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Research report

Inhibition of JNK phosphorylation reverses memory deficit induced by β-amyloid (1–42) associated with decrease of apoptotic factors

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia that is degenerative and terminal disease. The main reason of the disease is still unknown. β-amyloid (Aβ) plaques are the important hallmarks of memory impairment in patients suffering from AD. Aggregation of these plaques in the hippocampus appears during the development of the disease. One of the prominent factors having crucial impact in this process is MAPK. JNK, as a member of MAPK family has a pivotal role, especially in cell survival. We hypothesized that JNK may have beneficial effect on the process of memory improvement. Hence, we performed Morris water maze to investigate the possible impact of JNK inhibitor on spatial memory in Aβ-injected rats. Our data indicated that intracerebroventricular administration of SP600125, a JNK inhibitor, could significantly decrease escape latency and increase time spent in target quadrant, in treatment group. Furthermore, we evaluated some of the apoptotic factors in the hippocampus of the treated rats. Based on our data, the inhibitor led to the significant decrease in the amount of caspase-3, TUNEL positive cells, cyclooxygenase-2 and increase in Bcl-2/Bax ratio. Given the possible neuroprotective effects of SP600125 on Aβ-induced memory impairment and apoptosis, our results may open a new avenue for the treatment of AD.

1. Introduction

Alzheimer's disease (AD) is a multi-factorial neurodegenerative disease characterized by progressive synaptic loss and neuronal death with gradual cognitive decline. The illness starts insidiously with early signs of patchy memory loss and gradually progresses to impaired language comprehend dementia and ultimately death. The pathological characteristics of AD include accumulation and deposition of β-amyloid (Aβ) peptides in brain parenchyma (senile plaques) and cerebral vessels and the formation of neurofibrillary tangles [45]. One of the main hypotheses about the pathogenesis of AD, the Aβ hypothesis, is supported by a number of epidemiological, genetic and experimental studies [53,55]. Deposition of Aβ peptides in the brain and cerebral vessels results in neuroinflammation and neurovascular inflammation [9,26,49]. Loss of the normal physiological functions of Aβ is also thought to contribute to neuronal dysfunction [1]. $A\beta_{42}$, a major component of amyloid plaques in the AD brain, is an Aβ peptide with 42 amino acids that is produced by the amyloidogenic pathway [6].

Mitogen-activated protein kinases (MAPKs) are serine–threonine kinases that mediate intracellular signaling associated with a variety of cellular activities including cell proliferation, survival, death, and transformation [12,25,51]. JNK is a major cellular stress response protein induced by oxidative stress and plays an important role in AD, and its activation is considered as an early event in AD [67]. Activated JNK is found in the hippocampal and cortical regions of individuals with severe AD and localized with neurofibrillar alterations [66,67]. Aβ peptides induce JNK signaling which mediates Aβ toxicity and adverse effects on long-term potentiation in the hippocampus [3,30,52,60]. Application of Aβ peptides triggers the JNK signaling pathway resulting in phosphorylation of c-Jun [1,41,65]. A very recent study has demonstrated the possibility of JNK activity inhibition on providing therapeutic benefit in the context of AD [5].

Thus, due to the above-stated reasons, in this study we tried to investigate the effects of JNK inhibitor, SP600125, on behavioral response of rats in order to demonstrate the practical effect of JNK inhibitor on spatial memory. Besides, we studied some of the apoptotic factors such as caspase-3, Bax, and Bcl-2 that we expected to get impact from JNK.

2. Materials and methods

2.1. Animals

Thirty-five adult male albino Wistar rats (Pasteur Institute, Tehran, Iran) weighing 210–280 g were used in these experiments. Animals were housed in groups of three per cage in a 12/12 h light/dark cycle (light on between 7:00 a.m. and 7:00 p.m.)

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with free access to chow and tap water. The animals were randomly allocated to different experimental groups. Each animal was used only once. Rats were habituated to their new environment and handled for 1 week before the experimental procedure was started. All experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 1996) and were approved by the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences.

2.2. Drugs

Antibodies directed against Caspase-3, Bax, Bcl-2, Cyclooxygenase-2 (COX-2), p-JNK, and β-actin were obtained from Cell Signaling Technology. Electrochemiluminescence (ECL) kit was provided from Amersham Bioscience, USA. Aβ (1–42) and SP600125 were obtained from Sigma–Aldrich (St. Louis, MO). TUNEL (Apoptag plus peroxidase in situ Apoptosis detection) kit was gotten from Chemicon.

2.3. Preparation of beta-amyloid peptide 1–42 ($A\beta_{1-42}$) and fibrilization

The Aβ1–42 was dissolved, and aliquots were stored at −20 °C until use. Aliquots of Aβ1–42 at a concentration of 200 ng/μl prepared in Phosphate Buffer Saline (PBS 0.1 M) were incubated for 5 days at 37 °C. On the test day, PBS was added to the solution to reach the final concentration of 10 ng/μl.

2.4. Stereotaxic surgery

Rats were anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (100 mg/kg), and placed into stereotaxic device (Stoelting, USA). An incision was made along the midline, the scalp was retracted, and the area surrounding bregma was cleaned and dried. In addition, lidocaine with epinephrine solution (0.2 ml) was injected in several locations around the incision. Microinjections were performed by 30-gauge injector cannula. Polyethylene tube (PE-10) was used to attach injector cannula to the 5 μl Hamilton syringe. For intracerebroventricular (ICV) administration of JNK inhibitor solution, 30-gauge injector cannula was aimed at the lateral ventricle (stereotaxic coordinates: incisor bar -3.3 mm, 0.5 mm posterior to the bregma, 1.5 mm lateral to the sagittal suture and 4 mm down from top of the skull) [39]. For intra-hippocampal administration, stainless steel guide cannulae (23-gauge), 6 mm in length, were aimed at the CA1 area of hippocampus (stereotaxic coordinates: incisor bar −3.3 mm, 3.8 mm posterior to the bregma, ±3.2 mm lateral to the sagittal suture and 2.7 mm down from top of the skull) bilaterally [39]. Cannulae were secured with jewelers' screws and dental acrylic cement. After the cement was completely dried and hardened, two stainless steel stylets were used to occlude the guide cannulae. Penicillin-G 200,000 IU/ml (0.2–0.3 ml/rat, single dose, intramuscular) was administered immediately after surgery. Animals received a total volume of 5 μl JNK inhibitor into the left or right ventricle, and 3 μl/side Aβ microinjection into the CA1. All microinjections were performed slowly over a period of 60 s, and injection needles were left in place for an additional 60 s to facilitate diffusion of the drugs.

2.5. Behavioral test: Morris water maze (MWM)

2.5.1. Apparatus

The water maze that was used has been described extensively [27,42,46]. Briefly, it consisted of a dark circular pool (140 cm in diameter and 55 cm high) filled with water (20 ± 1 °C) to a depth of 25 cm. A transparent Plexiglas platform (11 cm diameter) was located 1 cm below the water surface in the center of one of the arbitrarily designed north-east (NE), south-east (SE), south-west (SW) or north-west (NW) orthogonal quadrants. The platform provided the only escape from the water. Many extra-maze cues such as racks, a window, a door, bookshelves and pictures on the walls surrounded the room where the water maze was performed. These were kept in fixed positions with respect to the swimming pool to allow the rat to locate the escape platform hidden below the water surface. The position of the animal was monitored by a camera that was mounted above the center of the pool. Animal displacement was recorded using a 3CCD camera (Panasonic Inc., Japan) placed 2 m above the MWM apparatus and locomotion tracking was measured by ethovision software (version XT7), a video tracking system for automation of behavioral experiments (Noldus Information Technology, the Netherlands).

In these series of experiments, escape latency and swimming speed as well as time spent in the target quadrant were recorded during 60 s, in both probe and training trials.

2.5.2. Habituation

Twenty-four hours prior to the start of training, rats were habituated to the pool by allowing them to perform a 60 s swimming without the platform.

2.5.3. Procedure

19 days after surgery, the behavioral tests were started. The single training session consisted of eight trials with four different starting positions that were equally distributed around the perimeter of the maze [11].

Each rat was placed in the water facing the wall of the tank at one of the four designated starting points (north, east, south and west) and was allowed to swim and find the hidden platform located in the SW quadrant (target quadrant) of the maze. Each of four starting positions was used twice in eight training sessions; their order was randomized. During each trial, each rat was given 60 s to find the hidden platform. After mounting the platform, the animals were allowed to remain there for 20 s, and were then placed in a holding cage for 30 s until the start of next trial. After completion of training, the animals were returned to their home cages until the probe trial 24 h later (on the test day). In the probe trial the hidden platform was removed and the animals were released from the north location and allowed to swim freely for 60 s. After the probe trial, the platform was elevated above the water surface and placed in the different position (SE quadrant) and rats were allowed to swim freely for 120 s in order to test their visual ability. All of experiments were conducted between 9:00 and 13:00.

2.6. Western blot analysis

Western blot analysis was carried out using protein extract 7 days after Aβ injection. For this purpose, the hippocampi were homogenized in lysis buffer containing complete protease inhibitor cocktail. Then, the total proteins were electrophoresed in 12% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. Immunoreactive polypeptides were detected by chemiluminescence using enhanced ECL reagents and subsequent autoradiography. Quantification of the results was performed by densitometric scan of films. Data analysis was done by Image. J., measuring integrated density of bands after background subtraction. Protein concentrations were determined according to Bradford's method [4]. Standard plot was generated using bovine serum albumin.

2.7. Immunostaining

To detect cells undergoing apoptosis, we used the technique of Terminal-Transferase dUTP Nick End labeling (TUNEL). After killing, brains were removed, two hemispheres separated and rapidly fixed in formalin 10% for 24 h. The tissues were processed and paraffin embedded. The blocks were cronally sectioned by microtome. Sections (10 μm) were mounted on slides and a proteinase K digestion (20 μg/ml) was carried out for 15 min. Endogenous hydrogen peroxidase activity was quenched in 3% hydrogen peroxide. After a series of rinsing, nucleotides labeled with digoxigenin were enzymatically added to DNA by terminal deoxy nucleotidyl transferase enzyme (TdT). The incubation was carried out for 60 min and the labeled DNA was detected using anti-digoxigenin-peroxidase for 30 min. Addition of the chromogen diaminobenzidine tetra hydrochloride (DAB) resulted in a brown reaction product that was evaluated by light microscopy. Positive and negative controls were carried out on slides from the same block. Incubation without TdT served as the negative control. For TUNEL staining, 10 fields were chosen from each groups (4 groups) and the percent of TUNEL-positive cells were calculated according to this relation: %TUNEL-positive neurons = (TUNEL-positive neurons/TUNEL-positive neurons (brown) + normal neurons(green)) × 100.

2.8. Experimental design

In the present study, animals were divided into four groups: (i) Aβ-injected group, which received unilateral ICV administration of DMSO (5 μl/rat) 4 h before the bilateral intra-CA1 injection of Aβ (30 ng/3 μl PBS per side), without receiving any treatment; (ii) Vehicle group, that only received carriers [DMSO (5 μl/rat) in lateral ventricle and PBS (3 μl/side) in both CA1 regions]; (iii) JNK inhibitor group, which received ICV infusion of SP600125 (30 μg/5 μl 1% DMSO in PBS) with PBS injection (3 μl/side) in CA1; and (iv) treatment group which received ICV administration of SP600125 (30 μg/5 μl 1% DMSO in PBS) 4 h prior to intra-hippocampal Aβ (30 ng/3 μl PBS per side) injection. The aforementioned groups entered two experimental protocols: behavioral experiments and molecular studies.

2.9. Statistics

Data were expressed as mean ± SEM (standard error of mean) and processed by commercially available software GraphPad Prism® 5.0. One-way analysis of variance (ANOVA) and randomized block model followed by post-hoc analysis (Newman–Keuls test) were used. On the other hand, the mean value of training data for the first and second four trials was compared by paired student *t*-test. *P*-value less than 0.05 ($P < 0.05$) was considered to be statistically significant.

3. Results

3.1. Behavioral results

3.1.1. SP600125, a JNK specific inhibitor, had influence on spatial learning in MWM

Data obtained in training session showed that there is a significant difference between the first and second four trials in escape latency in all experimental groups, except in Aβ-injected rats which was not significant [$t_{(4)} = 1.0570$, $P = 0.3502$; Fig. 1A]. The swimming speed did not show any significant alteration between the first and