Neurobiology of Aging 34 (2013) 2421–2430

Contents lists available at SciVerse ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Short-term treatment with tolfenamic acid improves cognitive functions in Alzheimer's disease mice

Gehad M. Subaiea [a], Lina I. Adwan [a], Aseef H. Ahmed [b], Karen E. Stevens [c], Nasser H. Zawia [a,b,*]

[a] Neurodegeneration Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI, USA
[b] Interdisciplinary Neuroscience Program, University of Rhode Island, Kingston, RI, USA
[c] Department of Psychiatry, University of Colorado, Anschutz Medical Campus, Aurora, CO, USA

ARTICLE INFO

Article history:
Received 1 February 2013
Received in revised form 25 March 2013
Accepted 1 April 2013
Available online 30 April 2013

Keywords:
Alzheimer's disease
Tolfenamic acid
AD transgenic mouse model
Learning and memory
Morris water maze
Y-maze

ABSTRACT

Tolfenamic acid lowers the levels of the amyloid precursor protein (APP) and amyloid beta (Aβ) when administered to C57BL/6 mice by lowering their transcriptional regulator specificity protein 1 (SP1). To determine whether changes upstream in the amyloidogenic pathway that forms Aβ plaques would improve cognitive outcomes, we administered tolfenamic acid for 34 days to hemizygous R1.40 transgenic mice. After the characterization of cognitive deficits in these mice, assessment of spatial learning and memory functions revealed that treatment with tolfenamic acid attenuated long-term memory and working memory deficits, determined using Morris water maze and the Y-maze. These improvements occurred within a shorter period of exposure than that seen with clinically approved drugs. Cognitive enhancement was accompanied by reduction in the levels of the SP1 protein (but not messenger RNA [mRNA]), followed by lowering both the mRNA and the protein levels of APP and subsequent Aβ levels. These findings provide evidence that tolfenamic acid can disrupt the pathologic processes associated with Alzheimer's disease (AD) and are relevant to its scheduled biomarker study in AD patients.

1. Introduction

Alzheimer's disease (AD) represents the most prevalent neurodegenerative disease in the elderly. During the course of the disease, memory, cognitive performance, and other daily activities are all impaired as a result of extensive neuronal loss (Berg et al., 1993; Braak and Braak, 1997; Nelson et al., 2012). AD is characterized by the presence of neuropathologic deposits consisting of extracellular senile plaques of amyloid beta (Aβ) core and intraneuronal neurofibrillary tangles, especially in the cerebral cortex, hippocampus, subcortical nuclei, and amygdala (Ballard et al., 2011; Harrington, 2012; Reddy et al., 2010; Selkoe, 2001).

The amyloid precursor protein (APP) is processed by the beta-site APP-cleaving enzyme 1 (BACE1) and γ-secretase to generate various Aβ peptide isoforms that can accumulate resulting in the formation of the insoluble aggregates of amyloid plaques (Querfurth and LaFerla, 2010; Shoji et al., 1992; Urbanc et al., 1999; Zhang et al., 2012). $Aβ_{1–40}$ and $Aβ_{1–42}$ are the major generated isoforms with $Aβ_{1–42}$ found to be more aggregative triggering amyloid plaque formation (Finder and Glockshuber, 2007; Naslund et al., 2000). Accumulation of Aβ into amyloid plaques initiates a pathologic cascade resulting in synaptic dysfunction and neuronal death that contribute to the neurodegeneration observed in AD according to the amyloid hypothesis (Hardy and Higgins, 1992; Selkoe, 2001). However, other studies suggest that Aβ-soluble oligomers and aggregates are the toxic species and that in AD patients soluble Aβ levels highly correlate with disease severity markers (Kroth et al., 2012; McLean et al., 1999).

Food and drug administration (FDA)—approved drugs for treatment of AD include 4 cholinesterase inhibitors and 1 *N*-methyl-D-aspartate receptor antagonist. However, these medications are not disease modifying, and they do not stop the progression of AD (Ozudogru and Lippa, 2012). Current research focuses on interventions that target Aβ production and aggregation and the production of hyperphosphorylated tau (Gotz et al., 2012; Ozudogru and Lippa, 2012; Roberson and Mucke, 2006); however, no therapeutic strategy has explored more upstream interventions at the transcriptional level. Specificity protein 1 (SP1) coactivates the transcription of APP, BACE1, and tau genes (Christensen et al., 2004; Docagne et al., 2004; Heicklen-Klein and Ginzburg, 2000), and consequently, changes in its levels can alter the downstream pathways related to amyloidogensis (Adwan et al., 2011; Basha et al., 2005) and tau pathology (unpublished data). Tolfenamic acid, a nonsteroidal anti-inflammatory drug (NSAID), induces the degradation of SP1 protein (Abdelrahim et al., 2006), and data from our

* Corresponding author at: Neurodegeneration Laboratory, Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, 7 Greenhouse Road, Kingston, RI 02881, USA. Tel.: +1 (401) 874 5909; fax: +1 (401) 874 5787.
E-mail address: nzawia@uri.edu (N.H. Zawia).

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http://dx.doi.org/10.1016/j.neurobiolaging.2013.04.002

laboratory have shown that the treatment of wild-type C57BL/6 mice with tolfenamic acid lowered the levels of cerebral SP1 and the expression of AD-related Sp1 target genes such as APP (Adwan et al., 2011).

Transgenic mouse models of AD are useful for elaborating mechanisms involved in the development and progression of AD. In addition, they allow for testing of new therapies in vivo to provide more accurate data for testing in human clinical trials (Duff and Suleman, 2004; Hock and Lamb, 2001). Our previous studies were conducted in wild-type C57BL/6 mice that do not exhibit AD pathology; so, we could not assess for cognitive function improvement associated with an amyloidogenic pathway. Thus, we decided to examine the ability of tolfenamic acid to lower AD proteins, including SP1, APP, and Aβ, and to examine whether such reductions are commensurate with improvements in cognitive functions in a mouse model of AD. Because the endogenous APP promoter is largely driven by SP1 (Docagne et al., 2004), we chose to study the effects of tolfenamic acid (which degrades SP1) in the hemizygous R1.40 mice, a genomic-base transgenic mouse model that harbors the Swedish mutation APPK670N/M671L, driven by the human APP promoter (Lamb et al., 1993).

Therefore, after the characterization of learning and memory impairment in female hemizygous R1.40 transgenic mice, 5 and 50 mg/kg/d tolfenamic acid was administered to female R1.40 mice aging between 14 and 21 months via oral gavage for 34 days and learning and memory functions were assessed in the Morris water maze (MWM) and the Y-maze. On day 35, mice were euthanized and AD-associated proteins including SP1, APP, and soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were assessed in the frontal cortex, which displays extensive AD pathology in this animal model (Kulnane and Lamb, 2001; Lehman et al., 2003).

2. Methods

2.1. Animal model

The transgenic mouse model R1.40 was used for this study. The rationale for choosing this transgenic line relies on the fact that the mutant human APP gene is driven by its endogenous human promoter unlike most of other transgenic lines, which use hamster PrP or murine Thy-1 promoters (Hock and Lamb, 2001). The human promoter region has numerous CpG boxes that SP1 binds to whilst activating gene expression. Thus, R1.40 is an ideal mouse model to conduct experiments on, as tolfenamic acid lowers SP1 and thus alters upstream transcriptional pathways of APP. These transgenic mice, B6.129-Tg(APPSw)40btla/J, were obtained from the Jackson laboratory (Bar Harbor, ME, USA), and colonies of hemizygous and homozygous strains were established in-house. R1.40 is a genomic-based transgenic mouse model that was developed by Bruce T. Lamb; it uses a yeast artificial chromosome that contains the full 400-kb human APP gene and flanking sequence of approximately 250 kb to harbor the Swedish mutation APPK670N/M671L, including the human transcriptional regulatory elements needed for proper spatial and temporal expression (Hock and Lamb, 2001; Lamb et al., 1997; Reaume et al., 1996). The developed hemizygous R1.40 line shows a significant increase in APP and Aβ production as early as 3 months of age with Aβ plaque deposition occurring at 24–26 months of age compared with the wild-type mice. Furthermore, the mnemonic deficits in R1.40 were similar to those observed in AD (Hock et al., 2009; Lamb et al., 1999).

To establish R1.40 transgenic mice colonies, mice were bred and genotyped in-house at the University of Rhode Island (URI). To ensure the accuracy of genotyping results, 2 genotyping techniques were performed: standard polymerase chain reaction (PCR) followed by gel electrophoresis on 1.5% agarose gel and the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA; for details, see Supplementary data). Animals of mixed genotypes were housed in standard mouse cages in the URI animal quarter rooms with a 12:12 hour light-dark cycle (light on at 6:00 AM, light off at 6:00 PM). Temperature was maintained at 22 ± 2 °C with humidity levels of 55% ± 5%, and food and water were available for mice ad libitum. The University of Rhode Island Institutional Animal Care and Use Committee approved all protocols including the breeding and genotyping methods. Animals were under continuous supervision by a URI veterinarian during the entire study and during drug administration.

2.2. Assessment of cognitive deficits in hemizygous R1.40 transgenic mice

To characterize memory and cognitive deficits in the hemizygous, R1.40, transgenic mouse model, behavioral testing in mazes that are reliant on the integrity of the hippocampus and brain cortex was conducted using the MWM and spontaneous alternations in the Y-maze. Preliminary studies showed cognitive deficits in both male and female mice, and the drug under study was active in both genders. In this study, we decided to use a single gender to minimize any possible influence or interference that may be created because of the use of mixed genders in the experiments. Thus, female, hemizygous, APP transgenic ($n = 19$) and female, control wild-type ($n = 18$) groups of ages ranging between 9 and 20 months were used.

2.2.1. Morris water maze

We have tested the mice in the hidden version of the MWM. In this task, the mice had to locate the hidden platform by learning multiple spatial relationships between the platform and the distal extramaze cues (Gulinello et al., 2009; Laczo et al., 2009; Vorhees and Williams, 2006). The apparatus consisted of a white 48″ diameter pool that is 30″ in height and was filled with water to a depth of 14″. The water was kept opaque by the addition of white, nontoxic liquid washable paint. The pool was surrounded by distinct fixed visual cues that the animals used to navigate to reach the escape platform. A clear Plexiglas platform 10 cm^2 was kept submerged 0.5 cm below the surface of the water. The temperature of the water was maintained at 25 ± 2 °C during all experiments in the water maze. On day 15 of tolfenamic acid administration, mice received a habituation trial in which they were allowed to swim freely for 60 seconds. On the following day and for a total of 8 days, mice received training sessions of 3 trials daily. The starting position for each trial was randomly assigned between the 4 possible positions (1 per quadrant), whereas the platform position was fixed in each trial. Each animal was allowed to swim until they found the immersed hidden platform or for a maximum duration of 60 seconds. If the mouse failed to locate the platform, it would be gently guided to sit on the platform for a maximum duration of 30 seconds. Mice were also left to sit on the platform for a maximum of 10 seconds on successful trial. After completion of the 8 acquisition sessions, probe trials for up to 60 seconds on day 1 and day 11 after the last day of training were performed to assess long-term memory retention by studying the preference of the mice for the correct quadrant that previously contained the hidden platform. The swim paths and latencies to locate the platform and time spent in quadrants were videotaped and tracked with a computerized video-tracking system (ObjectScan; Clever Sys., Inc, Reston, VA, USA), and the resultant data were analyzed.

2.2.2. Spontaneous alternation in the Y-maze

The spontaneous alternation ratio, defined as the percentage of the number of arm entries different from the previous 2 entries