β-Amyloid fragment 25–35 selectively decreases complex IV activity in isolated mitochondria

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Abstract Defects in mitochondrial oxidative metabolism, in particular decreased activity of cytochrome *c* oxidase, have been demonstrated in Alzheimer's disease, and after the expression of the amyloid precursor protein (APP) in cultured cells, suggesting that mitochondria might be involved in β-amyloid toxicity. Recent evidence suggests that the proteolysis of APP to generate β-amyloid is at least in part intracellular, preceding the deposition of extracellular fibrils. We have therefore investigated the effect of incubation of isolated rat brain mitochondria with the β-amyloid fragment 25–35 (100 μM) on the activities of the mitochondrial respiratory chain complexes I, II–III, IV (cytochrome *c* oxidase) and citrate synthase. The peptide caused a rapid, dose-dependent decrease in the activity of complex IV, while it had no effect on the activities on any of the other enzymes tested. The reverse sequence peptide (35–25) had no effect on any of the activities measured. We conclude that inhibition of mitochondrial complex IV might be a contributing factor to the pathogenesis of Alzheimer's disease.

Key words: Amyloid; Alzheimer's disease; Mitochondrion; Respiratory chain; Cytochrome oxidase; Free radical

1. Introduction

Deposition of amyloid β peptide (Aβ) in the brain is a characteristic hallmark of the pathology of Alzheimer's disease (AD) and some other neurodegenerative disorders. It is a constituent of senile plaques, in the form of aggregated insoluble fibrils surrounded by dystrophic neurites and activated microglia and astrocytes [1]. Aβ has been demonstrated to be neurotoxic to primary cultures of neurones and several neuronal cell lines, but only when forming β sheets which aggregate into fibrils [2]. The mechanism of Aβ toxicity is not known, although a potentiation of glutamate toxicity and disruption of calcium homeostasis have been observed in cultured neurones [3]. It has been assumed that Aβ produces its effects at the level of the plasma membrane, since amyloid deposits are classically extracellular. However, growing evidence is showing that the aberrant, amyloidogenic processing of the amyloid precursor protein (APP) also takes place intracellularly in neurones [4], where it may encounter favourable conditions for aggregation (i.e. high local concentration, lower pH, small amounts of free radicals), and form fibrils which would be deposited, without being degraded, after the cell's death [5]. An intracellular Aβ-binding protein localised in the endoplasmic reticulum and mitochondria and mediating Aβ toxicity has been identified [6,7].

Incubation with aggregated Aβ also decreases the cellular redox activity in cultures of primary neurones, astrocytes, HeLa and PC12 cells, as measured by the chemical reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) [8–10]. The site of reduction of this dye seems to be at least in part mitochondrial, at the level of succinate dehydrogenase, although other NADH/NADPH-linked processes might be involved [11]. The decrease in MTT reduction is detectable before the cells show other signs of degeneration, as measured by lactate dehydrogenase release or decreased [^{3}H]thymidine incorporation [10], suggesting a possible direct effect on mitochondria which may initiate the neurotoxic cascade.

Defects in mitochondrial oxidative metabolism have been demonstrated in AD, including decreased activity of mitochondrial complex IV in post-mortem brain, particularly in the cortical areas most affected by the disease [12,13]; this is unlikely to be due to a loss of mitochondria, since complex IV activity was decreased in AD samples even when expressed in relation to the activity of the mitochondrial marker citrate synthase [14], and when measured in isolated mitochondria [15]. Mitochondria are one of the major cellular sources of superoxide, and the production of superoxide increases when the flow of reducing equivalents through complex IV is inhibited [16]. Lipid peroxidation and free radical damage have been found to be increased in the cerebral cortex in AD [17], as well during normal aging and in Down's syndrome, which are also accompanied by the deposition of amyloid plaques [18].

The APP gene has been overexpressed in cultured human muscle cells, causing mitochondrial structural abnormalities and decreased complex IV activity [19]. Aβ has been shown to initiate lipid peroxidation in synaptosomal membranes [20], increase oxidative stress in cultured neurones [21] and lower synaptosomal ATP levels [22]. In some studies, antioxidants such as vitamin E, propyl gallate and nordihydroguaiaretic acid were shown to protect cultured neurones from Aβ toxicity [23,24], although another group have failed to confirm these findings [25]. Disruption of energy metabolism and generation of reactive oxygen species favour the aberrant metabolism of APP to generate amyloidogenic fragments [26] and promote aggregation of Aβ [27]. Aβ and superoxide therefore seem to potentiate the formation of each other and result in cellular toxicity.

The fragment of the Aβ peptide consisting of the sequence of amino acids 25–35 has also been shown to have the neurotoxic properties of the full-length sequence, and it has the

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Abbreviations: Aβ, amyloid β peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

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experimental advantage of rapidly aggregating upon solubilisation in aqueous buffers [28]. In order to test the hypothesis that amyloid peptides might damage cells by impairing mitochondrial respiratory chain function, we have studied the effects of the 25–35 fragment on isolated mitochondria from rat brain, and measured the activities of complex I, complexes II–III and complex IV. Citrate synthase activity was also measured as a marker of mitochondrial mass. Two types of control conditions were used: mitochondria were incubated in the presence of a peptide composed of the same amino acids as the amyloid fragment 25–35 but in the reverse sequence (35–25), or with the same volume of water, the vehicle in which the peptides were dissolved.

2. Materials and methods

All chemicals and enzymes used were supplied by The Sigma Chemical Company (Poole, UK), BDH (Dagenham, UK) or Boehringer Mannheim (Lewes, UK). CoQ1 was the kind gift of the Eisai Chemical Company (Tokyo, Japan).

Non-synaptic brain mitochondria were isolated from adult male Wistar rats (B&K, Hull, UK; approx. 250–300 g), using the method of Lai and Clark [29]. The purified mitochondrial fraction was resuspended in isolation medium (320 mM sucrose, 1 mM potassium EDTA, 10 mM Tris-HCl, pH 7.4) at 4°C at a protein concentration of ~1 mg/ml. The preparations were freeze-thawed three times before the assays to allow rapid access of the substrates and cofactors to the enzymes.

The amyloid peptide fragment 25–35 and the reverse sequence peptide 35–25 were dissolved in deionised double distilled water at the concentration of 1 mM and kept in aliquots at −70°C.

A hundred microlitres of the mitochondria were incubated with 10 μl of vehicle, or with the same volume of peptide solution to give the desired final concentration. Incubations were carried out at 20°C for the indicated times, and the samples immediately added to a cuvette for the enzyme activity measurement.

The activities of mitochondrial complex I [30], complex II–III [31], complex IV [32] and citrate synthase [33] were measured spectrophotometrically at 30°C.

Mitochondrial protein concentration was measured using the method of Lowry et al. [34], with bovine serum albumin used as a standard.

One-way ANOVA was used for statistical analysis. Where a significant difference was detected, the Tukey test for multiple comparisons was applied. Correlation was evaluated by calculating the correlation coefficient (r), and the significance was tested using published tables of critical values of the r distribution.

3. Results

Fig. 1 shows the activities of mitochondrial complex I, complexes II–III, complex IV and citrate synthase after 5 min incubation with 100 μM peptides. There was no significant difference between the specific activities in the three groups for complex I, complex II–III or citrate synthase. However, there was a significant difference in the activity of complex IV ($P < 0.001$). The two control groups were not different from each other, whereas the group incubated with the 25–35 peptide was significantly lower (−37%, $P < 0.05$). Similar results were obtained when activities were expressed in relation to the activity of citrate synthase, taken as a marker of the mitochondrial matrix (−35.5%, $P < 0.005$).

Fig. 2 shows the titration of the activity of complex IV with various concentrations of the 25–35 peptide. The activity of the enzyme is inhibited by the peptide in a dose-dependent way. The graph shows a significant linear correlation between the logarithm of peptide concentration and activity

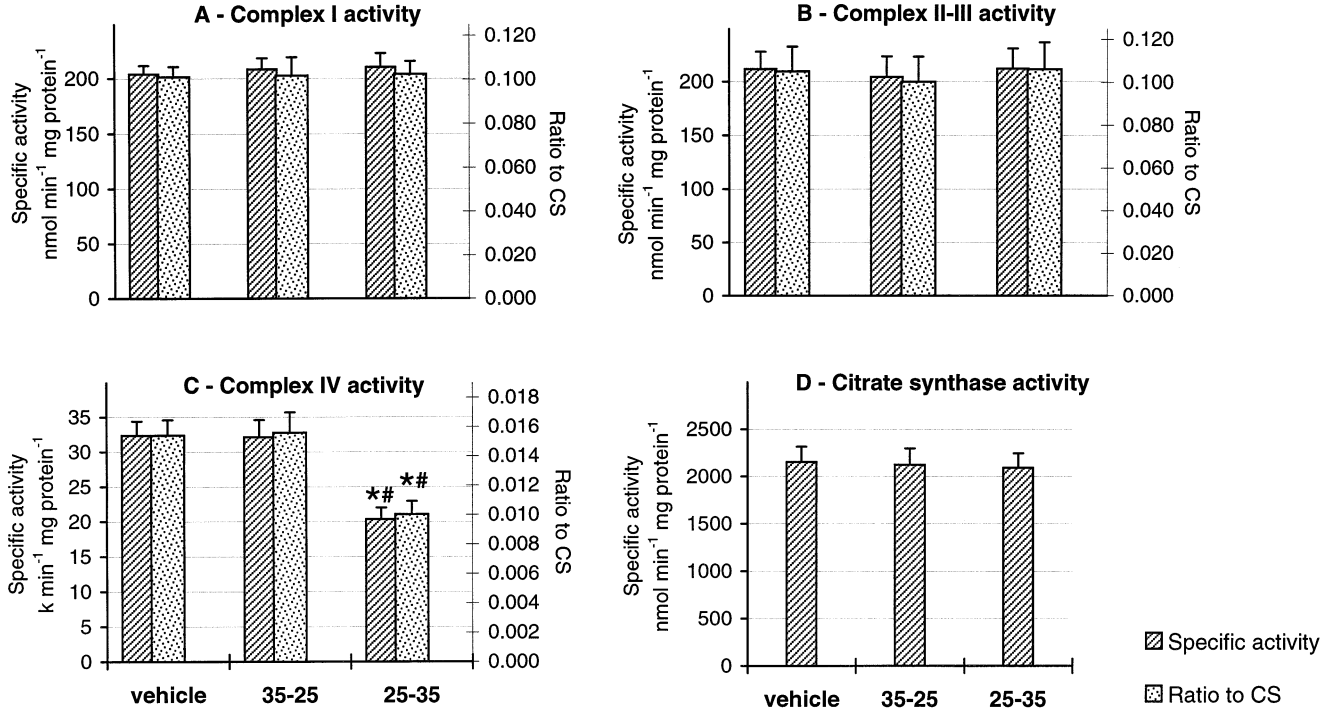

Fig. 1. Enzyme activities of respiratory chain complexes I (A), II–III (B), IV (C) and citrate synthase (D) in mitochondria incubated for 5 min in the presence of water (vehicle), 100 μM reverse sequence peptide (35–25) or 100 μM amyloid fragment 25–35 (25–35). Data are the means ± S.E.M. of $n = 7$–8 experiments. Enzyme activities are shown as specific activity (hatched column; expressed in nmol min^{-1} mg $protein^{-1}$, except complex IV activity, which is expressed in k min^{-1} mg $protein^{-1}$) and as ratio to citrate synthase activity (dotted column). *$P < 0.05$ vs vehicle; #$P < 0.05$ vs 35–25, Tukey test.

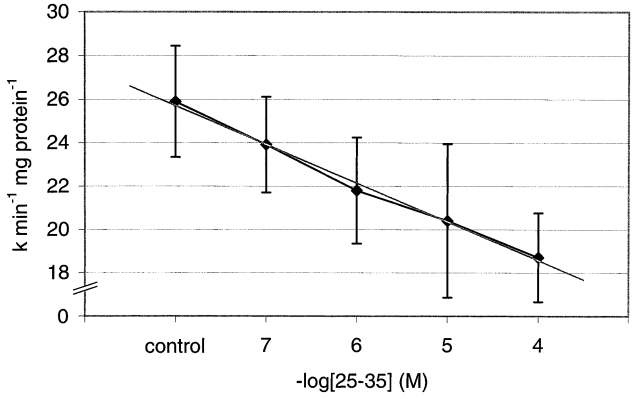

Fig. 2. Enzyme activity of complex IV of the mitochondrial respiratory chain in the presence of various concentrations of amyloid 25–35 peptide, or reverse sequence peptide (control). Each point is the mean ± S.E.M. of 3–11 experiments. Activity is expressed in k min^{-1} mg $protein^{-1}$.

($r = 0.9973$; $P < 0.001$). The calculated apparent K_i is approximately 100 mM.

Fig. 3 shows the time course of complex IV activity inhibition by incubation with 100 μM amyloid fragment 25–35. The specific activity decreased in a biphasic manner, very rapidly at first, and subsequently at a much slower rate over the following 30 min of incubation.

A hundred micromolar 25–35 peptide did not affect ferrocytochrome *c* autooxidation or ferricytochrome *c* autoreduction in the absence of mitochondrial sample (not shown).

4. Discussion

We performed a short-term incubation of isolated mitochondria in the presence of the amyloid fragment 25–35 with the rationale that if a direct effect was taking place involving, as has been suggested, perturbation of lipid membranes [35], it would be very rapid. The concentration of peptide we initially used (100 μM) may appear high compared to reported experiments on cultured cells where the peptides were present for hours or days, however: (1) although the aggregation of the 25–35 peptide is reported to be immediate in solution, we sought to optimise the conditions for aggregation (a prerequisite for toxicity), which increases with peptide concentration; (2) we aimed at emulating the scenario in which the amyloid generated intracellularly may be locally very concentrated. The data show that the 25–35 peptide can specifically decrease mitochondrial complex IV activity by acting directly on isolated mitochondria. This effect was observed also at lower concentrations of peptide, in a dose-dependent manner, and took place very rapidly (less than 1 min).

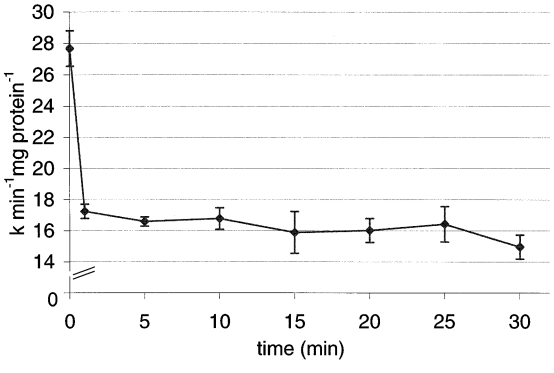

Fig. 3. Time course of complex IV activity inhibition by incubation with 100 μM amyloid fragment 25–35. Each point is the mean ± S.E.M. of three experiments. Activity is expressed in k min^{-1} mg $protein^{-1}$.

Complex IV activity is particularly sensitive to the fluidity and composition of the membrane lipid environment; in particular bound cardiolipin, phosphatidylcholine and phosphatidylethanolamine are essential for activity [36]. It has been shown that these lipids can undergo peroxidation, causing loss of complex IV activity [37,38]. Aggregates of Aβ interact with lipid membranes [35] perturbing their structure. Intracellular Aβ may modify mitochondrial membranes, and inhibit complex IV, thereby increasing the production of superoxide at complexes I–III [16]. This would induce lipid peroxidation which further damages the activity of complex IV, and promote the aggregation of more Aβ [27]. Increased lipid peroxidation and decreased complex IV activity are found in brain tissue from AD patients [12,13,17]. Mitochondrial dysfunction would also impair calcium homeostasis and generally reduce the availability of energy for cellular functions, such as glutamate uptake: this could also explain the increased sensitivity to excitatory amino acid toxicity in cells treated with the peptide [3].

In summary, these results support the possibility of a central role of mitochondria in the neurodegenerative process induced by the amyloid peptide, by a direct inhibition of the activity of mitochondrial complex IV.

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