhibition. Complex IV activity was not affected by reduced or oxidized DOPAC with a 15 min pre-incubation, which is in accordance with Jana et al. [30] who reported only marginal effects on complex IV activity by 400 μM DOPAC in rat brain mitochondria after a 2 h pre-incubation.

Another important finding from this study shows that in intact mitochondria exposed to elevations in reduced DA, as might occur in the cytosol of cells experiencing loss of DA homeostasis, the DA can access the interior of mitochondria to inhibit ETC function. This finding is supported by the work of van Laar et al. [31] who reported that intact brain mitochondria exposed to $^{14}$C-DAQ covalently bound to multiple subunits of complex I (75 kDa and 30 kDa subunits) and complex III (ubiquinol-cytochrome c reductase core protein 1 and Rieske Fe–S protein). Our work expands on these findings to show that such interactions can lead to loss of ETC activities, specifically complexes I and III. The findings are further supported by Berman and Hastings [3], who reported that intact mitochondria exposed to DAQ, but not DAH caused an initial uncoupling via a large increase in resting state 4 respiration and decrease in active state 3 respiration followed by increased swelling of mitochondria. The current study exposed intact mitochondria to DA in its reduced form, whereas van Laar et al. [31] exposed intact mitochondria to oxidized DA. In our characterization of the effects of oxidized and reduced DA on mitochondrial ETC function, only oxidized DA inhibited complexes I and III. Since intact mitochondria were exposed to DA in the presence of ascorbate, it is unlikely that DA was oxidized prior to uptake. This would indicate that both the reduced and oxidized forms of DA may gain entry into mitochondria although it is uncertain whether this would involve similar or different mechanisms of transport as the uptake mechanism remains to be investigated. DA oxidation occurs more readily at a pH above 7.4. The mitochondrial matrix pH has been reported to be 7.98 in HeLa cells and 7.91 in rat cardiomyocytes [32]. Therefore, we would speculate that as reduced DA gains access to the mitochondrial matrix, it would likely oxidize and increase the potential to interact with ETC proteins.

While much of the focus to date on catechols and mitochondrial function has centered on DA, it is noteworthy that DOPAC has a similar potency to DA as well as similar sites of interaction ([19] and the current work). MAO is located on the outer mitochondrial membrane and MAO metabolism of DA to DOPAC occurs rapidly once DA is elevated. Elevation of DOPAC in the cytosol with its production in close proximity to mitochondria raises the possibility that DOPAC may be more deleterious to mitochondrial function when DA homeostasis is lost. In support of this, Mosharov et al. [33] demonstrated that basal levels of total catechols in rat chromafin cells were 50–500 μM and increased 5-fold when challenged with amphetamine to increase cytosolic levels. Further, while DA levels were short lived, catechol derivatives such as DOPAC were elevated in the cytosol for hours following the amphetamine challenge. Our past findings of low mM $K_{ms}$ for DA and DOPAC for inhibition of respiration in intact mitochondria and low to moderate μM $K_{ms}$ for inhibition of complex I and III activities in mitochondrial membranes directly exposed to catechols would suggest that the findings from this study have physiological relevance to ongoing cell damage in PD and further indicate that loss of DA homeostasis could put mitochondria at risk for accumulating damage either through elevation in DA or DOPAC. Future studies thus need to address if DOPAC can access the mitochondrial matrix similar to DA.

## 5. Conclusions

In conclusion, the findings from this study suggest that many of the inconsistencies in the literature with regard to the sensitivity of ETC complexes to inhibition by DA or DOPAC are due to the oxidation state of the catechols at the time of exposure. Except for complex II, the reduced catechols show no inhibitory effect on ETC activities. Further, the presence of the reduced catechols and in particular DAH can produce artifacts that can either mask inhibition (complex II and III) or mimic inhibition (complex IV). Thus, care must be taken to ensure that non-ETC related redox chemistry is taken into account. It was further observed that pre-incubation time is only a factor when the redox status of the catechol changes from reduced to oxidized. In contrast with the reduced catechols, oxidized DA and DOPAC

inhibited complexes I and III. These studies support that mitochondria may be a downstream target for damage by catechols when elevated in the cytosol and may contribute to the ongoing progressive damage associated with PD.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbabio.2011.03.013.

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