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Amyloid-β 25 – 35 peptide induces expression of monoamine oxidase B in cultured rat astrocytes[1]

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KEY WORDS Alzheimer disease; astrocytes; amyloid beta-protein; monoamine oxidase; monoamine oxidase inhibitors; oxidative stress

ABSTRACT

AIM: To investigate the effects of amyloid-β 25 – 35 peptide (Aβ 25 – 35) on monoamine oxidase (MAO) expression and activity in primary cultured rat astrocytes. **METHODS**: Immunocytochemistry was used to observe the morphological changes in astrocytes. Fluorescence spectrophotometry was used to measure the activity of MAO in astrocytes. The expression of MAO in astrocytes was assayed by RT-PCR. **RESULTS**: Aβ 25 – 35 induced a reactive morphological change in cultured rat astrocytes which was accompanied by increased immunoreactivities for glial fibrillary acidic protein. Treatment with Aβ 25 – 35 resulted in an elevation of MAO activity in a dose- and time-dependent manner. Aβ 25 – 35-induced enhancement of MAO activity was of type B (MAO-B). The increase in MAO-B activity appeared to be due to an increase in 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 Aβ 25 – 35 also led to a substantial increase in MAO-B mRNA level in the astrocytes. **CONCLUSION**: Aβ 25 – 35 is able to selectively induce MAO-B expression in rat astrocytes and that the upregulation of MAO-B in Aβ 25 – 35-stimulated astrocytes may play an important role in the pathogenesis of Alzheimer's disease.

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive cognitive decline. The well-studied pathological features of AD include loss of neurons, formation of neurofibrillary tangles and senile plaques, and numerous alterations of neurotransmitter systems in the central nervous system (CNS). Evidence has shown that the activity of monoamine oxidase B (MAO-B) is upregulated in the brain of AD patients compared with controls[1,2]. In analogy to the proposed role of MAO-B in Parkinson's disease, the up-regulation of MAO-B in plaque-associated astrocytes in AD might, indirectly, be a potential source of cytotoxic free radicals that play an important role in the pathogenesis of AD[3]. However, the mechanism by which the activity of MAO-B is upregulated is poorly understood.

Recently, evidence has shown that amyloid beta-peptide (Aβ), the major constituent of the senile plaques, has central roles in the pathogenesis of AD[4,5]. Aβ is directly neurotoxic and can increase neuronal susceptibility to other toxic agents[6,7]. However, the actions of Aβ on glial cells are less well studied although evidence has shown that Aβ could induce astrocytes into reactive astrocytosis in culture and elevate the expression of a variety of bioactive molecules in the astrocytes around amyloid plaques[8].

In the present studies, the effects of Aβ 25 – 35, the active fragment of Aβ, on MAO-B activity and expression in cultured rat astrocytes were studied.

MATERIALS AND METHODS

Materials Aβ 25 – 35 (Sigma Chemical Co. St Louis, MO) was dissolved in distilled water, and stored in aliquots at – 20 ℃. To obtain the aggregatory form of Aβ 25 – 35, the peptide solution was placed in an incubator at 37 ℃ for 7 days[9]. Kynuramine, 4-hydroxyquinoline and dibutyryl cyclic AMP (dBcAMP) were purchased from Sigma Chemical Co. Clorgyline and se-

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legiline were from RBI. Streptavidin biotinylated peroxidase complex kit (ABC kit) was obtained from Sino-American Biotechnology Company (Shanghai).

Astrocyte cultures Cultures of rat cortical type I astrocytes were obtained as described by McCarthy and de Vellis[10]. In brief, 1-2-d-old Wistar rats were decapitated, and the brains. After removing the meninges, the cerebral cortex was dissected. The tissue was cut into small fragments, exposed to trypsin (0.25 % in PBS) digestion for 20 min at 37 ℃ and mechanically dissociated in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal calf serum (FCS). The resultant cell suspension was filtered through Nitex mesh (40 μm) and planted (one brain in one 75-cm^2 flask). Before experiment, cultures were passaged by trypsinization and replanted in 12-well plates with a seeding of 5×10^5 cells/well. This treatment yields a highly purified culture of astrocytes, consisting of 98 % astrocytes, as determined by glial fibrillary acidic protein (GFAP) immunostaining.

Morphological observation and immunocytochemistry Change in cell morphology was assessed by microscopic examination. Cultures were fixed with 4 % paraformaldehyde, permeabilized with 0.2 % Triton X-100 and used for immunocytochemical examination. After overnight incubation at 4 ℃ with monoclonal antibodies to GFAP, cells were stained with an ABC kit according to the manufacturer's instructions. Immunoreactivity was visualized with DAB and 0.01 % hydrogen peroxide.

Assay of MAO activity MAO activity was measured by monitoring the conversion of kynuramine to 4-hydroxyquinoline by the method of Morinan and Garratt[11]. The cultured cells were lysed with freeze-thawing and homogenized in 1 mL of potassium phosphate buffer (pH 7.2) (10 mmol·L^{-1}). The homogenate was centrifuged at 1000 × g for 10 min to remove cell debris and nuclei. The supernatant was used for enzymatic assay. An aliquot of 250 μL of the supernant was incubated with 50 μL of kynuramine (150 μmol·L^{-1}) at 37 ℃ for 15 min. For MAO inhibition test, each aliquot of 250 μL supernatant was incubated with clorgyline (MAO-A inhibitor, 0.1 μmol·L^{-1}) or selegiline (MAO-B inhibitor, 0.1 μmol·L^{-1}) for 30 min at 37 ℃ before the addition of kynuramine. The reaction was terminated by the addition of 100 μL of perchloric acid (0.6 mol·L^{-1}). The mixture was centrifuged at 11 600 × g for 1 min to remove precipitated proteins. An aliquot of 350 μL of the supernatant was transferred to test tubes containing 2 mL of NaOH (1 mol·L^{-1}). After mixing, the fluorescence intensity as 4-hydroxyquinoline was measured at an excitation wavelength of 315 nm and an emission wavelength of 380 nm using a fluorescence spectrophotometer (Hitachi, 650-10 s). For kinetic analysis, samples were incubated with various concentrations of kynuramine (6.25 – 100 nmol·L^{-1}) under identical conditions. The concentration of product was calculated from a standard curve of 4-hydroxyquinoline (0 – 10 nmol·L^{-1}) carried through the assay procedure. The concentration of the protein was measured by the method of Bradford[12].

RNA isolation and reverse-transcription RNA was isolated from 2×10^6 cells with TRIzol Reagent (GIBCO/BRL Life Technologies, Inc) according to the procedure provided in the instruction manual. The total RNA samples were dissolved in 20 μL RNase-free water and then electrophoresed on 1.2 % agarose gels to determine the intactness. For cDNA synthesis, 1 μL (1 μg) total RNA suspension was added to a reverse transcription mixture containing 10 μL 2 mmol·L^{-1} each of dNTPs (Forward, Shanghai), 1 μL (1.6 μg) Oligo $(dT)_{15}$ primer (Sangon, Canada), 1 μL (20 U) ribonuclease inhibitor (Promega, USA), 1 μL (20 U) M-MuLV reverse transcriptase (MBI, USA) and 4 μL 5 × reaction buffer (Tris-HCl 250 mmol·L^{-1}, KCl 250 mmol·L^{-1}, $MgCl_2$ 20 mmol·L^{-1}, DTT 50 mmol·L^{-1}). The mixture was incubated at 37 ℃ for 90 min and then reverse transcriptase was inactivated by heating the reaction mixture at 95 ℃ for 10 min.

PCR procedures The oligonucleiotide primers for MAO-B were the same as reported by Carlo *et al*[13]. One μL reverse transcription (RT) solution was added to PCR mixture containing 5 μL of 10 × buffer, 2 μL of 2 mmol·L^{-1} dNTPs, 1 μL (10 pmol) each of the primer and 0.5 μL (1 U) of Taq DNA polymerase in a total volume of 50 μL. PCR was performed on a thermal cycler PTC-150 (MJ, USA) with the following thermocycle parameters: a 5-min initial denaturation at 94 ℃ followed by 35 cycles of 1 min at 94 ℃, 45 s at 50 ℃, 1 min at 72 ℃, and finally a 10-min extension at 72 ℃. To ensure that reverse transcriptase efficiencies were comparable between test groups, β-actin cDNA was amplified by another PCR. The sense and antisense primers for β-actin were 5′-TTGTAACCAACTGGGACGATA-3′ and 5′-GATCTTGATCTTCATGGTGCT-3′ respectively. After a 5-min initial denaturation, the reaction of the mixture was subjected to amplification for 35 cycles as follows:

45 s at 94 ℃, 45 s at 60 ℃, 90 s at 72 ℃, and a 10-min extension at 72 ℃. Each of the PCR products (5 μL) was run on an ethidium bromide-stained 2 % agarose gel. The optical density of each band were measured by Gel Doc 1000 Video Gel Documentation System (Bio-Rad, Hercules, CA). In preliminary experiments, different numbers of cycle were performed to obtain data within the linear range of PCR amplification.

Statistical analysis Statistical analysis of the data for multiple comparisons was performed by ANOVA followed by Dunnett's test. For single comparison, the significance of differences between groups was determined by *t*-test.

RESULTS

Effect of Aβ 25 – 35 and dBcAMP on astrocytic morphology In 5 % FCS-containing medium, the astrocytes remained flat and polygonal and possessed few processes. When cells were treated with Aβ 25 – 35 (1 μmol·L^{-1}) or dBcAMP (250 μmol·L^{-1}) for 48 h, a morphological transformation was found (Fig 1). The flat and polygonal astrocytes differentiated to process-bearing stellate cells with enhanced GFAP immunoreactivity, resembling the reactive astrocytes found around senile plaques.

Effect of Aβ 25 – 35 and dBcAMP on MAO expression in astrocyte cells In untreated astrocytes, MAO activity remained at a constant level within the 48 h incubation. When cultures were exposed to Aβ 25 – 35 (1 – 10 mmol·L^{-1}) for 48 h, the MAO activity was obviously enhanced (Fig 2). However, no change was found in the MAO activity after 48 h treatment with dBcAMP (50, 250, or 500 μmol·L^{-1}) although dBcAMP and Aβ 25 – 35 induced a similar morphological transformation in astrocytes (Fig 1 and 2). The effect of Aβ 25 – 35 treatment was also time-dependent (Fig 3). Kinetic analysis showed that the maximum reaction velocity (V_{max}) of MAO in the astrocytes increased significantly after 48 h incubation with Aβ 25 – 35, while the K_m was unchanged (Fig 4). Neither Aβ 25 – 35 nor dBcAMP had effect on the cell viability when measured by MTT assay (data not shown).

Both clorgyline and selegiline inhibited MAO activity in astrocytes, suggesting that the astrocytes contain both isoenzymes of MAO, namely MAO-A and MAO-B (Fig 5). However, Aβ 25 – 35-induced increase in MAO activity was attenuated only by selegiline, whereas clorgyline showed little effect (Fig 5). The results suggest that MAO-B was the main isoform induced by Aβ 25 – 35.

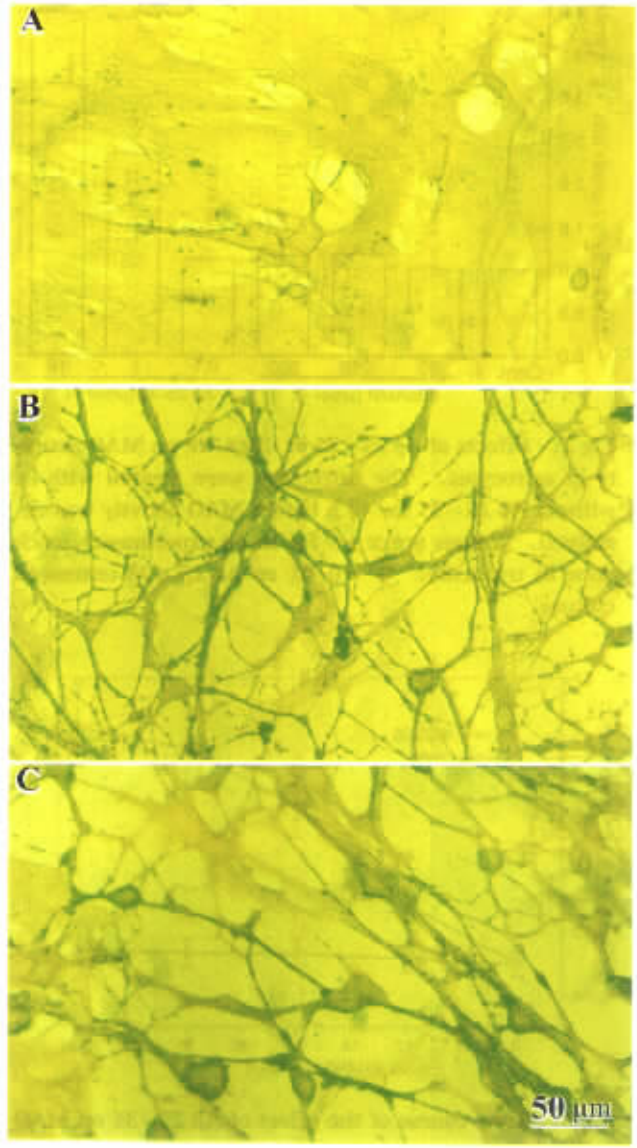

Fig 1. Effects of Aβ 25 – 35 or dBcAMP on morphology of cultured astrocytes. The cells were fixed 48 h after addition of Aβ 25 – 35 1 μmol·L^{-1} or dBcAMP 250 μmol·L^{-1} and stained with monoclonal antibody to astrocyte-specific marker GFAP. A pronounced morphological change was found. The flat and polygonal cells differentiated to the process-bearing stellate cells with enhanced GFAP immunoreactivity. (A) Control; (B) Aβ 25 – 35; (C) dBcAMP. Magnification × 200.

When MAO-B mRNA level was measured by RT-PCR, an increase in the MAO-B mRNA level was also found after astrocytes were treated with Aβ 25 – 35

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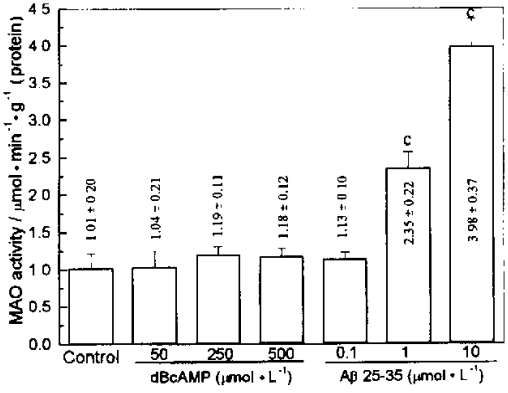


Fig 2. Effects of Aβ 25 – 35 or dBcAMP on MAO activity in astrocytes. The astrocytes were treated with or without Aβ 25 – 35 for 48 h before MAO activity was examined. Values are $\bar{x} \pm s$ for three experiments, each done in triplicate. $^cP < 0.01$ *vs* Aβ 25 – 35-untreated culture.

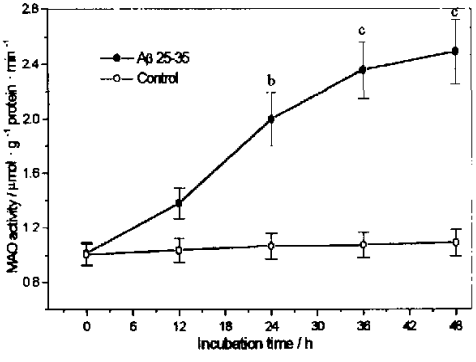


Fig 3. Time course of the effect of Aβ 25 – 35 on MAO activity in astrocytes. The astrocytes were treated with Aβ 25 – 35 (1 μmol·L^{-1}) for different time before MAO activity was examined. Values are $\bar{x} \pm s$ for three experiments, each done in triplicate. $^bP < 0.05$; $^cP < 0.01$ *vs* corresponding Aβ 25 – 35-untreated cultures.

(Fig 6). A slight increase in MAO-B mRNA level was found when the astrocytes were treated with Aβ 25 – 35 for 12 h. When astrocytes were treated with Aβ 25 – 35 for 48 h, a marked increase in the MAO-B mRNA level was detected.

DISCUSSION

Recent evidence has shown that Aβ 25 – 35 and Aβ 1 – 42 can stimulate a reactive phenotype in astrocyte cultures[14-17]. The main finding in our study is that

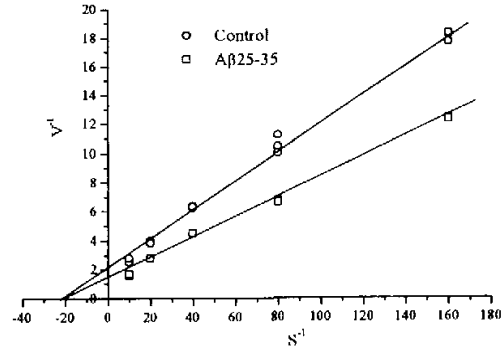


Fig 4. Lineweaver-Burk inverse plot of initial velocity (V, μmol·min^{-1}·g^{-1}) as a function of substrate concentration (S, μmol·L^{-1}) for MAO activity in astrocytes. MAO activity of astrocytes was measured after 48 h of incubation with or without Aβ 25 – 35 (1 μmol·L^{-1}). Each point represents a single value from a representative experiment. Kinetic parameters: control: $K_m = 0.052 \pm 0.004$ μmol·L^{-1}, $V_{max} = 0.508 \pm 0.026$ μmol·min^{-1}·g^{-1}(protein); Aβ 25 – 35 treated cells: $K_m = 0.051 \pm 0.004$ μmol·L^{-1}, $V_{max} = 0.746 \pm 0.045$ μmol·min^{-1}·g^{-1}(protein), $P = 0.001$ *vs* control.

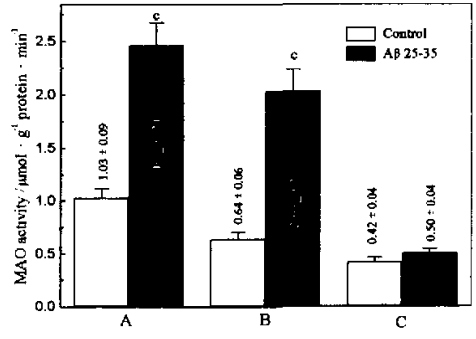
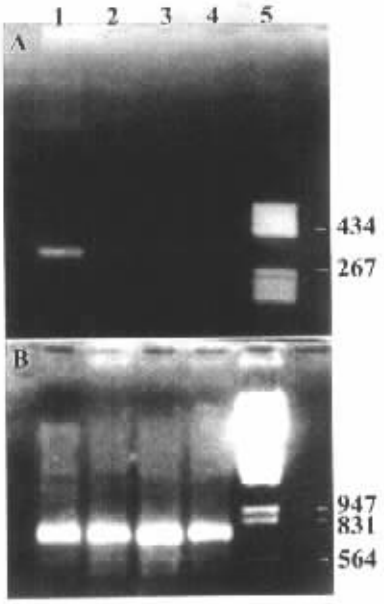


Fig 5. Effects of MAO inhibitors on Aβ 25 – 35-induced elevation of MAO activity in astrocytes. A) Astrocytes were incubated with or without Aβ 25 – 35 (1 μmol·L^{-1}) for 48 h, and MAO activity was measured. B) Astrocytes were incubated with or without Aβ 25 – 35 (1 μmol·L^{-1}) for 48 h, and then the MAO activity was measured after 30 min exposure to clorgyline (0.1 μmol·L^{-1}). C) Astrocytes were incubated with or without Aβ 25 – 35 (1 μmol·L^{-1}) for 48 h, and then the MAO activity was measured after 30 min exposure to selegiline (0.1 μmol·L^{-1}). Values are $\bar{x} \pm s$ for three experiments, each done in triplicate. $^cP < 0.01$ *vs* corresponding Aβ 25 – 35 untreated cultures.

chronic treatment with Aβ 25 – 35 induces a reactive



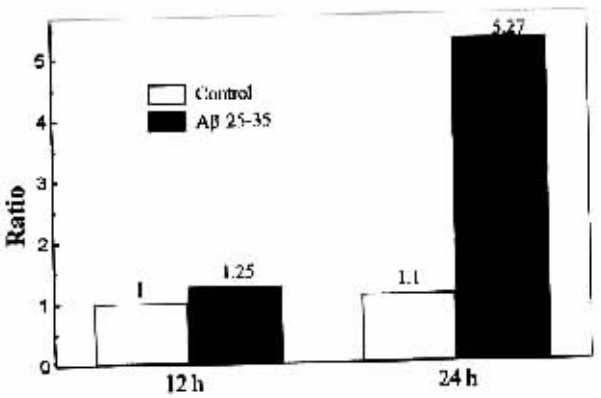


Fig 6. Effect of Aβ 25 – 35 on MAO-B mRNA level. RT-PCR was performed at 12 h and 48 h after the rat astrocytes were incubated with or without Aβ 25 – 35 (1 μmol·L⁻¹). A). RT-PCR using primers specific for about 300-bp fragment of MAO-B cDNA. Lane 1: 48 h incubation with Aβ 25 – 35; Lane 2: 48 h incubation without Aβ 25 – 35; Lane 3: 12 h incubation with Aβ 25 – 35; Lane 4: 12 h incubation without Aβ 25 – 35; Lane 5: DNA marker, pBR322/Hae Ⅲ. B). RT-PCR using primers specific for about 700-bp fragment of β-actin cDNA. Lane 1: 48 h incubation with Aβ 25 – 35; Lane 2: 48 h incubation without Aβ 25 – 35; Lane 3: 12 h incubation with Aβ 25 – 35; Lane 4: 12 h incubation without Aβ 25 – 35; Lane 5: DNA marker, λDNA/EcoR 1 + Hind Ⅲ. C) Semiquantification of A and B. Ratio = 10 × $OD_{MAO-B}/OD_{β-actin}$.

morphological change and an elevation in MAO-B expression in cultured rat astrocytes.

The role of MAO-B in astrocytes has received special attention, because of its possible involvement in neurodegenerative disorders, such as Huntington's disease, Parkinson's disease, and AD[18,19,20]. MAO-B is the predominant form of MAO in the human brain[21,22], and it is specifically located in glial cells and serotoninergic neurons. Enhanced expression of MAO-B has been reported in AD brains in particular in plaque-associated astrocytes[1,2]. Evidence has shown that the upregulation of MAO-B could lead to an excessive deamination of biogenic amines, via an overproduction of H_2O_2, contributing to cytotoxic free radical formation and oxidative stress[23], which is similar to its proposed role in Parkinson's disease[24].

Evidence has shown that Aβ is able to directly cause neuronal death by both apoptotic and necrotic mechanism[6]. Our observation that Aβ 25 – 35 increases MAO-B expression in astrocytes raises the possibility that additional mechanisms of Aβ-induced neurodegeneration exist which depend on the presence of glial cells. Supporting this idea is a recent report[25] showing that treatment of rat astrocytes with Aβ 1 – 42 induces activation and up-regulation of selective glial mRNA and proteins, such as the inflammatory cytokine interleukin-1β and inducible nitric oxide synthase (iNOS). All these data together with our results suggest that a consequence of Aβ-induced glial activation and up-regulation of selective proteins such as interleukin-1β, iNOS, and MAO-B can influence Aβ neurotoxicity in AD.

The mechanism by which Aβ 25 – 35 up-regulates MAO-B expression in astrocytes is unknown. However, it is interesting to note that dBcAMP treatment of astrocytes did not lead to an increase in MAO-B activity while it induced a morphological transformation with enhanced GFAP immunoreactivity[26]. It is clear that the array and degree of cellular responses in the activated glia cell depend on a number of variables, including the activating stimulus[27].

In summary, our results demonstrate that Aβ 25 – 35 is able to selectively induce MAO-B expression in rat astrocytes. These findings support the hypothesis that Aβ-induced glial activation and up-regulation of selective proteins can influence Aβ neurotoxicity in AD. We suggest that Aβ-induced upregulation of MAO-B may lead to an excessive deamination of biogenic amines and cytotoxic free radical formation via an overproduction of H_2O_2, which is similar to its proposed role in Parkinson's disease[23,24]. Although evidence has shown that both Aβ 25 – 35 and Aβ 1 – 42 can induce a stellate-shaped, process-bearing cell morphology, with increases in GFAP

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and bFGF immunoreactivity and glutamine synthetase activity[14-17], further studies are needed to see whether Aβ 1-40 or 1-42 have the same effect.

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淀粉样β蛋白25-35诱导大鼠星形胶质细胞表达单胺氧化酶B[1]

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关键词 阿尔采末病；星形胶质细胞；淀粉样β蛋白；单胺氧化酶；单胺氧化酶抑制剂；氧化应激

目的：观察淀粉样β蛋白25-35（Aβ 25-35）对大鼠星形胶质细胞内单胺氧化酶活性及表达的影响．**方法**：用免疫组织化学方法观察Aβ 25-35对大鼠星形胶质细胞形态的影响；用荧光分光光度法检测MAO的活性；用RT-PCR观察Aβ25-35对MAO表达的影响．**结果**：Aβ 25-35可诱导星形胶质细胞呈现激活形态，伴有胶质原纤维酸性蛋白染色增强．Aβ 25-35可使星形胶质细胞内MAO-B活性增强，并呈剂量和时间依赖性．经RT-PCR检测发现，MAO-B活性增强是由于MAO-B mRNA表达升高所致．**结论**：Aβ 25-35可选择性上调大鼠星形胶质细胞内MAO-B的表达，该作用在阿尔采末病的病理过程中可能具有重要意义．

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