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N-iminoethyl-L-lysine improves memory and reduces amyloid pathology in a transgenic mouse model of amyloid deposition

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ABSTRACT

A large body of evidence suggests the importance of inflammation and oxidative or nitrosative stress in Alzheimer's disease (AD) pathogenesis. Inflammatory stimuli upregulate transcription of inducible nitric oxide synthase (iNOS), which can lead to the production of nitric oxide and other reactive nitrogen species. We previously found that genetic deletion of iNOS in mice overexpressing the amyloid precursor protein (APP) and presenilin-1 (PS1) reduced mortality, nitrosative stress, amyloid plaque burden, microgliosis, astrocytosis, and peri-plaque tau phosphorylation. We therefore examined the effects of N6-(1-iminoethyl)-L-lysine (L-NIL), a pharmacological iNOS inhibitor, or D-NIL, its enantiomeric control, in a transgenic mouse model of amyloid deposition. Tg19959 mice carry human APP with two mutations and develop amyloid plaques and memory impairment starting at 3–4 months of age. Mice were given L-NIL or D-NIL in the drinking water from 1 month of age and assessed behaviorally and histopathologically at 8 months of age. We found that L-NIL administration reduced disinhibition in the elevated plus maze, improved spatial memory performance in the Morris water maze, and decreased cortical amyloid deposition as well as microglial activation in 8-month-old Tg19959 mice. These findings are consistent with previous reports demonstrating that iNOS inhibition ameliorates AD pathogenesis.

1. Introduction

Many important neurotransmitters and neuromediators are altered in Alzheimer's disease (AD), including acetylcholine (Pakaski and Kalman, 2008) and amino acids (Yenkoyan et al., 2009). Nitric oxide (NO) is another crucial element in the brain due to its numerous physiological functions, including neurotransmission, immune defense, platelet reactivity, and vasorelaxation (Dawson et al., 1992; Guix et al., 2005). There is a large body of evidence demonstrating the involvement of NO-related pathways in AD and other dementias (Togo et al., 2004). NO increases production of reactive nitrogen species (Dawson et al., 1992; Dawson and Dawson, 1996) and activation of inflammatory pathways (Brown and Bal-Price, 2003).

In 1996, inducible nitric oxide synthase (iNOS) was identified in tangle-bearing neurons in human Alzheimer's disease brains (Vodovotz et al., 1996). Confirming this observation, elevated iNOS expression was found in neurons and glia predominantly in the cortex (Fernandez-Vizarra et al., 2004) and in the hippocampus of AD patients (Lee et al., 1999). Transgenic mouse models of amyloid deposition also showed increased iNOS and NO levels (Rodrigo et al., 2004) and nitrosative stress in proximity to Aβ deposits (Luth et al., 2001; Luth et al., 2002). Increasing iNOS expression elevates the production of NO and NO-derived reactive nitrogen species that can be detrimental to cells (Malinski, 2007). Thus, iNOS inhibition has been suggested as a therapeutic target to reduce NO production and nitrosative stress, especially in AD pathogenesis. In transgenic mice carrying mutations in both the amyloid precursor protein (APP) and presenilin-1 (PS1), genetic deletion of iNOS ameliorated AD pathology by decreasing mortality and, at late stage, by reducing amyloid plaque burden, microgliosis, astrocytosis, nitrotyrosine levels, and peri-plaque tau phosphorylation (Nathan et al., 2005). Genetic deletion of iNOS in AD mouse models has been very helpful in understanding the importance of iNOS/NO-related pathways in AD pathogenesis. However, in the context of drug therapy to prevent disease progression, there is a need for more clinically relevant studies in vivo. We have therefore proceeded to test a pharmacological inhibitor of iNOS as a potential therapeutic approach.

In the current study, we administered N6-(1-iminoethyl)-L-lysine (L-NIL), a pharmacological iNOS inhibitor, in a transgenic mouse model of amyloid deposition. L-NIL is 28-fold more selective for iNOS than for the other NOS isoforms and has an IC_{50} of 3.3 μM (Moore et al., 1994; Stenger et al., 1995). Vehicle (drinking water) was used as control. As an additional control, we used the optical enantiomer D-NIL, a very poor inhibitor of iNOS with an IC_{50} of

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336 μM (Moore et al., 1994). L-NIL was given in the drinking water to Tg19959 mice from 1 to 8 months of age. Tg19959 mice overexpress human APP with two mutations, KM670/671NL and V717F. They develop amyloid deposition at approximately 3–4 months of age, starting in the neocortex, the amygdala, and the hippocampus (Li et al., 2004a), similar to the related TgCRND8 mice (Chishti et al., 2001). At the same age, reactive microglia and activated astrocytes surround amyloid plaques (Dudal et al., 2004). Mice also exhibit spatial learning and memory impairments in the Morris water maze (Chishti et al., 2001). Moreover, amyloid pathology and behavioral deficits increase with age (Hyde et al., 2005).

2. Experimental procedures

2.1. Animals

Tg19959 mice were obtained from Dr. George Carlson (McLaughlin Research Institute, Great Falls, MO, USA). Mice were maintained on a hybrid C57BL6/SJL background. Only female offspring were used for the study. Transgenic AD females typically exhibit greater pathology than males (Hirata-Fukae et al., 2008; Schafer et al., 2007; Wang et al., 2003). Starting at 1 month of age, mice received 4 mM L-NIL, 4 mM D-NIL (inactive enantiomer), or vehicle (water) in the drinking water. At 8 months of age, mice were assessed behaviorally and a sub-group of the same animals was euthanized for histological assays.

All experiments were conducted in accordance with the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College.

2.2. Materials

L-NIL dihydrochloride (100% pure) and D-NIL dihydrochloride (99.9% pure) were synthesized for this project as follows. Reaction of L-(or D)-N^2-(t-butoxycarbonyl)lysine with ethyl acetimidate hydrochloride in water in the presence of sodium hydroxide led to L-(or D)-N^6-(1-iminoethyl)-N^2-(t-butoxycarbonyl)lysine, which was deprotected with aqueous hydrochloric acid at room temperature. As assessed by elemental analysis, ^{1}H NMR, ^{13}C NMR, liquid chromatography–mass spectroscopy and optical rotation, both preparations contained the authentic compounds at a purity of 100% for L-NIL and 99.9% for D-NIL. For L-NIL, these results included: ^{1}H NMR (D_2O, 500 MHz): δ 3.81 (1H, t), 3.35 (2H, t), 2.29 (3H, s), 1.95 (2H, m), 1.76 (2H, m), 1.52 (2H, m); ^{13}C NMR (D_2O, 125 MHz): 174.91, 164.92, 54.90, 42.09, 30.24, 26.70, 22.05, 18.79; elemental analysis: C 34.0%, H 8.1%, N 15.9%, Na 3.8%, total halogens as Cl 19.7%; MS: 186 (M−1); optical rotation: $[a]_d^{20}$ = +16.4 (C = 1.0, 80% acetic acid in water). Compounds were administered to mice in the drinking water acidified with HCl to pH 2.7 to retard microbial growth. The drinking water used as vehicle was also acidified to pH 2.7. Liquid chromatography–mass spectrometry demonstrated that the compounds were stable for at least a week under these conditions.

2.3. Behavioral procedure

Exploratory behavior was assessed in the open-field (45 cm × 45 cm; height: 20 cm). Distance traveled was recorded during 5 min per day and averaged over 3 days using a video tracking system (Ethovision 3.0, Noldus Technology, Attleborough, MA, USA).

Anxiety was evaluated in the elevated plus maze. The apparatus consisted of four arms (length: 30 cm; width: 5 cm; height: 39 cm) in a cross-shaped form and a central region (5 cm × 5 cm). Two of these arms were enclosed on three sides by walls (height: 15 cm) whereas the other arms were not. The two enclosed arms faced each other, as did the two open arms. At the beginning of the test, mice were placed in the central region. Time spent in the open arms was measured during a single trial of 5 min.

Spatial learning and memory were assessed in the Morris water maze (diameter: 120 cm; height of the wall: 60 cm). To familiarize the animals with the task, each mouse was placed on the platform (diameter: 10 cm) in the center of the basin for 20 s. Then, 4 practice trials were performed, where animals were released 3–5 cm away from the platform and were allowed to find it within 20 s. For each trial, they were forced to stay 20 s on the platform before being removed. This procedure was repeated the next day.

During the acquisition period, extra-maze visual cues were arranged in the room. The hidden platform was located in the middle of the northwest (NW) quadrant. Each day, mice were placed next to and facing the wall of the basin in 4 starting positions: north, east, south, and west, corresponding to four successive trials. The duration of a trial was 60 s with an inter-trial interval of 20 min. Whenever the mouse failed to reach the platform within 60 s, it was placed on the platform by the experimenter for 5 s. Latencies and distances before reaching the platform were recorded for 5 days (Ethovision 3.0, Noldus Technology, Attleborough, MA, USA). After each trial, animals were placed in a plastic holding cage to keep them warm and dry.

A probe trial was assessed 1 h after the last trial of the acquisition period, removing the platform from the pool. Mice were released on the north side for a single trial of 60 s, during which the percent time spent in each quadrant was measured.

The visible platform version was performed during 2 days with 4 trials per day. In this cued version, a pole (13 cm) was added on the platform and its location was changed between each trial. The duration of a trial was 60 s with an inter-trial interval of 20 min. Latencies and distances before reaching the platform were recorded and averaged.

2.4. Immunohistochemistry

To study amyloid deposition and microglial activation, a sub-group of mice assessed behaviorally was euthanized by transcardiac saline perfusion. Brains were post-fixed in 4% paraformaldehyde in 1 mM phosphate buffer pH 7.4 for 24 h and stored in cryoprotectant. Retrosplenial/motor cortex was analyzed from bregma −1.06 to bregma −1.94. The CA1/dentate region was analyzed from bregma −1.34 to bregma −2.7.

For the amyloid deposits, brain sections were pretreated with 50% formic acid for 5 min before labeling with anti-Aβ42 rabbit polyclonal antibody AB5078P (1:1000, Chemicon, Temecula, CA, USA). For the microglial activation, adjacent sections were also labeled with anti-CD-40 rat monoclonal antibody (1:100, Serotec, Raleigh, NC, USA).

Immunolabeling was detected by the avidin–biotin complex peroxidase method and visualized after DAB (diaminobenzidine) incubation for 5 min (Vector, Burlingame, CA, USA). For each animal, 5 sections were analyzed. Using NIH Image 1.63 (National Institutes of Health, Bethesda, MD, USA), the percentage areas occupied by AB5078P immunoreactive amyloid plaques and by CD-40 immunoreactive reactive microglia per 0.75 mm^2 were calculated. Two fields per section were analyzed. The threshold was set at 50.

2.5. Statistics

For the body weight, open-field and elevated plus maze testing, and immunohistochemistry, one-way ANOVA was used to analyze experimental groups ((wild-type or Tg19959 mice) × (vehicle, D-NIL or L-NIL)). Fisher PLSD post hoc test (Fisher) was used for further pair-wise analyses between treatments.

For the acquisition period of the Morris water maze, we used repeated measures ANOVA to account for the 5 days of training. Fisher test was used for further pair-wise analyses between mouse groups.

For the probe trial of the Morris water maze, we used a one-way ANOVA for each of the six experimental groups to compare their performances in the 4 quadrants of the pool. Fisher test was used for further pair-wise analyses between quadrants (Statview 5.0.1, SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. L-NIL or D-NIL did not affect body weight

To ensure that D-NIL and L-NIL did not affect water intake, body weights were recorded in a sub-group of mice at 8 months of age prior to behavioral testing (Fig. 1). We did not observe any

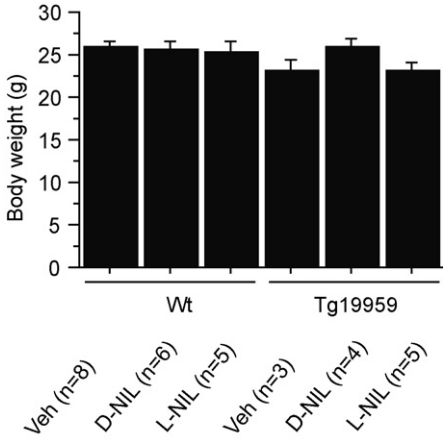

Fig. 1. Effect of L-NIL administration on body weight. Data are expressed as means ± standard errors. Neither L-NIL nor D-NIL affected body weight in Tg19959 mice.