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Inhibition of rat brain mitochondrial electron transport chain activity by dopamine oxidation products during extended in vitro incubation: Implications for Parkinson's disease

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Abstract

Several studies on mitochondrial functions following brief exposure (5–15 min) to dopamine (DA) in vitro have produced extremely variable results. In contrast, this study demonstrates that a prolonged exposure (up to 2 h) of disrupted or lysed mitochondria to DA (0.1–0.4 mM) causes a remarkable and dose-dependent inhibition of complex I and complex IV activities. The inhibition of complex I and complex IV activities is not prevented by the antioxidant enzyme catalase (0.05 mg/ml) or the metal-chelator diethylenetriaminepentaacetic acid (0.1 mM) or the hydroxyl radical scavengers like mannitol (20 mM) and dimethyl sulphoxide (20 mM) indicating the non-involvement of ·OH radicals and Fenton's chemistry in this process. However, reduced glutathione (5 mM), a quinone scavenger, almost completely abolishes the DA effect on mitochondrial complex I and complex IV activities, while tyrosinase (250 units/ml) which catalyses the conversion of DA to quinone products dramatically enhances the former effect. The results suggest the predominant involvement of quinone products instead of reactive oxygen radicals in long-term DA-mediated inactivation of complex I and complex IV. This is further indicated from the fact that significant amount of quinones and quinoprotein adducts (covalent adducts of reactive quinones with protein thiols) are formed during incubation of mitochondria with DA. Monoamine oxidase A (MAO-A) inhibitor clorgyline also provides variable but significant protection against DA induced inactivation of complex I and complex IV activities, presumably again through inhibition of quinoprotein formation. Mitochondrial ability to reduce tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in presence of a respiratory substrate like succinate (10 mM) is also reduced by nearly 85% following 2 h incubation with 0.4 mM DA. This effect of DA on mitochondrial function is also dose-dependent and presumably mediated by quinone products of DA oxidation. The mitochondrial dysfunction induced by dopamine during extended periods of incubation as reported here have important implications in the context of dopaminergic neuronal death in Parkinson's disease (PD).

Keywords: Dopamine; Mitochondria; Electron transport chain; Quinones; Oxygen radicals; Parkinson's disease

1. Introduction

Based on several lines of evidence, mitochondrial dysfunction has been implicated in the dopaminergic neuronal death in PD [1,2]. The analysis of post mortem brain tissue has indicated a decreased complex I activity in substantia nigra region of parkinsonian patients compared to that of normal controls [3,4]. The toxin 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) which produces parkinsonism in experimental animals and human beings has been shown to inhibit mitochondrial respiration and complex I (NADH-ubiquinone reductase) activity through its metabolite 1-methyl-4 phenyl pyridinum ion (MPP+) which is

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accumulated in dopaminergic neurons through dopamine reuptake system [5,6]. Rotenone, a specific inhibitor of NADH-ubiquinone reductase, causes degeneration of nigral dopaminergic neurons after intracerebral administration in experimental animals and the rotenone model is being used extensively to understand the pathogenesis of Parkinson's disease [7,8]. It is, however, not clear at this moment what leads to mitochondrial dysfunction in nigral dopaminergic neurons in idiopathic PD. Although mitochondrial genomic alterations may contribute to complex I defects in PD, it is generally accepted that oxidative damage mediated by DA oxidation products play a central role in this process [9–12]. Dopamine can undergo autoxidation or monoamine oxidase (MAO)-catalyzed oxidation to produce active oxygen species and reactive quinones, which are potential tissue damaging species [13]. It has been shown from in vitro studies that DA oxidation products can induce various forms of mitochondrial dysfunctions, such as mitochondrial swelling, decreased electron transport chain (ETC) activity and altered mitochondrial membrane potential during short-term (5–15 min) incubation in vitro [11,12,14]. However, in other studies with brief exposure of mitochondria to DA, the results have not been reproducible [15,16]. On the other hand, not many reports are available on the effects of DA on mitochondrial functions during extended periods of incubation. We have observed earlier that prolonged incubation (up to 2 h) of rat brain synaptosomal–mitochondrial fraction with DA results in the covalent cross-linking of membrane proteins and formation of quinoprotein adducts (protein–cysteinyl catechols) which are mediated by DA-derived quinones without any involvement of toxic oxygen radicals [17]. It was, therefore, thought interesting to investigate further the effect of DA on mitochondrial ETC activity during prolonged incubation (up to 2 h) and to elucidate the role of reactive quinones and active oxygen species in this process. The autoxidation of DA near the physiological pH of 7.4 is extremely slow and the extended period of incubation allows a sufficient accumulation of toxic quinone products.

2. Materials and methods

2.1. Materials

All common chemicals were of analytical grade. Dopamine and bovine serum albumin (BSA) were obtained from E. Merck (Germany). Catalase, cytochrome *c*, clorgyline, deprenyl hydrochloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), EGTA., phenyl-methanesulphonyl fluoride (PMSF), diethylenetriaminepentacetic acid (DTPA) and tyrosinase were purchased from Sigma Chemical Co.(USA). NADH, GSH, mannitol, dimethyl sulphoxide (DMSO), sodium dodecyl sulphate (SDS), HEPES, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), dimethylformamide, trichloroacetic acid (TCA), nitroblue tetrazolium (NBT) and sucrose were from Sisco Research Laboratory (Bombay).

2.2. Animals

Albino rats of Charles–Foster strain kept on laboratory chow and water ad libitum were used in this study. The animals were maintained as per the guidelines of the Animal Ethical Committee of our institute.

2.3. Isolation of rat brain mitochondria

Rat brain mitochondria were isolated following the method published earlier [11]. Briefly, the brain from one adult rat was homogenized in 10 ml of buffer A (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/ml BSA, pH 7.4). The homogenate was brought to 30 ml with the same buffer followed by centrifugation at $2000 \times g$ for 3 min at 4 °C. The supernatant was preserved and the pellet resuspended in 10 ml of buffer A followed by recentrifugation as earlier. The supernatants were pooled and centrifuged in 4 tubes at $12{,}000 \times g$ for 8 min. The pellet in each tube containing synaptosomes and mitochondria was treated with 10 ml of buffer A containing 0.02% digitonin to lyse the synaptosomes. The mitochondria were pelleted down by centrifugation at $12{,}000 \times g$ for 10 min. The mitochondrial pellet was washed again in buffer A without EGTA and BSA and resuspended in an appropriate buffer for further experimentation. For the measurement of complex I and complex IV activities and quinoprotein formation, mitochondria were resuspended in 50 mM phosphate buffer, pH 7.4, kept frozen at −20 °C in several aliquots and used within 3 days. In case of MTT reduction assay, mitochondria were resuspended in isotonic buffer B (145 mM KCl, 50 mM sucrose, 5 mM NaCl, 1 mM EGTA, 1 mM magnesium chloride, 10 mM phosphate buffer, pH 7.4) and used immediately in the experiments.

2.4. Assay of citrate synthase for mitochondrial integrity

The mitochondria suspended in buffer B were checked for membrane integrity by assaying citrate synthase activity before and after treatment with 0.1% Triton X-100 to obtain the latency value of citrate synthase and ratios exceeding 10 were considered indicative of good membrane integrity [18]. The reaction measured the release of free coenzyme A from acetyl CoA by citrate synthase and DTNB was used to react with free thiol groups of coenzyme A.

2.5. Measurement of quinones and quinoprotein adducts

Dopamine was incubated in 50 mM phosphate buffer, pH 7.4 for 2 h in the presence or absence of rat brain mitochondria with or without GSH (5 mM) or clorgyline (10 μM). Quinone formation was monitored by absorbance change at 480 nm at the end of the incubation after correcting for

turbidity due to presence of mitochondria as adopted from Graham et al. [19]. Quinoprotein formation was measured by the NBT/glycinate assay in the TCA-precipitated and delipidated mitochondrial protein as described previously [17].

2.6. Mitochondrial enzyme assay

Frozen and thawed samples of mitochondria suspended in 50 mM phosphate buffer, pH 7.4 (200 μl containing 160–360 μg protein) were incubated with varying concentrations of dopamine (100–400 μM) for up to 2 h with or without other additions such as catalase (50 μg/ml), mannitol (20 mM), DMSO (20 mM) or reduced glutathione (5 mM) or clorgyline (10 μM) at 37 °C in 50 mM phosphate buffer, pH 7.4 in a total volume of 400 μl. For some experiments, mitochondria were incubated with dopamine (400 μM) and tyrosinase (250 units/ml) for a shorter period of time (15 min) in the presence or absence of reduced glutathione (5 mM). At the end of the incubation, the mitochondrial membranes were washed with an excess of ice-cold 50 mM phosphate buffer, pH 7.4, collected by centrifugation at 4 °C and resuspended in the same buffer. Aliquots of mitochondrial suspension were used for measurement of complex I and complex IV activities.

The activity of complex I was assayed by using ferricyanide as the electron acceptor as adapted from Hatefi [20]. The assay system at 30 °C contained O.17 mM NADH, 0.6 mM ferricyanide, triton X-100 (0.1% v/v) in 50 mM phosphate buffer pH 7.4. The reaction was initiated by the addition of mitochondrial suspension (10–30 μg protein) to the sample cuvette and the rate of oxidation of NADH was measured by the decrease in absorbance at 340 nm [18]. Complex I assayed by this method (NADH-ferricyanide reductase) is not rotenone-sensitive since the latter compound acts at the O_2-side and not the substrate side of the flavoprotein from where ferricyanide accepts electrons [21]. The activity of complex IV was assayed by following the oxidation of reduced cytochrome *c* (ferrocytochrome *c*) at 550 nm. Reduced cytochrome *c* (50 μM) in 10 mM phosphate buffer pH 7.4 was added in each of two 1 ml cuvettes. In the blank cuvette, ferricyanide (1 mM) was added to oxidize ferrocytochrome *c* and the reaction initiated in the sample cuvette by the addition of mitochondrial suspension (10–30 μg protein).The rate of decrease of absorbance at 550 nm was measured at room temperature. The activity of the enzyme was calculated from the first order rate constant taking into account the concentration of reduced cytochrome *c* in the cuvette and the amount of mitochondrial protein added [22].

2.7. MTT reduction assay

The freshly prepared mitochondria were incubated in isotonic medium (buffer B) in presence or absence of dopamine varying from 100 to 400 μM with or without reduced glutathione (5 mM) for 2 h in a total volume of 400 μl. In some experiments, tyrosinase was added in the incubation mixture to cause rapid oxidation of dopamine when the incubation time was shortened to 15 min and DA concentration kept at 50 μM. The incubation was terminated by the addition of an excess of ice-cold buffer B followed by centrifugation at 4 °C to pellet the mitochondria. The pellet was resuspended in isotonic buffer B (300 μl). An aliquot of mitochondrial suspension (200 μl) was added to 800 μl of buffer B containing 10 mM succinate and MTT (0.5 mg/ml) and kept at 37 °C for 15 min. The samples were quenched with 500 μl of lysis buffer (45% dimethylformamide and 10% SDS, pH 4.7). The absorbance was read after 5 min and the difference in absorbance values at 550 nm and 620 nm noted [10].

2.8. Protein estimation

The protein was estimated after solubilizing the membranes in 1% SDS by the method of Lowry et al. [23].

3. Results

3.1. Assay of citrate synthase in purified mitochondria from rat brain

The enzyme citrate synthase was assayed in the purified mitochondrial preparation in the presence or absence of detergent triton X-100 in 5 sets of experiments in different days. The latency value ratio was found to be more than 10 in each case indicating good membrane integrity of our mitochondrial preparation (data not shown).

3.2. Inhibition of complex I and complex IV activity by DA

The data presented in Fig. 1 show that DA in concentrations ranging from 100 to 400 μM caused dose-dependent inhibition of mitochondrial complex I and complex IV activities during an extended period of in vitro incubation. At the concentration of 400 μM of DA, complex I and complex IV activities were reduced by nearly 45% and 55%, respectively (Fig. 1). The extent of inhibition of complex I and complex IV by DA in various sets of experiments was found to be reasonably similar in different sets of experiments. The effects of metal-chelators, antioxidant enzymes, hydroxyl radical scavengers and the quinone scavenger reduced glutathione (GSH) on DA-mediated inhibition of complex I and complex IV were investigated. The metal chelator DTPA (0.1 mM) or the antioxidant enzyme catalase (50 μg/ml) failed to prevent inactivation of complex I and complex IV by DA during in vitro incubation up to 2 h (Fig. 2). Mannitol (20 mM) and DMSO (20 mM) which are potent scavengers of ·OH radicals also did not prevent DA inhibition of respiratory chain complex I and complex IV activities to any noticeable extent (Fig. 2). In fact, DA induced inhibition of complex IV activity is slightly more pronounced in presence of mannitol or DMSO (Fig. 2).

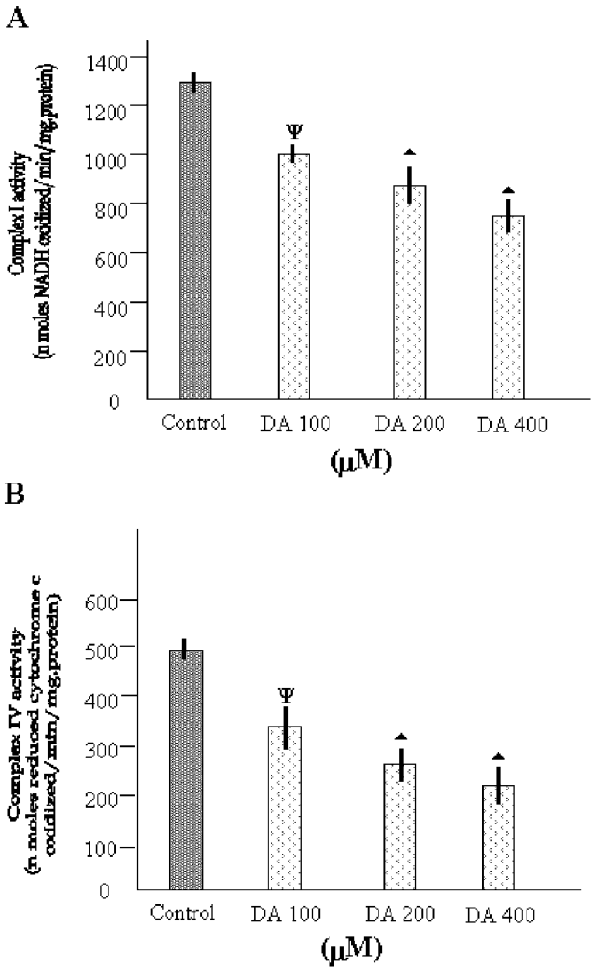

Fig. 1. Dose-dependent inhibition of mitochondrial complex I and complex IV activities by dopamine. Rat brain mitochondria were incubated without (control) or with DA (100–400 μM) for 2 h at 37 °C followed by the measurement of complex I and complex IV activities as described in Materials and methods. The values are means ± SEM of 4 observations for complex I (A) and 5 observations for complex IV (B). Ψ$P < 0.01$, ▲$P < 0.001$ vs. control; Student's t test, paired. The activities of complex I and complex IV in unincubated mitochondria were 1330 ± 13 nmol NADH oxidized/min/mg. protein and 480 ± 16 nmol reduced cytochrome c oxidized/min/mg protein, respectively, which were similar to that in control incubated samples.

However, reduced glutathione (5 mM) almost completely abolished the DA effect (Fig. 2). When added in the absence of DA in the incubation mixture, reduced glutathione (5 mM) or DTPA (0.1 mM) or catalase (50 μg/ml) or mannitol (20 mM) or DMSO (20 mM) did not have any noticeable effect on mitochondrial complex I and complex IV after 2 h of incubation (results not shown).

3.3. Quinone and quinoprotein formation during incubation of mitochondria with DA

Dopamine (400 μM) during in vitro incubation produced quinone products as measured by absorbance change at 480 nm and in presence of co-incubated mitochondria the formation of quinones was further enhanced (Table 1). Reduced glutathione (5 mM), when present in the incubation mixture along with DA and brain mitochondria, abolished completely the increase in absorbance at 480 nm. Under similar conditions, clorgyline (10 μM) also partially prevented the formation of quinone oxidation products (Table 1). Further, a significant formation of quinoprotein adducts (protein-cysteinyl catechols) in mitochondrial proteins was observed during incubation of rat brain mitochondria with DA (400 μM) for 2 h (Table 1). Reduced glutathione (5 mM) prevented quinoprotein formation nearly completely while clorgyline (10 μM) also inhibited the process significantly (Table 1). Tyrosinase (250 units/ml) rapidly converts DA (50 μM) to quinone products with a dramatic increase in absorbance at 480 nm and formation of quinoprotein adducts in co-incubated mitochondria within 15 min (data not shown).

3.4. Effects of MAO-inhibitors and tyrosinase on DA inhibition of complex I and complex IV

For some initial experiments with MAO-inhibitors, we added a combination of 10 μM clorgyline (MAO-A inhibitor) and 2 μM deprenyl (MAO-B inhibitor) in the incubation medium, since DA was a substrate for both MAO-A and MAO-B. However, the combination of clorgyline and deprenyl prevented DA-inactivation of complex I and complex IV activities partially and approximately to the same extent as clorgyline (10 μM) alone did (results not shown) and, therefore, in all the subsequent experiments with MAO-inhibitors, only clorgyline (10 μM) was used. As shown in Table 2, MAO-A inhibitor clorgyline (10 μM) provided partial but statistically significant protection against DA-mediated inactivation of complex I and complex IV during 2 h incubation of mitochondria with DA (400 μM). Clorgyline (10 μM) alone in the absence of DA did not have any effect on mitochondrial complex I and complex IV activities (data not shown).

When mitochondria were incubated for a short period (15 min) with DA (400 μM) no noticeable inhibition of complex I and complex IV activities occurred (Table 3). However, in presence of tyrosinase (250 units/ml) in the incubation mixture DA caused a very significant inhibition of complex I and complex IV activities up to approximately 35% and 60%, respectively, within 15 min and the inhibition was completely prevented by 5 mM GSH (Table 3). Tyrosinase alone, however, did not have any effect on complex I and complex IV activities in the absence of added DA in the incubation mixture during 15-min incubation (data not shown).

3.5. Inhibition of mitochondrial MTT reduction by DA

Intact rat brain mitochondria suspended in isotonic buffer pH 7.4 containing 10 mM succinate caused reduction of MTT as measured by absorbance increase at 550 nm due

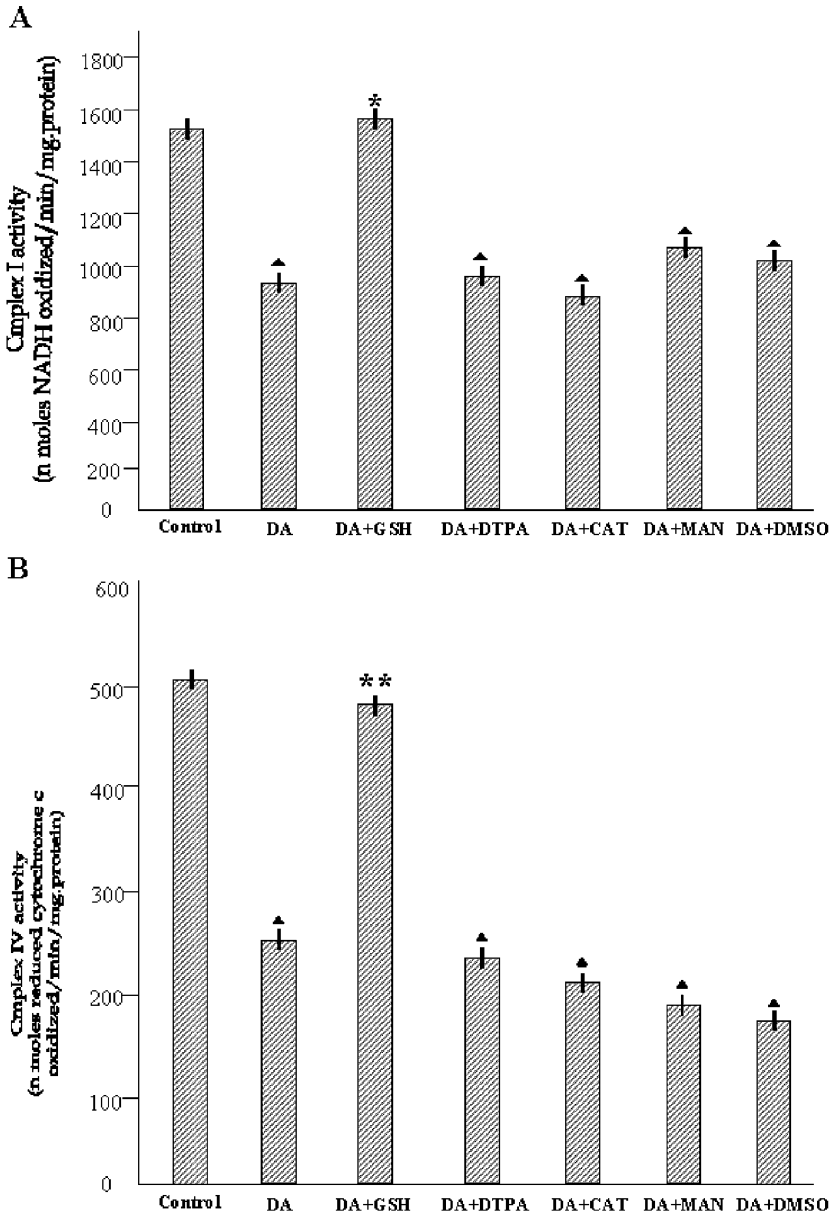

Fig. 2. Effects of antioxidants, radical and quinone scavengers on DA-mediated inhibition of mitochondrial complex I and complex IV activities. Rat brain mitochondria were incubated without (control) or with DA (400 μM) in the presence or absence of GSH (5 mM) or DTPA (0.1 mM) or CAT (catalase, 50 μg/ml) or MAN (mannitol, 20 mM) or DMSO (20 mM) for 2 h at 37 °C. Complex I and complex IV activities were measured in mitochondrial suspensions at the end of the incubation as described in Materials and methods. The values are means ± SEM of 6 observations for complex I (A) and 4 observations for complex IV (B). Statistical significance was calculated by Student's *t* test paired. ▲$P < 0.001$ vs. control, *$P < 0.001$ vs. DA; ♦$P < 0.01$ vs. control, **$P < 0.01$ vs. DA.

to formation of formazan. However, when mitochondria were preincubated with DA for 2 h, subsequent reduction of MTT by mitochondria in succinate containing isotonic buffer was inhibited in dose-dependent manner with more than 85% inhibition noticed with 400 μM DA (Fig. 3). Reduced glutathione (5 mM) nearly completely and clorgyline (10 μM) only marginally protected against DA induced inhibition of mitochondrial MTT reduction (Fig. 3). Neither GSH (5 mM) nor clorgyline (10 μM) in the absence of added DA had any effect on mitochondrial MTT reduction (Fig. 3). An exposure of intact mitochondria to DA (50 μM) for a short period (15 min) did not result in any significant inhibition of MTT reduction (Fig. 4). However, in presence of tyrosinase (250 units/ml) in the incubation mixture DA (50 μM) led to a very dramatic decrease of mitochondrial MTT reduction (up to nearly 70%) within 15 min (Fig. 4). The effect of tyrosinase in turn was again abolished by reduced glutathione in the incubation mixture (Fig. 4). Tyrosinase or GSH alone had no effect on MTT reduction in the absence of DA (Fig. 4). In this set of experiments with DA and tyrosinase, the concentration of DA was restricted to 50 μM in order to minimize the formation of intense brown to black pigments from DA by the action of tyrosinase. The pigments tend to deposit on mitochondria

Table 1
Production of quinones and quinoprotein adducts during incubation of mitochondria with DA

Incubation mixture	Quinones (absorbance at 480 nm)	Quinoprotein adducts (absorbance at 520 nm)
DA in buffer alone	0.17	–
Mitochondria in buffer alone	–	0.066
Mitochondria + DA	0.31	0.210
Mitochondria + DA + GSH	0.08	0.072
Mitochondria + DA + Clorgyline	0.20	0.102

Dopamine (400 μM) was incubated alone or with rat brain mitochondria in the presence or absence of reduced glutathione (5 mM) or clorgyline (10 μM) in 50 mM phosphate buffer, pH 7.4 at 37 °C for 2 h followed by the measurement of quinones and quinoprotein adducts as described in Materials and methods. For measurement of quinone production by absorbance at 480 nm, the turbidity due to mitochondria was corrected by using the appropriate control. Values are from one representative experiment repeated four times.

and interfere with the measurement of coloured formazan developed from MTT reduction.

4. Discussion

From an analysis of post mortem brain samples of PD patients and studies on animal models of parkinsonism, the inactivation of mitochondrial complex I activity has been implicated in the death of dopaminergic neurons in Parkinson's disease [2,3,7]. In this context, the results of the current study may be of significance as it shows the inhibition of mitochondrial ETC at various levels by oxidation products of DA. In particular, we have demonstrated that dopamine inhibits rat brain mitochondrial complex I and complex IV activities in a dose-dependent manner during prolonged incubation (up to 2 h) in vitro with no noticeable inhibition seen during a shorter exposure period of 15 min (Fig. 1 and Table 3). Autoxidation or MAO catalyzed oxidation of DA leads to production of several deleterious products like H_2O_2, oxygen radicals and reactive quinones, which can cause dysfunction of mitochondria. Under our experimental conditions, H_2O_2 and oxygen radicals apparently do not play a significant role in DA-mediated inactivation of complex I and complex IV. This is indicated from the fact that the antioxidant enzyme catalase and the metal-chelator, DTPA, fail to prevent DA-induced inactivation of complex I and complex IV (Fig. 2). The inability of hydroxyl radical scavengers like DMSO and mannitol to prevent DA inhibition of complex I and complex IV activities further confirms the non-involvement of ·OH radicals in this process (Fig. 2). On the other hand, tyrosinase which causes rapid oxidation of dopamine to quinone products leads to a dramatic acceleration of DA-mediated inactivation of complex I and complex IV (Table 3). Further, the quinone scavenger reduced glutathione (5 mM) almost completely protects the enzymes from inactivation by DA (Fig. 2 and Table 3). These findings clearly indicate that quinone products derived from DA-oxidation are responsible for the inhibition of complex I and complex IV of mitochondrial ETC. In the absence of any added tyrosinase, quinones can be formed from the autoxidation of DA which is, however, very slow at pH 7.4 and only during an extended incubation (up to 2 h) of mitochondria with DA a sufficient build-up of the damaging quinones take place to produce inactivation of ETC enzymes. The role of quinones is further supported by the fact that significant formation of quinone products and quinoprotein adducts (protein-cysteinyl catechols) occur during in vitro incubation of mitochondria with DA (Table 1). Reduced glutathione scavenges reactive quinones derived from DA and almost completely abolishes the increase in absorbance at 480 nm

Table 2
Effect of clorgyline on DA induced inhibition of rat brain mitochondrial complex I and complex IV activities during in vitro incubation

Incubation mixture	Complex I activity (nmol of NADH oxidized/min/mg of protein)	Complex IV activity (nmol of reduced cytochrome *c* oxidized/min/mg protein)
Mitochondria alone	1600 ± 139	404 ± 35
Mitochondria + DA	997 ± 86*	175 ± 12*
Mitochondria + DA + clor	1125 ± 89**	303 ± 20**,***

Rat brain mitochondria were incubated in the presence or absence of DA (400 μM) with or without clor (clorgyline, 10 μM) for 2 h at 37 °C. At the end of the incubation mitochondrial complex I and complex IV activities were measured as detailed in Materials and methods. The results are the means ± SEM of 10 observations for complex I and 8 observations for complex IV. Statistical significance was calculated by Student's *t* test, paired.

* $P < 0.001$ vs. control.

** $P < 0.001$ vs. mitochondria + DA.

*** $P < 0.01$ vs. mitochondria alone.

Table 3
Dopamine effects on mitochondrial respiratory enzymes in presence of tyrosinase during short-term (15 min) incubation

Incubation mixture	Complex I activity (as % of control)	Complex IV activity (as % of control)
Mitochondria + DA	98.3 ± 5.85	93.3 ± 6.54
Mitochondria + DA + Tyr	66.7 ± 8.37*	37.2 ± 1.78**
Mitochondria + DA + Tyr + GSH	90.1 ± 4.72s	89.9 ± 5.74s

Aliquots of rat brain mitochondrial preparations were incubated without (control) or with DA (400 μM) or DA (400 μM) + Tyr (tyrosinase, 250 units/ml) or DA (400 μM) + Tyr (tyrosinase, 250 units/ml) + GSH (5 mM) for 15 min at 37 °C. Mitochondrial complex I and complex IV activities were assayed after the incubation as described in Materials and methods. Each value expressed as percentage of control represents mean ± SEM of 4 observations. Statistical significance was calculated by Student's *t* test, paired. *$P < 0.01$ vs. control; **$P < 0.001$ vs. control; s'$P < 0.01$ vs. mitochondria + DA + Tyr.

Control values (100%) for complex I and complex IV were 1520 ± 42 nmol NADH oxidized/min/mg protein and 455 ± 19 nmol reduced cytochrome *c* oxidized/min/mg protein, respectively.

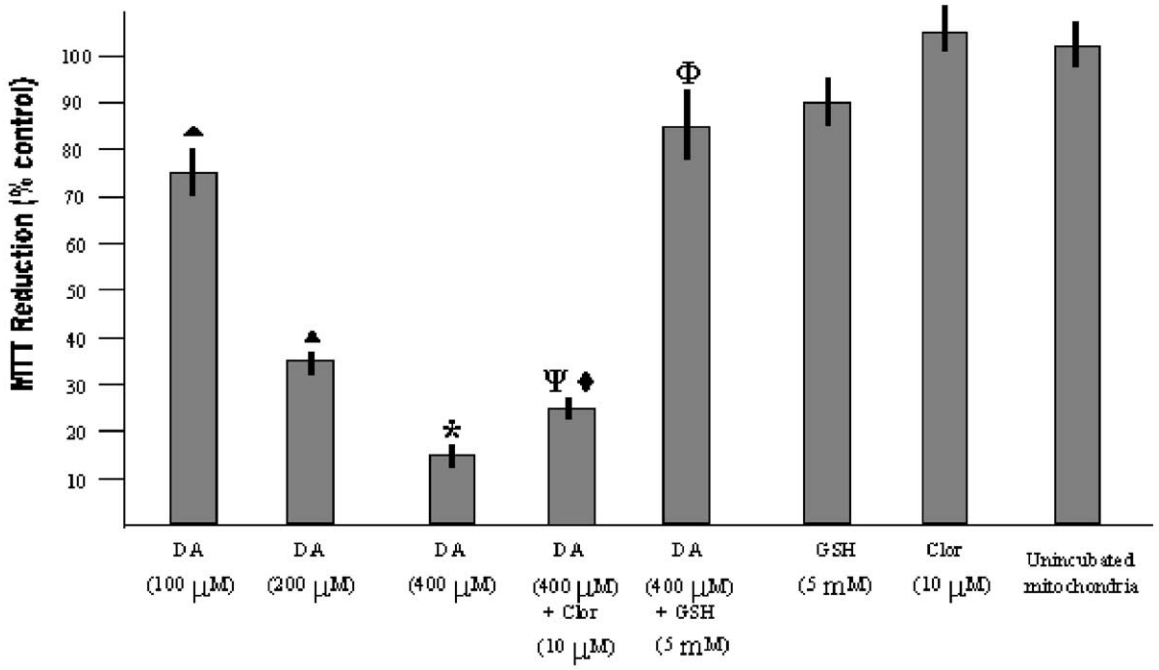

Fig. 3. Dose-dependent inhibition of mitochondrial MTT reduction by DA. Intact mitochondria from rat brain were incubated in isotonic buffer without (control) or with DA (100–400 μM) or with DA (400 μM) plus GSH (5 mM) or DA (400 μM) plus clor (clorgyline, 10 μM) for 2 h at 37 °C followed by the measurement of mitochondrial MTT reduction in presence of 10 mM succinate. Each value expressed as percentage of control represents mean ± SEM of 4 observations. Values for unincubated mitochondria, mitochondria plus GSH (5 mM) only or mitochondria plus clorgyline (10 μM) only are also presented. Statistical significance was calculated by Student's *t* test, paired. ▲$P < 0.05$, ♦$P < 0.01$, *$P < 0.001$ vs. control; Ψ$P < 0.02$, Φ$P < 0.001$ vs. DA (400 μM).

due to quinones and further markedly inhibits the formation of protein-cysteinyl catechols (Table 1). These effects of GSH account for its protective action on complex I and complex IV from DA induced inactivation. It is important to consider, however, the other possible ways by which GSH may produce its protective effects on

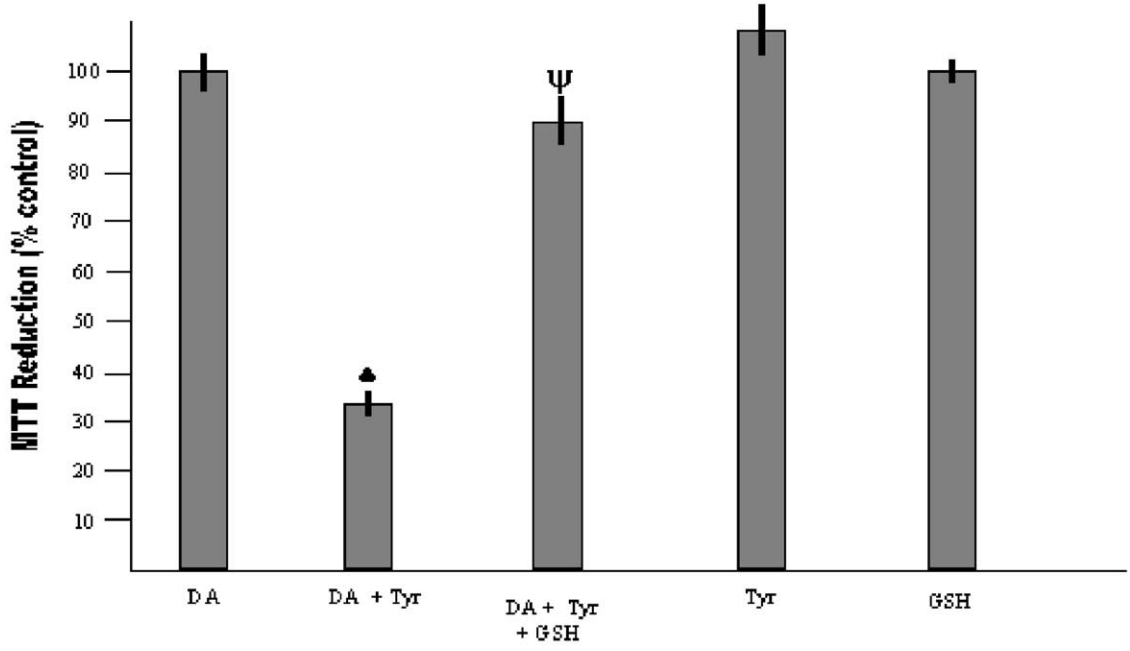

Fig. 4. Effect of enzymatic oxidation of DA on mitochondrial MTT reduction. Rat brain mitochondria were incubated without (control) or with DA (50 μM) or with DA (50 μM) plus Tyr (tyrosinase, 250 units/ml) or with DA (50 μM) plus Tyr (tyrosinase, 250 units/ml) plus GSH (5 mM) for 15 min at 37 °C. The mitochondria were washed and collected by centrifugation and used for MTT reduction assay. Values (expressed as percentage of control) are means ± SEM of 4 observations. Values for mitochondria plus Tyr (250 units/ml) only and mitochondria plus GSH (5 mM) only are also presented. ♦$P < 0.01$ vs. control; ψ $P < 0.01$ vs. DA + Tyr; Student's *t* test, paired.

mitochondrial respiratory complexes. In particular, mitochondrial glutathione peroxidase can catalyze the decomposition of MAO generated H_2O_2 in presence of reduced glutathione, which gets converted to the oxidized form (GSSG). The detoxification of H_2O_2 in this way may not be of much significance, since under our conditions of incubation any role of H_2O_2 and oxygen radicals in the inactivation mechanism of complex I and complex IV has been excluded already. However, GSSG formed in the glutathione peroxidase reaction may interact with protein thiols to produce glutathione–protein mixed disulphides. It cannot be established from the results of our study whether glutathione–protein mixed disulphides formation has any role in preventing the damaging effects of DA oxidation products on mitochondrial complex I and complex IV. Further, GSH alone has no effect on mitochondrial complex I and complex IV activities in the absence of DA and, therefore, the protective action of GSH on DA-inhibition of complex I and complex IV is likely to be mediated by some interaction of GSH with DA or its oxidation products. Our results clearly depict a complete agreement between quinone scavenging function of GSH and its protection of complex I and complex IV activities from DA induced inhibition implying that the quinone products of DA are responsible for the latter phenomenon.

The effect of MAO-A inhibitor clorgyline on DA inhibition of complex I and complex IV extends our interpretation much further. The enzyme MAO-A bound to mitochondrial outer membrane, converts DA to 3,4 dihydroxyphenylacetic acid (DOPAC) and releases H_2O_2 as a by-product. Both DOPAC and H_2O_2 can have deleterious effects on mitochondria [24,25]. Under our experimental conditions, any role of H_2O_2 can be excluded since catalase is ineffective in preventing DA effects. However, DOPAC undergoes autoxidation to produce quinone derivatives and also forms protein–cysteinyl adducts and thus is capable of inhibiting mitochondrial complex I and complex IV activities [24–26]. Moreover, it has been suggested that DOPAC and other aromatic carboxylate compounds may directly affect mitochondrial function through some still unspecified mechanism [25]. Our present results clearly show that MAO-A inhibitor clorgyline partially but significantly inhibits formation of quinone derivatives and quinoprotein adducts during incubation of mitochondria in the presence of DA (Table 1). This may be partly because of the fact that in the absence of any added tyrosinase, the autoxidation rate of DOPAC is higher than that of DA as has been shown in an earlier study [26]. Moreover, DOPAC-quinone may have higher sulphydryl reactivity than dopamine-quinone. It is, therefore, entirely possible that the partial protection of complex I and complex IV from DA induced inactivation in presence of clorgyline as seen in our study (Table 2) is related to the inhibition of quinoprotein adduct formation, although it cannot be completely ruled out that clorgyline protection at least partly reflects the loss of DOPAC mediated direct inhibition of complex I and complex IV. It is further important to compare the protective actions of GSH and clorgyline on DA-mediated inactivation of complex I and complex IV in order to understand the involvement of DA-derived and DOPAC-derived quinones in the process. Reduced glutathione completely abolishes the increase in absorbance at 480 nm during incubation of DA in the presence of mitochondria indicating that GSH scavenges quinones derived from both DA and DOPAC (Table 1) and this results in the complete inhibition of quinoprotein formation and near total protection of complex I and complex IV activities against DA-mediated inactivation (Table 1 and Fig. 2). On the other hand, clorgyline can prevent the formation of DOPAC-quinones, but not DA-quinones and, consequently, it has only partial protection against DA-mediated quinoprotein formation and inactivation of complex I and IV (Table 1 and Table 2).

The degree of protection by clorgyline is quantitatively less in case of complex I than in complex IV, although the reasons for this difference is not clear from the present data. Thus, taking in to consideration the effects of catalase, DTPA, radical scavengers, GSH and the MAO-A inhibitor, the most likely mediators of DA induced inactivation of complex I and complex IV activities are not ROS but the quinone oxidation products of DA and DA-catabolite DOPAC. It is, however, difficult to deduce the relative contributions of DA-quinones and DOPAC-quinones in the inactivation mechanism of mitochondrial respiratory chain enzymes in the absence of more quantitative information such as the actual amount of DOPAC formed from DA during the incubation period and also the relative rates of autoxidation of DA and DOPAC.

In order to assess the mitochondrial electron transport chain activity in complex II and complex III after exposure to DA, succinate supported MTT reduction assay with intact mitochondria has been performed. It is suggested that MTT reduction occurs predominantly between cytochrome *c* and cytochrome *a* and to a lesser extent between one Fe-S center of complex II and the site of inhibition by antimycin A [10,27]. Our results show a remarkable and dose-dependent inhibition of succinate-supported respiration as measured by MTT reduction in intact mitochondria following an extended period (2 h) of incubation with DA (Fig. 3). As in the case of complex I and complex IV inactivation, reduced glutathione (5 mM) prevents to a great extent the inhibitory effect of DA on mitochondrial MTT reduction by scavenging the reactive quinones and inhibiting the formation of quinoprotein adducts (Fig. 3 and Table 1). The involvement of quinone products that are formed slowly from DA autoxidation at pH 7.4 also explains why a short exposure (15 min) to DA alone does not affect mitochondrial MTT reduction ability (Fig. 4). However, in the presence of tyrosinase which catalyses oxidation of DA to quinone derivatives, DA-mediated inhibition of mitochondrial MTT reduction sets in rapidly implicating again the involvement of quinone products in this process (Fig. 4). Further, MAO-A inhibitor clorgyline provides a marginal

but statistically significant protection against DA-induced impairment of mitochondrial MTT reduction ability which again is probably related to partial inhibition of quinone and quinoprotein formation by clorgyline. The marginal protection may reflect the susceptibility of the electron transport chain components to the residual quinones available in the system. In an earlier study, DA (500 μM) has been shown to inhibit MTT reduction (up to approximately 28%) by intact mitochondria during an incubation period varying from 15 min to 1 h with greater degree of inhibition noticeable during the early phase of incubation [10]. The inhibition is attributed to the action of H_2O_2 generated from MAO-catalyzed oxidation of DA and the involvement of glutathione–protein mixed disulphides has been suggested in the process [10,28]. The results of our study differ in several ways from those of the earlier reports with regard to the onset and extent of inhibition and the predominant involvement of quinone products instead of H_2O_2 [10,28]. The reasons for this difference are not obvious, but any role of H_2O_2 in inhibiting mitochondrial MTT reduction cannot be established from our results since MAO-A inhibitor provides only marginal protection, which again can be ascribed to its inhibitory action on quinoprotein formation. Since catalase or DTPA was unlikely to pass through intact mitochondrial membrane, we did not test their effects on DA-mediated inhibition of mitochondrial MTT reduction and it is, therefore, difficult to ascertain the contribution of H_2O_2 and oxygen radicals in the latter process. However, H_2O_2 (500 μM) added in the incubation mixture in the absence of DA can inhibit mitochondrial MTT reduction by approximately 15% at the end of 1 h of incubation (data not shown). Putting together these observations, we would tend to believe that DA inhibition of mitochondrial MTT reduction under our conditions of incubation is predominantly mediated by quinone oxidation products without any major contribution from H_2O_2 derived from MAO-catalyzed oxidation of DA.

Several other investigators have examined the effects of DA during short-term exposure (5–15 min) on various parameters of mitochondrial functions such as mitochondrial respiration, activity of complex I, mitochondrial swelling and membrane potential [11,14–16]. An inhibition of respiration (measured by oxygen uptake or MTT reduction) and a decrease in complex I activity in incubated mitochondria following brief exposure to DA have been reported which is attributed entirely or partly to the deleterious actions of H_2O_2 and oxyradicals [11,12,14,25]. However, the results are somewhat controversial since others have failed to reproduce these under comparable incubation conditions and DA concentrations [15,16]. Further, in some studies, an enormously high concentration of DA (10 mM) has been used in vitro to produce moderate inhibition of complex I activity within 10 min of incubation and increasing slowly up to 1 h [29]. The information on the effects of DA on mitochondrial respiratory complexes other than complex I is scanty, although L-DOPA has been shown to inhibit complex IV activity in NB 69 cells but without any action on complex I or complex II–III activities [30]. On the other hand, there are reports that chronic L-DOPA administration leads to a reduction in complex I activity without any alteration in the activities of complex II and complex IV in rat brain [29].

In contrast to such studies with brief exposure of mitochondria to DA, our results indicate inhibitory effects of DA on mitochondrial electron transport chain at several levels only after an extended period of incubation that are mediated almost entirely by quinone products derived from DA and its catabolite DOPAC. We have shown before that DA-derived quinones can cause protein cross-linking and quinoprotein adduct formation in rat brain mitochondrial–synaptosomal fraction and such effects may be instrumental in impairing mitochondrial respiratory chain activity as seen in the current study [17]. Our results, however, do not rule out the possibility of milder or reversible damage to mitochondria by DA-derived oxyradicals under different conditions of incubation such as a brief exposure to lower concentrations of DA. However, the inhibitory action of DA on mitochondrial ETC during prolonged incubation in vitro as presented in our work may be of more physiological relevance as in the endogenous scenario in dopaminergic neurons mitochondria will be continuously exposed to quinone products derived from DA and DOPAC. Although post mortem studies in PD have revealed a deficiency of complex I activity in substantia nigra, not many reports are available on the status of other respiratory complexes in this disease condition [23].Our present work as well as other in vitro studies indicating DA induced mitochondrial dysfunctions at various levels emphasize the need for a more elaborate study of mitochondrial functions in post mortem brains of PD patients.

It is tempting to speculate from the results of our study that the mitochondrial function would be somewhat compromised in dopaminergic neurons and any further exposure to mitochondrial toxin like MPTP or rotenone may therefore, lead to death of such neurons as reported in several experimental animals [7,31]. Furthermore, the effect of clorgyline in preventing quinoprotein adduct formation indicates that the protective action of MAO inhibitors against DA-mediated damage in several systems variously reported by different groups may actually reflect a dual mechanism of action of MAO inhibitors in preventing both H_2O_2 production and quinoprotein formation. Our data on DA-mediated inhibition of mitochondrial ETC have other important implications. A deficient mitochondrial ETC activity will lead to neuronal ATP depletion leading to an impairment of Na^+, K^+-ATPase activity with associated toxic consequence [2,32,33]. Our earlier study has shown that DA oxidation products can also directly inactivate synaptosomal membrane associated Na^+, K^+-ATPase in rat brain [34]. Thus, a progressively increasing impairment of Na^+, K^+-ATPase may develop in nigral dopaminergic neurons in course of time and this may

play a critical role in the degeneration of such neurons in Parkinson's disease.

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