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RAPID COMMUNICATION

Mitochondria deficient in complex I activity are depolarized by hydrogen peroxide in nerve terminals: relevance to Parkinson's disease

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Abstract

Deficiency of complex I in the respiratory chain and oxidative stress induced by hydrogen peroxide occur simultaneously in dopaminergic neurones in Parkinson's disease. Here we demonstrate that the membrane potential of *in situ* mitochondria ($\Delta\Psi$m), as measured by the fluorescence change of JC-l (5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide), collapses when isolated nerve terminals are exposed to hydrogen peroxide (H_2O_2,100 and 500 µM) in combination with the inhibition of complex I by rotenone (5 nM−1 µM). H_2O_2 reduced the activity of complex I by 17%, and the effect of H_2O_2 and rotenone on the enzyme was found to be additive. A decrease in $\Delta\Psi$m induced by H_2O_2 was significant when the activity of complex I was reduced to a similar extent as found in Parkinson's disease (26%). The loss of $\Delta\Psi$m observed in the combined presence of complex I deficiency and H_2O_2 indicates that when complex I is partially inhibited, mitochondria in nerve terminals become more vulnerable to H_2O_2-induced oxidative stress. This mechanism could be crucial in the development of bioenergetic failure in Parkinson's disease.

Keywords: complex I, hydrogen peroxide, mitochondria, mitochondrial membrane potential, oxidative stress, Parkinson's disease.

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The deficiency of mitochondrial complex I (NADH: ubiquinone oxidoreductase) activity is believed to be a critical factor in the development of Parkinson's disease (PD), a condition characterized by a selective degeneration of the nigro-striatal dopaminergic neurones (for a review see Beal 1995).

A significant and specific reduction in the activity and amount of complex I (Schapira *et al*. 1990; Mizuno *et al*. 1998) was found postmortem in the substantia nigra, in platelets (Parker *et al*. 1989; Haas *et al*. 1995) and skeletal muscle mitochondria of patients with PD (see Mizuno *et al*. 1998). The role of complex I deficiency in the pathogenesis of PD is supported by the fact that MPP^+, which is able to induce a Parkinsonian-like state, inhibits complex I (Ramsay *et al*. 1989).

However, it is unclear why primarily dopaminergic neurones are destroyed in PD. It may be crucial in this respect that mitochondria in dopaminergic neurones are exposed to an increased oxidative stress, particularly to an excess amount of hydrogen peroxide (H_2O_2). H_2O_2 is derived in dopaminergic neurones as a consequence of the activity of monoamine oxidase or of a nonenzymatic autoxidation of dopamine (Schapira 1994; see also Jakel and Maragos 2000). An excess H_2O_2 generation during dopamine metabolism is indicated by a dopamine-induced increase in the oxidized glutathione level observed in mesencephalic neurones (Spina and Cohen 1988), and a stimulation of pentose-phosphate pathway demonstrated in isolated nerve terminals (Hothersall *et al*. 1982).

The membrane potential of mitochondria ($\Delta\Psi$m) controls fundamental functions such as ATP synthesis and Ca^{2+} uptake; thus, determination of $\Delta\Psi$m could reflect the functional integrity and competence of mitochondria. When $\Delta\Psi$m is lost, mitochondria are no longer able to generate ATP and to accumulate Ca^{2+} from the cytosol, which could result in bioenergetic collapse and cell death (for a review see Nicholls and Budd 2000).

We have reported recently that $\Delta\Psi$m is unaltered by H_2O_2 in nerve terminals (Chinopoulos *et al*. 1999, 2000); however, when complex I is blocked by rotenone, H_2O_2 induces a gradual decrease in $\Delta\Psi$m (Chinopoulos *et al*. 1999). The relevance of this observation to PD is limited by the fact that complex I was completely blocked in these experiments, while in PD only ~30% decrease in the enzyme activity was demonstrated (Schapira *et al*. 1990).

A question bearing potential relevance to PD is whether H_2O_2 could contribute to a mitochondrial failure in neurones when

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Abbreviations used: CoQ_1, coenzyme Q_1; $\Delta\Psi$m, membrane potential of mitochondria; H_2O_2, hydrogen peroxide; JC-1, 5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; PD, Parkinson's disease.

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complex I is partially inhibited. Thus, in the present work we titrated the activity of complex I with different concentrations of rotenone, applied H_2O_2 when complex I was inhibited to varying extents and measured $\Delta\Psi$m of *in situ* mitochondria in isolated nerve terminals.

The results show that when complex I is inhibited to an extent found in PD and mitochondria are exposed to H_2O_2, $\Delta\Psi$m is gradually decreased. The increased vulnerability to oxidative stress of mitochondria exhibiting a reduced complex I activity could be an important underlying mechanism in the pathogenesis of PD.

Materials and methods

Preparation of synaptosomes

Isolated nerve terminals (synaptosomes) were prepared from brain cortex of guinea pigs as described elsewhere (Chinopoulos *et al.* 1999). Synaptosomes were suspended in 0.32 M sucrose (20 mg/mL protein), kept on ice, and for further manipulations 50 μL aliquots were incubated in a standard medium (in mM – 140 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 piperazine-*N*,*N*′-*bis*(2-ethaesufonic acid), pH 7.38 and 10 glucose) at 37°C.

Determination of $\Delta\Psi$m

Membrane potential of *in situ* mitochondria was determined by the fluorescence probe JC-1 (5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide) as described by Reers *et al.* (1991) and detailed previously (Chinopoulos *et al.* 1999). Fluorescence intensity was determined at 37°C in a PTI Deltascan fluorescence spectrophotometer. As H_2O_2 causes a non-specific change in the signal at 595 nm, which is unrelated to $\Delta\Psi$m (Chinopoulos *et al.* 1999), fluorescence at 535 nm was recorded, which reliably reflects changes in $\Delta\Psi$m (Di Lisa *et al.* 1995).

Determination of activity of complex I

For complex I assay the method described by Ragan *et al.* (1987) was used, which is based on the determination of the rate of conversion of NADH to NAD^+, coenzyme Q_1 (CoQ_1) being the electron acceptor. Samples of synaptosomes were incubated in 1-mL standard medium with different concentrations of rotenone or H_2O_2 (as shown in Fig. 1) for 25 min, and after freezing–thawing three times, 200 μL (200 μg) was transferred to an assay medium (in mM – 20 potassium phosphate, 0.2 NADH, 10 $MgCl_2$, 1 KCN; 2.5 mg fat-free bovine serum albumin; final volume 2 mL).

The absorbance at 340 nm was recorded in a GBC UV spectrophotometer at 30°C after the addition of 50 μM CoQ_1. The absorbance in a reference sample containing no CoQ_1 was also recorded.

Materials

Standard laboratory chemicals were obtained from SIGMA. JC-1 was obtained from Molecular Probes.

Statistics

Results are expressed as mean ± SEM values. Statistical significance was calculated using one-way ANOVA (Dunnett's test). Differences were considered significant at a level of $p < 0.05$.

Results

The activity of complex I in synaptosomes was measured in the presence of varying concentrations (5 nM−1 μM) of rotenone (Fig. l). The effect of rotenone at a given concentration was maximal after a 5-min incubation (data not shown), but in order to obtain a comparison with data on $\Delta\Psi$m (see below) incubation was carried out for 25 min.

It has been reported that H_2O_2 was without effect on complex I in submitochondrial particles prepared from bovine heart (Zhang *et al.* 1990) and in isolated rat brain mitochondria (Bates *et al.* 1994). We found that in isolated nerve terminals the activity of complex I was decreased by H_2O_2 (100 μM) from 50.4 ± 1.6 to 41.6 ± 0.1 nmol/min/mg (17% inhibition) during incubation for 25 min (Fig. 1). The effect of 100 μM H_2O_2 was maximal after incubation for 10 min and was quantitatively similar to that observed at 500 μM (date not shown). An additive effect on complex I was recorded when we applied rotenone and H_2O_2 together (Fig. 1). In the combined presence of 10 nM rotenone and 100 μM H_2O_2, complex I activity was reduced to 37.4 ± 0.2 nmol/min/mg, i.e. the enzyme was inhibited by 26%.

In accordance with earlier reports we found that $\Delta\Psi$m in synaptosomes was marginally decreased in the presence of 2 μM rotenone, which completely inactivates complex I, and established that for maintaining $\Delta\Psi$m under this condition the reverse function of the mitochondrial ATP synthase was required (Chinopoulos *et al.* 1999). Data on the relative fluorescence of JC-1 monitored at 535 nm in this study are demonstrated in Fig. 2. It is somewhat surprising that when complex I was inhibited by only 5% (5 nM rotenone), the ATP synthase had already made a contribution to H^+ extrusion, as indicated by the small rotenone-induced increase in JC-1 fluorescence observed after blocking the ATP synthase by oligomycin (Fig. 2A). At larger inhibition of complex I, a larger contribution from the ATP synthase is required for maintaining

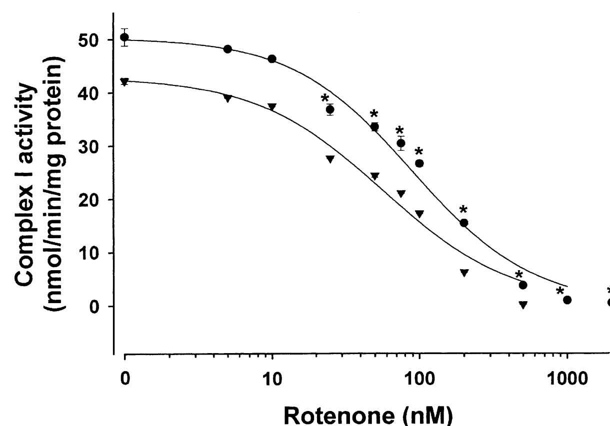

Fig. 1 Inhibition of complex I by rotenone in the absence (●) or presence of 100-μM H_2O_2 (▼). Data are mean ± SEM values of five experiments. Where SEM values are within the size of the symbols they are not shown. Values obtained in the presence of H_2O_2 and rotenone are significantly different from those measured with rotenone only. *Significantly different from the control.

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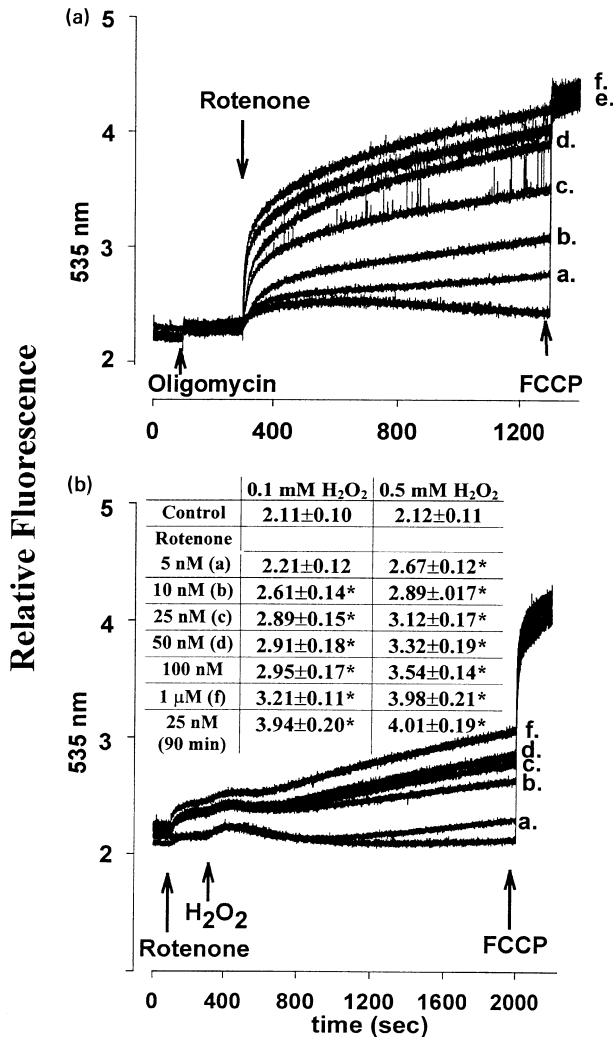

	0.1 mM H_2O_2	0.5 mM H_2O_2
Control	2.11±0.10	2.12±0.11
Rotenone		
5 nM (a)	2.21±0.12	2.67±0.12*
10 nM (b)	2.61±0.14*	2.89±.017*
25 nM (c)	2.89±0.15*	3.12±0.17*
50 nM (d)	2.91±0.18*	3.32±0.19*
100 nM	2.95±0.17*	3.54±0.14*
1 μM (f)	3.21±0.11*	3.98±0.21*
25 nM (90 min)	3.94±0.20*	4.01±0.19*

Fig. 2 Relative fluorescence of JC-1 monitored at 535 nm (10^5 counts) in the presence of rotenone and oligomycin (A) or rotenone and H_2O_2 (B). Oligomycin (for A; 10 μM), H_2O_2 (for B; 100 μM) and rotenone (for A and B) in 5 nM (a.), 10 nM (b.), 25 nM (c.), 50 nM (d.), 100 nM (e.) or 1 μM (f.) concentrations were applied as indicated. At the end of the incubation 1 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) was added to collapse ΔΨm. (A) A small increase in the fluorescence was induced by rotenone (1 μM) when added without oligomycin, which slowly recovered close to the baseline over an incubation for 20 min (bottom trace). Oligomycin itself was also generally without effect on JC-1 fluorescence, but in some experiments it induced a small hyperpolarization. Traces are representative of three independent experiments. (B) inset shows the quantitative data on the relative fluorescence monitored at the end of an incubation for 30 or 90 min (bottom values) with H_2O_2. Data represent mean ± SEM of four experiments. *Significantly different from the control. The bottom trace was recorded with H_2O_2 added without rotenone.

ΔΨm. This indicates that for the maintenance of ΔΨm, no spare in the proton pumping capacity of complex I exists, and a few percentage decrease in the activity could lead to a drop in ΔΨm if not compensated by the ATP synthase working in reverse.

The most important question addressed in this work was whether ΔΨm of *in situ* mitochondria is influenced by H_2O_2 when complex I is partially defective. For this, complex I was inhibited to varying extents by rotenone (5 nM−1 μM), and then H_2O_2 was given in 100- or 500-μM concentration (Fig. 2B). The addition of H_2O_2 (100 μM) after rotenone initiated a slow increase in the fluorescence, indicating a gradual decline in ΔΨm, whereas it had no effect itself, when added without rotenone. Quantitative data on the relative fluorescence recorded at the end of the incubation for 30 min (Fig. 2B inset) show that ΔΨm was significantly decreased by 100 μM H_2O_2 when applied in combination with ≥ 10 nM rotenone, and the higher the rotenone concentration the more pronounced was the effect of H_2O_2 on ΔΨm. Depolarization to larger extents were observed when H_2O_2 was given in 500-μM concentration.

It must be emphasized that ΔΨm was not stabilized at a lower value after the addition of H_2O_2 as indicated by the steady increase in JC-1 fluorescence. In synaptosomes incubated with 25 nM rotenone and 100 μM H_2O_2 for 90 min, a complete loss of ΔΨm was recorded (Fig. 2b inset, bottom values). This particular condition was interesting as the inhibition of complex I in the presence of 25 nM rotenone (25%; Fig. 1) is similar to that found in PD.

Discussion

We report in the present work an increased vulnerability to H_2O_2-induced oxidative stress of *in situ* mitochondria, in which the activity of complex I is reduced to a similar extent as found in PD. It is unclear whether mitochondrial failure in PD is primary, or rather is the consequence of cell damage, and often the argument for complex I deficiency to be secondary is that it is not severe enough to induce cell death by itself (see Mizuno *et al.* 1998). It appears from our study that, when this condition is combined with oxidative stress induced by H_2O_2, a severe mitochondrial deficiency could develop as indicated by a decline in ΔΨm. The importance of this finding is indicated by the fact that the combination of complex I deficiency and excess production of H_2O_2 occurs in dopaminergic neurones in PD.

It was reported by Davey *et al.* (1997, 1998) that the activity of complex I in isolated synaptic mitochondria needed to be reduced by 25% before respiration and ATP synthesis were compromised. A threshold inhibition of complex I at which oxidative stress by H_2O_2 starts to reduce ΔΨm is more difficult to establish. Taking into account the additive effects of rotenone and H_2O_2 on complex I, it can be established that the effect of 100 μM H_2O_2 on ΔΨm is significant when complex I is inhibited by 25% or more (at 10 nM rotenone, Fig. 1 and Fig. 2b). However, an inhibition of complex I to a smaller extent is sufficient for H_2O_2 to reduce ΔΨm when applied in higher concentration (500 μM; Fig. 2b). Thus, it appears that the higher the H_2O_2 concentration, the smaller the extent of complex I inhibition at which oxidative stress is able to compromise ΔΨm. This study also indicates (Fig. 2a) that at a

few percentage inhibition of complex I, the ATP synthase should already contribute to the maintenance of $\Delta\Psi$m.

The mechanism by which H_2O_2 inhibits complex I has not been addressed in this study; the oxidant might directly interact with the enzyme, or the accumulation of oxidized glutathione resulting from detoxification of H_2O_2 might contribute to the effect by forming protein mixed disulphides (Cohen *et al*. 1997).

100 μM was the smallest concentration of H_2O_2 used in this study. This concentration could be achieved under pathological conditions in the brain as was indicated by data obtained with microdialysis in the striatum during reperfusion (Hyslop *et al*. 1995). No data is available concerning the concentration of H_2O_2 in dopaminergic neurones in the brain. It is noteworthy, however, that the catecholamine concentration in the peripheral sympathetic neurones was estimated to be in the range of 600 μM–50 mM (for references see Cohen *et al*. 1997). Given the increased turnover of dopamine in the Parkinsonian brain (Hornykiewicz and Kish 1987), 100 μM H_2O_2 is probably an appropriate condition to model oxidative stress.

The question arises as to the mechanism by which H_2O_2, in combination with diminished complex I activity, gives rise to a loss of $\Delta\Psi$m. The effect of H_2O_2 on complex I is insufficient to explain the decrease in $\Delta\Psi$m. In this respect a crucial effect of the oxidant could be the inhibition of glyceraldehyde-3-phosphate dehydrogenase (Hyslop *et al*. 1988), by which ATP generation in the glycolysis could be impaired. We have reported previously that indeed H_2O_2 decreases the ATP level and ATP/ADP ratio in synaptosomes (Tretter *et al*. 1997). A reduction in the ATP level by H_2O_2 was also observed in the presence of mitochondrial inhibitors (Tretter *et al*. 1997) when ATP is presumed to be generated by the accelerated glycolysis (Kauppinen and Nicholls 1986), pointing to a site of action of H_2O_2 in the glycolysis. In intact nerve terminals when the ATP synthase functions in reverse, ATP produced in the glycolysis is hydrolysed (Scott and Nicholl 1980; see also Nicholls and Budd, 2000). Thus, by limiting the amount of ATP, H_2O_2 could restrict the reverse function of the ATP synthase, preventing the maintenance of $\Delta\Psi$m when complex I is inhibited. A direct effect of H_2O_2 on the ATP synthase seems to be unlikely given the report by Zhang *et al*. (1990), who found that both the ATP synthase and components of the respiratory chain are relatively resistant to H_2O_2.

It could be concluded that a 30% loss in complex I activity could be critical in nigro-striatal neurones where H_2O_2 is also produced in excess. The results presented here suggest that when complex I is inhibited, mitochondria become vulnerable to H_2O_2-induced oxidative stress resulting in a gradual loss of $\Delta\Psi$m. As the combined presence of complex I deficiency and H_2O_2-induced oxidative stress is characteristic in dopaminergic neurones in PD, the loss of $\Delta\Psi$m could be a crucial factor leading to a bioenergetic incompetence, and eventually cell death in the nigro-striatal dopaminergic neurones.

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