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Gradual Alteration of Mitochondrial Structure and Function by β-Amyloids: Importance of Membrane Viscosity Changes, Energy Deprivation, Reactive Oxygen Species Production, and Cytochrome *c* Release

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Intracellular amyloid beta-peptide (Aβ) accumulation is considered to be a key pathogenic factor in sporadic Alzheimer's disease (AD), but the mechanisms by which it triggers neuronal dysfunction remain unclear. We hypothesized that gradual mitochondrial dysfunction could play a central role in both initiation and progression of sporadic AD. Thus, we analyzed changes in mitochondrial structure and function following direct exposure to increasing concentrations of $A\beta_{1-42}$ and $A\beta_{25-35}$ in order to look more closely at the relationships between mitochondrial membrane viscosity, ATP synthesis, ROS production, and cytochrome *c* release. Our results show the accumulation of monomeric Aβ within rat brain and muscle mitochondria. Subsequently, we observed four different and additive modes of action of Aβ, which were concentration dependent: (i) an increase in mitochondrial membrane viscosity with a concomitant decrease in ATP/O, (ii) respiratory chain complexes inhibition, (iii) a potentialization of ROS production, and (iv) cytochrome *c* release.

KEY WORDS: Alzheimer's disease; amyloid-β peptide; threshold effect; oxidative phosphorylation; membrane fluidity; cytochrome *c* release.

INTRODUCTION

Extensive evidence suggest a central role for amyloid-β peptide (Aβ) overproduction in the pathogenesis of Alzheimer's disease (AD). In familial cases of AD, mutations in the amyloid-β peptide precursor (APP) or in genes that regulate its processing (PS1, PS2), are responsible for Aβ overproduction. However, in common late-onset sporadic form, such mutations are not present and the pathogenesis remains unknown. Moreover, the mechanisms by which the abnormal accumulation of Aβ in the cerebral cortex and the hippocampus could be responsible for neuronal dysfunction and cognitive decline are also unclear. A central controversy surrounds the indentity of the toxic form of Aβ *in vivo* (deposit, membrane bound, fibrils, soluble oligomers, intermediary forms) and whether extracellular Aβ deposition or intraneuronal Aβ overproduction precedes the disease process. According to the "amyloid cascade hypothesis" (Hardy and Higgins, 1992) extracellular Aβ (in the form of senile plaques)

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Abbreviations used: AD, Alzheimer's disease; Aβ, amyloid beta peptide; ADP, adenosine diphosphate; ATP, adenosine triphosphate; COX, cytochrome *c* oxidase; CS, citrate synthase; DPH, 1,6 diphenyl-1,3,5-hexatriene; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; J_{O_2}, respiratory rate; J_{ATP}, rate of ATP synthesis; η, viscosity; OXPHOS, oxidative phosphorylation; PTP, permeability transition pore; PS, presinilin; *r*, fluorescence anisotropy; RCR, respiratory control ratio; ROS, reactive oxygen species, SDH, succinate dehydrogenase; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

initiate a progressive synaptic and neuritic injury that leads to oxidative stress, widespread neuronal dysfunction, and, finally, cell death. Several analyses now contradict the "amyloid cascade hypothesis," with observations of synaptic and neuronal dysfunction appearing before senile plaque and tangle pathology (de la Torre, 2004). A recent alternative to account for the imperfect correlation between insoluble fibrillar amyloid deposits and AD progression considers that the pathogenic species are soluble diffusible Aβ oligomers generated before the senile plaques (Klein, 2002). However, this hypothesis still considers that neuronal dysfunction and degeneration are caused by extracellular Aβ at the synaptic level.

In contrast, recent studies on neurons in culture and autopsied AD brains have raised the possibility that neuronal dysfunction and degeneration could be caused by an intraneuronal gradual accumulation of Aβ rather than by an extracellular process (D'Andrea *et al*., 2001; Fernandez-Vizarra *et al*., 2004; Gouras *et al*., 2000; Skovronsky *et al*., 1998). This so-called "intracellular hypothesis" is based on cell biological studies that have first demonstrated that Aβ was generated within neurons (Li *et al*., 1999; Petanceska *et al*., 2000), essentially in the endoplasmic reticulum and the Golgi. Then, it was shown that Aβ was accumulated progressively inside these neurons (Fernandez-Vizarra *et al*., 2004) from where it triggered cell dysfunction before plaque formation and tangle pathology (Chui *et al*., 2001; Knauer *et al*., 1992; Oddo *et al*., 2003; Skovronsky *et al*., 1998; Takahashi *et al*., 2002; Turner *et al*., 1996; Wirths *et al*., 2001; Zhang *et al*., 2002). In the end, the accumulated Aβ could be massively released by neuronal lysis to form the senile plaque (D'Andrea *et al*., 2001).

In this "intracellular hypothesis," a large amount of evidence suggest that mitochondria could intervene in the mechanisms by which intraneuronal Aβ triggers neuronal dysfunction and degeneration. These include first the *in vivo* demonstration of Aβ accumulation within mitochondria of brains from AD patients (Fernandez-Vizarra *et al*., 2004; Lustbader *et al*., 2004), as well as the observation of mitochondrial structural abnormalities (Hirai *et al*., 2001). Second, impairement of mitochondrial oxidative phosphorylation was also extensively reported in the brain of AD patients (Chagnon *et al*., 1995), the degree of impairment being proportional to clinical disability (Blass, 2003). In particular, these observations consistently revealed a deficient mitochondrial respiration with a complex IV (cytochrome *c* oxidase) enzymatic defect. Similar observations were also reported on platelets from AD patients (Bosetti *et al*., 2002). However, it is important to note that complex IV activity defects are not specific to AD, and that mutations in mitochondrial or nuclear DNA genes encoding some of its subunits or assembly proteins are the cause of various neuromuscular disorders belonging to the group of mitochondrial diseases (Darin *et al*., 2003). Interestingly in AD such mutations are not found, and several observations suggest that a direct interaction of Aβ with mitochondrial membranes could be the cause of complex IV inhibition *in vivo* (Canevari *et al*., 1999; Mancuso *et al*., 2003). Taken together, these observations raised the possibility that intraneuronal accumulation of Aβ in mitochondrial membranes could impair organellar functions and participate in the physiopathology of AD.

To analyze the mechanisms involved, studies were performed on mitochondria isolated from rat brain and other tissues. They evidenced a deleterious direct action of micromolar Aβ concentrations on mitochondrial respiration (Canevari *et al*., 1999), ATP synthesis (Moreira *et al*., 2003), and the activity of various enzymes involved in energy production (Casley *et al*., 2002a,b; Gibson *et al*., 1998; Hensley *et al*., 1994; Shoffner, 1997). The mechanisms evidenced include a direct inhibition of enzyme activity (Canevari *et al*., 1999), a perturbation of mitochondrial membrane properties (Muller *et al*., 2001), and the set-up of a vicious cycle of oxidative damage. However, their relative importance in Aβ mitochondrial toxicity, as well as their time course, were never analyzed. Other investigations performed on animal models of AD (Bales *et al*., 1997; Holcomb *et al*., 1998), neuron or neuron-like cell lines treated with Aβ (Casley *et al*., 2002a,b; Pereira *et al*., 1998) also revealed a defect in mitochondrial energy production. However, in these studies the presence of Aβ in the mitochondrion was unfortunately never investigated, and the sequence of mitochondrial alterations not assessed. In addition, the tumor-derived cellular models generally used in these studies derive energy for growth essentially from glycolysis rather than mitochondrial oxidative phosphorylation, so that the depletion of ATP levels observed in these cells treated with extracellular Aβ is more likely to result from the inhibition of glycolysis by Aβ (Bigl and Eschrich, 1995) rather than OXPHOS impairment. These transformed cells also present high mitochondrial membrane potential and high cytosolic level of ROS, so that a minor oxidative insult such as the ROS generated by Aβ itself could suffice to initiate a vicious cycle of ROS damage with subsequent mitochondrial impairment and apoptosis.

Thus, this situation does not allow yet to propose a unifying model to explain the deleterious effect of Aβ on mitochondrial function, and the role of this organelle in the pathogenesis of AD. In particular, to understand the

relationships between mitochondrial membrane fluidity changes, ATP synthesis, ROS production, and cytochrome *c* release in response to Aβ accumulation would be of great value to evaluate the relative importance of energy deprivation, oxidative damage, and apoptosis in the disease process.

For this reason we determined the *in vitro* effect of increasing concentrations of $A\beta_{25-35}$ and $A\beta_{1-42}$ on mitochondria isolated from rat brain and muscle with identical experimental conditions. This is the first study to compare the effect of two Aβ peptides, used at various concentrations chosen according to previous studies (see Discussion), on critical oxidative phosphorylation parameters, and to observe the consequences of OXPHOS impairment in mitochondria isolated from different tissues. We observed initially the mitochondrial accumulation of $A\beta_{25-35}$ and $A\beta_{1-42}$ following a short-time incubation with toxic aggregates of Aβ and subsequently, a progressive impairment of both physical and functional properties of the mitochondrial inner membrane, according to four different and additive mechanisms. From the sole changes observed in membrane viscosity, brain mitochondria appeared twice more sensitive to Aβ than muscles. However, when we looked at the effect of Aβ on ATP synthesis both tissues presented a similar level of inihibition. Likewise, no significant difference was observed between brain and muscle when we looked at the effect of Aβ on respiratory chain complexes activity, ROS generation, or cytochrome *c* release. Taken together, our data suggest the idea of an early and progressive alteration of mitochondrial function along the course of intracellular Aβ accumulation.

EXPERIMENTAL

Chemicals

$A\beta_{1-42}$, $A\beta_{25-35}$, and $A\beta_{35-25}$, trypsin, antimycin, carboxyatractyloside (CATR), cyanide (KCN), oligomycin, rotenone, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), iodoacetate, and EDTA were purchased from Sigma. ATP Bioluminescence Assay Kit HS II was purchased from Boehringer Mannheim. DPH was obtained from Molecular Probes Inc.

Aβ Solutions

$A\beta_{1-42}$ (toxic), $A\beta_{25-35}$ (toxic), and $A\beta_{35-25}$ (scrambled-nontoxic) were first solubilized in dimethylsulfoxide (DMSO) at an initial concentration of 10 mM and stored at −80°C. All peptides were dissolved in PBS at a concentration of 1 mM. $A\beta_{1-42}$ was stored for 5 days at 37°C to allow aggregation, while $A\beta_{25-35}$ was aged by incubation for 24 h at 37°C. Aggregation state (data not shown) was monitored using the congo red analysis as described previously (Klunk *et al*., 1989). To take into account the large variability in the use of Aβ (Cotman *et al*., 1992; May *et al*., 1992) we followed the guidelines reported in Methods in Enzymology Vol. 309 (1999), concerning Aβ solubilization, aging, and utilization. In addition, before each experiment we verified both the integrity of mitochondrial preparations by polarography (RCR determination), and the toxicity of the Aβ solution by determining its effect on mitochondrial respiratory rate and COX activity. Then it could be used for Western Blotting or membrane fluidity studies.

Mitochondrial Incubation with Aβ

To study the effect of Aβ on mitochondrial respiration, ATP synthesis, enzyme activities, cytochrome *c* release, and membrane fluidity, mitochondria were incubated with $A\beta_{1-42}$ or $A\beta_{25-35}$ at different concentrations ranging from 10 to 100 μM (see below) for 20 min at 25°C. For each experiment, control samples were prepared in identical conditions with the scrambled control peptide $A\beta_{35-25}$ used at 10 and 100 μM. In each experiment detailed in the Results section, no significant effect ($P < 0.05$) was observed on the different parameters analyzed (J_{O2}, J_{ATP}, cytochrome *c* release . . .) after addition of this control peptide (data not shown).

Animals

Male Wistar rats weighing 250 g having free access to water and standard laboratory diet were used for this study. Experimental animals were sacrificed by cervical shock and decapitation.

Preparation of Rat Muscle Mitochondria

Rat muscle mitochondria were isolated by differential centrifugation. Muscle from two hindlegs were collected in Isolation Medium I (210 mM mannitol, 70 mM sucrose, 50 mM Tris/HCl pH 7.4, 10 mM EDTA) and digested by trypsin (0.5 mg/g of muscle) for 30 min. The reaction was stopped by addition of trypsin inhibitor (soja bean 3:1 inhibitor to trypsin). The homogenate was

centrifuged at 1000 × *g* for 5 min. The supernatant was strained through gauze and centrifuged at 7000 × *g* for 10 min. The resulting pellet was resuspended in ice-cold Isolation Medium II (225 mM mannitol, 75 mM sucrose, 10 mM Tris/HCl pH 7.4, 0.1 mM EDTA) and a new series of centrifugations (1000 × *g* and 7000 × *g*) was performed. The last mitochondrial pellet was resuspended into a minimum volume of Isolation Medium II in order to obtain a mitochondrial concentration between 50 and 80 mg/mL. Protein concentration was measured by the Biuret method using bovine serum albumin as standard.

Preparation of Rat Brain Mitochondria

Brain mitochondria were isolated from whole brain. Rats were sacrificed by decapitation without stunning and the brains were removed and homogenized in Isolation Buffer (250 mM sucrose, 10 mM Tris/HCl pH 7.4, and 0.5 mM K+ EDTA). The homogenate was centrifuged at 1000 × *g* for 5 min. The supernatant was strained through gauze and recentrifuged at 7000 × *g* for 10 min. The resulting pellet was resuspended in ice-cold Isolation Buffer and a new series of centrifugations (1000 × *g* and 7000 × *g*) was performed. The crude mitochondrial pellet was resuspended in a final volume of 10 mL in 3% Ficoll Medium (3% Ficoll, 250 mM sucrose, 10 mM Tris/HCl pH 7.4, and K^+ 0.5 mM EDTA). This suspension was carefully layered onto 20 mL of 6% Ficoll Medium (6% Ficoll, 250 mM sucrose, 10 mM Tris/HCl pH 7.4, and 0.5 mM K^+ EDTA) and centrifuged for 30 min at 11,500 × *g*. The mitochondrial pellet was resuspended in Isolation Medium and centrifuged for 10 min at 12,500 × *g*. The mitochondria were made up to a concentration of about 50 mg of protein per mL in the isolation buffer.

Respiration Measurements

Mitochondrial oxygen consumption was monitored at 30°C in a 1 mL thermostatically controlled chamber equipped with a Clark oxygen electrode (Oxy 1, Hansatech), in Respiration Buffer: 75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM Tris Phosphate, 10 mM Tris/Hcl pH 7.4, 50 μM EDTA, plus respiratory substrates (10 mM pyruvate in the presence of 10 mM malate or 25 mM succinate). The mitochondrial concentration used for respiration measurements was 1 mg/mL, and state 3 was obtained by addition of 2 mM ADP. The respiratory rates were expressed in ngatom O/min/mg proteins. The respiratory control ratio (RCR) is defined as the ratio of state 3 (in the presence of ADP) to state 4 (in the absence of ADP) respiratory rates.

ATP Synthesis Recording

Mitochondrial ATP synthesis was followed in the cuvette of the oxygraph containing mitochondria (1 mg) in 1 mL of respiratory buffer plus respiratory substrates (see above). Steady-state ATP synthesis was initiated by adding 2 mM ADP, and was recorded during 2 min as follows: every 30 s after ADP addition, 10 μL aliquots were withdrawn, quenched in 100 μL DMSO, and diluted in 5 mL of ice-cold distilled water. For each sample collected from the cuvette, the quantity of ATP was measured by bioluminescence in a Luminoskan using the ATP monitoring reagent (ATP Bioluminescence Assay Kit HS II) from Boehringer Mannheim. Standardization was performed with known quantities of standard ATP provided with the kit (5, 10, 15, 20, 25 pmoles) measured in the same conditions. The rate of ATP synthesis was calculated using a linear regression. Rates were expressed in nmol ATP/min/mg of mitochondrial protein.

ATP/O Calculation

The efficiency of mitochondrial ATP production is given by the number of moles of ATP produced by the F_1F_o-ATPsynthase per gatom of oxygen consumed by the respiratory chain. This so-called ATP/O ratio can be calculated accurately from the data obtained during respiration and ATP synthesis measurements as the ratio of mitochondrial ATP synthesis rate over mitochondrial respiratory rate (state 3), determined simulteanously in the same experimental conditions as explained above.

ROS Generation Measurement

The rate of H_2O_2 production in mitochondria incubated with amyloid-β peptides was determined by fluorimetry using *p*-hydroxyphenylacetate (PHPA) on a Jobin–Yvon fluorometer. This nonfluorescent substrate is oxidized to the stable fluorescent product 2,2′-dihydroxybiphenyl-5,5′-diacetate by the enzymatic reduction of H_2O_2 by horseradish peroxidase (Hyslop and Sklar, 1984). The excitation wavelength was set at 315 nm and the signal recorded at 425 nm. Standardization was performed with known quantities of H_2O_2 measured in the same conditions. The results are expressed in pmol H_2O_2/min/mg. To take into account differential topology

of H_2O_2 production we added external SOD (20 U/mL) when precised in the text.

Cytochrome *c* Release Assay

Cytochrome *c* release was followed on energized isolated mitochondria (1 mg/mL), incubated in the same conditions as for the respiration and ATP synthesis measurements (respiratory buffer described above plus 10 mM pyruvate and 10 mM malate). Total cytochrome *c* content was determined using 0.1% deoxycholate. After incubation with or without Aβ, the samples were centrifuged at 10,000 × *g* for 10 min, and the supernatant and pellet analyzed for the presence of cytochrome *c* (as well as porin) by Western Blotting (see below) using an anticytochrome *c* polyclonal mouse antibody (Zymed Laboratories Inc.).

Determination of Aβ Accumulated in Isolated Mitochondria

After exposure to $A\beta_{1-42}$ mitochondria (500 μg/500 μL) were centrifuged at 10,000 × *g* for 10 min, and the pellet was analyzed for the presence of Aβ by Western Blotting (see below) using a polyclonal antiamyloid-β peptide rabbit antibody (Zymed Laboratories Inc.), catalog number 71-5800. The immunogen used was a 30 amino-acid synthetic peptide derived from the full-length (1-43) Aβ peptide. The specificity of the antibody toward Aβ is guaranteed by the manufacturer (Zymed Inc.) and was verifed against synthetic $A\beta_{1-40}$, human brain sections and ^{35}S methionine-labeled Aβ secreted by transfected cells in culture. Moreover, we also verified by Western-Blotting the presence of signal against synthetic $A\beta_{25-35}$ and $A\beta_{1-42}$ as well as the absence of signal when this antibody was used at the recommended concentration on rat muscle mitochondria. Another criteria for selection of this antibody was the fact that it had also been chosen by other laboratories working in the field (Fukuchi *et al.*, 1998).

Western-Blotting

Samples were diluted into SDS-PAGE tricine sample buffer (BioRad) containing 2% β-mercaptoethanol by incubation for 30 min at 37°C, and separated on a 15% SDS-polyacrylamide mini-gel (Bio-Rad) at 150 V. Proteins were transferred electrophoretically to 0.45 μm polyvinylidine difluoride (PVDF) membranes for 2 h at 100 mA in CAPS buffer (3.3 g CAPS, 1.5 L 10% methanol, pH 11) on ice. Membranes were blocked overnight in 5% milk-PBS + 0.02% azide and incubated for 3 h with the primary antibodies. The protein loading was verified using a polyclonal antiporin antibody (Calbiochem). After three washes with PBS-0.05% Tween 20, the membranes were incubated for 2 h with horseradish peroxidase-conjugated goat antimouse (Biorad) diluted in 5% milk-PBS. This secondary antibody was detected using the chemiluminescent ECL Plus™ reagent (Amersham). The signal was quantified by densitometric analysis using Image J (NIH) sofware.

Enzymatic Determination

Assays of all respiratory chain enzyme activities were carried out spectrophotometrically on a double wavelength $UVMC^2$ spectrophotometer from SAFAS (Monaco), using standardized and reproducible methods. All activities are expressed in nmol/min/mg.

Complex I (NADH Ubiquinone Reductase)

The oxidation of NADH by complex I was recorded using the ubiquinone analogue decylubiquinone (Sigma D-7911) as electron acceptor. The basic assay medium (35 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM KCN, pH 7.2) was supplemented with 2.5 mg/mL BSA, 5 μg/mL antimycin A, 65 μM UQ_1decylubiquinone, and 0.13 mM NADH in a final volume of 1 mL. Enzyme activity was measured by starting the reaction with 50 μg of mitochondrial protein. The decrease in absorption due to NADH oxidation was measured at 340 nm in both the absence and presence of rotenone 5 μg/mL. The difference ΔI in both activities gives the rotenone-sensitive activity of complex I. The extinction coefficient used for NADH was 6.22 mM^{-1} cm^{-1}.

Complex II (Succinate Dehydrogenase)

The assay was performed by following the decrease in absorbance at 600 nm resulting from the reduction of 2,6-dichlorophenolindo-phenol (DCPIP) in 1 mL of medium containing 60 mM KH_2PO_4 (pH 7.4), 3 mM KCN, 20 μg/mL rotenone, 20 mM succinate, and 10 μg mitochondrial protein. The reaction was initiated by the addition of 1.3 mM phenazine methasulfate (PMS) and 0.18 mM DCIP. The extinction coefficient used for DCIP was 21 mM^{-1} cm^{-1}.

Complex III (Ubiquinol Cytochrome c Reductase)

The oxidation of 6.5 mM decylubiquinol by complex III was determined using cytochrome *c* (III) as an electron acceptor. The assay was carried out in basic medium supplemented with 2.5 mg/mL BSA, 15 μM cytochrome *c*(III), and 5 μg/mL rotenone. The reaction was started with 10 μg of mitochondrial protein and the enzyme activity was measured at 550 nm. The extinction coefficient used for cytochrome *c* was 18.5 mM^{-1} cm^{-1}.

Complex IV (Cytochrome c Oxidase)

Two method were used for determining cytochrome *c* oxidase activity were used: Initially, cytochrome *c* oxidase activity was determined spectrophotometrically using cytochrome *c*(II) as substrate. The oxidation of cytochrome *c* was monitored at 550 nm at 30°C. The exctinction coefficient used for cytochrome *c* was 18.5 mM^{-1} cm^{-1}. In the second method, we monitored cytochrome *c* oxidase activity by inhibiting the rest of the respiratory chain with rotenone and antimycin and using 3 mM ascorbate and 0.5 mM TMPD as an electron donor system. The respiratory rate was monitored with the polarographic method described above.

Citrate Synthase

The reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by citrate synthase (CS) at 412 nm (extinction coefficient 13.6 mM^{-1} cm^{-1}) was followed in a coupled reaction with CoA and oxaloacetate. A reaction mixture of 0.2 M Tris-HCl, pH 8.0, 0.1 mM acetyl-CoA, 0.1 mM DTNB, and 5–20 μg of muscle or brain mitochondrial protein was incubated at 30°C for 5 min. The reaction was initiated by the addition of 0.5 mM oxaloacetate, and the absorbance change monitored for 5 min.

ATPase Activity

Complex V ATP hydrolysis activity was determined spectrophotometrically by following the decrease of NADH absorption (340 nm) at 30°C, using an ATP-regenerating system as reported by Bosetti *et al.* (2002). Time-dependent ATPase activity was determined in the presence or absence of 1 μg/mL oligomycin to measure the oligomycin sensitive-ATPase (OS-ATPase).

Membrane Fluidity and Viscosity

Mitochondrial membrane fluidity changes were reported by measuring fluorescence anisotropy changes of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH). In order to incorporate the probe into mitochondrial membrane, 0.2 mg/mL of mitochondria were incubated with DPH (4 μM final) for 20 min at 25°C in the dark. DPH-incorporated mitochondria were incubated with various concentrations of $A\beta_{25-35}$, $A\beta_{1-42}$, and $A\beta_{35-25}$ as described above. Fluorescence anisotropy (r) was measured at 30°C on a Jobin–Yvon JY3NE1 fluoromiter, using excitation and emission wavelengths of 360 and 450 nm, respectively. The fluorescence anisotropy was calculated from emission intensities through a polarizer whose polarization axis was oriented parallel (I_{VV}) and perpendicular (I_{VH}) to the vertical polarization of excitation light. These intensities were corrected from light diffusion measured on mitochondria without DPH. To account for differences in the response of the photomultiplier to polarization of the emission beam and optical bias, we calculated a correction factor $G = I_{HV}/I_{HH}$ (Lakowicz, 1983). I_{HH} and I_{HV} are intensities measured with horizontal polarization of the excitation beam and horizontal or vertical polarization of the emission beam, respectively. The value of anisotropy was thus obtained from the relation: $r = (I_{VV} - G\,I_{VH)}/(I_{VV} + 2G\,I_{VH})$. This anisotropy ($r$) is inversely correlated with the mobility of the acyl chains of fatty acids in the membrane hydrocarbon core, so that a decreased mobility of DPH in the mitochondrial membrane is indicated by an increased value of anisotropy. To better evaluate physical changes in mitochondrial membranes we calculated the mitochondrial membrane viscosity (η). Indeed, Perrin's equation rewritten for anisotropy is: $r^{-1} = r_0^{-1}(1 + RT\tau/\eta V)$. We obtain $\eta = (RT\tau/V)[(r_0/r) - 1]^{-1}$ with $r_0 = 0.362$ the fundamental anisotropy of DPH calculated from the fundamental polarization P_0 (0.46), V the molar hydrodynamic volume (113 cm^3), and τ (10 ns) the lifetime of the probe.

RESULTS

Mitochondrial Preparations

First, we verified mitochondrial membrane integrity and subsequent normal oxidative phosphorylation functioning by determining state 3 respiratory rate (J_{O_2}), respiratory control ratio (RCR), and ATP/O value, following the isolation procedure. Mean values obtained for these parameters on at least four different

mitochondrial preparations, were 134.17 ± 8.57, 3.33 ± 0.51, and 2.91 ± 0.13 ngatomO/min/mg in brain respectively, compared to 212.0 ± 20.14, 4.21 ± 0.49, and 3.16 ± 0.15 ngatomO/min/mg in muscle. All these values correspond to normal control values, as obtained by different authors (Rossignol *et al*., 2000).

Effect of Aβ on Mitochondrial Respiration and COX Activity

In brain mitochondria (Fig. 1(A)), 20 μM Aβ_{25-35} inhibited state 3 respiration (using pyruvate-malate as substrate) by $27 \pm 6.0\%$ with no significant effect on state 4 respiratory rate nor COX activity (measured by polarography using ascorbate-TMPD and endogenous cytochrome *c* as substrates). When Aβ_{25-35} was used at 60 μM, the inhibition of COX activity reached $38 \pm 6.3\%$, associated with a significant effect on state 4 and state 3 respiration (respectively $41 \pm 10.4\%$ and $64 \pm 8.6\%$ inhibition), $P < 0.05$. Using 20 μM Aβ_{1-42}, we observed a stronger inhibition of state 4 and state 3 respiration (respectively 64 ± 9.3 and $81 \pm 7.5\%$ inhibition) as well as for COX activity ($79 \pm 5.8\%$ inhibition). Similar effects were observed with 20 μM Aβ_{1-42} when succinate was used as respiratory substrate, i.e, state 4 was decreased by $76 \pm 14\%$, state 3 by $82 \pm 11\%$, and COX activity by $83 \pm 9.6\%$. On isolated muscle mitochondria (Fig. 1(B)), we observed a similar inhibition of mitochondrial respiration and COX activity by Aβ_{25-35} and Aβ_{1-42}, as compared to brain mitochondria. For instance, in muscle, 20 μM Aβ_{25-35} inhibited state 3 respiration by $24 \pm 8.3\%$ with no significant effect on state 4 respiratory rate and COX activity, as observed in brain. Likewise, 60 μM Aβ_{25-35} decreased state 4 respiratory rate by $43 \pm 6.6\%$, state 3 by $50 \pm 9.0\%$, and COX activity by $49 \pm 8.4\%$. Finally, using 20 μM Aβ_{1-42}, we observed a stronger inhibition of state 4 and state 3 respiration (respectively $64 \pm 9.3\%$ and $75 \pm 10\%$) compared to Aβ_{25-35}, as well as for COX activity ($61 \pm 9.6\%$ inhibition).

Effect of Aβ on Mitochondrial Respiratory Coupling

Calculation of the respiratory control ratio (RCR) revealed a significant effect of Aβ peptides on the coupling of mitochondrial respiration. For instance, on brain mitochondria respiring with pyruvate-malate, the RCR value was 2.84 ± 0.39 after control incubation as compared to 2.16 ± 0.12 with 20 μM Aβ_{25-35}, 1.42 ± 0.36 with 60 μM Aβ_{25-35}, and 1.42 ± 0.52 with 20 μM Aβ_{1-42}. On succinate, the RCR value was equal to 2.57 ± 0.19 after control incubation as compared to 1.77 ± 0.54 after incubation with Aβ_{1-42} 20 μM. Similar results were obtained with muscle mitochondria. For instance, using pyruvate-malate as respiratory substrate, the RCR value was 3.96 ± 0.51 after control incubation as compared to 3.52 ± 0.13 with Aβ_{25-35} 20 μM, 3.46 ± 0.21 with Aβ_{25-35} 60 μM, and 2.74 ± 0.32 with Aβ_{1-42} 20 μM. On succinate, the RCR value in muscle mitochondria was equal to 2.57 ± 0.28 after control incubation as compared to 1.68 ± 0.33 with Aβ_{1-42} 20 μM.

Effect of Aβ on Mitochondrial ATP Synthesis

Concomitant to the respiration measurements detailed above, we analyzed the effect of 20–60 μM Aβ_{25-35} and 20 μM Aβ_{1-42} on mitochondrial ATP synthesis rate (oligomycin sensitive), on brain and muscle isolated mitochondria respiring on pyruvate-malate in the presence of ADP (Fig. 2(A)). First, we observed a large difference in the control rate of ATP production between brain and muscle mitochondria ($J_{ATP} = 262.00 \pm 16.56$ and 554.33 ± 41.91 nmoles ATP/min/mg, respectively). On brain, the rate of ATP production (Fig. 2(A)) strongly decreased after incubation with 20 μM Aβ_{25-35} ($J_{ATP} = 93.59 \pm 5.14$ nmoles ATP/min/mg). This inhibition was further observed after incubation with 60 μM Aβ_{25-35} ($J_{ATP} = 26.16 \pm 5.33$ nmoles ATP/min/mg), and 20 μM Aβ_{1-42} ($J_{ATP} = 9.75 \pm 1.78$ nmoles ATP/min/mg). Similar results were observed with muscle mitochondria (Fig. 2(A)). For instance, in muscle, the rate of ATP synthesis was inhibited by $75 \pm 9.0\%$ after incubation with 20 μM Aβ_{25-35}, $86 \pm 9.0\%$ with 60 μM Aβ_{25-35}, and $94 \pm 11\%$ with 20 μM Aβ_{1-42}, $P < 0.05$.

Effect of Aβ on Mitochondrial Efficiency

ATP/O measured in brain and muscle mitochondria (Fig. 2(B)) after control incubation (ATP/O $= 2.79 \pm 0.10$ and 2.90 ± 0.2, respectively) revealed no significant difference between tissues, as opposed to what can be observed separately at the level of mitochondrial respiration and ATP synthesis where muscle mitochondria show stronger values when compared to brain mitochondria. In both tissues, incubation with 20 μM Aβ_{25-35} decreased the ATP/O ratio by half. This value was further dramatically decreased after incubation with 60 μM Aβ_{25-35} (ATP/O $= 0.85 \pm 0.18$ in brain and 0.80 ± 0.08 in muscle) or 20 μM Aβ_{1-42} (ATP/O $= 0.58 \pm 0.15$ in brain and 0.17 ± 0.07 in muscle).

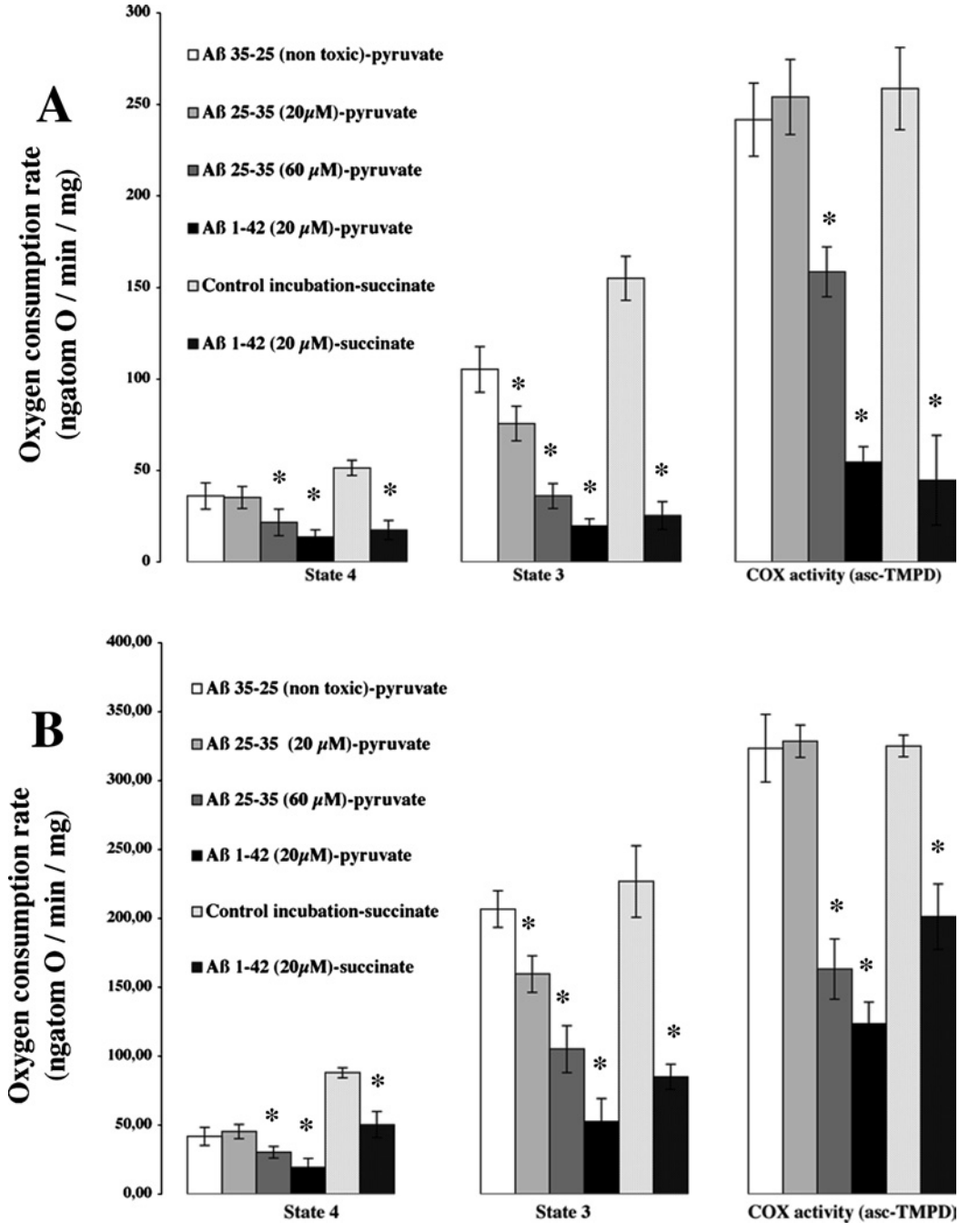

Fig. 1. Effect of Aβ on mitochondrial respiration and COX activity: Mitochondrial respiration at state 4 and state 3 were measured by polarography using either pyruvate-malate or succinate as respiratory substrates. Cytochrome *c* oxidase activity (COX) was also measured by polarography at the end of each run, using ascorbate-TMPD as substrate. These experiments were performed both on brain (A) or muscle (B) isolated mitochondria. The oxygen consumption rate was first recorded using pyruvate-malate as substrate after control incubation with 60 μM Aβ_{35-25} (white bars) or after incubation for 20 min at 25°C in the presence of 20 μM Aβ_{25-35} (grey bars), 60 μM Aβ_{25-35} (dark grey bars), and 20 μM Aβ_{1-42} (black bars). Similar experiments were performed using succinate as respiratory substrate after control incubation (dashed grey bars), or after incubation for 20 min at 25°C in the presence of 20 μM Aβ_{1-42} (dashed black bars). All the results are expressed in ngatom O/min./mg and represent the mean value $\pm$ SD of at least three separate experiments performed on different mitochondrial preparations. The symbol (*) indicates that the value is statistically different from the control incubation value, using the Student's *t* test with $P < 0.05$.

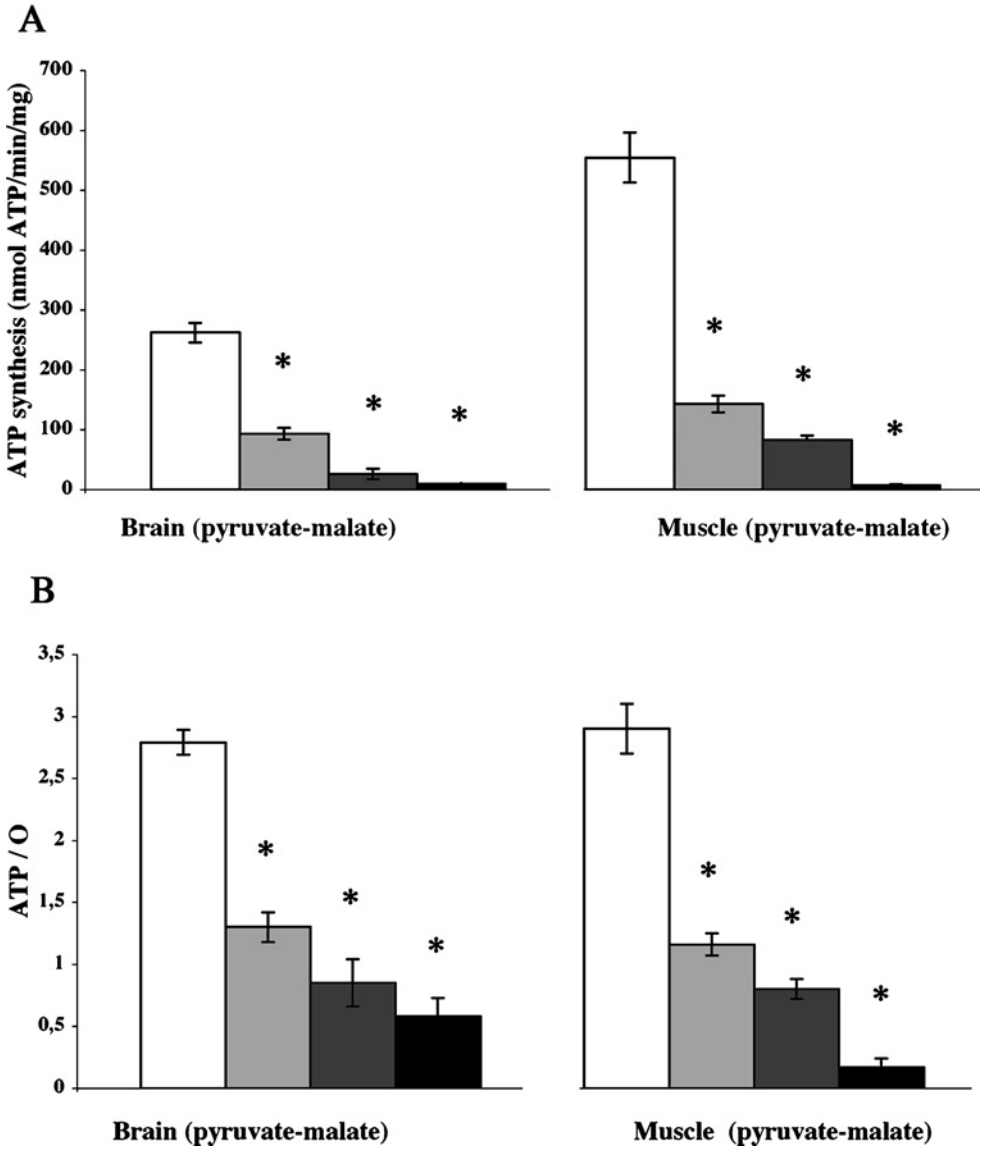

Fig. 2. Effect of Aβ on mitochondrial ATP synthesis and ATP/O ratio: (A) The rate of mitochondrial ATP synthesis was measured concomitantly to the determination of mitochondrial respiration at state 3, using pyruvate-malate as respiratory substrates on brain- and muscle-isolated mitochondria after control incubation with 60 μM Aβ_{35-25} (white bars), or after incubation for 20 min at 25°C in the presence of 20 μM Aβ_{25-35} (grey bars), 60 μM Aβ_{25-35} (dark grey bars), and 20 μM Aβ_{1-42} (black bars). The rate of ATP synthesis was expressed in nmol ATP/min/mg and represents the mean value $\pm$ SD of at least three separate experiments performed on different mitochondrial preparations. (B) The ATP/O ratio (no unit) was calculated as described in the Methods section. The symbol (*) indicates that the value is statistically different from the control incubation value, using the Student's *t* test with $P < 0.05$.

Effect of Aβ on Respiratory Chain Complexes Activity

On brain and muscle mitochondria, we initially verified that Aβ peptides had no effect on citrate synthase activity (CS), which was used to standardize assay results (Letellier *et al.*, 2000). No effect of Aβ on CS activity has also been reported by other groups (Canevari *et al.*, 1999; Casley *et al.*, 2002a,b). We observed tissue differences in enzyme activities (normalized

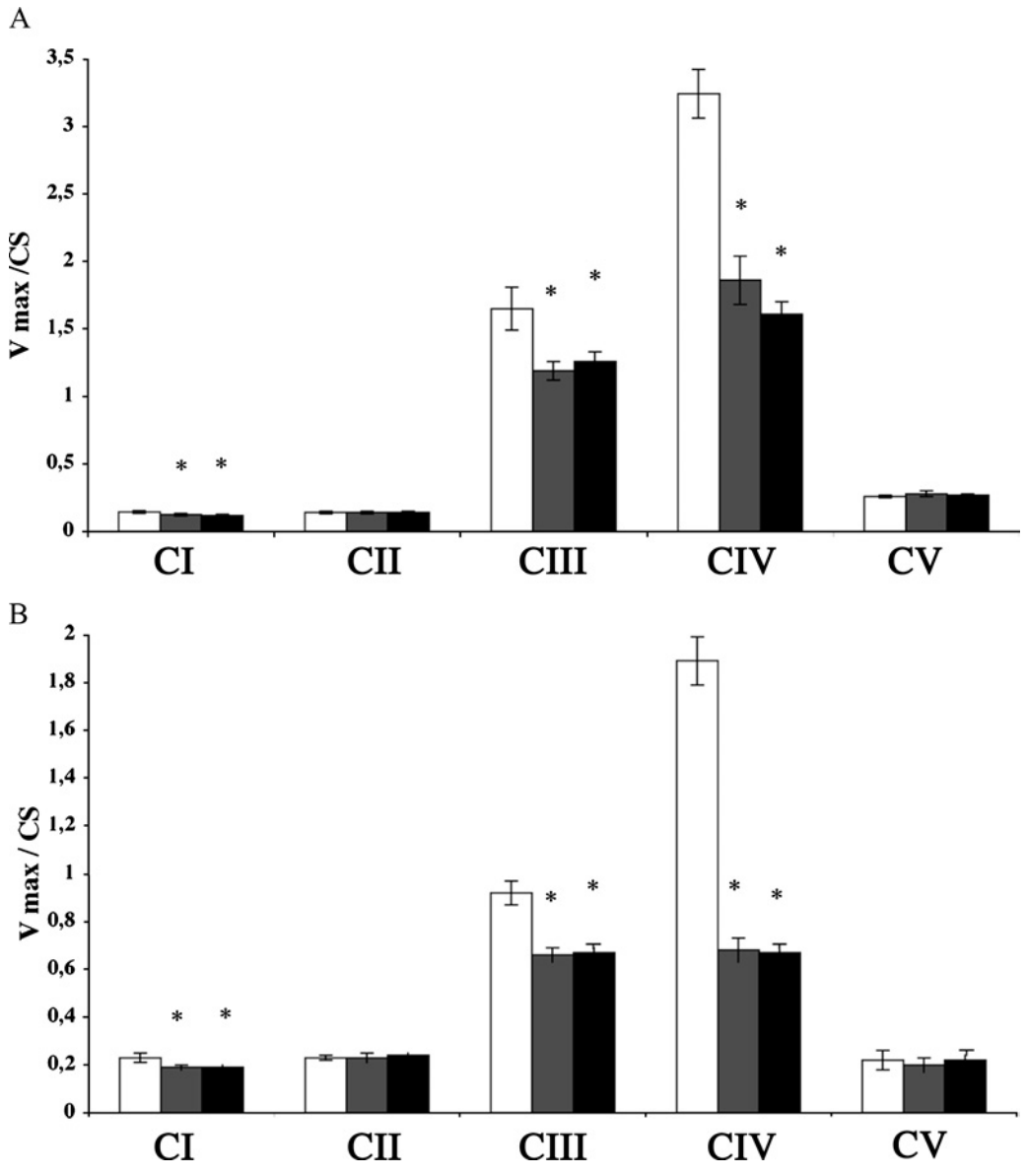

Fig. 3. Effect of Aβ on respiratory chain complexes activity: The activity of respiratory chain complexes I, II, III, IV, and V was measured spectrophotometrically on mitochondria isolated from brain (A) or muscle (B). Raw activity values (V_{max}) were normalized to that of citrate synthase (CS) to take into account variations in the amount of mitochondrial and nonmitochondrial protein contamination between samples. The data represent V_{max}/CS determined after control incubation with 60 μM Aβ_{35-25} (white bars), or after incubation for 20 min at 25°C in the presence of 60 μM Aβ_{25-35} (dark grey bars) and 20 μM Aβ_{1-42} (black bars). Each value represents the mean ± SD of at least three separate experiments performed on different mitochondrial preparations and the symbol (*) indicates that the value is statistically different from the control incubation value, using the Student's *t* test with $P < 0.05$.

to CS) between brain and muscle after control incubation (Fig. 3(A) and (B), respectively). For instance, complex I/CS $= 0.14 \pm 0.01$ in brain vs. 0.23 ± 0.02 in muscle, complex II/CS $= 0.13 \pm 0.01$ vs. 0.24 ± 0.01, complex III/CS $= 1.65 \pm 0.16$ vs. 0.92 ± 0.05, and complex IV/CS (COX) $= 3.24 \pm 0.18$ vs. 1.89 ± 0.12. Complex V/CS (F_1F_0-ATPase) activity was similar in both tissues (0.26 ± 0.01 in brain vs. 0.22 ± 0.04 in muscle). Incubation of mitochondria with 20 μM Aβ_{25-35} produced no effect on all activities assayed in brain and

muscle mitochondria (data not shown). In brain mitochondria, 60 μM Aβ_{25-35} inhibited complex I activity by $13 \pm 2\%$, complex III by $28 \pm 4\%$, and COX by $43 \pm 6\%$ (Fig. 3(A)), $P < 0.05$. In muscle, we observed similar results, i.e, complex I was inhibited by $11 \pm 3\%$, complex III by $26 \pm 3\%$, and COX by $64 \pm 5\%$. In both tissues, complex II and F_1F_o-ATPase remained unaffected by Aβ_{25-35} and Aβ_{1-42} at concentrations as high as 100 μM. In brain mitochondria, incubation with 20 μM Aβ_{1-42} led to a similar inhibition of respiratory chain complexes activity as compared to 60 μM of Aβ_{25-35}, i.e, $17 \pm 3\%$ inhibition on complex I, $24 \pm 4\%$ on complex III, and $51 \pm 6\%$ on COX. Similar results were observed with muscle mitochondria (Fig. 3(B)).

Effect of Aβ on Mitochondrial Membrane Fluidity

Changes in mitochondrial membrane fluidity were analyzed after control incubation or exposure to 20–100 μM of Aβ_{25-35} and 5–20 μM of Aβ_{1-42}, by measuring DPH fluorescence anisotropy (r) on brain and muscle isolated mitochondria (Fig. 4). Initially, we observed that DPH fluorescence anisotropy measured in the absence of Aβ was higher in brain mitochondria ($r = 0.178 \pm 0.013$) than muscle ($r = 0.141 \pm 0.02$) indicating a stronger membrane rigidity in brain. Mitochondrial exposure to 20 μM Aβ_{25-35} was followed by an increase in DPH anisotropy in brain ($r = 0.327 \pm 0.020$) and in muscle ($r = 0.200 \pm 0.016$), indicating an important decrease in membrane fluidity in both tissues. This effect was dependent on the concentration Aβ_{25-35}, which was nearly maximal at 100 μM (Fig. 4). Brain mitochondria appeared to be more sensitive to the effect of Aβ_{25-35} on membrane fluidity compared to muscle since they reached maximal anistropy value with lower concentration of amyloid-β peptides. Finally, exposure to 10 μM Aβ_{1-42} induced an increase in DPH anisotropy, similar to the effect of 60 μM Aβ_{25-35} on both brain and muscle mitochondria (Fig. 4). This effect was nearly maximal when 20 μM of Aβ_{1-42} was used. Again, brain mitochondria appeared more sensitive to the effect

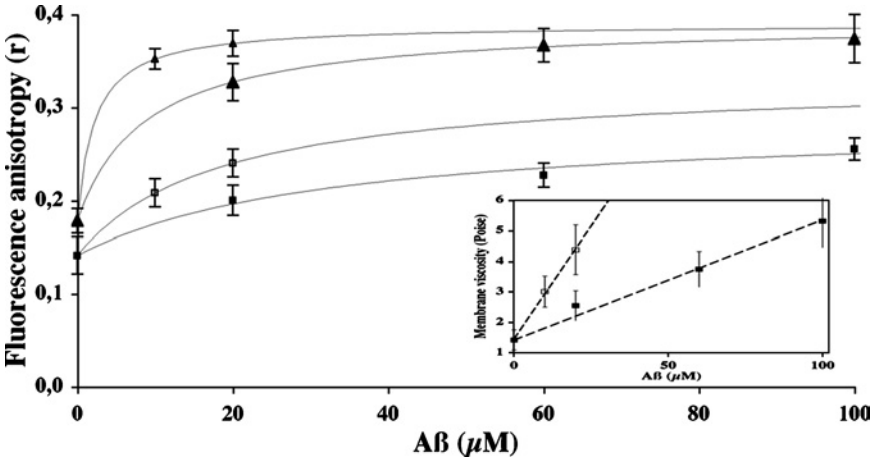

Fig. 4. Effect of Aβ on mitochondrial membrane fluidity: Mitochondrial membrane fluidity changes were analyzed on brain (triangles) and muscle (squares) mitochondria, by measuring changes in DPH fluorescence anisotropy (r) following exposure for 20 min at 25°C in the presence of Aβ_{25-35} used at 20, 60, and 100 μM (filled symbols) or Aβ_{1-42} used at 10 and 20 μM (open symbols). Each point represents the mean value $\pm$ SD of at least 30 separate determinations, on three different experiments performed on different mitochondrial preparations. Mitochondrial membrane viscosity values were calculated from the fluorescence anisotropy values. An example (insert) is given for the results obtained in muscle mitochondria following control incubation, or after exposure for 20 min at 25°C in the presence of Aβ_{25-35} (white squares) or Aβ_{1-42} (black squares), as detailled in the Methods section. In each experiment, control incubation with the scrambled peptide Aβ_{35-25} used at the same concentrations as that of the toxic species had no effect on mitochondrial membrane fluidity (data not shown). Notice that mitochondrial membrane viscosity varies linearly within the range of Aβ concentrations used, while anisotropy signal exhibits an apparent saturation profile. This is due to the fact that anisotropy does not vary linearly with viscosity changes, and this is why we chose to discuss changes in membrane physical properties in terms of viscosity rather than fluorescence anisotropy as generally reported.

of $A\beta_{1-42}$ on membrane fluidity when compared to muscle. The calculation of mitochondrial membrane viscosity from DPH fluorescence anisotropy values show, in the absence of $A\beta$, a 2-fold higher viscosity in brain ($\eta = 2.15 \pm 0.31$) when compared to muscle ($\eta = 1.42 \pm 0.33$) with ($P < 0.005$). In brain, incubation with $A\beta_{1-42}$ led to a linear increase in viscosity (Fig. 4 insert) 8-fold stronger as compared to $A\beta_{25-35}$ (4-fold in muscle). In addition, in comparison to muscle, brain mitochondria appeared much more sensitive to $A\beta$ peptides from the changes observed in anisotropy (and, therefore, membrane viscosity) as they reached maximal values characteristic of a rigid membrane.

Effect of $A\beta$ on Mitochondrial ROS Production

We investigated whether inhibition of mitochondrial energy production by $A\beta_{25-35}$ and $A\beta_{1-42}$ was associated with an increase in reactive oxygen species (ROS) generation. To this end, we measured the rate of mitochondrial hydrogen peroxide (H_2O_2) production using succinate as respiratory substrate on mitochondria isolated from both brain and muscle (Fig. 5). Following control incubation, brain and muscle mitochondria did not generate detectable H_2O_2 levels. However, the addition of antimycin A 0.75 nM, a concentration that fully inhibits complex III, induced a strong increase in the rate of H_2O_2 production. The rate of H_2O_2 production measured in the presence of antimycin A was 785 ± 71 pmol H_2O_2/min/mg in brain and 1380 ± 129 in muscle. Mitochondria incubated with 60 μM $A\beta_{25-35}$, and 20 μM $A\beta_{1-42}$ alone did not generate detectable H_2O_2 levels. However, after exposure to 60 μM $A\beta_{25-35}$ and 20 μM $A\beta_{1-42}$, we observed a strong concomitant increase in the rate of H_2O_2 production measured in the presence of antimycin A. This underscores a potentializing effect of $A\beta$ peptides on the magnitude of ROS production. Indeed, the rate of H_2O_2 produced in the presence of antimycin A after incubation with 60 μM $A\beta_{25-35}$ was 1300 ± 157 pmol/min/mg in brain and 2350 ± 187 pmol/min/mg in muscle. A similar potentializing effect was obtained after incubation with 20 μM $A\beta_{1-42}$ with a rate of H_2O_2 production (measured in the presence of antimycin) equal to 1185 ± 168 pmol/min/mg in brain and 2349 ± 174 pmol/min/mg in muscle.

Effect of Antioxidants on $A\beta$-Mediated Mitochondrial Dysfunction

To verify that inhibition of mitochondrial respiration observed after short-time β-amyloid exposure was not due to ROS oxidative damage, we reconducted the polarographic analysis with brain mitochondria preincubated with three different antioxidants (L-ascorbate 300 μM, GSH 2 mM, α-tocopherol 300 μM). These antioxidants and their concentration were chosen according to their high lipophilicity and their already proven efficacy on cell systems exposed to $A\beta$ (Behl *et al.*, 1994).

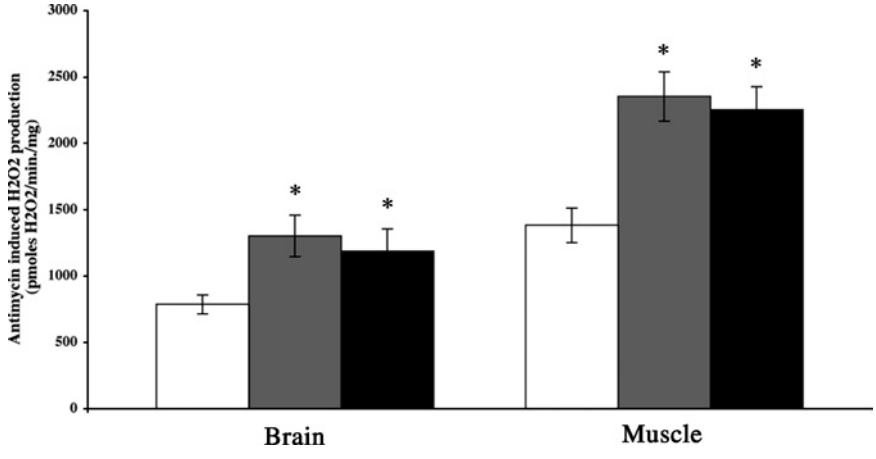

Fig. 5. Effect of $A\beta$ on mitochondrial ROS production: Mitochondrial ROS production was measured in the presence of exogenous SOD, catalase inhibitor, and antimycin A on brain- and muscle-isolated mitochondria after control incubation with 60 μM $A\beta_{35-25}$ (white bars), or after exposure to 60 μM $A\beta_{25-35}$ (dark grey), or 20 μM $A\beta_{1-42}$ (black). Each value represents the mean $\pm$ SD of at least three separate experiments performed on different mitochondrial preparations and the symbol (*) indicates that the value is statistically different from the control incubation value, using the Student's *t* test with $P < 0.05$.

In these conditions, measurement of mitochondrial respiration with succinate or pyruvate-malate as substrate, in the presence of ADP (state 3) did not reveal any protective effect of these antioxidants when $A\beta_{1-42}$ 20 μM was used (data not shown). These results are consistent with the absence of ROS production observed after exposure to Aβ. Similar results were obtained on mouse brain and liver mitochondria incubated with $A\beta_{1-42}$ 50 μM (Kim *et al*., 2002).

Effect of Aβ on Mitochondrial Cytochrome *c* Release

One possible mechanism by which Aβ could trigger neurodegeneration in AD might be the induction of the mitochondrial apoptotic pathway that occurs essentially through mitochondrial membrane permeabilization and cytochrome *c* release. To verify this possibility we measured the effect of $A\beta_{25-35}$ and $A\beta_{1-42}$ on the release of cytochrome *c* from brain and muscle mitochondria by Western-Blot (Fig. 6). We determined the total content of cytochrome *c* present in brain and muscle mitochondria after complete solubilization with deoxycholate (Schwerzmann *et al*., 1986; Vijayasarathy *et al*., 1998), using the porin signal to take into account variations in mitochondrial protein loading. Then, the cytochrome *c* released from the mitochondria (normalized to the porin signal) was compared to the total cytochrome *c* content (Fig. 6). In brain and muscle mitochondria we detected the presence of low levels of cytochrome *c* released during mitochondrial isolation ($2.56 \pm 0.58\%$ of the total in brain and $3.07 \pm 0.63\%$ in muscle mitochondria) (Fig. 6). Incubation with 20, 60, and 100 μM $A\beta_{25-35}$ induced a significant release of cytochrome *c*, corresponding to 7.15 ± 0.98, 9 ± 1.42, and $13 \pm 2.04\%$ of total, respectively ($P < 0.05$). After exposure to 20 and 60 μM $A\beta_{1-42}$, brain mitochondria released nearly 12 ± 2.61 and about $34 \pm 3.44\%$ of total cytochrome *c*, respectively. Similar results were obtained in muscle mitochondria (data not shown).

Accumulation of Aβ in Mitochondria After Incubation

To investigate whether Aβ was accumulated in mitochondrial membranes after the incubation procedure,

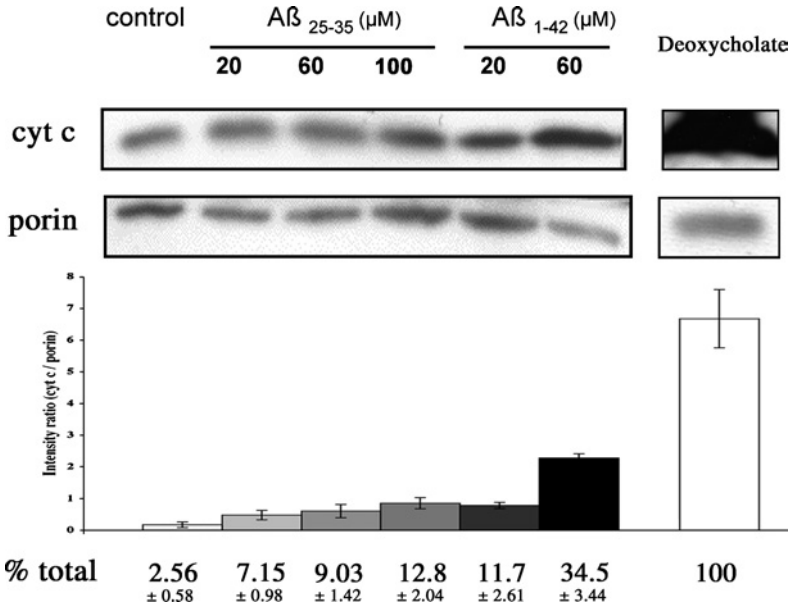

Fig. 6. Effect of Aβ on mitochondrial cytochrome *c* release: The release of cytochrome *c* was analyzed by Western Blot on brain mitochondria after exposure to Aβ and centrifugation as detailed in the Methods section. Control incubation was performed with 100 μM $A\beta_{35-25}$. Other incubations were performed with 20, 60, and 100 μM $A\beta_{35-25}$ and also, 20 and 60 μM $A\beta_{1-42}$. On the blots, the signal of cytochrome *c* measured in the supernatant was normalized to the porin signal of the pellet using Image J software (NIH). The intensity signal ratio (cyt *c*/porin) was compared to that obtained on mitochondria solubilized with deoxycholate 0.1% that set the value to 100%. Each value represents the mean $\pm$ SD of at least three separate experiments performed on different mitochondrial preparations.

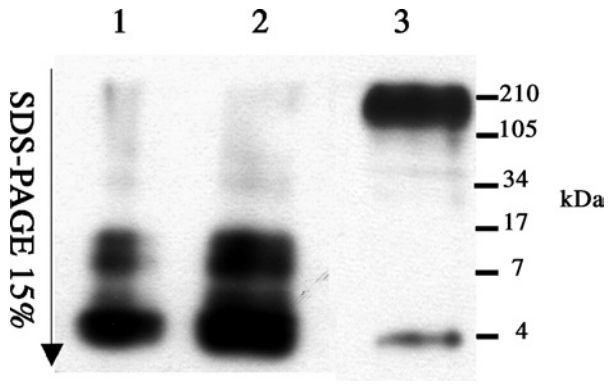

Fig. 7. Aβ accumulation in rat brain and muscle-isolated mitochondria: The presence of amyloid-beta peptide in mitochondria was analyzed by Western Blot after exposure to Aβ and centrifugation. Incubation was performed with toxic aggregates of 20 μM Aβ_{1-42}. Lane 1 contains brain mitochondria exposed to Aβ, lane 2 muscle mitochondria exposed to Aβ, and lane 3 contains the solution of Aβ used for these incubations. No accumulation was noticed when incubation was performed using 100 μM of the scrambled peptide Aβ_{35-25} (data not shown).

and prior to the functional studies detailed above, we performed a SDS-PAGE on the mitochondrial pellet followed by a Western-Blot analysis using an antibody raised against Aβ. In this manner, we observed the presence of Aβ_{1-42} both in brain and muscle mitochondria (Fig. 7). The amyloid-beta peptide accumulated in the mitochondria was present essentially in monomeric form, but we also noticed the presence of trimers and tetramers. On the opposite, the Aβ solution used for the incubation contained essentially large oligomers (>200 KDa), in coexistence with a fraction of monomer.

DISCUSSION

A large amount of evidence suggests that the abnormal intraneuronal accumulation of amyloid-beta peptides (Aβ) could be an early contributor to the pathogenicity of sporadic Alzheimer's disease (Gouras *et al.*, 2000; Skovronsky *et al.*, 1998). However, the mechanisms by which Aβ could mediate neuronal dysfunction are unclear. We hypothesize that intraneuronal Aβ accumulation induces gradual mitochondrial dysfunction that in turn causes progressive neuronal dysfunction by multiple means including energy deprivation, oxidative stress, cytochrome *c* release, and ultimately, necrosis or apoptosis. However, numerous studies on the effect of Aβ on mitochondrial oxidative phosphorylation, membrane properties, ROS production, and cytochrome *c* release were never carried out all together, and several discrepancies exist due to conflicting results that do not allow a full understanding of the role of mitochondria in the pathogenesis of AD (Cotman *et al.*, 1992a,b). In order to address this question we performed an integrated analysis of the effect of Aβ on mitochondrial structure and function, strictly performed on the same model and under identical experimental conditions.

In this manner, we observed that Aβ_{25-35} (20 μM) induced a significant decrease in state 3 respiration (-30%) at low concentration, and a more important decline in ATP synthesis (-50%), without an effect on respiratory chain complexes activity, nor state 4 respiratory rate. We also measured a dramatic increase in mitochondrial membrane viscosity (more than 10-fold in brain) associated with a large decrease in ATP/O value, that provide an explanation to why only state 3 respiration, and not state 4, was affected by 20 μM Aβ_{25-35}. Indeed, the ATP synthesis that occurs during state 3 but not state 4 is highly dependent on membrane fluidity, as the efficiency of the F_1F_0-ATPsynthase is modulated by surrounding phospholipids (Solaini *et al.*, 1984). The important increase in membrane viscosity observed in the presence of Aβ is likely to have affected the F_0-mediated conformational changes in the F_1 catalytic subunits necessary for ATP synthesis, without effect on proton translocation (Penefsky, 1985). The resulting proton-slippage can explain the important alteration of ATP/O observed in the presence of 20 μM Aβ_{25-35}. In addition, the decrease in membrane fluidity induced by Aβ could also have an effect on the activity of other membranous proteins involved in the phosphorylation of ADP such as the adenine nucleotide translocator or the phosphate carrier. This membrane destabilizing property of Aβ can thus be regarded as the primary mechanism for mitochondrial toxicity at low concentration, and the accumulation of Aβ_{1-42} in rat brain mitochondria observed in our study can directly explain this phenomenon. It could also explain the alteration of membrane fluidity observed both at the level of mitochondria (Mecocci *et al.*, 1996) and total cellular hipoccampal membranes (Eckert *et al.*, 2000) taken from the brain of AD patients.

Our observations are also in agreement with a recent immunohistochemical study performed on human brain sections showing a progressive intraneuronal accumulation of Aβ directly related to the severity of AD, and the accumulation of Aβ in the mitochondrion (Fernandez-Vizarra *et al.*, 2004). Moreover, our results indicate a stronger effect of Aβ_{1-42} on OXPHOS when compared to its shorter homologue Aβ_{25-35}, correlating with a stronger rigidifying effect on mitochondrial membranes. This could be related to the higher hydrophobicity of Aβ_{1-42} and could also explain the higher toxicity of

$A\beta_{1-42}$ on cellular energy metabolism observed on cultured neuron-like cells, as compared to $A\beta_{25-35}$ (Pereira *et al*., 1998, 1999). It can be noticed from our results that incubation of the mitochondrial samples 30 min at 37°C in the presence of a reducing agent (β-mercaptoethanol) and an anionic detergent (SDS), followed by a 10 min spin at 10,000 × *g* was not sufficient to remove Aβ from mitochondrial membranes. This excludes a simple adsorption of Aβ at the surface of mitochondrial membranes and suggests a stronger interaction such as an insertion in the hydrophobic core of phospholipid bilayers as demonstrated previously on lipid vesicles, or on membranes reconstituted with lipids taken from AD patients' cortical gray matter (Waschuk *et al*., 2001). The decrease in mitochondrial membrane fluidity observed in our study (and others) is also consistent with the insertion of Aβ in the bilayer. Our results also show that the forms of Aβ preferentially accumulated in the mitochondrion are the monomer, dimer, and trimer. This raises important concerns about the form of Aβ toxic to the mitochondrion. Indeed, in our study, when mitochondria were incubated with monomeric Aβ only, no toxicity was observed on every parameter assayed. There had to be a coexistence of aggregated and monomeric Aβ in the incubation mix to observe mitochondrial toxicity. One possiblity could be that the interaction of Aβ oligomers with mitochondrial membranes inhibits the aggregation process and stabilizes the monomers that may be inserted thereafter. This mechanism was previously proposed to explain the insertion of Aβ within Golgi membranes (Waschuk *et al*., 2001). It was also shown on PC12 cells and human fibroblasts that Aβ aggregates are adsorbed to the cell surface, and that Aβ is subsequently internalized in the cytosol and accumulated inside the cell (Burdick *et al*., 1997; Knauer *et al*., 1992). In this case, our results indicate that mitochondrial membrane might be a target for therapy in AD.

The second mode of action of Aβ on mitochondrial function observed in our study was a direct inhibitory effect on respiratory chain complexes, ranging from 10% inhibition for complex I, 23% for complex III, and up to 51% for COX, with 20 μM $A\beta_{1-42}$. This effect, in combination with a large increase in membrane viscosity and a consecutive decrease in ATP/O (i.e, the first mode of action of Aβ), results in the rather complete inhibition of mitochondrial ATP synthesis (around 90% inhibition in brain). A similar dramatic energetic situation was obtained when brain mitochondria were incubated with 60 μM $A\beta_{25-35}$. In our study Complex IV was the most affected respiratory complex. A higher sensitivity to Aβ was already observed by other authors (Chagnon *et al*., 1995; Parker, 1991), and could be related to the deeper embedment of complex IV in the phospholipid bilayer, where Aβ accumulates. Our results are also in agreement with a study of Canevari *et al*. (1999) who purified complex IV and reported a decrease in the apparent kinetic parameters of this complex, with a Ki value of 50 μM for $A\beta_{25-35}$. Accordingly in our study, a 70% inhibition of COX activity was induced by 60 μM $A\beta_{25-35}$. We observed no effect of Aβ on complex II (SDH) and F_1F_0-ATPase activities. One possible explanation is that those activities are carried out by protein domains protruding in the matrix where Aβ may not be accumulated. The inhibition of complex III activity by $A\beta_{1-42}$ (20 μM) and $A\beta_{25-35}$ (60 μM) observed in our study was never reported before, and could be of particular importance when considering the mechanisms of ROS overproduction observed in AD brain.

In our study, a large decline in mitochondrial energy production induced by Aβ was not associated with an increase in mitochondrial ROS production, even in the the presence of exogenous SOD, catalase inhibitor, or in measurements performed during exposure to Aβ. This observation is in good agreement with a threshold for ROS production demonstrated by Sipos *et al*. (2003) who showed that the inhibition of complexes I and III must reach a critical value (16 and 70%, respectively) before any ROS production can be observed. In our study, the inhibition of complex I and III by $A\beta_{25-35}$ or $A\beta_{1-42}$ alone (10 and 25%, respectively) did not reach this threshold, and consequently did not produce ROS. Using antimycin titration curves of mitochondrial state 3 respiration and following superoxide production we obtained a threshold for ROS production by complex III of 75% (data not shown). On the other hand, we observed a strong increase in antimycin A-induced H_2O_2 production (+40%) when brain and muscle mitochondria were priorly incubated with $A\beta_{25-35}$ (60 μM) or $A\beta_{1-42}$ (20 μM). This suggests a potentializing effect of Aβ on mitochondrial H_2O_2 production that can be regarded as the third mode of action of Aβ on mitochondrial metabolism. One possible explanation for this observation could be that the Aβ-induced inhibition of cytochrome *c* oxidase may facilitate ROS production from complex I and III, as observed by Dawson *et al*., using azide to inhibit complex IV (Dawson *et al*., 1993). Consequently, in our study, we observed no protective effect for the various antioxidants assayed on Aβ-induced inhibition of mitochondrial respiration. This may not be the case in cell lines studies or on brain taken from AD patients where the longer exposure to Aβ could induce a larger inhibition of mitochondrial OXPHOS allowing to overpass the threshold for ROS production and initiate a vicious cycle of ROS damage. In these studies (Cardoso *et al*., 2004; Pereira *et al*., 1999), increased ROS levels (around +30–50%) were reported, as well as a beneficial effect of antioxidants (Behl *et al*., 1994). Our

results suggest that the situation may be different in the early steps of AD where the primary mechanisms for ROS generation, if any, might not be the inhibition of mitochondrial respiratory chain in neurons. Instead, the activation of microglia, potentialized by an Aβ-induced loss of GSH as observed in astrocytes, might lead to ROS generation (Canevari *et al*., 2004).

The fourth and last mode of action of Aβ on mitochondrial function suggested by our study is a release of cytochrome *c*, at high Aβ concentrations. We observed that 60 μM Aβ_{25-35} induced a release of 9% of total cytochrome *c*, that was increased up to 35% with 60 μM Aβ_{1-42}, indicating once again a higher mitochondrial toxicity of Aβ_{1-42}. The mechanisms involved could include PTP opening or might simply result from important physicochemical constraints imposed by the presence of Aβ in the lipid bilayer. Such constraints could lead to local membrane disruption, and disturb cytochrome *c*/cardiolipin interactions resulting in the release of a fraction of cytochrome *c*. Our results also show a correlation between increased membrane viscosity induced by Aβ, and the extent of cytochrome *c* released. Moreover in the mitochondrion, a minor fraction of cytochrome *c* is located in the intermembrane space, while the majority is sequestrated in the cristae (Scorrano *et al*., 2002). In our study, Aβ is likely to have favoured the release of the "loosely bound" pool of cytochrome *c*, since we observed that only a minor fraction of total cytochrome *c* was released when Aβ reached its maximum membrane destabilizing effect. A central question that remains to be answered is to understand to what extent this partial release of cytochrome *c* is sufficient to activate the apoptotic cascade. Indeed, it is widely proposed that Aβ neurotoxicity could be mediated via the initiation of mitochondrial apoptotic pathway based on cell culture studies, but this is a matter of controversy as several findings suggest a prevalance of necrotic death in AD brains (Canevari *et al*., 2004), thus indicating a possible early determinance of energy deprivation in the disease process. In addition, one has to consider not only the extent of cytochrome *c* released, but also the level of respiratory chain inhibition and the level of ROS produced to predict a commitment to apoptosis.

In summary, our results show the direct accumulation of Aβ into rat brain and muscle mitochondria, followed by a concentration-dependent progressive alteration of mitochondrial function. The concentration of Aβ toxic to the mitochondrion determined in our study and others, lies in the micromolar range. To validate the physiological relevance of our findings, one need to know the exact *in vivo* concentration of Aβ inside the neurons of AD patient brains, as well as that inside the mitochondrion. However, this is a very difficult task technically, and these values still remain unknown. What is known without speculations, is (1) the total content of amyloid peptides in the brain of AD patients (from 12.7 to 40.8 nmol/g wet brain (Lewis *et al*., 2004), (2) the concentration of extracellular Aβ cytotoxic for cells in culture (around 10 μM for Aβ_{25-35} and 2 μM for Aβ_{1-42}) (Behl *et al*., 1994; Casley *et al*., 2002a,b), and (3) the circulating concentration of Aβ in biological fluids (around 5 nM in the cerebrospinal fluids (Seubert *et al*., 1992)). However, these information cannot be used to evaluate the intraneuronal and mitochondrial Aβ content *in vivo*. Furthermore, Aβ is highly hydrophobic and preferentially accumulated in membraneous compartments of the cell such as the mitochondrion (Fernandez-Vizarra *et al*., 2004; Lustbader *et al*., 2004) where it could reach high local concentrations. In addition, given the existence of several contact sites between mitochondria and the endoplasmic reticulum or the Golgi (where Aβ is overproduced in pathological situation), it is highly possible that Aβ could reach concentrations in the micromolar range in the vicinity of mitochondria *in vivo*. This eventuality was already proposed by other authors (Canevari *et al*., 1999; Casley *et al*., 2002a,b; Moreira *et al*., 2002; Pereira *et al*., 1999). Moreover, β-amyloids are produced from the cleavage of a precursor protein (APP) by specific proteases located in membraneous subcellular compartments such as the E/R or the lysosomes, but also the mitochondria (Hansson *et al*., 2004; Leissring *et al*., 2004). In addition, APP is targeted to mitochondria (Anandatheerthavarada *et al*., 2003) so that Aβ could be produced directly in mitochondrial membranes and reach micromolar concentrations. Another indication that Aβ could reach micromolar concentrations in mitochondria *in vivo* is that, in our study, respiratory chain complex IV was inhibited to the same extent as what is consistently observed in the brain of AD patients (Canevari *et al*., 1999).

Taken together, our data allow to propose a model (see Fig. 8) where individuals with a lower mitochondrial threshold value (Rossignol *et al*., 2003), could be more prone to develop a toxic-Aβ pathology. This is consistent with lower levels of respiratory chain complexes proteins, and mRNAs observed in AD brains (Chandrasekaran *et al*., 1996). Interestingly, we did not observe any significant difference in the mode of action of Aβ between brain and muscle mitochondria, suggesting that intracellular Aβ overproduction could affect all tissues, although in a mitochondrial threshold-dependent manner (Rossignol *et al*., 1999; Sipos *et al*., 2003). For instance, one can notice that in brain mitochondria the absolute value of mitochondrial ATP synthesis is about half that of muscle mitochondria.

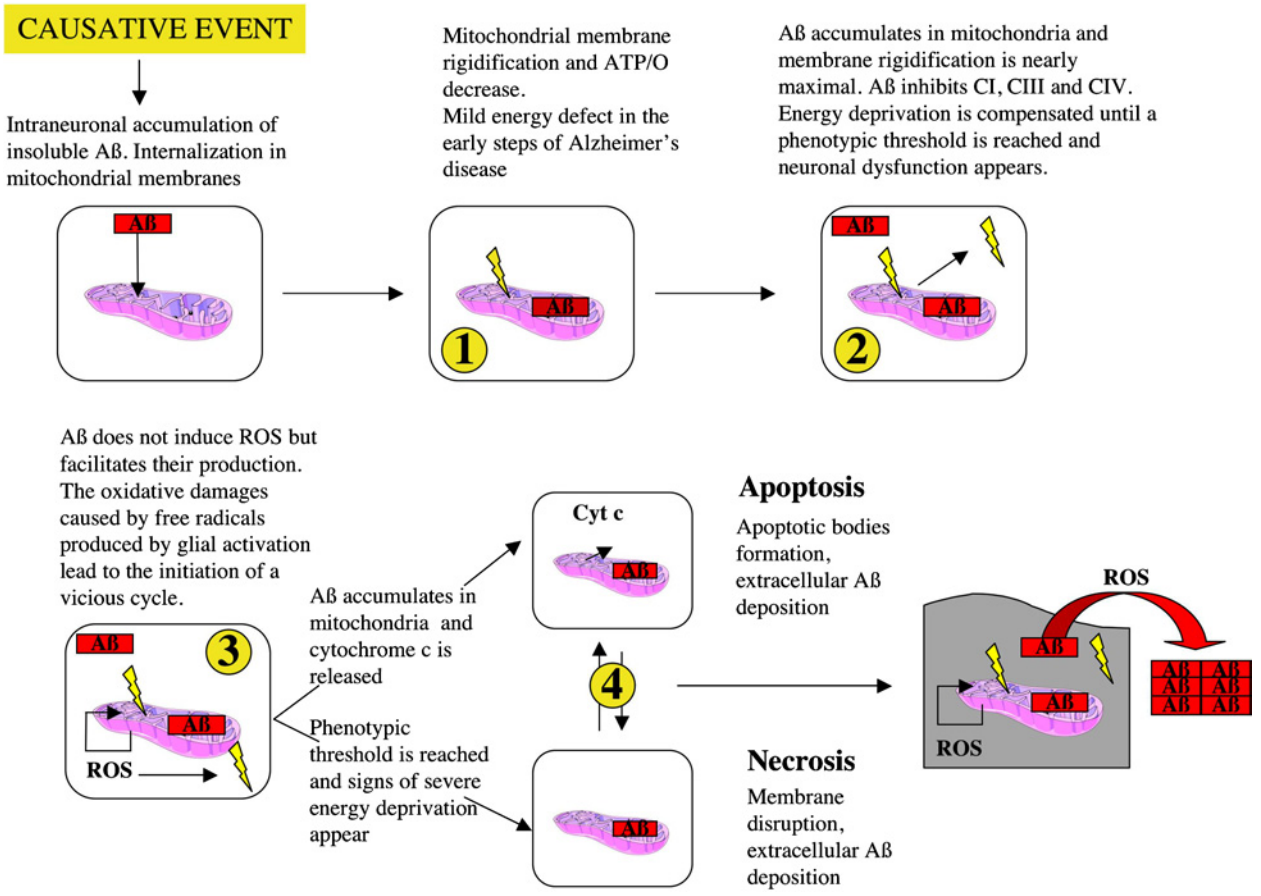

Fig. 8. Model of gradual alteration of mitochondrial structure and function by Aβ: In the first step, intracellular Aβ is abnormally overproduced and accumulates within mitochondrial membranes. This leads to an increase in mitochondrial membrane viscosity and a subsequent decrease in energy production that could be particularly important in the first stage of Alzheimer's disease, possibly during the Mild Cognitive Impairment state period that precedes pathology. When the inhibition of mitochondrial respiratory chain has reached a phenotypic threshold, the sudden drop in energy production could lead to neuronal dysfunction and impairement of cognitive capacities. When the inhibition of mitochondrial function has reached a phenotypic threshold (Rossignol *et al*., 2003), neuronal dysfunction and impairement of cognitive capacities could appear. The further accumulation of Aβ within mitochondrial membranes induces cytochrome *c* release and possibly, neuron apoptosis. However, even the deprivation of ATP production could suffice to kill the neurons by necrosis, and this remains to be clarified. In the end, the cell membrane is disrupted and Aβ deposited extracellularly, although Aβ deposition can also result from secretion in the course of intracellular Aβ accumulation.

Therefore, brain cells could be more affected by the inhibition of oxidative phosphorylation by Aβ than muscle cells. Moreover, there is also a threshold value under which a further decrease in mitochondrial ATP production triggers cell dysfunction, and we have previously shown that this threshold can vary between tissues (Rossignol *et al*., 1999).

Therefore, a lower biochemical and phenotypic threshold in brain than muscle could also explain in part why this tissue is more affected. In addition, several other parameters could participate the higher sensitivity of the brain to Aβ observed *in vivo*. They include for instance differences in intracellular Aβ concentration between tissues, the type of Aβ oligomer, tissue energy demand and supply (the prefential use for a given energy substrate, oxygenation), the balance between glycolytic versus OXPHOS capacity, the antioxidant defense system, and the mitochondrial bioenergetic features (isoforms, stoichiometry…). This could also explain why only some parts of the brain are severely affected while Aβ deposits can be found in multiple tissues. Finally, the abnormal accumulation of hydrophobic peptides in mitochondrial membranes with subsequent deleterious effect on organellar structure and function might not be specific to Aβ and Alzheimer's disease. For instance, the accumulation of huntingtin or

alpha-synuclein in the mitochondrion was proposed to play a role in sporadic Parkinson's (Trimmer *et al*., 2000) and Huntington's (Panov *et al*., 2002) diseases, respectively. Moreover, replicated evidences also indicate that this phenomenon could concern other neurodegenerative disorders such as the bipolar disorder (Kato and Kato, 2000) or even schizophrenia (Ben-Shachar and Laifenfeld, 2004).

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