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Effects of amyloid-beta peptides on hydrogen peroxide-metabolizing enzymes in rat brain *in vivo*

YURY G. KAMINSKY, & ELENA A. KOSENKO

Institute of Theoretical and Experimental Biophysics, RAS, Pushchino, Russia

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Abstract
Amyloid-β (Aβ) peptides are components of senile plaques initiating degeneration of brain neurons in Alzheimer's disease. They increase reactive oxygen species generation that may exceed the defensive capacity of cells. To test the hypothesis, this study investigated the *in vivo* effects of Aβ peptides on mitochondrial and non-mitochondrial enzymic sources of reactive oxygen species and antioxidant enzymes in rat brain. Continuous intracerebroventricular infusion of both Aβ_{25-35} and Aβ_{1-40} for up to 14 days stimulated the hydrogen peroxide (H_2O_2) generation in isolated neocortex mitochondria. Infusion of Aβ_{1-40} led to an increase in Mn-superoxide dismutase activity and a decrease in activities of catalase and glutathione peroxidase in mitochondria, to elevation of activities of Cu,Zn-superoxide dismutase and aldehyde oxidase, forwarded the conversion of xanthine dehydrogenase to xanthine oxidase and corresponding increase in the rate of H_2O_2 formation in the cytosol. Thus, Aβ peptides increase H_2O_2-formation and H_2O_2-forming enzyme activities and inhibit H_2O_2-consuming enzyme activities in mitochondria and cytosol *in vivo*. These studies suggest that disbalance between H_2O_2-generating and H_2O_2-metabolizing enzyme activities can contribute to oxidative stress underlying neurodegeneration and neuronal death in Alzheimer's disease.

Keywords: *Amyloid-β peptide, hydrogen peroxide, antioxidant enzymes, xanthine oxidase, monoamine oxidase, aldehyde oxidase, neocortex mitochondria*

Abbreviations: *AD, Alzheimer's disease; Aβ, amyloid-β; SOD, superoxide dismutase; MAO, monoamine oxidase; XO, xanthine oxidase; XDH, xanthine dehydrogenase; ROS, reactive oxygen species; $O_2^{\cdot-}$, superoxide radical; H_2O_2, hydrogen peroxide; PMSF, phenylmethylsulphonyl fluoride.*

Introduction

Amyloid-β (Aβ) peptides are proteolytic products of amyloid precursor protein and main components of amyloid deposits known as extracellular senile plaques initiating slow degeneration of brain neurons in Alzheimer's disease (AD) and other neurodegenerative disorders.

One concept of Aβ peptide-initiated neuronal death resides in the disruption of calcium homeostasis [1], on the ability of the peptide to form Ca^{2+}-permeable pores in neuronal membranes [2] resulting in an excessive Ca^{2+} influx and induction of neurotoxic cascades. It is possible that, during Aβ peptide exposure, a Ca^{2+}-mediated increase in reactive oxygen species (ROS) generation may exceed the defensive capacity of cells and thus lead to cell death [3]. The above studies have been carried out using cultured neurons, aortic endothelial cells and isolated liver mitochondria and the sources of the ROS have not been clearly identified. Moreover, it should be taken into account that the responses in

Correspondence: Yury Kaminsky, DSc, Institute of Theoretical and Experimental Biophysics, RAS, Institutskaya ul. 3, Pushchino, 142290 Russia. Fax: +74 96 77 33 05 53. Email: kaminsky@iteb.ru

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cultured neurons may be different than in neurons in the whole brain where they are interacting with other cell types (e.g. astrocytes) that modulate its function.

The main calcium-dependent mechanisms that may lead to increased ROS formation include the following: calcium entering the neuron is taken up by mitochondria and causes a depolarization of the inner mitochondrial membrane. This leads to altered function of the mitochondrial respiratory chain and to increased formation of superoxide radical (O_2^-) [4]. This radical is then transformed by Mn^{2+}-superoxide dismutase (Mn-SOD) to hydrogen peroxide (H_2O_2) within the mitochondrial matrix. H_2O_2 may also be generated by monoamine oxidase (MAO), an enzyme located in the outer mitochondrial membrane [5]. Aldehyde oxidase and xanthine oxidase (XO), as well as Cu,Zn-SOD, seem to be the potential sources of H_2O_2 in brain cytosol. Thus, there are a number of mitochondrial and non-mitochondrial pathways that may generate ROS in the brain, especially under some pathological conditions. To test the hypothesis, here we analyse the contribution of the enzymes above to the production of ROS in rat brain following continuous infusion of Aβ peptides *in vivo*. We showed that intracerebroventricular infusion of aggregated A$\beta_{1–40}$ induced increases in activity of H_2O_2-generating enzyme Mn-SOD and decreased activities of H_2O_2-consuming enzymes catalase and glutathione peroxidase in rat brain neocortex mitochondria. Also, infusion of A$\beta_{1–40}$ led to increases in XO, Cu,Zn-SOD and aldehyde oxidase activities in the brain cytosolic fraction.

Materials and methods

Materials

β-phenylethylamine, *tert*-butylhydroperoxide, allopurinol, NAD, NADPH, EDTA, EGTA, *p*-nitrotetrazolium blue, methylene blue, xanthine, xanthine oxidase, horseradish peroxidase, dl-dithiothreitol, phenylmethylsulphonyl fluoride (PMSF), GSH, GSSG, scopoletin, pterine, isoxanthopterin, antimycin A, potassium cyanide, potassium ferricyanide, sodium azide, A$\beta_{25–35}$ and A$\beta_{1–40}$ were from Sigma Chemical Co. (USA); sucrose and Mops were from Serva (Germany); Sephadex G-25 (particle size 10–40 μM) was from Bio-Rad Lab. (USA); potassium mercaptosuccinate and Triton X-100 were from Fluka (Switzerland); thioflavin T was from Molecular Probes (Eugene, OR). Other chemicals were commercially available.

A$\beta_{25–35}$ and A$\beta_{1–40}$ were used as commercial (non-treated) and aggregated forms.

Animals

Male Wistar rats weighing 200–250 g were used. Food and water were available *ad libitum*. The experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery

According to the stereotaxic atlas [6], an infusion cannula was implanted into the rat right cerebral ventricle (0.8 mm posterior, 1.5 mm lateral to the bregma and 4.5 mm below the surface of the cranium) under uretane (800 mg/kg, intraperitoneally) and ketamine (500 mg/kg, intramuscularly) anaesthesia using a stereotaxic apparatus, as previously described [7]. In preliminary experiments, the injection site was checked by administering trypan blue.

Stock solutions of A$\beta_{25–35}$ and A$\beta_{1–40}$ (1 mg/ml) were prepared in the sterile water. Rats were injected intracerebroventricularly with A$\beta_{25–35}$ or A$\beta_{1–40}$ continuously for up to 14 days, essentially as described by Olariu et al. [8]. The infusion cannula was attached to a mini-osmotic pump and 4 days later 15 μl of 0.9% NaCl containing an Aβ peptide began to be infused at a dose of 3–3.7 ng/h/rat or 70–90 ng/day/rat for up to 14 days.

Control animals received a 0.9% NaCl injection (15 μl/ventricle).

Preparation of the brain cytosolic fraction and mitochondria

Generally rats were killed by decapitation on days 1–14 after the start of A$\beta_{25–35}$ or A$\beta_{1–40}$ infusion. The brains were quickly removed from the skull, the neocortex was dissected out, washed and homogenized in nine volumes of the buffer, containing 0.25 M sucrose, 0.5 mM EGTA, 10 mM Tris-HCl, pH 7.4 [9]. The mitochondrial and cytosolic fractions were isolated as described by Kosenko et al. [10]. Non-synaptic mitochondria were isolated from the total mitochondrial fraction [11] and used in the experiments within 60 min of isolation.

Determination of hydrogen peroxide

The rate of H_2O_2 production by isolated mitochondria was monitored fluorimetrically by scopoletin fluorescence in the presence of peroxidase as described by Loschen et al. [12] except that sodium azide and potassium mercaptosuccinate, inhibitors of catalase and glutathione peroxidase, respectively, were included in the incubation mixture. Mitochondria (0.5 mg/ml) were added to a medium containing 0.25 M sucrose, 10 mM KCl, 10 mM Mops, pH 7.3, 0.5 mM EGTA, 5 mM potassium succinate, 2 μM antimycin A, 50 μM sodium azide, 50 μM potassium mercaptosuccinate and 0.25 μM scopoletin. The reaction was initiated by addition of peroxidase (6 IU/ml). Assays were performed at 25°C in a

volume of 1 ml; H_2O_2 (0.5–1.0 μM) was added at the end of the reaction as a standard. H_2O_2 standards were prepared daily by dilution of 30% H_2O_2. The concentration of H_2O_2 was calculated by measuring the absorbance at 240 nm and using a molar absorption coefficient of 39.4 $M^{-1} \times cm^{-1}$ [13].

To estimate the *in vitro* effects of Aβ_{25-35} and Aβ_{1-40} on H_2O_2 production, rat neocortex non-synaptic mitochondria were incubated for 10 min at 37°C with the Aβ peptide, then the H_2O_2 generation was measured as above.

Determination of enzyme activities

Activities of H_2O_2-generating monoamine oxidase, xanthine oxidase and aldehyde oxidase, antioxidant enzymes catalase, total SOD, Cu,Zn-SOD, Mn-SOD and glutathione peroxidase were measured in the cytosolic fraction and purified non-synaptic mitochondria from neocortex of control rats and animals treated with Aβ peptides.

For determination of glutathione peroxidase (EC 1.11.1.9) and SOD (EC 1.15.1.1) activities, mitochondria were disrupted by osmotic shock in 10 mM phosphate buffer (pH 7.4, 10 min at 4°C) and three freezing/thawing cycles. The suspension was centrifuged at 140 000 × g for 20 min and supernatant was used as the source of enzymes.

MAO (EC 1.4.3.4) activity was measured in intact mitochondria, while catalase (EC 1.11.1.6) activity was measured in mitochondria in the presence of 1% Triton X-100. It was found in preliminary experiments that catalase activity was of mitochondrial origin as it was not determined in mitochondria in the absence of Triton X-100.

For the measurements of xanthine:NAD^+ oxidoreductase (EC 1.17.1.4, xanthine dehydrogenase, XDH), XO (EC 1.17.3.2) and aldehyde oxidase (EC 1.2.3.1) activities, rat brain homogenate was prepared as follows. Brains were chopped rapidly with scissors and the chopped material was frequently washed with cold 0.9% NaCl to remove blood. The tissue was homogenized manually in cold medium containing 50 mM KH_2PO_4, pH 7.4, 1 mM EDTA, 10 mM DL-dithiothreitol, 0.5 mM PMSF. The homogenate was centrifuged at 16 000 × g for 30 min. The supernatant was rapidly chromatographed using Sephadex G-25 (0.8 × 4 cm, Bio-Rad Lab., particle size 10–40 μM) and centrifugal force (900 × g, 2 min) to remove endogenous low molecular weight inhibitors [14].

Glutathione peroxidase activity was determined by Lawrence and Burk's [15] method with *tert*-butylhydroperoxide as a substrate except that 5 mM GSH was used instead of 1 mM because the K_m value for GSH was found previously to be 2.5 mM for the brain mitochondrial enzyme [16].

Total SOD activity was determined by the inhibition of the reduction of *p*-nitrotetrazolium blue in the presence of the xanthine-xanthine oxidase system [17]. Mn-SOD activity was calculated as the difference between the total activity (Cu,Zn-SOD plus Mn-SOD) and the activity measured in the presence of the Cu,Zn-SOD inhibitor potassium cyanide (1 mM). One unit of SOD activity was defined as the amount of SOD required to inhibit by 50% the rate of *p*-nitrotetrazolium blue reduction.

Catalase activity was measured with H_2O_2 by measuring the decrease in absorption at 240 nm as described by Aebi [13] and was expressed in terms of the first order reaction rate constant as s^{-1} per mg of mitochondrial protein.

MAO activity in intact mitochondria was assayed by the measurement of H_2O_2 production [16]. Mitochondria (0.5 mg protein/ml) were added to the incubation medium containing 0.25 M sucrose, 10 mM KCl, 10 mM Mops, pH 7.3, 0.5 mM EGTA; 0.2 mM β-phenylethylamine was used as a substrate of the MAO-B isoenzyme. The reaction was monitored spectrophotometrically at 240 nm.

XDH and XO activities were measured fluorimetrically [14]. XO in the supernatant was assayed by the oxidation of pterin (10 μM) to the fluorescent product isoxanthopterin ($\lambda_{ex} = 345$ nm excitation, $\lambda_{em} = 390$ nm emission) while the sum of XDH+XO activity was determined with methylene blue (10 μM) as an electron acceptor [16]. The incubation mixture consisted of 50 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA and 10 μM allopurinol. Known amounts of isoxanthopterin (0.12 μM; $\varepsilon_{336} = 13$ mm^{-1} cm^{-1} at 20°C) used as internal standard were added at the end of the reaction and the fluorescent signals were used in calculations.

Aldehyde oxidase activity was measured in the 16 000 × g supernatant spectrophotometrically at 420 nm with phthalazine as substrate and potassium ferricyanide ($\varepsilon_{420} = 2.08$ mM^{-1} cm^{-1}) as electron acceptor [18].

Thioflavine T dye binding

For the aggregation studies, the Aβ peptides were diluted (from the stock solution of 1 mg/ml) in sterile 0.1 M phosphate buffer, pH 6.5, to 0.1 mg/ml and incubated for a period from 10 min to 5 days at 37°C under continuous shaking, which led to the formation of amyloid fibrils [19]. A 100 μl sample was mixed with 900 μl of 25 μM thioflavine T in 25 mM phosphate buffer pH 6.5. The fluorescence emission was measured immediately after mixing using excitation and emission wavelengths of 440 and 482 nm, respectively, and the SFM-25 spectrofluorimeter (Kontron Instrument, Korea). No separation of aggregates from free amyloids was made as monomeric

and dimeric Aβ peptides did not induce thioflavine T fluorescence [19].

Statistics

Differences among groups were analysed by one-way ANOVA followed by Student *t*-test to determine statistical significance using the Prizm 4.0 software. Comparison between two experimental groups was based on two-tailed *t*-test. $p < 0.05$ was considered statistically significant.

Results

Infusion of A$\beta_{25–35}$ and A$\beta_{1–40}$ increases hydrogen peroxide production by brain mitochondria

The rate of H_2O_2 generation by isolated neocortex mitochondria from rats injected with commercial A$\beta_{25–35}$ was 0.286 nmol/min.mg on day 1 and increased to 0.328 nmol/min.mg (by 26%, $p = 0.0062$) on day 14 after the start of A$\beta_{25–35}$ infusion (Figure 1).

The same dosage of commercial A$\beta_{1–40}$ did not affect H_2O_2 generation by mitochondria isolated on days 2–7.

Similar results were obtained *in vitro* (Figure 2). Pre-incubation of control mitochondria with commercial 1 μM A$\beta_{25–35}$ for 10 min stimulated H_2O_2 generation by 14% ($p = 0.0197$) and that with 10 μM A$\beta_{25–35}$ by 56% ($p < 0.0001$). Commercial A$\beta_{1–40}$ did not affect the H_2O_2 generation by mitochondria at concentrations ranged from 10–100 μM and pre-incubation time up to 30 min (Figure 2).

The data on the absence of the A$\beta_{1–40}$ effects seem to be unexpected as A$\beta_{1–40}$ is known to disturb brain mitochondrial function. According to the literature, Aβ peptides exhibited cytotoxicity in aggregated form [19] and the cytotoxicity was associated with their

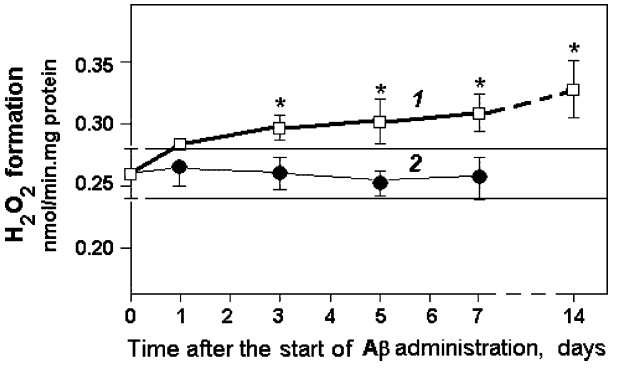

Figure 1. The effects of A$\beta_{25–35}$ and A$\beta_{1–40}$ infused *in vivo* on H_2O_2 generation by rat neocortex non-synaptic mitochondria. In control, the rate of H_2O_2 generation was 0.26 ± 0.02 nmol/min.mg of protein (these values are bounded by two horizontal lines). (1) A$\beta_{25–35}$; (2) A$\beta_{1–40}$. Values are the mean ± SEM of $n = 3$ with three measurements on each preparation. $^*p < 0.05$ as compared with control.

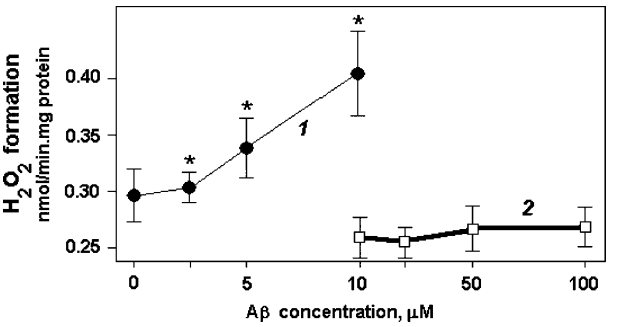

Figure 2. The effects of A$\beta_{25–35}$ and A$\beta_{1–40}$ added *in vitro* on H_2O_2 generation by rat neocortex non-synaptic mitochondria. (1) A$\beta_{25–35}$; (2) A$\beta_{1–40}$. Values are the mean ± SEM of $n = 5–6$. $^*p < 0.05$ as compared with control.

action on mitochondria [3]. The results shown in Figures 1 and 2 suggest that commercial A$\beta_{1–40}$ does not exert the expected effect as it can in unaggregated form. Therefore A$\beta_{25–35}$ and A$\beta_{1–40}$ solutions were exposed to conditions causing aggregation [19]. The Aβ peptides were incubated in phosphate buffer, pH 6.5, 0.1 mg/ml peptide, for a period from 10 min to 5 days. Self-association of the Aβ peptides was monitored by the fluorescence emission of thioflavine T at 482 nm. We found that 100% A$\beta_{25–35}$ was aggregated within the first 10 min following its solution in the phosphate buffer, while A$\beta_{1–40}$ did so only on day 5. There were almost no aggregated A$\beta_{1–40}$ during the first 24 h incubation and over 80% were aggregated on day 3 (Table I).

It is suggested that, under the conditions of Figures 1 and 2, A$\beta_{25–35}$ was infused to rats and added to the incubation in the aggregated state while A$\beta_{1–40}$ was infused and added in the free form.

In further experiments we used both Aβ peptides preliminarily exposed to conditions causing aggregation for 3 days.

Indeed, aggregated A$\beta_{1–40}$ caused stimulation of H_2O_2 production by rat brain mitochondria *in vivo* (Table II) and *in vitro* (Table III).

Although the fluorescence peak at 482 nm was observed on day 5 of incubation of 1 μM A$\beta_{1–40}$ in

Table I. A$\beta_{25–35}$ and A$\beta_{1–40}$ aggregation.

	Incubation period of an Aβ peptide in the solution			
Aβ species	10 min	24 h	3 days	5 days
A$\beta_{25–35}$	94.74	100	100	100
A$\beta_{1–40}$	3.40	3.55	83.65	96.58

Commercial Aβ peptides were solved in phosphate buffer (pH 6.5) to the final concentration of 0.1 mg/ml and pre-incubated at 37°C. At times indicated, 0.1 ml aliquots were taken and added into the spectral cuvette, mixed with 0.9 ml of phosphate buffer (pH 6.5) containing 25 μM thioflavine T and the fluorescence emission was monitored at 482 nm. Results of a typical experiment are given and expressed as% maximal fluorescence emission on day 5 of incubation.

Table II. Effects of chronic administration of aggregated Aβ_{1-40} on H_2O_2 generation and activities of Mn-SOD, catalase, glutathione peroxidase and MAO B in isolated neocortex mitochondria.

Parameter	Control	Aβ_{1-40}
H_2O_2 generation (nmol/min.mg protein)	0.26±0.02	0.31±0.01*
Mn-SOD (U/min.mg protein)	1.89±0.16	2.29±0.06*
Catalase (s^{-1}/mg protein $\times 10^4$)	3.05±0.01	1.89±0.18*
GSH peroxidase (nmol/min.mg protein)	62±3	38±2*
MAO-B (nmol/min.mg protein)	0.74±0.03	0.80±0.06

Rats were injected intracerebroventricularly with saline or aggregated Aβ_{1-40} at a dose of 1.8 μg/kg for 5 days and then neocortex non-synaptic mitochondria were isolated. H_2O_2 generation and activities of enzymes indicated were measured in mitochondria. Values are the mean±SD of $n=5$–6.
*Significant difference from the control, $p<0.05$.

phosphate buffer, pH 6.5, such concentration of aggregated Aβ_{1-40} did not induce significant stimulation of H_2O_2 production by mitochondria for 10 min ($p=0.2094$) (Table III). The higher concentration of aggregated Aβ_{1-40} and more its incubation time with mitochondria were used, the higher rate of H_2O_2 production by mitochondria was observed (Table III). Aggregated Aβ_{1-40} incubated with mitochondria at 20 μM or 100 μM for 20 min increased the rate of H_2O_2 production by 31% and 47%, respectively ($p<0.0001$).

Aβ_{1-40} administration results in alteration of activities of Mn-SOD, MAO, catalase and glutathione peroxidase

The H_2O_2 level in the mitochondrion depends on the activity of Mn-SOD, MAO, catalase and glutathione peroxidase.

We tested whether aggregated Aβ_{1-40} administration *in vivo* led to alterations of these enzyme activities in non-synaptic mitochondria isolated from rat brain. The activities of Mn-SOD and MAO-B appeared to increase by 21% ($p=0.0006$) and 8% ($p=0.076$), respectively, and catalase and glutathione peroxidase activities were both reduced by 38% ($p<0.0001$) after a 5 day-course of Aβ_{1-40} infusion (Table II).

We did not observe any H_2O_2 generation by Aβ peptides themself; H_2O_2 was not determined by our methods in the mitochondrial suspension pre-incubated with any Aβ peptide at 1–10 μM for up to 30 min in the absence of potassium succinate.

Aβ_{1-40} administration results in the conversion of xanthine dehydrogenase to xanthine oxidase

Cytosolic enzyme XO generates H_2O_2. In our experiments, most of the xanthine-oxidating enzyme (79%) in neocortex of control rats appeared to exist in the XDH form that does not generate ROS. In the neocortex cytosolic fraction, the activities of XDH and XO and the XO/XDH activity ratio were 10.2, 2.7 pmol/min.mg protein and 0.27, respectively (Table IV). Injection of rats with aggregated Aβ_{1-40} for 5 days did not change the XDH activity ($p=0.2205$), led to increasing the XO activity by 42% ($p=0.0008$) and the XO/XDH activity ratio by 55% ($p=0.003$) (Table IV).

Aβ_{1-40} administration increases Cu,Zn-SOD and aldehyde oxidase activities in brain cytosol

Aβ_{1-40} administration increased cytosolic Cu,Zn-SOD activity (by 23%, $p=0.0122$) and aldehyde oxidase activity (by 73%, $p=0.0001$) (Table IV).

Discussion

It has been proposed that oxidative stress and disruption of calcium homeostasis are chief factors and major causes of Aβ-induced toxicity to cells [20–22]. It has been known for a long time that mitochondria are major cellular generators of H_2O_2 [20] and mitochondrial failure plays a key role in the

Table III. The effects of aggregated Aβ_{25-35} and Aβ_{1-40} *in vitro* on H_2O_2 generation by rat neocortex non-synaptic mitochondria.

Aβ species	Aβ peptide concentration (μM)	Incubation time (min)	H_2O_2 generation (nmol/min.mg)
—		5	0.26±0.02
Aβ_{25-35}	1	5	0.29±0.01*
Aβ_{1-40}	1	5	0.28±0.02
	1	20	0.31±0.02*
	20	20	0.34±0.03*
	100	20	0.38±0.03*

Neocortex non-synaptic mitochondria were pre-incubated for 5 or 20 min at 37°C with aggregated Aβ_{25-35} or Aβ_{1-40} at concentration indicated, then the H_2O_2 generation was measured. Values are the mean±SD of $n=5$–6.
*Significant difference from the control, $p<0.05$.

Table IV. Effects of chronic administration of aggregated Aβ_{1-40} on xanthine oxidase (XO), xanthine dehydrogenase (XDH), SOD, aldehyde oxidase activity and the XO/XDH activity ratio in the rat neocortex cytosolic fraction.

Parameter	Control	Aβ_{1-40}
XO (pmol/min.mg protein)	2.7±0.09	3.83±0.35*
XDH (pmol/min.mg protein)	10.2±0.22	9.61±0.97
XO/XDH	0.27±0.01	0.41±0.06*
Cu,Zn-SOD (Units/min.mg protein)	8.7±1.0	10.67±1.20*
Aldehyde oxidase (pmol/min.mg protein)	40.5±4.3	70±5*

Rats were injected intracerebroventricularly with saline or aggregated Aβ_{1-40} at a dose of 1.8 μg/kg for 5 days. Then the neocortex cytosolic fraction was isolated and activities of enzymes indicated were measured. Values are the mean±SD of $n=4$–6.
*Significant difference from the control, $p<0.05$.

generation of ROS, resulting in oxidative damage to cellular compartments [4,23]. H_2O_2 levels were reported to be significantly increased in brain mitochondria of Tg2576 mice, an animal model of human Alzheimer's disease and directly correlated with levels of soluble Aβ, suggesting that Aβ peptides may be responsible for increased production of H_2O_2 in Alzheimer's disease progression in Tg2576 mice [24]. Aleardi et al. [25] analysed changes in mitochondrial function following direct exposure to increasing concentrations of $A_{25–35}$ and observed a potentiation of ROS accumulation within rat brain and muscle mitochondria. An increase in H_2O_2 levels was observed in isolated rat brain mitochondria exposed to $A_{1–40}$ [26].

Although production of H_2O_2 by mitochondria is increased under the influence of Aβ peptides, data on the *in vivo* and *in vitro* effects of A peptides on the actual rate of H_2O_2 generation by mitochondria, e.g. under blockade of such H_2O_2 consumers as catalase and glutathione peroxidase, are absent from the literature.

In our experiments, H_2O_2 production by brain mitochondria was measured in the presence of a catalase inhibitor sodium azide and glutathione peroxidase inhibitor potassium mercaptosuccinate, to exclude under-estimation of H_2O_2 generation. Under such conditions, the rate of H_2O_2 generation by brain mitochondria from rats injected with A$\beta_{25–35}$ was increased significantly as compared to controls but that has not happened with commercial A$\beta_{1–40}$. The latter was unexpected as A$\beta_{1–40}$ was known to cause an uncoupling of oxidative phosphorylation, a drop in mitochondrial membrane potential, the inhibition of complexes I, III and IV of the mitochondrial respiratory chain and an increase in H_2O_2 production [26–28]. According to the literature, Aβ peptides exhibited cytotoxicity in aggregated form [19] and the cytotoxicity was associated with their action on mitochondria [3]. *In vitro* studies have shown that A$\beta_{25–35}$ did not require ageing to aggregate, unlike the full-length peptide such as A$\beta_{1–40}$ [29,30] and formed fibrils immediately after solution [31]. Our results suggest that commercial A$\beta_{1–40}$ did not exert the expected effect as it was in unaggregated form. Under conditions causing aggregation, virtually all A$\beta_{25–35}$ became aggregated within the first 10 min following its solution in the phosphate buffer, while aggregation of A$\beta_{1–40}$ proceeded more slowly and achieved 100% only on day 5. The different effects on H_2O_2 formation between A$\beta_{25–35}$ and A$\beta_{1–40}$ can be explained on the basis of the ability of two peptides to form aggregates: A$\beta_{25–35}$ was seemingly injected to rats (Figure 1) and added to the incubation (Figure 2) in the aggregated state while A$\beta_{1–40}$ was injected and added in the monomeric form. The lack of A$\beta_{1–40}$ activity seen under conditions of Figures 1 and 2 was apparent and could scarcely be explained by 'over-aggregation' of the peptide [32] as after exposition to conditions causing aggregation for 3 days, A$\beta_{1–40}$ became active. Both aggregated A$\beta_{25–35}$ and aggregated A$\beta_{1–40}$ stimulated H_2O_2 production by rat brain mitochondria *in vivo* and *in vitro*. Much higher concentration of aggregated A$\beta_{1–40}$ and much longer incubation time were necessary for maximum acceleration of H_2O_2 production by mitochondria than those for A$\beta_{25–35}$.

A$\beta_{25–35}$ is often selected as a model for full-length Aβ peptide because it retains both its physical and biological properties, while its short length readily allows derivatives to be synthesized and studied [33]. A$\beta_{25–35}$ was reported two decades ago to be not present in biological systems [34]. However, Kaneko et al. [35] proposed an alternative natural means to produce A$\beta_{25–35}$. They suggested that racemization of serine residues of Aβ peptide may be involved in neurodegeneration and formation of senile plaques through escaping from the degradation process by brain proteases. A$\beta_{25–35}$ accumulates in AD brains in a racemized form (L- to D-Ser^{26}) [36]. Racemization of Ser and Asp in Aβ peptide, e.g. at Ser^{26}, is a typical age-dependent modification in AD and occurs as part of the normal ageing process [37,38], so A$\beta_{25–35}$ can be produced in aged AD brains when the soluble racemized A$\beta_{1–40}$ is proteolytically cleaved. Kubo et al. [36] have shown that A$\beta_{1–40}$ racemized at Ser^{26} ([D-Ser^{26}]A$\beta_{1–40}$) was easily converted by brain proteases to a truncated toxic fragment, [D-Ser^{26}]A$\beta_{25–35}$. Truncated [D-Ser^{26}]A$\beta_{25–35}$ was detected immunohistochemically in both senile plaques and neurons in AD-affected brains [39]. These results suggest that [D-Ser^{26}]A$\beta_{1–40}$, formed during ageing, becomes soluble, diffuses from senile plaques and proteolysed to [D-Ser^{26}]A$\beta_{25–35}$, which is toxic and may contribute to the neurodegeneration.

Thus, A$\beta_{25–35}$ appeared to be the endogenous peptide, forming in human brain naturally. It is smaller, better permeating cellular membranes and not less toxic than the full-length peptide. It can be widely used instead of the full-length peptide and have replaced other Aβ peptides in experiments in order to unify scientific works.

Are injected doses of Aβ peptides adequate in our model? The steady state A$\beta_{1–40}$ levels in cerebrospinal fluid were found to be 5–25 ng/ml (1–6 nM) in healthy volunteers and subjects diagnosed with AD [40], 20 ng/ml (5 nM) in Tg2576 mice, transgenic animal model of human AD [41], 5–10 ng/ml (1–2.3 nM) in the guinea pig, a non-transgenic model that has an Aβ peptide sequence identical to that of humans [42] and as much as 35–45 ng/g cerebral cortex tissue (12–15 nM) in 2–6-month old mutant PS cDKO mice [43]. In studying the exploratory behaviour and learning-memory ability, rats were bilaterally injected with A$\beta_{1–40}$ at 4 μg for each side into the hippocampus and 2 weeks later tested by

using open field, Y-maze and passive avoidance task [44]; it is indirect evidence for such that the high dose of A$\beta_{1–40}$ is atoxic.

A$\beta_{1–40}$ administered intravenously to mice was reported to be rapidly degraded with half-lives of 2.5–3.0 min. C-terminally as well as N-terminally degraded peptides in the bile of these mice were clearly identified 5 min after the injection and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry revealed that the end products were A$\beta_{6–39}$, A$\beta_{1–33}$, A$\beta_{4–36}$, A$\beta_{6–20}$, A$\beta_{3–35}$, A$\beta_{4–37}$, A$\beta_{1–37}$, A$\beta_{1–38}$, A$\beta_{1–39}$, A$\beta_{1–40}$ [45]. Therefore it would make allowances when injected doses of A$\beta_{1–40}$ were chosen.

In preliminary experiments, we tested A$\beta_{1–40}$ at doses from 0.1–100 ng/h per animal and found that 3–3.7 ng/h/rat (70–90 ng/day/rat = 20 pmol/day/rat) was enough to see the A$\beta_{1–40}$ effects described in the Results section. This dosage roughly corresponds to the physiological level of the Aβ peptide in cerebrospinal fluid and to the 1 ng/ml (0.23 nM) concentration increment in each 3 min with degradation of > 0.5 ng/ml in each 3 min.

Presently it is unknown what levels and injected doses of A$\beta_{25–35}$ are near-physiological ones. In some studies, rats received intracerebroventricular injections of A$\beta_{25–35}$ at 20 μg/day (15 nmol/day) for 7 days and levels of AChE were measured in the cerebral cortex and cerebrospinal fluid after 2 months [46]; it is indirect evidence for such that the high dose of A$\beta_{25–35}$ is atoxic.

Both H_2O_2 level and the rate of H_2O_2 production in the mitochondrion depends on the activity of the H_2O_2-generating enzymes Mn-SOD in the mitochondrial matrix and MAO in the outer mitochondrial membrane and of H_2O_2-consuming enzymes catalase and glutathione peroxidase.

SOD destroys radicals which are normally produced within the cells and which are toxic to biological systems. It catalyses the dismutation of superoxide to H_2O_2 and O_2. Mn-SOD is the mitochondrial matrix form of SOD binding one manganese ion per sub-unit and active as a tetramer.

We determined Mn-SOD activity in isolated non-synaptic mitochondria from the rat brain neocortex. Measurements showed that the activity of Mn-SOD was increased in A$\beta_{1–40}$-treated rats. These data are in line with literature results obtained early in the *in vitro* study. In cultured hippocampal neurons exposed to A$\beta_{1–40}$ an increase of Mn-SOD gene expression was observed [47]. Surprisingly, a significant decrease in Mn-SOD activity was registered in homogenized brain samples from APP/PS-1 mutant mice, double knock-in mouse model of AD, at the age of 3–14 months with no changes of the enzyme protein levels [48]. The cause of this difference is not clear and will be explored in the future.

MAO is the enzyme located on the cytoplasmic side of outer mitochondrial membrane, catalyses the oxidative deamination of biogenic and xenobiotic amines with the formation of H_2O_2 and ammonia and has important functions in the metabolism of neuroactive and vasoactive amines in the central nervous system and peripheral tissues. In the brain, there are two sub-types, MAO-A mainly in neurons [49] and MAO-B in glial cells [50]. MAO-A preferentially oxidizes biogenic amines such as 5-hydroxytryptamine, norepinephrine and epinephrine, while MAO-B preferentially degrades benzylamine and phenylethylamine. In our experiments, MAO-B activity in brain mitochondria from A$\beta_{1–40}$-treated rats tended to increase as compared to control, although the a difference between A$\beta_{1–40}$-treated and control animals was insignificant. Preliminary experiments using ELISA revealed some increase in amount of MAO-B in brain mitochondria from A$\beta_{1–40}$-treated rats (not shown). Similar results were obtained by other authors. The evidence for a protective effect of MAO-B inhibitors in the treatment of AD in humans confirmed indirectly that MAO-B activity was increased in AD brains [51,52]. Song et al. [53] investigated the effects of A$\beta_{25–35}$ on MAO-B expression and activity in primary cultured rat astrocytes and found that the increase in MAO-B activity was due to an increment of the number of enzyme molecules since kinetic analysis demonstrated a 1.5-fold increase in V_{max} with no change in K_m. Treatment with A$\beta_{25–35}$ also led to a substantial increase in MAO-B mRNA level [53].

Increase in Mn-SOD and MAO-B activities following A$\beta_{1–40}$ infusion can add to corresponding acceleration of H_2O_2 production by mitochondria.

Catalase serves to protect cells from the toxic effects of H_2O_2. It decomposes H_2O_2 to water and O_2. Glutathione peroxidase protects cells and enzymes from oxidative damage, catalysing the reduction of H_2O_2, lipid peroxides and organic hydroperoxides, by glutathione, in that way removing H_2O_2 and other potentially toxic peroxides.

To now, five glutathione peroxidase types were identified in mammals: cytosolic, phospholipid hydroperoxidase, plasma, gastrointestinal and, in humans, glutathione peroxidase, which is restricted to the olfactory system [54]. Mitochondrial glutathione peroxidase is absent from this list. Similarly, catalase is generally assumed to be a peroxisomal enzyme. However, we were able to find both catalase and glutathione peroxidase activities in highly purified rat brain and liver mitochondria [55–57] as well as in rat heart mitochondria [58]. It is known that intracellular Aβ peptides are present in mitochondria from brains of AD patients and transgenic mice with targeted neuronal over-expression of mutant human amyloid precursor protein. Mitochondria might be an important target of Aβ peptides [59]. Aβ peptides progres-

sively accumulate in mitochondria and are associated with diminished enzymatic activity of respiratory chain complexes III and IV and a reduction in the rate of oxygen consumption [60]. They can affect mitochondrial catalase and glutathione peroxidase activities.

We determined these enzyme activities in isolated non-synaptic mitochondria from rat brain neocortex. Both catalase and glutathione peroxidase activities in rat neocortex mitochondria were reduced significantly on the 5th day of A$\beta_{1–40}$ infusion. These results obtained *in vivo* are in coincidence with data of *in vitro* studies on cellular enzymes. Exposure of the rat pheochromocytoma line PC12 cells to A$\beta_{25–35}$ caused a significant reduction in activities of glutathione peroxidase and catalase [61]. Changes of antioxidant enzyme activities in rat brain mitochondria can contribute to the corresponding acceleration of mitochondrial H_2O_2 production described above.

There is no data in the literature available on the effects of Aβ peptides on XO and XDH activities in any brain preparation. In healthy tissue, xanthine-oxidizing enzyme is present as NAD^+-dependent dehydrogenase (XDH) [62] which oxidizes hypoxanthine to xanthine and then to urate but does not generate H_2O_2. Under many pathological conditions XDH may be converted to XO irreversible by proteolysis or reversible through the oxidation of sulphydryl groups [62]. Cytosolic enzyme XO generates H_2O_2 via xanthine oxidation to urate. We measured activities of both enzymes, XO and XDH, in the neocortex cytosolic fraction and found that injection of rats with aggregated A$\beta_{1–40}$ for 5 days led to an increase in the XO activity and XO/XDH activity ratio. Our results are the first to indicate that continuous intracerebroventricular infusion of aggregated A$\beta_{1–40}$ to rats leads to conversion of neocortical XDH to XO associated with increase in XO activity. This can contribute to acceleration of cytosolic H_2O_2 production.

Cu,Zn-SOD is a cytosolic enzyme and binds one copper ion and one zinc ion per sub-unit and is active as a monomer. Cytosolic aldehyde oxidase catalyses oxidation of aldehydes to corresponding carboxylic acids with the formation of H_2O_2. Continuous intracerebroventricular infusion of aggregated A$\beta_{1–40}$ to rats resulted in an increase in the Cu,Zn-SOD activity in the neocortex cytosolic fraction. These data are in the line with the only literature result; in studies on cultured hippocampal neurons, Aksenov et al. [47] have demonstrated an increase in Cu,Zn-SOD gene expression in cells exposed to A$\beta_{1–40}$.

There is no data in the literature on the effects of the Aβ peptide on aldehyde dehydrogenase activity in any brain preparation. Our results are first to indicate that chronic intracerebroventricular infusion of A$\beta_{1–40}$ to rats leads to the dramatic increase in aldehyde oxidase activity and can, together with increased Cu,Zn-SOD activity, add to the increase in the rate of cytosolic H_2O_2 production in the brain neocortex.

According to the results presented, we propose a possible mechanism for increased ROS generation in both mitochondria and the cytosol of rat neocortex under the influence of Aβ peptide (Figure 3).

The Aβ peptide increases the cytosolic and mitochondrial Ca^{2+} concentrations in the cell (either directly or via ammonia action on NMDA receptors [63]) that in turn leads to stimulation of the O_2^- formation in the respiratory chain, MAO-B and Mn-SOD activities in mitochondria, Cu,Zn-SOD and aldehyde oxidase activities and XDH to XO conversion in the cytosol, to inhibition of mitochondrial catalase and glutathione peroxidase activities. All of these events cause massive ROS accumulation and oxidative stress development in the cell.

In summary, we showed that continuous infusion of aggregated A$\beta_{1–40}$ into rat brain induced increases in activity of H_2O_2-generating enzyme Mn-SOD, decreases activities of H_2O_2-consuming enzymes catalase and glutathione peroxidase in brain neocortex mitochondria. As well, infusion of A$\beta_{1–40}$ led to an increase in XO, Cu,Zn-SOD and aldehyde oxidase activities in the brain cytosolic fraction.

These studies suggest that alterations of H_2O_2-metabolizing enzyme activities can contribute to oxidative stress underlying neurodegeneration and neuronal death in Alzheimer's disease.

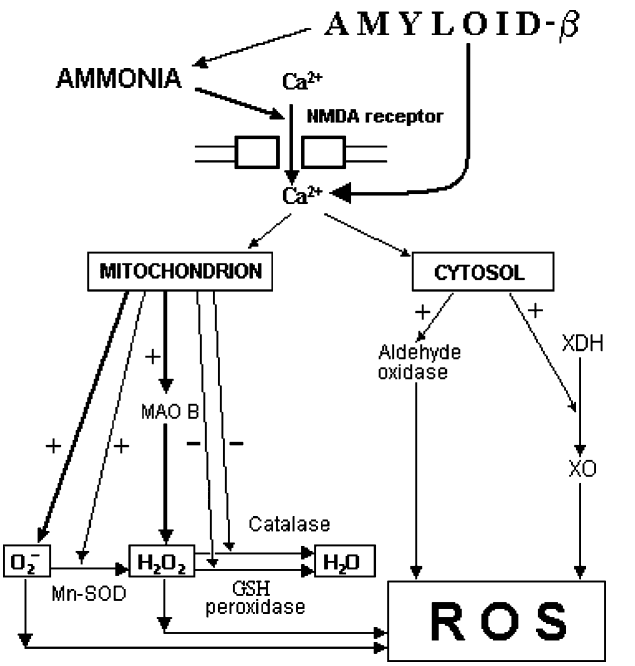

Figure 3. Scheme illustrating the sequence of events following an Aβ peptide injection. Activating and inhibiting effects correspond to '+' and '−'.

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