Energy Thresholds in Brain Mitochondria

POTENTIAL INVOLVEMENT IN NEURODEGENERATION*

(Received for publication, October 6, 1997, and in revised form, January 29, 1998)

Gavin P. Davey‡, Stefan Peuchen, and John B. Clark
From the Department of Neurochemistry, Institute of Neurology, Queen Square, London WC1N 3BG, United Kingdom

Decreases in mitochondrial respiratory chain complex activities have been implicated in neurodegenerative disorders such as Parkinson's disease, Huntington's disease, and Alzheimer's disease. However, the extent to which these decreases cause a disturbance in oxidative phosphorylation and energy homeostasis in the brain is not known. We therefore examined the relative contribution of individual mitochondrial respiratory chain complexes to the control of NAD-linked substrate oxidative phosphorylation in synaptic mitochondria. Titration of complex I, III, and IV activities with specific inhibitors generated threshold curves that showed the extent to which a complex activity could be inhibited before causing impairment of mitochondrial energy metabolism. Complex I, III, and IV activities were decreased by approximately 25, 80, and 70%, respectively, before major changes in rates of oxygen consumption and ATP synthesis were observed. These results suggest that, in mitochondria of synaptic origin, complex I activity has a major control of oxidative phosphorylation, such that when a threshold of 25% inhibition is exceeded, energy metabolism is severely impaired, resulting in a reduced synthesis of ATP. Additionally, depletion of glutathione, which has been reported to be a primary event in idiopathic Parkinson's disease, eliminated the complex I threshold in PC12 cells, suggesting that antioxidant status is important in maintaining energy thresholds in mitochondria. The implications of these findings are discussed with respect to neurodegenerative disorders and energy metabolism in the synapse.

Mitochondria are known to be integrally involved in many cellular mechanisms, such as Ca\(^{2+}\) homeostasis (1), programmed cell death (2–5), ischemic delayed neuronal death (6), and excitotoxic neuronal death (7–9). Additionally, mitochondrial dysfunction is characteristic of several neurodegenerative disorders (10) and also the aging process (11). Evidence for mitochondria being a site of damage in neurodegenerative disorders is partially based on reductions in respiratory chain complex activities in Parkinson's disease (12–14), Alzheimer's disease (15), and Huntington's disease (16). Such defects in respiratory chain complex activities in mitochondria are thought to underlie defects in energy metabolism and cellular degeneration (17, 18).

Parkinson's disease is characterized by a selective decrease in dopamine in the striatum caused by a degeneration of dopaminergic neurons in the zona compacta of the substantia nigra (19, 20). In addition to a reduction in complex I activity in Parkinson's disease, decreased levels of glutathione have also been found in postmortem examination of the substantia nigra (21–23). This suggests an increased oxidative stress involvement in Parkinson's disease, as GSH is present in millimolar concentrations in mammalian cells and is considered to be a major antioxidant in the brain, capable of protecting cells from damage caused by free radicals (24). The reduction in GSH levels is believed to be a primary event in Parkinson's disease because in incidental Lewy body disease (thought to be presymptomatic Parkinson's disease) GSH is depleted in the absence of a deficiency in complex I activity (25).

In an experimental model of rat brain mitochondria of nonsynaptic origin it was found that threshold exists (26) whereby complex activities need to be reduced by at least 60% before major changes in ATP synthesis and oxygen consumption occur. In this study we examine the relationship between individual respiratory chain complexes and oxidative phosphorylation (rates of respiration and ATP synthesis) in synaptic mitochondria energized with NAD-linked substrates and discuss the consequences for maintenance of energy metabolism in the synapse. In order to observe the consequences of GSH depletion on mitochondrial function, the catecholaminergic PC12 cell line is depleted of GSH, partly imitating which is thought to occur in idiopathic Parkinson's disease and the effect on the complex I threshold is measured.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were supplied by either BDH, Dagenham, Essex, UK, or Sigma Chemical Company, Poole, United Kingdom. The Eisai Chemical Company, Tokyo, Japan, supplied ubiquinone-1. The animals used were adult (250 g) male Wistar rats, supplied by B and K Universal, Aldbrough, Hull, UK.

Cell Culture—Rat pheochromocytoma-derived PC12 cells (27) were cultured in Dulbecco's modified Eagle's medium containing 5% horse serum and 5% fetal bovine serum, which was changed every 2–3 days. The cells were incubated in an atmosphere containing 5% CO\(_2\) at 37 °C.

Mitochondria—Mitochondria were prepared from the heart, liver, and brain of 25-day-old rats according to the method of Lai and Clark (28) and were resuspended in isolation medium (320 mm sucrose, 1 mm K\(^+\)EDTA, 10 mm Tris-HCl, pH 7.4). Mitochondria routinely had a respiratory control ratio of approximately 5 with glutamate and malate as substrates. Protein concentration was determined by the method of Lowry et al. (29) using bovine serum albumin as the standard.

Inhibitor Titration Experiments—Oxygen consumption or respiration (JO\(_2\)) rates in mitochondria were measured using a Clark-type electrode (Yellow Springs Instruments Co., Yellow Springs, OH) fitted into the top of a 250-μl capacity water-jacketed Perspex incubation vessel.
chamber. An electromagnetic stirrer and bar flea were used to mix the incubation medium. In a typical experiment, mitochondria (0.125 mg) were pre-incubated (2 min, 30 °C) in respiration medium (final volume, 250 μl; 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM phosphate-Tris, 10 mM tri-HCl, and 50 μM EDTA, pH 7.4) containing bovine serum albumin (0.125 mg). Depending on the complex under study, rotenone (0–150 pmol) was used to inhibit complex I, myxothiazol (0–60 pmol) to inhibit complex III, and KCN (0–75 nmol) to inhibit complex IV. After 5 min of incubation with the inhibitor, state 4 respiration was generated with glutamate (10 mM) and malate (5 mM) for 2 min; then state 3 respiration was induced by the addition of ADP (500 μM) and allowed to continue for 3 min before samples were taken for ATP production measurement and for complex activity measurement.

Analysis of ATP and GSH—Mitochondrial samples were perchloric acid (60% v/v)-extracted and the pH adjusted to 6 with 1 M K₂HPO₄ for ATP analysis. PC12 cell samples were perchloric acid (60% v/v)-extracted, and the pH was adjusted to 2.5 with 5 M NaOH for GSH analysis. ATP and GSH were separated from other nucleotides using isotropic ion-paired reverse-phase high performance liquid chromatography. A Beckman System Gold was used, and the separation performed at 30 °C with a Hichrom S502D column (25 cm × 4.6 mm) (Hichrom, Reading, Berkshire, UK). For ATP, the method was based on that of Ingebretsen et al. (30) with UV detection at 254 nm. The mobile phase consisted of 60 mM orthophosphoric acid, 2% methanol, and 80 mM triethylamine (pH 6.0); the flow rate was 1 ml/min. For GSH, the method was based on that of Harvey et al. (31) with electrochemical detection (ESA Coulomb electrochemical detector; E₁ = 250 mV, E₂ = 800 mV). The mobile phase consisted of 10 mM NaH₂PO₄, 25 mM sodium octyl sulfate, 1% acetonitrile, pH 2.7; the flow rate was 1 ml/min.

Respiratory Chain Complex Assays—All assays were performed at 30 °C. Prior to analysis, samples were freeze-thawed and gently shaken three times to ensure mitochondrial lysis was complete. Complex I activity was determined using a modification of the method of Ragan et al. (32), and followed the oxidation of NADH at 340 nm using ubiquinone-1 as the electron acceptor. Complex III activity was determined using a modification of the method of Ragan et al. (32), and followed the oxidation of decylubiquinol with cytochrome c (33) and was expressed as a fractional change in pathway flux of a metabolic network under the global flux of that pathway. The flux control coefficient can be defined using a modification of the method of Ragan et al. (32), and followed the oxidation of NADH at 340 nm using ubiquinone-1 as the electron acceptor. Complex III activity was determined using a modification of the method of Ragan et al. (32), and followed the oxidation of decylubiquinol with cytochrome c (33) and was expressed as a fractional change in pathway flux of a metabolic network under the global flux of that pathway. The flux control coefficient can be defined using a modification of the method of Ragan et al. (32), and followed the oxidation of decylubiquinol with cytochrome c (33) and was expressed as a fractional change in pathway flux under considering. For oxidative flux (respiration) in mitochondria

\[
D_{Vc} = \frac{dD_{Vc}}{dI} (\text{Inhibitor}) \quad (\text{Eq. 1})
\]

where \(C_{Vc}\) is the flux control coefficient of the mitochondrial complex under investigation, \(dD_{Vc}/dI\) is the rate of change of complex activity (individual step) and \(dD_{Vc}/dI\) is the rate of change of respiration (global flux), at low concentrations of the complex inhibitor.

**RESULTS**

**Complex I Threshold Effect**—The common pattern present in the experiments with synaptic mitochondria was the abrupt decrease in complex activity as the respective inhibitor concentration was increased, while rates of respiration and ATP synthesis stayed within 85–100% of their control rates. The difference in the shapes of the curves generates the threshold effect which demonstrates how far the enzymatic activity of these complexes can be reduced before oxidative phosphorylation is compromised and significant decreases in ATP production and oxygen respiration occur. In the case of complex I, titration with rotenone initially resulted in a linear relationship between inhibitor concentration and the activity of that complex (Fig. 1). Titration of ATP respiration in mitochondrial energized with NAD-linked substrates stayed within 90% and 100% of the control rates and decreased appreciably at amounts of rotenone greater than 5 pmol. The rates of overall oxidative flux and ATP synthesis were expressed as a function of the amount of inhibition of complex I activity and a threshold curve was generated (Fig. 2). When complex I activity was decreased further than 25% of the control activity both parameters of oxidative phosphorylation decreased at a rate linearly proportional to the rate of complex I inhibition.

**Complex III Threshold Effect**—The effect of titrating complex III activity with myxothiazol on the rates of state 3 respiration and ATP synthesis is shown in Fig. 3. As myxothiazol concentration was increased (0 to 15 pmol), complex III activity decreased in a linear fashion while the rates of respiration and ATP synthesis stayed between 85 and 100% of the control values and then decreased sharply at amounts of myxothiazol greater than 15 pmol. The 80% threshold observed for complex III (Fig. 4) was greater than that found for complex I (Fig. 2); however, there was a gradual decline (between 100 and 70% of the control) in rates of respiration and ATP synthesis before...
this threshold was reached.

**Complex IV Threshold Effect**—Complex IV activity was titrated with KCN and the effect on rates of state 3 respiration and ATP synthesis is shown in Fig. 5. Increasing the KCN concentration (0 to 0.2 nmol) caused a linear decrease in complex IV activity, rapidly decreasing to approximately 50% of the control values. The rates of respiration and ATP synthesis linearly decreased over this range of KCN concentration but stayed between 85 and 100% of the control levels. Generation of a threshold curve showed that complex IV activity must be decreased by approximately 70% before a rapid decline in the rates of respiration and ATP synthesis occurred (Fig. 6).

**Flux Control Coefficients**—Inhibitors were used to titrate the activities of complexes I, III, and IV. The initial slope of the respiration inhibition profile was calculated and expressed as a ratio to the initial slope of the complex activity profile as described in the equation for calculating flux control coefficients under “Experimental Procedures.” The same procedure was carried out for all three inhibitors (Figs. 1, 3, and 5) and Table I shows the flux control coefficients for all three complexes, with complex I having the higher control on oxygen consumption and ATP synthesis in synaptic mitochondria.

**Complex I Threshold Effects in PC12 Cells**—Incubation of PC12 cells with L-BSO for 24 h resulted in a 80% decrease in GSH levels (control PC12 cells, 18.2 ± 2.1 nmol of GSH/mg of protein; L-BSO treated PC12 cells, 4.0 ± 0.3 nmol of GSH/mg of protein). Rotenone-insensitive complex I activities were subtracted from total complex I activities and the resulting rotenone-sensitive complex I activities were plotted against respiration rate and ATP synthesis data.
ratiom rates (Fig. 7). During 0–40% inhibition of complex I activity, oxygen consumption rates were reduced to approximately 80% of the control rate. Further inhibition resulted in a threshold effect similar to that found in Fig. 2 for synaptic mitochondria, and oxygen consumption rates decreased rapidly as a higher proportion of complex I activity was inhibited. Depletion of GSH in the l-BSO-treated PC12 cells to 20% of the control level abolished the threshold effect, and oxygen consumption decreased in a linear fashion proportional to the inhibition of complex I activity.

**DISCUSSION**

We investigated the involvement of respiratory chain complexes in oxidative phosphorylation in synaptic mitochondria that were energized with NAD-linked substrates. Of the complexes tested, complex I possessed the highest flux control coefficient (0.29) for oxygen consumption and ATP synthesis. Metabolic thresholds existed whereby 25, 80, and 70% inhibition of complex I, III, and IV activities, respectively, were required before ATP synthesis and respiration were severely compromised. Additionally, depletion of GSH abolished the threshold effect for complex I in PC12 cell mitochondria, thus having possible ramifications for the process of neurodegeneration in Parkinson’s disease.

When complex I activity was inhibited by approximately 25% there was an abrupt decrease in rates of respiration and ATP synthesis (Fig. 2). This is in contrast to the threshold of 72% found for complex I that is present in nonsynaptic mitochondria (26). The significance of this finding is important in that the reported decrease of 40% in complex I activity in Parkinson’s disease patients (12–14) would extrapolate to a 35–40% inhibition of ATP synthesis and respiration in the synaptic mitochondria model (Fig. 2). Although cultured astrocytes appear capable of surviving extensive periods of anaerobic conditions, presumably by glycolytic mechanisms, the same cannot be said about neurons where, following inhibition of aerobic metabolism, neuronal glycolysis cannot compensate for loss of mitochondrial ATP synthesis (36–38), so it would seem possible that a 40% inhibition of mitochondrial oxidative phosphorylation would compromise energy homeostasis in the synapse.

Synaptic and nonsynaptic mitochondria have similar respiratory control ratios suggesting no major differences introduced during the mitochondrial isolation techniques. However, synaptic mitochondria have a 36% lower complex I activity than nonsynaptic mitochondria (39), therefore subtraction of this difference from the 72% nonsynaptic complex I threshold may partly account for the lower complex I threshold observed in synaptic mitochondria.

Threshold effects were also seen for complexes III and IV in synaptic mitochondria, whereby once the activities were reduced by 80% (Fig. 4) and 70% (Fig. 6), respectively, rates of respiration and ATP synthesis sharply declined. In contrast to the different complex I thresholds present in synaptic and nonsynaptic mitochondria, complex III and IV thresholds in synaptic mitochondria are similar to those found in nonsynaptic mitochondria (26). These threshold effects are not restricted to brain mitochondria and have been observed in rat muscle mitochondria for complex IV (40) and III (41), where the activity must be reduced by approximately 70 and 60%, respectively, before major changes in oxidative phosphorylation occur. The results shown in this study imply that the threshold effects for complexes III and IV will allow rat brain mitochondria to maintain near-optimal levels of oxidative phosphorylation even if their complex activities are reduced by up to 70%. Decreases in complex IV activity of up to 30% of the control activities reported in Alzheimer’s disease (15, 42), Huntington’s disease (16), and in aged rhesus monkey (17) brain would produce less than a 10% decrease in respiration and ATP synthesis in the synaptic mitochondria model (Fig. 6), thereby implying that a complex IV deficiency alone would not lead to a reduction in energy stores.

Previous studies have shown that respiratory chain complexes are involved in the control of mitochondrial respiration and that the distribution of the control indices of these complexes may be different, depending on the tissue from which the mitochondria were isolated (43–46). In the case of synaptic mitochondria, complex I has a flux coefficient of 0.29 (Table I) and is approximately twice that found in nonsynaptic mitochondria (26), suggesting that a large proportion of the control of oxidative phosphorylation rests with complex I in synaptic mitochondria. Additionally, complex III and IV share some of the control having coefficients of 0.2 and 0.14, respectively. However, according to the summation theory for flux control (34), the sum of all the control coefficients in any pathway is equal to unity; so in the case of the three complexes described above the total is 0.63. Other systems known to contribute to the control of mitochondrial respiration are: adenine translocator (43, 47), phosphate carrier and calcium (48, 49), proton leak (50), ADP-regenerating system and dicarboxylate carrier (43).

Complex I thresholds were monitored in the PC12 cell line and compared with in vitro preparations of synaptic and nonsynaptic mitochondria. PC12 cells were grown in suspension which did not allow for dendritic growth, thus providing a system where mitochondria were primarily of cell body origin.

**Table I**

**Flux control coefficients in synaptic mitochondria**

The flux control coefficients for complexes I, III, and IV were calculated as described under “Experimental Procedures,” using the data in Figs. 1, 3, and 5.

| Respiratory chain complex | Flux control coefficients |
|---------------------------|---------------------------|
| Complex I                 | 0.29                      |
| Complex III               | 0.20                      |
| Complex IV                | 0.15                      |

**Fig. 7.** Complex I threshold effects in PC12 cells. PC12 cells were incubated for 18 h in the presence (○) and absence (●) of the GSH-depleting compound, l-BSO. After addition of ADP, respiration rates and complex I activities were measured and expressed as percentages of their control activities (control cells, 15.2 ± 1.0 nmol of O$_2$/min/mg; l-BSO treated cells, 14.9 ± 1.5 nmol of O$_2$/min/mg, 8.6 ± 0.8 nmol/min/mg). The percent inhibition of complex I activity was calculated and a threshold curve generated for the control PC12 cells. Data are mean ± S.E. (bars) values of three experiments. Where no error bar is shown, the S.E. falls within the size of the symbol.
As shown in Fig. 7, oxygen respiration exhibited a threshold effect at 40% inhibition of complex I activity, suggesting that PC12 cell mitochondria have a different complex I threshold to that found in synaptic and non-synaptic mitochondria. As previously stated, nonsynaptic mitochondria which are of neuronal and astrocyte cell body origin, have a complex I threshold of 72%, while synaptic mitochondria have a complex I threshold of 25%, suggesting heterogeneous complex I thresholds in mitochondria of different origin.

Depletion of GSH in PC12 cells reduces complex I activity and also abolishes the threshold effect. The mechanism by which GSH causes removal of the complex I threshold in mitochondria is not known. GSH is an antioxidant which protects mitochondria from lipid peroxidation (51) and when depleted may render complex I susceptible to free radical attack. Previously, depletion of GSH has been shown to cause enlargement and degeneration of brain mitochondria (52) and a decrease in complex IV activity in purified brain mitochondrial preparations (53). GSH also plays a role in protection of sympathetic neurons in vivo from the effects of the neurotoxin, 1-methyl-4-phenylpyridinium (54) and when mesencephalic cultures are treated with 1-BSO, toxicity is potentiated upon exposure to the succinate dehydrogenase inhibitor, malonate (55). These results suggest that, under conditions of GSH depletion, mitochondria are more vulnerable to metabolic insult, which results in a compromise in energy metabolism.

The finding of a reduced complex I threshold may have implications for processes involved in neuronal degeneration in Parkinson's disease. GSH depletion is a primary event in incidental Lewy body disease (thought to be presymptomatic Parkinson's disease), and as such may cause a reduction in complex I activity in the dopaminergic neurones of the substantia nigra which are selectively destroyed in idiopathic Parkinson's disease. This finding, in conjunction with a constitutive metabolic deficiency in dopaminergic neurones from the substantia nigra (56, 57) may account, in part, for the selective vulnerability of the nigrostriatal dopamine pathway to neurodegeneration. In addition, heterogeneity of mitochondrial thresholds may exist in different types of neurons and may be involved in specific neuronal death in selectively vulnerable brain regions.

Threshold effects have been described in mitochondrial diseases (58, 59) and may be related to the balance between normal and mutant mtDNA. If the expression of this heteroplasmy of mtDNA is at the level of a given respiratory complex enzyme, then these may reinforce threshold effects observed in mitochondrial metabolism (40, 41). Whether or not respiratory chain complex activities would be reduced sufficiently to seriously compromise oxidative phosphorylation would depend on the type of mitochondria affected. Due to the heterogeneous nature of brain mitochondria in which complex I thresholds in synaptic mitochondria (this study) are different to those in non-synaptic mitochondria (26), it may be possible that degeneration preferentially occurs in synapses. And in the case of a neurodegenerative disorder such as Parkinson's disease, a primary depletion of antioxidant such as glutathione may render certain neurons more susceptible to degeneration.