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β-Amyloid inhibits integrated mitochondrial respiration and key enzyme activities

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Abstract

Disrupted energy metabolism, in particular reduced activity of cytochrome oxidase (EC 1.9.3.1), α-ketoglutarate dehydrogenase (EC 1.2.4.2) and pyruvate dehydrogenase (EC 1.2.4.1) have been reported in post-mortem Alzheimer's disease brain. β-Amyloid is strongly implicated in Alzheimer's pathology and can be formed intracellularly in neurones. We have investigated the possibility that β-amyloid itself disrupts mitochondrial function. Isolated rat brain mitochondria have been incubated with the β-amyloid alone or together with nitric oxide, which is known to be elevated in Alzheimer's brain. Mitochondrial respiration, electron transport chain complex activities, α-ketoglutarate dehydrogenase activity and pyruvate dehydrogenase activity have been measured. β-Amyloid caused a significant reduction in state 3 and state 4 mitochondrial respiration that was further diminished by the addition of nitric oxide. Cytochrome oxidase, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase activities were inhibited by β-amyloid. The K_m of cytochrome oxidase for reduced cytochrome *c* was raised by β-amyloid. We conclude that β-amyloid can directly disrupt mitochondrial function, inhibits key enzymes and may contribute to the deficiency of energy metabolism seen in Alzheimer's disease.

Keywords: Alzheimer's disease, amyloid, energy metabolism, mitochondria, nitric oxide.
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Aggregated β-amyloid peptide (Aβ) is the core component of brain neuritic plaques that are a defining feature of Alzheimer's disease (AD). Aβ is a 39–42 amino acid peptide which is thought to play a critical role in the pathogenesis of AD. There is strong evidence that excess Aβ production is sufficient to cause AD, including the fact that mutations in the amyloid precursor protein gene that lead to excessive production of Aβ are sufficient to give rise to an autosomal dominant form of AD (Citron *et al*. 1992). However the precise mechanism by which Aβ may contribute to neurodegeneration in AD is yet to be elucidated. Soluble Aβ monomers aggregate to form antiparallel β-pleated sheets as shown by circular dichroism studies (Pike *et al*. 1995). Aβ neurotoxicity requires aggregation with both aggregation and toxicity being inhibited by Congo red (Lorenzo and Yankner 1994).

There is considerable evidence implicating elevated oxidative stress in both the pathology of Alzheimer's disease and also the mechanism of toxicity of Aβ. A wide range of studies have shown markers of oxidative stress to be elevated in brain with Alzheimer's disease: lipid peroxidation, protein oxidation, DNA oxidation and glycooxidation (for review see Praticó and Delanty 2000). Treatment of cultured neurones with Aβ increases markers of oxidative stress such as protein carbonyls and lipid peroxidation products (Harris *et al*. 1995; Mark *et al*. 1997). It has been claimed that Aβ can spontaneously generate peptide radicals in solution (Hensley *et al*. 1994). However, this observation has been recently challenged and it has been shown that Aβ only generates free radicals in the presence of metal ions (Dikalov *et al*. 1999; Turnbull *et al*. 2001). Nevertheless Aβ, with associated trace levels of iron and copper, is able to generate reactive oxygen species (Huang *et al*. 1999), which may in turn oxidize cellular lipids, proteins and DNA.

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Abbreviations used: Aβ, β-amyloid peptide; AD, Alzheimer's disease; BSA, bovine serum albumin; C-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt; FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone; α-KGDHC, α-ketoglutarate dehydrogenase complex; LDH, lactate dehydrogenase; PDHC, pyruvate dehydrogenase complex; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

Defects in energy metabolism are a consistent feature of AD-affected brain. In addition recent studies have shown impaired energy metabolism in Aβ-treated neuronal cells. Decreased regional cerebral metabolic rates for glucose have been reported in affected brain regions of Alzheimer's patients even when corrected for brain atrophy (Ibanez *et al*. 1998). The activities of key enzymes of glycolysis and the tricarboxylic acid cycle, pyruvate dehydrogenase complex (PDHC) and α-ketoglutarate dehydrogenase complex (α-KGDHC), are lower in brain taken post mortem from Alzheimer's patients compared with controls (Sorbi *et al*. 1983; Butterworth and Besnard 1990; Mastrogiacoma *et al*. 1996). There is also a reduction in the activity of cytochrome oxidase (Kish *et al*. 1992; Mutisya *et al*. 1994; Parker *et al*. 1994). Sims *et al*. (1987) have reported partial mitochondrial uncoupling in neocortical brain tissue homogenate obtained from patients with Alzheimer's disease. Studies with cultured PC12 cells have also demonstrated Aβ-induced inhibition of respiratory chain complexes and reduction of cellular ATP levels (Pereira *et al*. 1998; Pereira *et al*. 1999). Inhibition of the respiratory chain, in particular cytochrome oxidase, can contribute to oxidative stress by production of reactive oxygen species as a result of electron leak at complexes I and III. Conversely, oxidative stress can contribute to impaired energy metabolism by damaging enzymes. Cytochrome oxidase has been shown to be susceptible to free radical-induced damage via oxidation of the phospholipid cardiolipin (Paradies *et al*. 1998). Pyruvate dehydrogenase and α-ketoglutarate dehydrogenase were both found to be very sensitive to inhibition by the lipid peroxidation product 4-hydroxynonenal (Humphries and Szweda 1998).

Recent studies have indicated that intracellular production of Aβ occurs in neurones (Turner *et al*. 1996; Tienari *et al*. 1997) where it aggregates to form an insoluble pool (Skovronsky *et al*. 1998). $A\beta_{1-42}$ is formed at the ER and intermediate compartment (Cook *et al*. 1997). Since neuronal mitochondria are thought to be in close association with ER (Perkins *et al*. 1997), it is reasonable to suppose that the local concentration of Aβ will be higher in the proximity of mitochondria than in the cell as a whole. Therefore it is important to consider the direct effects of Aβ on mitochondria as a possible contributing factor to neurodegeneration in AD. Studies in our laboratory (Canevari *et al*. 1999) and others (Parks *et al*. 2001) have demonstrated that the Aβ specifically inhibits cytochrome oxidase in mitochondria isolated from rat brain and liver, respectively. The latter study concluded that Aβ was capable of inducing mitochondrial permeability transition that could be blocked by cyclosporin A. In the present study we have investigated the effect of $A\beta_{25-35}$ and $A\beta_{1-42}$ on integrated mitochondrial function by measuring state 3 and state 4 respiration rates utilizing glutamate + malate and succinate. We have also studied the effect of the shortened Aβ fragment, $A\beta_{25-35}$, on individual mitochondrial enzymes, in isolated brain mitochondria and in purified form, in order to ascertain whether inhibition is a direct effect on the enzyme or is mediated by effects on mitochondrial lipid.

$A\beta_{25-35}$ is a widely used substitute for full-length $A\beta_{1-40/42}$. It contains the residues essential for aggregation and toxicity including the methionine 35 (Pike *et al*. 1995). $A\beta_{25-35}$ aggregates rapidly to form a β-pleated sheet structure without the need for 'aging' procedures required for $A\beta_{1-40/42}$ aggregation. The toxicity of $A\beta_{25-35}$ is very similar to that of $A\beta_{1-42}$ on hippocampal neuronal cultures (Pike *et al*. 1993).

There is evidence for increased nitric oxide (NO) production in AD (for a review see Law *et al*. 2001). Increased levels of nitrotyrosine (Smith *et al*. 1997) and nitric oxide synthase positive astrocytes adjacent to Aβ-containing plaques (Wallace *et al*. 1997) have been found in AD-affected brain tissue. Aβ activates microglia *in vitro* leading to elevated NO production (Ii *et al*. 1996). Glial-derived NO may exacerbate oxidative stress caused by Aβ, worsening the toxic insult to which neurones are subjected. NO is also a potent inhibitor of respiration (Brown and Cooper 1994). Superoxide, whether formed by Aβ or due to electron leak from the respiratory chain, may react rapidly with NO to form peroxynitrite, a potent oxidant that severely disrupts neuronal energy metabolism (Bolaños *et al*. 1995). Therefore in this study we have also explored the effects of Aβ in conjunction with authentic NO, on brain mitochondrial function.

Materials and methods

Materials

The β-amyloid peptides $A\beta_{1-42}$ and $A\beta_{25-35}$ were used in this study. The latter aggregates rapidly in solution and shares most of the biological properties of the full-length aggregated Aβ. The soluble, non-aggregating reverse sequence peptide, $A\beta_{35-25}$, was used as a control. All peptides were purchased from Bachem UK Ltd. (Merseyside, UK). The peptides were dissolved in double-distilled deionized water at a concentration of 5 mM ($A\beta_{25-35}$ and $A\beta_{35-25}$) or 1 mM ($A\beta_{1-42}$). The $A\beta_{1-42}$ solution was 'aged' by incubation at room temperature for 24 h, followed by one freeze–thaw cycle. Aliquots were stored at − 70°C. Aggregation of $A\beta_{25-35}$ and $A\beta_{1-42}$ was verified by the thioflavin-T fluorometric assay (LeVine 1993). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) was obtained from Molecular Probes Europe BV (Leiden, the Netherlands). Unless otherwise stated all other chemicals were obtained from Sigma Chemical Company (Poole, UK).

Mitochondria

Non-synaptic brain mitochondria were isolated from male Wistar rats (Tuck and Son, Battlesbridge, Essex, UK) weighing approximately 250–300 g, using the method of Lai and Clark (1979). The mitochondrial fraction was resuspended in respiration buffer (100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM Tris-

phosphate, pH 7.4) and stored on ice prior to measurement of respiration rate.

Oxygen and NO electrodes

Oxygen consumption rates were measured polarographically using a Clark-type O_2 electrode housed in a Perspex, water-jacketed chamber with a volume of 250 μL maintained at 30°C. The electrode was connected to a chart recorder.

For some experiments O_2 tension was measured in parallel with NO concentration. For these a glass water-jacketed O_2 electrode chamber (Rank Brothers, Bottisham, Cambridge, UK) was modified to allow the simultaneous insertion of a 2-mm shielded NO electrode (World Precision Instruments, Sarasota, FL, USA) via the stopper. The stopper was constructed from the oxygen-impermeant ceramic, Micor® (RS, Radio Spares, UK). The NO electrode was connected to an ISO-NO I NO meter. Data was collected using a MacLab™ data collection and analysis system (ADI Instruments, Castle Hill, New South Wales, Australia). The chamber volume was set at 800 μL and the temperature maintained at 37°C.

NO solutions were prepared as in Sharpe and Cooper (1998), with slight modification. Crude NO gas was prepared in a Kipps apparatus by the addition of 2 M H_2SO_4 to solid $NaNO_2$. The NO was then purified by serial passage through three 20% NaOH traps, followed by a solid NaOH drying trap and then a dry ice trap. The NO was dissolved in double-distilled deionized water that had previously been deoxygenated.

Measurement of respiration rates

Ten microlitres of mitochondrial suspension was added to the chamber to give final concentration of 0.4 mg protein/mL. Final concentrations of 10 mM glutamate and 5 mM malate were added as substrates in some experiments. In other experiments respiration was supported by 10 mM succinate in the presence of 2 μM rotenone (to block complex I). Bovine serum albumin (BSA; 2 mg/mL) was added to prevent uncoupling of mitochondria by free fatty acids. 100 μM ADP was added to stimulate state 3 respiration. Carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP; 1 μM) was added to uncouple mitochondria.

In experiments involving addition of NO, 25–30 μL of mitochondrial suspension was added to the chamber to give an exact final concentration of 0.5 mg protein/mL. NO (3 μM) was added as a single dose. In order to completely remove NO, the NO scavenger carboxy-PTIO (30 μM) was added.

Enzyme activity determination

Following measurement of respiration rate, the mitochondrial suspension was removed from the electrode chamber and frozen in liquid nitrogen until used to measure the activities of the mitochondrial complexes. The mitochondrial suspension was subjected to three freeze–thaw cycles to disrupt membranes and expose enzymes. All enzyme assays were performed at 30°C. Complex I (EC 1.6.99.3) (Ragan *et al*. 1987), complexes II–III (EC 1.8.3.1) (King 1967), complex IV (cytochrome oxidase) (EC 1.9.3.1) (Wharton and Tzagoloff 1967) and the matrix enzyme citrate synthase (EC 4.1.3.7) (Shepherd and Garland 1969) were measured spectrophotometrically. α-KGDHC (EC 1.2.4.2) activity was measured using the method of Lai and Cooper (1986). Pyruvate dehydrogenase complex (PDHC; EC 1.2.4.1) activity was measured using method 3 of Elnageh and Gaitonde (1988). Purified porcine heart PDHC and α-KGDHC were purchased from Sigma UK.

Purified bovine cytochrome oxidase (EC 1.9.3.1) (Sigma, UK) activity was determined by measuring O_2 consumption polarographically in the oxygen electrode chamber. Enzyme (5 nM) was incubated in 100 mM potassium phosphate buffer, pH 7.0, 0.015% (w/v) *n*-dodecyl α-D-maltoside, 37°C, using 20 mM sodium ascorbate and 300 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) as an electron donor system (Sharpe and Cooper 1998).

Nitrotyrosine ELISA

Nitrotyrosine was measured using an ELISA kit (ZID 7500 A, TCS Cellworks Ltd, Bucks, UK) with nitrated BSA as standard. This method uses a rabbit anti-nitrotyrosine primary antibody and a donkey anti-rabbit IgG antibody coupled to horse radish peroxidase as secondary antibody. Standard curve of nitrated BSA was linear over the concentration range tested. The assay was performed as per manufacturers' instructions.

Protein content determination

Protein concentration was determined by the method of Lowry *et al*. (1951) using BSA as protein standard.

Statistical analysis

Data is presented as mean ± standard error of the mean (SEM). One- or two-way ANOVA was used for statistical comparisons. Where significant differences were detected, the Tukey post-test (for multiple comparisons) or the Dunnett's post-test (for multiple comparisons against control) were applied. Curve fitting was performed, where appropriate, using non-linear regression. Data analysis and statistical comparisons were performed using Graphpad PRISM (Graphpad Software Inc., CA, USA).

Results

Effects of Aβ on mitochondrial respiration

$A\beta_{25-35}$ and $A\beta_{1-42}$ significantly inhibited mitochondrial respiration. Figure 1 shows typical traces of oxygen consumption by isolated mitochondria utilizing glutamate and malate as substrates following 10 minute incubation at room temperature with the control peptide $A\beta_{35-25}$ (Fig. 1a) and $A\beta_{25-35}$ (Fig. 1b). The mitochondria were well coupled as shown by the clear increase in respiration rate following addition of ADP. Average respiration rates for mitochondria incubated with $A\beta_{35-25}$, $A\beta_{25-35}$, and $A\beta_{1-42}$ are shown in Table 1. State 3 and state 4 respiration was significantly inhibited by both $A\beta_{25-35}$ and $A\beta_{1-42}$ for both glutamate + malate and succinate as substrates. Neither peptide had any significant effect on RCR, though RCR was lower when succinate was used as substrate compared with glutamate + malate. It is clear from this data that Aβ does not uncouple mitochondria.

Effects of NO combined with Aβ on mitochondrial respiration

Nitric oxide is a potent inhibitor of respiration and is believed to be involved in the pathogenesis of AD. In order to

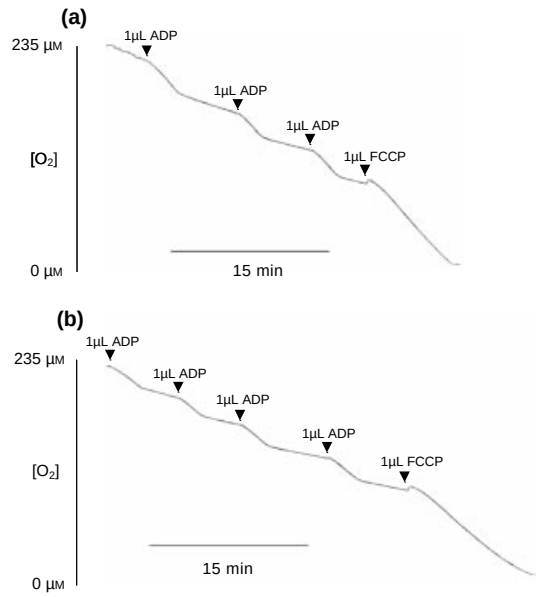

Fig. 1 Effect of Aβ on mitochondrial respiration. Representative oxygen electrode traces are shown for isolated mitochondria (0.4 mg protein/mL final concentration) at 30°C with glutamate and malate as substrates. Mitochondria were incubated with 50 μM $A\beta_{35-25}$ (a) or 50 μM $A\beta_{25-35}$ (b) for 10 min at room temperature. State 3 respiration was stimulated by addition of 100 μM ADP (final concentration). State 4 respiration followed the phosphorylation of all the ADP added. FCCP (1 μM) was added to uncouple mitochondria.

Table 1 Respiration rates of treated mitochondria in the presence of glutamate plus malate, or succinate plus rotenone

	$A\beta_{35-25}$	$A\beta_{25-35}$	$A\beta_{1-42}$
Glutamate/malate			
State 3	67.2 ± 3.2	46.5 ± 5.4*	51.6 ± 2.4*
	100%	69%	77%
State 4	20.6 ± 2.0	11.0 ± 1.7*	12.6 ± 1.7*
	100%	53%	61%
Uncoupled	67.8 ± 2.3	48.5 ± 5.2*	57.4 ± 1.3
	100%	72%	85%
RCR	3.30 ± 0.2	4.27 ± 0.16	4.26 ± 0.51
Succinate			
State 3	138.9 ± 16.8	86.0 ± 4.2*	84.1 ± 10.3*
	100%	62%	61%
State 4	100.2 ± 10.5	64.9 ± 3.9*	68.7 ± 3.5*
	100%	65%	69%
Uncoupled	128.3 ± 13.6	67.1 ± 9.2*	76.9 ± 9.0*
	100%	52%	60%
RCR	1.38 ± 0.03	1.32 ± 0.03	1.21 ± 0.09

State 3, state 4 and uncoupled respirations rates are given in nmol(O)/min/mg protein for mitochondria incubated for 10 min with $A\beta_{35-25}$, $A\beta_{25-35}$ and $A\beta_{1-42}$ all at 50 μM. The percentage change compared to control is also shown. The respiratory control ratio (RCR) is given for each condition. Mean ± SEM, $^*p < 0.05$ compared with $A\beta_{35-25}$-treated, $n = 3$.

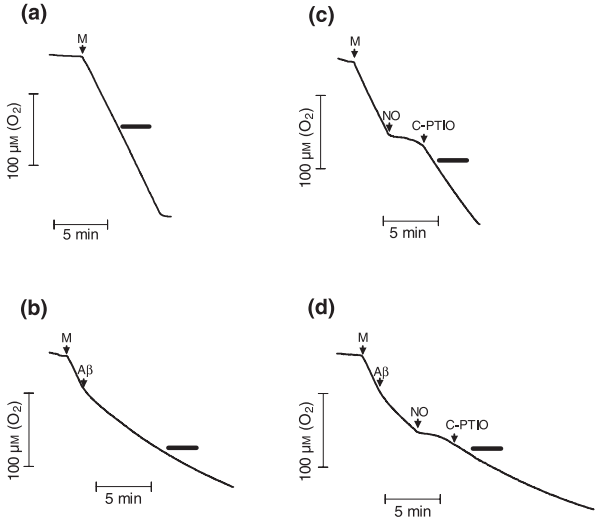

Fig. 2 Effects of Aβ and NO on state 4 mitochondrial respiration. Typical oxygen electrode traces are shown for isolated mitochondria (0.5 mg protein/mL final concentration) at 37°C with glutamate and malate as substrates. M indicates addition of mitochondria. Measurement of respiration rate was taken over a period of 3 min as indicated by dark bars. (a) Control mitochondria. (b) 8 μL of 5 mM $A\beta_{25-35}$ was added to the electrode chamber containing mitochondria to give a final concentration 50 μM. (c) Mitochondria had 3 μM NO added at approximately 50% maximal O_2 tension. NO was completely removed by addition of 30 μM of the NO scavenger carboxy-PTIO. (d) $A\beta_{25-35}$ was added as in (b), followed by a bolus addition of 3 μM NO at approximately 50% O_2 tension and then 30 μM C-PTIO was added to remove any residual NO.

investigate the effects of NO on Aβ-induced inhibition of respiration separate experiments were performed in a modified chamber that permits recording of O_2 tension in parallel with NO concentration. Figure 2 shows typical oxygen electrode traces for the action of 50 μM $A\beta_{25-35}$ and/or 3 μM NO on isolated, non-synaptic rat brain mitochondria. Mitochondria treated with vehicle or reverse peptide displayed linear oxygen consumption until oxygen tension was ≤ 2 μM (Fig. 2a). $A\beta_{25-35}$ (50 μM) induced a rapid and significant inhibition of state 4 respiration within 1 minute, that appeared to increase in magnitude over subsequent minutes (Fig. 2b). Addition of 3 μM NO initially abolished mitochondrial respiration. Following the decay of NO and the addition of the NO scavenger C-PTIO, mitochondrial respiration showed partial recovery (Fig. 2c). This indicates that a substantial proportion (43%) of the NO-mediated inhibition of respiration is irreversible in brain mitochondria. When NO was added to the mitochondria in the presence $A\beta_{25-35}$ (Fig. 2d), respiration after NO decay was significantly lower than that with either treatment alone. No differences were seen between mitochondria incubated with the reverse peptide, $A\beta_{35-25}$ and those incubated without peptide.

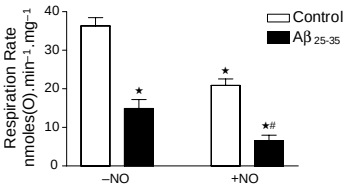

Fig. 3 State 4 respiration rates determined from six independent experiments, as described in Fig. 1, are shown for control mitochondria (left open bar, □) and mitochondria treated with $A\beta_{25-35}$ (50 μM) (left solid bar, ■), also NO (3 μM) alone (right open bar, □) or together with $A\beta_{25-35}$ (50 μM) (right solid bar, ■). Values are expressed as nanomoles O_2/min/mg protein corrected for background O_2 consumption due to the electrode. ANOVA, Tukey's *post-hoc* test, $n = 6$. $^*p < 0.05$ versus control, $\#p < 0.05$ versus all other treatments. Mean ± SEM.

Figure 3 shows average respiration rates from six independent experiments calculated from oxygen traces over a 3 minute time interval indicated by the dark bars on Fig. 2. Both 50 μM $A\beta_{25-35}$ and 3 μM NO caused significant inhibition of respiration (down 59% and 43%, respectively). The combination of 50 μM $A\beta_{25-35}$ and 3 μM NO caused an average 82% inhibition of respiration. Control respiration rate was faster than that shown in Table 1 due to the higher temperature used in these experiments (37°C versus 30°C) and the omission of the 10 min incubation at room temperature.

In order to establish whether peroxynitrite formation played a significant role in the effects of NO in combination with Aβ, mitochondrial suspension that had been treated with 50 μM $A\beta_{25-35}$ and/or 3 μM NO was tested for the presence of nitrotyrosine, a marker of peroxynitrite. Nitrotyrosine ELISA of the mitochondrial sample gave the following values (in ng nitrotyrosine per mg protein): 6 ± 1 (control), 8 ± 1 ($A\beta_{25-35}$), 16 ± 2 (NO) and 13 ± 1 ($A\beta_{25-35}$ + NO). Therefore peroxynitrite formation from NO was not enhanced by the presence of $A\beta_{25-35}$.

Effects of Aβ and NO on respiratory chain complexes

Incubation of mitochondria for 20 min with $A\beta_{25-35}$ (50 μM) at 37°C had no effect on either complex I activity (Fig. 4a), or activities of complexes II–III (Fig. 4b), while complex IV activity was inhibited by 50% (Fig. 4c). A bolus addition of NO (3 μM) did not affect the activity of any of the complexes; NO did not alter the effect of $A\beta_{25-35}$ on any of the complexes. There was no significant change in the specific activity of citrate synthase with any treatment condition. No differences were seen between mitochondria incubated with the reverse peptide, $A\beta_{35-25}$ and those incubated without peptide.

Direct effects of Aβ on mitochondrial enzymes

$A\beta_{25-35}$ inhibited purified cytochrome oxidase in a dose-dependent manner (Fig. 5a). The IC_{50} for $A\beta_{25-35}$ on cytochrome oxidase turnover was approximately 60 μM. $A\beta_{25-35}$ (50 μM) and $A\beta_{1-42}$ (50 μM) shifted the apparent

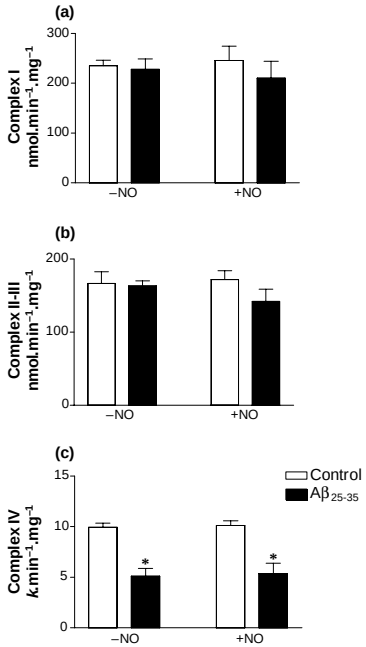

Fig. 4 Effects of Aβ and NO on mitochondrial respiratory chain complex activities. Enzyme activities expressed as nmol/min/mg protein are shown for complex I (a), complexes II–III (b) and complex IV (expressed as rate constant *k*/min/mg protein (c). The following treatment conditions are shown: control mitochondria (left open bars, □); mitochondria treated with $A\beta_{25-35}$ (50 μM) (left solid bar, ■); NO (3 μM) alone (right open bar, □); NO (3 μM) together with $A\beta_{25-35}$ (50 μM) (right solid bar, ■). ANOVA, Tukey's *post-hoc* test, $n = 4$, $^*p < 0.05$ versus control. Mean ± SEM.

K_m for cytochrome *c* oxidation by purified cytochome oxidase from 44 ± 3 μM to 88 ± 6 μM and 136 ± 10 μM, respectively (Fig. 5b).

Aβ caused a dose-dependent decrease in α-KGDHC activity in isolated brain mitochondria (Fig. 6a); the IC_{50} for $A\beta_{25-35}$ on α-KGDHC was approximately 20 μM. In addition Aβ inhibited purified α-KGDHC in a dose-dependent fashion with an IC_{50} of approximately 100 μM (Fig. 6b). Inhibition was not due to direct oxidation of NADH by $A\beta_{25-35}$ as the peptide had no effect on the activity of lactate dehydrogenase (LDH), an enzyme also measured by monitoring NADH concentration spectrophotometrically at 340 nm (results not shown).

$A\beta_{25-35}$ caused a dose-dependent inhibition in PDHC in both isolated mitochondria (Fig. 7a) and the purified enzyme (Fig. 7b). The IC_{50} for $A\beta_{25-35}$ on PDHC in brain mitochondria was approximately 70 μM while that for purified PDHC was approximately 90 μM.

Discussion

The present study describes the effects of Aβ alone and together with NO, on the respiratory function of mitochondria

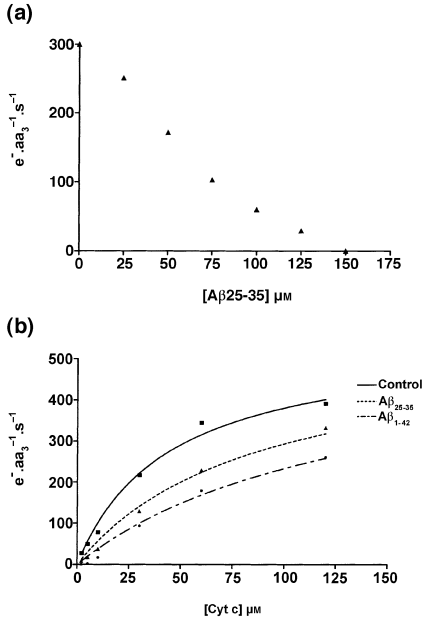

Fig. 5 Effect of $A\beta_{25-35}$ on purified cytochrome oxidase activity. (a) Cytochrome oxidase activity was measured in the presence of $A\beta_{25-35}$ at a range of concentrations up to 150 μM. (b) The enzyme activity was also measured at a range of cytochrome *c* concentrations in the absence of peptide (■), the presence of 50 μM $A\beta_{25-35}$ (▲), or the presence of 50 μM $A\beta_{1-42}$ (●). Enzyme activity is expressed as electrons per aa_3 centre per second. Curve fit was to the Michaelis–Menten equation using non-linear regression.

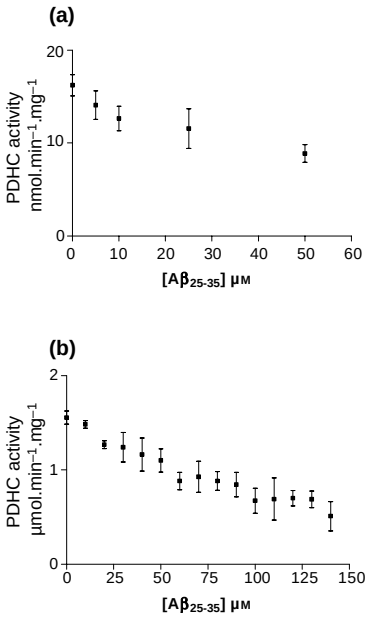

Fig. 7 Effect of $A\beta_{25-35}$ on pyruvate dehydrogenase complex (PDHC) activity. The activity of PDHC was measured following 30 min incubation of brain mitochondria with varying concentrations of $A\beta_{25-35}$ (a). Activity of purified PDHC was measured in the same manner (b). Activity was measured at 30°C over a period of 10 min ANOVA, Tukey's *post-hoc* test, $n = 3$, $^*p < 0.05$, $^{**}p < 0.01$ compared with control. Mean ± SEM.

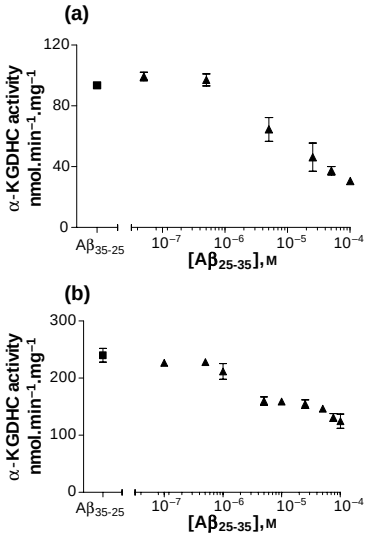

Fig. 6 Effect of $A\beta_{25-35}$ on α-ketoglutarate dehydrogenase complex (α-KGDHC) activity. The activity of α-KGDHC was measured following 30 min incubation of brain mitochondria with varying concentrations of $A\beta_{25-35}$ (a). The reverse peptide $A\beta_{35-25}$ (50 μM) was used as control. Activity of purified α-KGDHC was measured in the same manner, also following 30 min incubation with varying concentrations of $A\beta_{25-35}$ (b). Activity was measured at 30°C over a period of 10 min ANOVA, Tukey's *post-hoc* test, $n = 3$, $^*p < 0.05$ versus control. Mean ± SEM.

isolated from rat whole brain. $A\beta_{25-35}$ and $A\beta_{1-42}$ at 50 μM caused a significant drop in state 3 and state 4 respiration. The mitochondrial enzyme complexes cytochrome oxidase, α-KGDHC and PDHC were inhibited significantly by $A\beta_{25-35}$, with or without mitochondrial membrane present. NO caused a further inhibition of mitochondrial respiration, potentiating the effects of $A\beta_{25-35}$.

Aβ inhibits mitochondrial respiration

The mechanism by which Aβ inhibits integrated mitochondrial respiration is not known. By measuring state 3 and state 4 respiration, we have shown that Aβ does not cause mitochondrial uncoupling. This was in contrast to the results of Sims *et al.* (1987), who reported an elevated rate of respiration in the absence of ADP in neocortical homogenate derived from Alzheimer's patients. It is possible that agents other than Aβ that accumulate during AD, for example oxidized lipids, may cause mitochondria to become partially uncoupled. The decreased respiration rate following treatment of mitochondria with Aβ can be explained in part by the observed inhibition of cytochrome oxidase. The outer mitochondria membrane is relatively permeable and should afford access of Aβ to cytochrome oxidase. The contribution of α-KGDHC inhibition to the inhibition of

respiration is difficult to assess. Although we have shown that Aβ inhibits α-KGDHC in permeabilized mitochondria and in purified form, it is unclear whether Aβ could penetrate the inner mitochondria membrane to gain access to α-KGDHC in intact mitochondria. Aβ inhibited succinate-supported mitochondrial respiration to a similar extent as glutamate + malate-supported respiration. Since utilization of glutamate requires α-KGDHC activity, whilst utilization of succinate does not, this result appears to suggest that inhibition of α-KGDHC does not contribute significantly to the inhibition of respiration. However the electron flux through the respiratory chain when utilizing succinate is faster for a given mitochondrial membrane potential than it is when utilizing glutamate + malate. Therefore, inhibition of cytochrome *c* binding to cytochrome oxidase by Aβ would be expected have a greater effect on succinate-supported respiration. Our results suggest that inhibition of cytochrome oxidase is the dominant mechanism by which Aβ inhibits mitochondrial respiration, but we cannot rule out a contribution by inhibition of other enzymes such as α-KGDHC and PDHC for substrates upstream of these enzymes.

Mechanism of enzyme inhibition by Aβ

It has been proposed that Aβ exerts its effects by the production of free radicals, which in turn cause damage to membranes and protein subunits (Hensley *et al*. 1994). In the present study we have shown that the inhibitory effect of $A\beta_{25-35}$ on cytochrome oxidase, α-KGDHC and PDHC occurs also in the absence of biological membranes. This finding argues that $A\beta_{25-35}$ directly interacts with these enzyme complexes, rather than through an effect on the membrane microenvironment such as oxidation of cardiolipin or via the production of the toxic lipid-derived species 4-hydroxynonenal, as has been shown to occur in cultured hippocampal neurones (Mark *et al*. 1997). The direct effects of $A\beta_{25-35}$ on these enzyme complexes could be mediated by binding of the peptide to the protein subunits. The apparent K_m of cytochrome oxidase for reduced cytochrome *c* was raised (Fig. 5b), indicating that $A\beta_{25-35}$ may act as an inhibitor for the cytochrome *c* binding. Parker and Parks (1995) have reported that cytochrome oxidase purified from AD brain showed a loss of one of the two kinetically identifiable binding sites for reduced cytochrome *c*. Such structural changes were thought to be the result of Aβ binding to or altering the reduced cytochrome *c* high affinity binding site. We suggest that $A\beta_{25-35}$ may bind in a non-specific manner to the cytochrome oxidase complex preventing access of reduced cytochrome *c* to its binding site on cytochrome oxidase. It is tempting to speculate that a similar mechanism of non-specific binding may explain the inhibition of α-KGDHC and PDHC. For example, Aβ may prevent the motility of lipoic acid required for both enzymes to function. Direct peptide–protein interaction has been suggested to explain the inhibition of phosphofructokinase by Aβ (Bigl and Eschrich 1995).

An alternative explanation for the effects of $A\beta_{25-35}$ on the enzyme complexes is via the production of reactive oxygen species, that in turn damage protein subunits and/or essential cofactors. Any such mechanism cannot depend on the formation of lipid peroxidation products as $A\beta_{25-35}$ caused inhibition even in the absence of lipid membranes. We have determined that $A\beta_{25-35}$ does not cause oxidation of reduced cytochrome *c* (results not shown), however, oxidation of protein subunits cannot be ruled out. $A\beta_{25-35}$ has been shown to inactivate glutamine synthetase and creatine kinase rapidly (within minutes) at concentrations similar to that used in the present study (Hensley *et al*. 1994). The inactivation of the two enzymes was suggested to be the result of Aβ-derived free radicals attacking the proteins. Inhibition of cytochrome oxidase would be expected to increase the production of superoxide and hydrogen peroxide by increasing electron leak from upstream components of the respiratory chain such as ubiquinone and the cytochrome b_{566} in complex III. This additional source of reactive oxygen species could further damage mitochondrial enzymes leading to a vicious cycle.

The concentrations required to inhibit cytochrome oxidase, α-KGDHC and PDHC (IC_{50}s in the micromolar range) may appear high in comparison to the low levels of Aβ found in the CSF of AD patients. However the local concentration of Aβ in the vicinity of mitochondria may be considerably higher. An insoluble pool of $A\beta_{1-42}$ formed at the ER/intermediate compartment could result in Aβ aggregates forming in close proximity to mitochondria. If the hydrophobic Aβ aggregates insert into mitochondrial membrane the effective concentration of Aβ experienced by mitochondrial enzymes would be much greater than the average cytosolic concentration.

There is evidence for a systemic cytochrome oxidase deficiency in Alzheimer's disease. Platelets from AD patients show reduced cytochrome oxidase activity despite having no interaction with amyloid plaques (Parker *et al*. 1990). However, a genetic defect that gives rise to a systemic loss of cytochrome oxidase activity does not rule out additional inhibition of brain cytochrome oxidase activity by Aβ. We propose that individuals with a deficiency in cytochrome oxidase of a genetic origin may be particularly vulnerable to Aβ and therefore predisposed to AD. Similarly our results suggest that Aβ-induced inhibition of α-KGDHC and PDHC may explain the reduced activity of these enzyme complexes in AD brain tissue, but do not rule out other factors that may contribute to their reduced activity such as genetic abnormalities and environmental factors.

NO, Aβ and mitochondrial respiration

NO caused an immediate and partially reversible inhibition of mitochondrial respiration. At low concentrations, NO has been proposed to act as a reversible inhibitor of mitochondrial respiration, by competing with oxygen at the binuclear centre of cytochrome oxidase (Brown and Cooper 1994). Higher concentrations of NO have been shown to cause inhibition of respiration that is not completely reversible. Brown and collaborators (Brown *et al*. 1998) investigated the effects of macrophage-derived NO on the respiration of cultured fibroblasts. NO (0.5 μM) inhibited respiration almost completely. Close examination of the data presented shows that when NO was removed from solution with oxyhaemoglobin, respiration only partially recovered to approximately 85% of the pre-NO rate. This is consistent with the irreversible inhibition reported in the present study. Astrocytes, stimulated with cytokines to produce endogenous NO for a period of 18 h, have decreased respiration rate (Brown *et al*. 1995). This NO-induced inhibition was only partially reversible upon treatment with the nitric oxide synthase inhibitor, N^G-methylarginine. Cytochrome oxidase is one possible site for the irreversible inhibition caused by NO. This has been reported by Sharpe and Cooper (1998), who examined the effects of NO on purified cytochrome oxidase under steady state turnover. They showed that 1 μM NO caused an irreversible inhibition of the enzyme by some 15%. However in the present study NO (3 μM) did not cause a decrease in the v_{max} of any of the respiratory chain complexes when measured independently (Fig. 4). It appears that the irreversible component of inhibition of respiration by NO in non-synaptic brain mitochondria is not due to permanent damage to the respiratory chain complexes, but rather an effect on the intact mitochondria. Irreversible inhibition due to NO may have important implications *in vivo* under pathological conditions where NO concentration may reach micromolar levels.

The combined treatment of mitochondria with $A\beta_{25-35}$ and NO caused a greater inhibition of respiration than either treatment alone. We examined the possibility that peroxynitrite formation was involved by measuring levels of nitrotyrosine, a marker for peroxynitrite, in mitochondrial sample. NO elevated nitrotyrosine above control levels, but $A\beta_{25-35}$ did not cause in further elevation in the presence of NO. Peroxynitrite is a potent inhibitor of the respiratory chain (Radi *et al*. 1994; Bolaños *et al*. 1995), yet the combination of $A\beta_{25-35}$ and NO did not inhibit respiratory chain complexes apart from the inhibition of complex IV equal to that seen with $A\beta_{25-35}$ alone. Therefore we suggest that NO and Aβ inhibit respiration independently, not via peroxynitrite formation. The combined effect is a drastic reduction in respiratory rate.

The effects of Aβ alone and in the presence of NO reported here would be expected to be detrimental to cellular energy metabolism. In particular, neurones rely almost exclusively on oxidative phosphorylation to meet their energy demands and therefore are very susceptible to mitochondrial damage. The observed deficits in energy metabolism of the AD brain, namely impaired activity of cytochrome oxidase, α-KGDHC and PDHC may all be attributed, at least in part, to intracellular Aβ interacting with neuronal mitochondria. The ability of NO to further exacerbate Aβ-induced damage to mitochondria may have important implications *in vivo* as any condition which elevates brain NO levels, such as infection, ischaemic episodes or head trauma could potentially accelerate neuronal damage in Alzheimer's patients.

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