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Quinone and oxyradical scavenging properties of N-acetylcysteine prevent dopamine mediated inhibition of Na^+, K^+-ATPase and mitochondrial electron transport chain activity in rat brain: Implications in the neuroprotective therapy of Parkinson's disease

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Abstract
Dopamine oxidation products such as H_2O_2 and reactive quinones have been held responsible for various toxic actions of dopamine, which have implications in the aetiopathogenesis of Parkinson's disease. This study has shown that N-acetylcysteine (0.25–1 mM) is a potent scavenger of both H_2O_2 and toxic quinones derived from dopamine and it further prevents dopamine mediated inhibition of Na^+,K^+-ATPase activity and mitochondrial respiratory chain function. The quinone scavenging ability of N-acetylcysteine is presumably related to its protective effect against dopamine mediated inhibition of mitochondrial respiratory chain activity. However, both H_2O_2 scavenging and quinone scavenging properties of N-acetylcysteine probably account for its protective effect against Na^+,K^+-ATPase inhibition induced by dopamine. The results have important implications in the neuroprotective therapy of sporadic Parkinson's disease since inactivation of mitochondrial respiratory activity and Na^+,K^+-ATPase may trigger intracellular damage pathways leading to the death of nigral dopaminergic neurons.

Keywords: *Na^+,K^+-ATPase, N-acetylcysteine, quinone, electron transport chain, mitochondria, Parkinson's disease*

Introduction

The cause of dopaminergic neuronal death in sporadic Parkinson's disease (PD) is not clear, but mitochondrial dysfunction, oxidative damage and protein aggregation are some of the key events that probably activate apoptotic and exitotoxic pathways in nigral dopaminergic neurons, causing their eventual death [1–3]. A body of evidence has been accumulated from the studies in several experimental model systems to implicate dopamine (DA) oxidation products as the major damaging species within dopaminergic neurons [4–6]. The enzymatic or non-enzymatic oxidation products of DA include H_2O_2 and other reactive oxygen species (ROS) as well as semiquinones and quinones which have been shown to cause mitochondrial damage, protein modification and inactivation of key enzymes in various experimental systems and thus their role in the pathogenesis of sporadic PD has

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been substantiated [7–13]. The scavengers of ROS and quinones are, therefore, considered as putative neuroprotective agents for patients of sporadic PD.

It has been shown in our earlier studies that DA can cause inactivation of electron transport chain (ETC) complexes at several levels during extended *in vitro* incubation which are mediated by toxic quinones without any major involvement of ROS [8]. On the other hand in rat brain crude synaptosomal fraction a dose-dependent inhibition of Na^+,K^+-ATPase has been noticed during prolonged exposure to DA *in vitro* and both reactive quinones and H_2O_2 generated from DA oxidation are implicated in the process [14]. The mitochondrial ETC inactivation and Na^+, K^+-ATPase inhibition mediated by quinones and/or H_2O_2 may have an important role in the dopaminergic neuronal death in sporadic PD. Thus, it was thought interesting to look for agents that can scavenge both H_2O_2 and quinones and to examine the effects of such agents on DA mediated ETC inactivation and Na^+,K^+-ATPase inhibition. N-acetylcysteine, a well-known antioxidant, has been used extensively in experimental systems to prevent damage caused by ROS [15,16]. On the other hand, the free thiol group of N-acetylcysteine can presumably take part in adduct formation with DA-derived quinones. Thus, N-acetylcysteine has been tested in our experimental system for protective action against DA mediated injury.

Materials and methods

Chemicals

All common chemicals were of analytical grade. Dopamine and bovine serum albumin (BSA) were obtained from E. Merck (Germany). N-acetylcysteine was from ICN (Ohio, USA). EGTA, cytochrome c, 3-(4,5-dimethylthiozol-2-yl)-2,5-phenyltetrazolium bromide (MTT) and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (USA). Ouabain and xylenol orange were from Acros (Belgium). ATP-disodium salt, NADH, mannitol, sodium dodecyl sulphate (SDS), HEPES, digitonin, dimethylformamide, sucrose and all other common chemicals were obtained from Sisco Research Laboratory (Bombay, India).

Animals

Albino rats of Charles–Foster strain (4–6 months) kept on commercial 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. The animals were sacrificed by cervical dislocation and the brain from each animal was dissected out cleanly, made free of blood vessels and kept in a petridish over ice.

Measurement of H_2O_2 production

The production of H_2O_2 from DA (400 μM) during *in vitro* incubation in the presence or absence of N-acetylcysteine (0.25–1 mM) in 50 mM phosphate buffer, pH 7.4 was measured by reacting an aliquot (100 μl) of the incubation mixture with 900 μl of peroxide assay reagent consisting of ammonium ferrous sulphate (250 μM), H_2SO_4 (25 mM) and xylenol orange (100 μM) [17]. The absorbance was read after 45 min of incubation at room temperature at 580 nm. The H_2O_2 production was calculated from a standard curve using pure H_2O_2 (10–100 μM).

Measurement of quinone formation

Quinone formation from DA was measured spectrophotometrically by the increase in absorbance at 303 nm after incubating DA (400 μM) in 50 mM phosphate buffer, pH 7.4 at 37°C in the presence or absence of N-acetylcysteine (0.25–1 mM) as described earlier [18].

Preparation of rat brain crude synaptosomal fraction (P_2 pellet)

The brain tissue was homogenized in nine volumes of 320 mM sucrose and 5 mM imidazole at pH 7.4 containing 0.1 mM phenylmethylsulphonyl fluoride. The crude synaptosomal fraction (P_2 pellet) was prepared by differential centrifugation [19]. The pellet was resuspended in 25 mM Tris buffer, pH 7.4.

Isolation of rat brain mitochondria

Mitochondria were isolated from rat brain by a method based on differential centrifugation and digitonin treatment as published earlier [8]. The final mitochondrial pellet was resuspended in an appropriate buffer depending on subsequent experimental procedures. Further, isolated mitochondria were checked for membrane integrity by assaying citrate synthase activity in the presence or absence of 0.1% Triton-X 100 [20].

Measurement of mitochondrial ETC activity after dopamine exposure

A frozen aliquot of rat brain mitochondria in 50 mM phosphate buffer, pH 7.4 was thawed and an aliquot further incubated in the same buffer at 37°C for 2 h in the presence or absence of DA (100–400 μM) with or without the addition of N-acetylcysteine (0.25–1 mM) at a final mitochondrial protein concentration of 1.0–1.5 mg/ml. At the end of the incubation the mitochondria were washed twice with an excess of 50 mM phosphate buffer, pH 7.4, and collected by centrifugation at 4°C. The final mitochondrial pellet was resuspended in the same buffer to a final protein concentration of ~1.0 mg/ml for the assay of

complex I and complex IV. For the measurement of mitochondrial MTT reduction after DA exposure, freshly isolated mitochondria were used and an identical incubation and washing protocol was followed except that 50 mM phosphate buffer was replaced by isotonic buffer A (145 mM KCl, 50 mM sucrose, 1 mM EGTA, 1 mM $MgCl_2$, 10 mM phosphate buffer, pH 7.4).

The activity of complex I (NADH: ubiquinone oxidoreductase) was assayed by using ferricyanide as the electron acceptor instead of ubiquinone in a system containing 0.17 mM NADH, 0.6 mM ferricyanide and Triton X-100 (0.1% v/v) in 50 mM phosphate buffer, pH 7.4 at 30°C [20,21]. 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. Complex I as measured by this method in our study is, however, insensitive to rotenone since the latter acts at the O_2 side and not at the substrate side of the flavoprotein from where ferricyanide accepts electrons [8]. A control assay was run in which ferricyanide was omitted from the assay mixture and KCN (1 mM) added to it and any oxidation of NADH by non-mitochondrial NADH oxidase that uses molecular oxygen as the electron acceptor was taken into account during calculation of complex I activity [22,23].

The activity of complex IV was assayed by measuring the rate of decrease of absorbance at 550 nm at room temperature following the oxidation of reduced cytochrome c. Reduced cytochrome c (50 μM) in 10 mM phosphate buffer pH 7.4 was taken in each of two 1 ml cuvettes. In the reference (blank) cuvette, ferricyanide (1 mM) was added to oxidize reduced cytochrome c and the reaction initiated in the sample cuvette by the addition of mitochondrial suspension (10–30 μg of protein) and monitored by the absorbance change at 550 nm. The activity of the enzyme was calculated from the first order rate constant [24].

For the measurement of mitochondrial MTT reduction ability, an aliquot of mitochondrial suspension (200 μl) in isotonic buffer A was added to 800 μl of the same buffer 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 15 min and the difference in absorbance values at 550 nm and 620 nm noted [25]. In some experiments for the assessment of MTT reduction ability, complex II inhibitors like 3-nitropropionic acid (1 mM) or malonate (5 mM) or complex III inhibitor like antimycin A (1 mM) was added to the incubation mixture containing control mitochondria (unexposed to dopamine) in the presence of 10 mM succinate and MTT (0.5 mg/ml).

Measurement of Na^+,K^+-ATPase activity in crude synaptosomal fraction after DA exposure

An aliquot of rat brain crude synaptosomal fraction in 25 mM Tris buffer, pH 7.4, was diluted and further incubated in the same buffer at 37°C for 2 h in the presence or absence of DA (200–400 μM) with or without the addition of N-acetylcysteine (100 μM–1 mM) at a final synaptosomal protein concentration of 1.0–1.5 mg/ml. At the end of the incubation the synaptosomes were washed twice with an excess of 25 mM imidazole buffer, pH 7.4 and pelleted by centrifugation at 4°C. The final synaptosomal pellet was resuspended and diluted in the imidazole buffer to a final protein concentration of 0.4–1.2 mg/ml for the assay of Na^+, K^+-ATPase activity.

An aliquot of synaptosomal suspension (100 μl containing 40–120 μg protein) was used for measurement of Na^+, K^+-ATPase activity in a reaction mixture (1 ml) containing 100 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$ and 3 mM ATP in 30 mM imidazole, pH 7.4, in the presence or absence of 3 mM ouabain as adapted from a published procedure [26]. The incubation was terminated after 10 min by the addition of 5% ice cold TCA and the liberated inorganic phosphate was estimated, as described earlier [14]. The activity of the enzyme was expressed as μmoles of inorganic phosphate liberated/mg protein/h. Ouabain sensitive ATPase activity was taken as the measure of Na^+, K^+-ATPase activity.

The K_m and V_{max} of the enzyme were determined using different substrate concentrations (0.2–5.0 mM ATP). The K_m and V_{max} were calculated using direct linear plot with the help of Biosoft-Enzpack software.

Protein estimation

The protein was estimated after solubilizing the membranes in 1% SDS by the method of Lowry et al. [27].

Results

Production of H_2O_2 and quinones during DA autoxidation

The slow autoxidation of DA in 50 mM phosphate buffer, pH 7.4, for 2 h leads to an accumulation of H_2O_2 and quinones as shown in Table I. Addition of catalase (50 μg/ml) in the incubation medium resulted in nearly complete removal of H_2O_2, while N-acetylcysteine also scavenged H_2O_2 generated in the medium in a graded manner and 92% scavenging is noticed at N-acetylcysteine concentration of 1 mM (Table I). In the tube containing DA with added H_2O_2 (100 μM), the measurement of final H_2O_2 concentration in the reaction mixture showed an additional expected increase of nearly 66 μM which validated our H_2O_2 assay method (Table I). The accumulation of quinone products over 2 h from

Table I. Production of quinones and H_2O_2 from dopamine. DA (400 μM) was incubated in 50 mM phosphate buffer, pH 7.4 for 2 h in the absence or presence of catalase (50 μg/ml) or N-acetylcysteine (0.25–1 mM) or H_2O_2 (100 μM) followed by the measurement of quinones and H_2O_2 produced as described in the text. The values are mean ± SEM of four observations. In the tube containing DA with 100 μM H_2O_2, an additional production of H_2O_2 was observed to an extent of 65.83 μM. Statistical significance was calculated by Student's *t*-test paired.

Incubation mixture	Quinone production (absorbance at 303 nm)	H_2O_2 production (μM)
DA	0.176 ± 0.002	66.51 ± 5.44
DA + Catalase (50 μg/ml)	0.176 ± 0.002	1.92 ± 0.16*
DA + N-acetylcysteine (250 μM)	0.074 ± 0.002*	11.23 ± 1.02*
DA + N-acetylcysteine (500 μM)	0.054 ± 0.002*	6.15 ± 0.41*
DA + N-acetylcysteine (1 mM)	0.045 ± 0.002*	5.45 ± 0.32*
DA + H_2O_2 (100 μM)	0.180 ± 0.003	165.83 ± 5.33

*$p < 0.001$ vs DA.

incubated DA was, however, unaffected by the presence of catalase in the medium, but the former could be effectively scavenged by N-acetylcysteine (0.25–1 mM).

Effect of N-acetylcysteine on DA induced mitochondrial respiratory chain inhibition

DA in the concentration of 400 μM led to a pronounced inhibition of rat brain mitochondrial ETC activity at several levels (Figure 1). In particular, mitochondrial complex I and complex IV activities were decreased by ~35% and 47%, respectively, during incubation with DA over a period of 2 h and such DA mediated inhibition was prevented in a graded manner by the presence of N-acetylcysteine (0.25–1 mM) in the incubation mixture (Figure 1). Likewise, succinate-supported mitochondrial MTT reduction ability was decreased by ~80% following exposure to DA (400 μM) for 2 h and the phenomenon was prevented conspicuously and in a dose-dependent manner by N-acetylcysteine (0.25–1 mM) present in the incubation mixture (Figure 1). It is to be emphasized that N-acetylcysteine alone in the absence of added dopamine had no effect on mitochondrial ETC activity (data not shown).

Moreover, extent of inhibition of mitochondrial ETC activity was dose-dependent with respect to DA (100–400 μM) and N-acetylcysteine (1 mM) could prevent the phenomenon at each concentration of DA (data not shown).

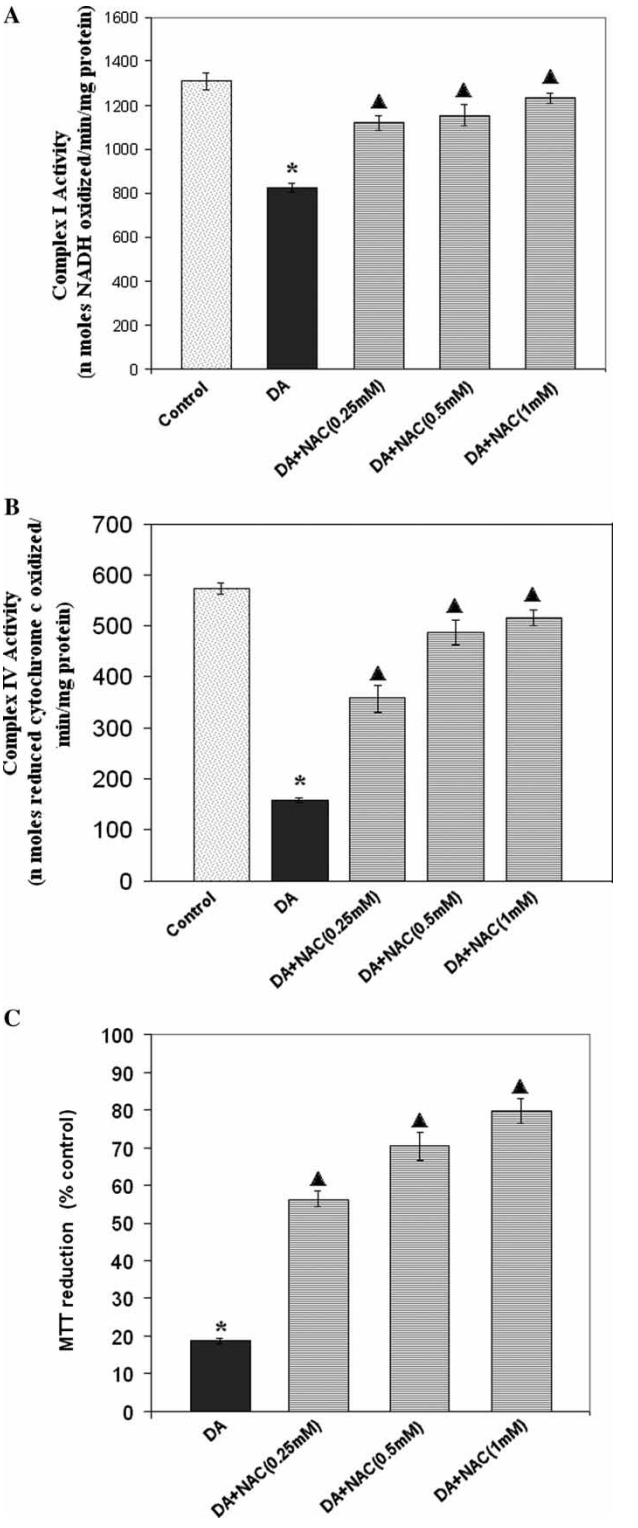

Figure 1. Effects of N-acetylcysteine on DA induced inhibition of rat brain mitochondrial ETC activity. Rat brain mitochondria were incubated without (control) or with DA (400 μM) in the presence or absence of N-acetylcysteine (0.25–1 mM) for 2 h at 37°C. The activities of complex I (A), complex IV (B) and succinate supported MTT reduction (C) were measured in samples at the end of the incubation as described in the text. The values are the means ± SEM of six observations. Statistical significance was calculated by Student's *t*-test paired. *$p < 0.001$ vs control and ▲$p < 0.01$ vs DA.

Changes in Na^+,K^+-ATPase activity of rat brain synaptosomes after exposure to DA and N-acetylcysteine

Rat brain synaptosomal Na^+, K^+-ATPase activity was inhibited very significantly by DA (200–400 μM) during incubation up to 2 h (Figure 2). The inactivation of Na^+,K^+-ATPase activity by DA resulted in decreased V_{max} and K_m values of the enzyme as calculated from a direct linear plot (Figure 2). Other linear transformations of Michaelis Menten curve (Double-reciprocal plot, Hanes plot, etc.) yielded similar results (data not shown). More interestingly, N-acetylcysteine (0.25–1 mM) present in the incubation mixture prevented the DA mediated inhibition of synaptosomal Na^+,K^+-ATPase activity in a dose-dependent manner (Figure 2). However, N-acetylcysteine itself in the absence of DA did not have any effect on Na^+,K^+-ATPase activity (data not presented).

Discussion

N-acetylcysteine is known to react with several members of reactive oxygen species including H_2O_2 and the kinetics of N-acetylcysteine oxidation by H_2O_2 has also been studied in much detail [28,29]. Our data are in conformity with earlier studies and indicate that N-acetylcysteine is a powerful scavenger of H_2O_2 (Table I). Thiol containing compounds such

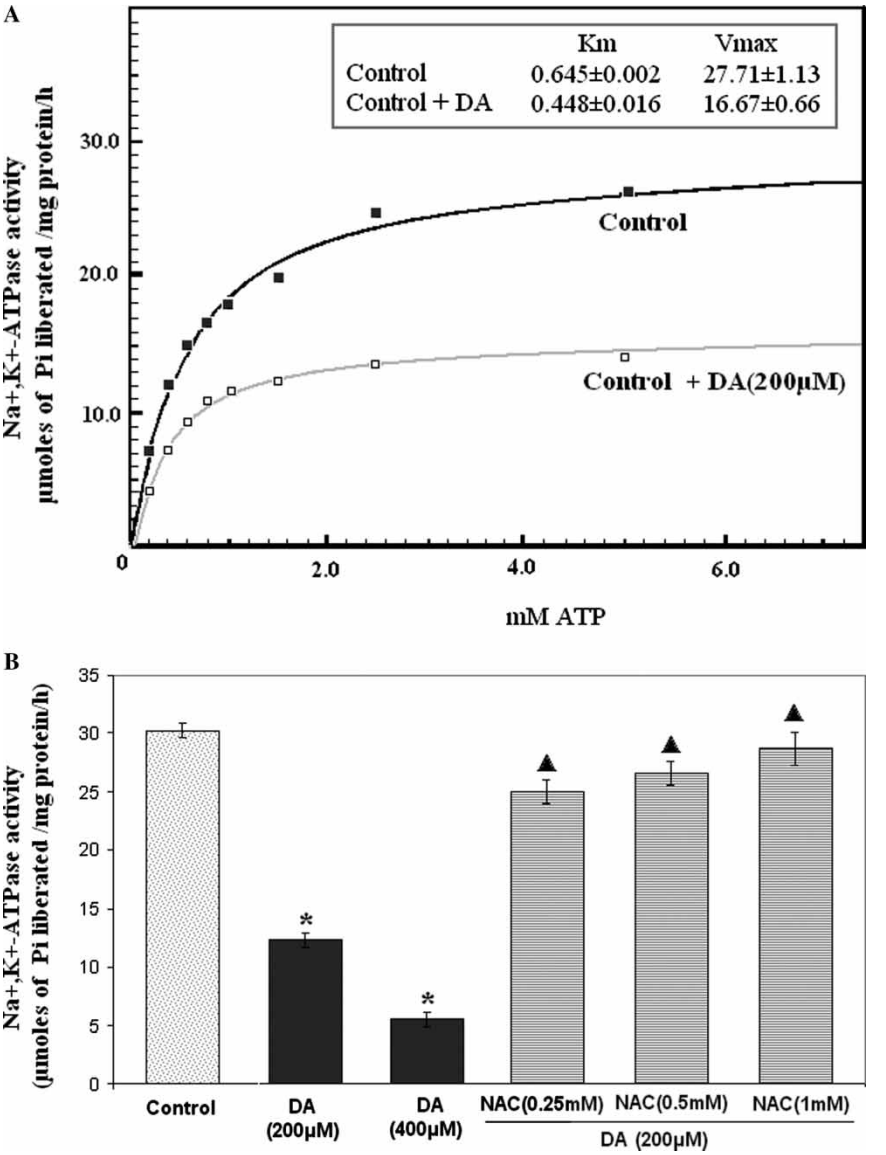

Figure 2. Effect of N-acetylcysteine on DA mediated inhibition of rat brain synaptosomal Na^+, K^+-ATPase activity. Rat brain synaptosomes were incubated without (control) or with DA (200 or 400 μM) in the presence or absence of N-acetylcysteine (0.25–1 mM) for 2 h at 37°C followed by the measurement of activity and kinetic parameters of Na^+, K^+-ATPase as described in the text. (A) Michaelis-Menten plots of Na^+, K^+-ATPase in control and DA treated synaptosomal membranes (representative data). K_m and V_{max} values determined by direct linear plot are the means ± SEM of six observations. (B) Activity of Na^+, K^+-ATPase (means ± SEM of six observations) was determined in synaptosomes incubated only in buffer (control) or with DA (200 μM or 400 μM) or with DA (200 μM) plus N-acetylcysteine (0.25–1 mM). Statistical significance was calculated by Student's *t*-test paired. $^\star p < 0.001$ vs control and $^\blacktriangle p < 0.01$ vs DA.

as reduced glutathione, cysteines, etc. are known to form conjugates with quinones [30]. Likewise, N-acetylcysteine forms nucleophilic monoaddition and diaddition adducts with quinones of which the major product is 5-S-N-acetylcysteinyl dopamine [31]. Thus, N-acetylcysteine is an effective scavenger of quinones as has been seen under our experimental conditions (Table I). N-acetylcysteine is, therefore, chosen for further investigation for its possible effect on quinone and/or ROS mediated damage to rat brain subcellular components during DA exposure.

Under our experimental conditions both in the present as well as in the earlier studies rat brain mitochondria or crude synaptosomes have been exposed to moderate concentrations of dopamine (50–400 μM) for a prolonged period (up to 2 h) and then analysed for various DA induced damage [8,12,14,18]. Mitochondrial functions have been assessed by determining complex I (NADH: ubiquinone oxidoreductase) and complex IV (cytochrome c oxidase) activities along with mitochondrial MTT reduction ability (Figure 1). Reduction of MTT by isolated mitochondria during succinate supported respiration takes place in the region of complex II and complex III and can be blocked by inhibitors of complex II and complex III [32]. We have also verified that MTT reduction by rat brain mitochondria in the presence of succinate (10 mM) can be blocked significantly by complex II inhibitors like malonate and 3-nitropropionic acid or by a complex III inhibitor like antimycin A (data not presented). Thus, MTT reduction has been taken as a simple indirect assessment of mitochondrial complex II–III activity during succinate supported respiration.

In our earlier study it was shown that DA causes a dose-dependent inhibition of mitochondrial complex I and complex IV activities along with a corresponding decreased mitochondrial succinate supported MTT reduction during *in vitro* incubation of brain mitochondria up to 2 h and the phenomenon has been shown to be primarily mediated by toxic quinones and not by ROS [8]. In the present study, DA has been shown to produce similar inhibition of rat brain mitochondrial ETC activity but interestingly the phenomenon can be strikingly prevented by N-acetylcysteine (Figure 1). The protective effect of N-acetylcysteine on mitochondria as seen here can be reasonably attributed to its quinone scavenging property since the involvement of ROS has been ruled out under our experimental conditions [8]. Further, some studies have demonstrated that N-acetyl cysteine directly enhances Complex I activity of aged mice brain mitochondria *in vitro*, presumably through protection of free thiol groups [33]. However, in this study with adult rat brain mitochondria we have failed to observe any direct rate enhancement effect of N-acetylcysteine on mitochondrial respiratory complexes (data not shown).

In our experimental model, mitochondria are exposed to DA (50–400 μM) concentration for a prolonged period of 2 h in contrast to many other studies where brain mitochondria are briefly exposed to DA (5 μM–20 mM) for 5–15 min [34–37]. However, the results in the latter studies have often been very variable [10,36–39] and, therefore, it has been thought prudent to utilize an extended incubation protocol with a different concentration of DA which provides more consistent and reproducible results. It will be interesting to point out here that our results emphasizing the role of toxic quinones in DA induced mitochondrial dysfunctions are in agreement with a recent study where critical mitochondrial proteins including a subunit of complex I have been lost following *in vitro* exposure of isolated brain mitochondria to DA derived quinones as revealed by fluorescent labelling of proteins followed by 2D-gel electrophoresis and laser scanning [40]. The latter study has also hinted that quinone induced protein aggregation may be the cause for the loss of mitochondrial proteins as apparent in 2D-gels and the phenomenon may affect mitochondrial function and stability [40]. Our earlier published data have also indicated that quinone mediated protein aggregation and formation of quinoprotein adducts may lead to mitochondrial respiratory chain inhibition [8,12]. In the context of such findings, the quinone scavenging property of N-acetylcysteine attains an added significance.

The inactivation of Na^+, K^+-ATPase in DA treated synaptosomal membranes is associated with an altered K_m and V_{max}, presumably because of a structural modification of the enzyme protein (Figure 2). In contrast to DA mediated inhibition of mitochondrial ETC activity, our earlier study has shown that DA inhibits rat brain Na^+,K^+-ATPase through complex interactions of H_2O_2, metal ions and reactive quinones [14]. Since N-acetylcysteine is a potent scavenger of both H_2O_2 and quinones, the protective action of the former against DA mediated Na^+,K^+-ATPase inactivation as observed here is expected (Figure 2). It may be pointed out here that the value of Na^+,K^+-ATPase activity and the extent of inhibition caused by DA in different doses as reported here are somewhat different numerically from those published earlier by us, presumably because of the modification used in the present assay to get a better estimate of the initial velocity of Na^+,K^+-ATPase reaction.

Mitochondrial ETC inactivation by DA can lead to altered transmembrane potential and increased ROS production, which are linked to activation of apoptotic cascade [41]. On the other hand, ETC inactivation can bring about ATP depletion that is likely to impair Na^+,K^+-ATPase activity within the dopaminergic neurons and the process may be further aggravated by direct inhibition of Na^+,K^+-ATPase

by DA oxidation products. The suppression of Na^+, K^+-ATPase function by both these mechanisms in dopaminergic neurons can lead to partial membrane depolarization, NMDA receptor activation, influx of Ca^{2+}, altered neurotransmitter release and excitotoxic damage [42,43]. The importance of Na^+,K^+-ATPase in nigral dopaminergic neurons is of particular importance, as has been indicated from the recent observation that DJ-1 gene mutations which cause early onset Parkinsonism also make the nigral cells extremely vulnerable to Na^+,K^+-ATPase inhibition [44]. Thus, mitochondrial ETC inhibition and Na^+,K^+-ATPase inactivation by DA oxidation products may act in concert to bring about the death of dopaminergic neurons in sporadic PD. The protective action of N-acetylcysteine against DA mediated injury as presented here may be of considerable significance and it is interesting to note in this context that in cultured cells of neural origin or in animal models, DA or 6-hydroxydopamine induced apoptosis or neuronal degeneration can be inhibited by N-acetylcysteine [45–48]. N-acetylcysteine is already available in the market as a drug for several clinical conditions like paracetamol poisoning and chronic obstructive lung disease [49]. The neuroprotective action of this drug in PD is also under trial because of its known antioxidant property [50,51]. The present study has highlighted the quinone scavenging property of N-acetylcysteine along with its antioxidant property and thus it is likely to be a better neuroprotective agent in sporadic PD than other antioxidants.

One concern, however, remains as to the ability of N-acetylcysteine to cross the blood–brain barrier effectively. However, several recent studies have clearly indicated that N-acetylcysteine can accumulate in the brain in significant amounts after acute or chronic administration and produce therapeutic or pharmacological effects [52,53]. Thus, N-acetylcysteine appears to be a drug with potential neuroprotective action against sporadic PD and may also serve as a parent compound from which more potent drugs with both radical scavenging and quinone scavenging property may be developed.

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