ONLINE SUPPLEMENT

Cognitive deficit in amyloid beta-injected mice was improved by pretreatment with a low dose of telmisartan partly due to PPAR-gamma activation

Authors:
Kana Tsukuda, Masaki Mogi, Jun Iwanami, Li-Juan Min, Akiko Sakata, Fei Jing, Masaru Iwai, and Masatsugu Horiuchi

Department of Molecular Cardiovascular Biology and Pharmacology, Ehime University Graduate School of Medicine, Tohon, Ehime, Japan

Running title: Inhibition of cognitive deficit by telmisartan

*Correspondence: Masatsugu Horiuchi, MD, PhD, FAHA
Department of Molecular Cardiovascular Biology and Pharmacology
Ehime University, Graduate School of Medicine, Shitsukawa, Tohon, Ehime 791-0295, JAPAN
Tel: + 81-89-960-5249, Fax: + 81-89-960-5251,
E-mail: horiuchi@m.ehime-u.ac.jp
Materials and Methods
This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All of the animal studies were reviewed and approved by the animal studies committee of Ehime University.

Animals and Treatment
Adult male ddY mice (CLEA, Tokyo, Japan) were used in this study. Telmisartan, an angiotensin II type-1 receptor blocker (provided by Boehringer Ingelheim, Germany), and GW9662, a PPAR-γ antagonist (Sigma-Aldrich, St. Louis, MO), were administered to 8-week-old mice at a concentration of 0.0001% (estimated daily concentration 0.35 mg/kg) in drinking water. Aβ 1-40 (Peptide Institute, Osaka, Japan) was injected intracerebroventricularly at 200 pmol in 5 µl PBS after 2 weeks of drug treatment, while control mice received PBS injection alone. Intracerebroventricular injection was performed as described previously. Briefly, each mouse was fixed in a stereotactic frame, anesthetized, and a 28-gauge needle was inserted unilaterally 1 mm to the right of the midline, 0.5 mm posterior to the bregma and 3 mm deep. Systolic blood pressure was monitored in conscious mice by the tail-cuff method (MK-1030, Muromachi Co., Tokyo, Japan) as described previously. Blood pressure was measured twice before telmisartan treatment and at the end of each experiment. Mice were held in a small plastic holder on a warming pad that was thermostatically controlled at 37 °C. The mean systolic blood pressure of ten measurements in each group was determined.

Behavioral procedures
The Morris water maze test was performed in mice after 4 weeks of treatment with or without i.c.v. Aβ injection as described previously. The experimental apparatus consisted of a circular water tank (diameter, 120 cm) containing water at 25 ± 2 °C. An escape platform (diameter, 6 cm) was then submerged 1 cm below the water surface and placed at the midpoint of one quadrant. Before the first training trial, mice were placed in the pool water and allowed to remain on the platform for 10 s. Mice that did not find the platform within 120 s were placed on the platform for 10 s at the end of the trial. This procedure was repeated 5 times a day for 5 days. Swimming was video-tracked, and latency, path length, swim speed, and cumulative distance from the platform were analyzed by AnyMaze (Stoelting Co., Wood Dale, IL).

Real-Time PCR Method
mRNA was extracted from brain samples after homogenization in Sepazol (Nacalai Tesque Inc., Kyoto, Japan). Real-time quantitative RT-PCR was performed with a SYBR Green I kit (MJ Research, Inc.). PCR primers for tumor necrosis factor alpha (TNF-α) were 5'-CGAGTGACAAGCCTGTAGCC-3' (forward) and 5'-GGTGAGGAGCACGTAGTCG-3' (reverse), for nitric oxide synthase 2 (NOS2) were 5'-GGCAGCCTGTGAGACCTTTG-3' (forward) and 5'-TTGCATTGGAAGTGAAGCGTT-3' (reverse), and for monocyte chemotactic and activating factor 1 (MCP-1) were 5'-TTAACGCCCCACTCACCTGCTG-3' (forward) and
5’-GCTTCTTTGGGACACCTGCTGC-3’ (reverse).

**Measurement of Cerebral Blood Flow**
Cerebral blood flow was determined by laser speckle flowmetry (Omegazone, laser speckle blood flow imager, Omegawave), which obtains high-resolution 2D images in a matter of seconds, as described previously. Briefly, mice were anesthetized by intraperitoneal injection of 65 mg/kg Nembutal (Abbott Co., Abbott Park, IL) in saline. The skull was exposed and covered with plastic wrap. A 780 nm laser semiconductor laser illuminated the area of interest. Light intensity was accumulated in a charge-coupled device camera and transferred to a computer for analysis. Image pixels were analyzed to produce average perfusion values.

**Aβ Concentration Analysis**
Brain samples were obtained 1 or 4 weeks after Aβ injection. Brain tissue was initially homogenized in 1 ml TBS (50 mM Tris-HCl, pH7.6, 150 mM NaCl) containing protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were centrifuged at 100,000 g at 4 °C for 60 min. The precipitates were suspended with 500 µl of 2% sodium dodecyl sulfate (SDS) in TBS, and incubated at 37 °C for 15 min. Then, the homogenates were centrifuged at 100,000 g at 25 °C for 60 min. The pellet was further extracted with 1 ml of 70% formic acid by sonication. All supernatants were stored at -80 °C prior to analysis. Formic acid extracts were neutralized initially by 1:20 dilution in 1 M Tris (pH 10), and diluted as necessary using the reaction buffer in the kit. Aβ deposition in the brain was measured by ELISA (Human β Amyloid 1-40 ELISA Kit Wako II, Wako Chemical Industries, Osaka, Japan) according to the manufacturer’s protocol. The amount of Aβ was calculated by comparison with a standard curve of synthetic human Aβ 1-40.

**Statistical Analysis**
All of the data are expressed as mean ± SEM in the text and figures. Data were analyzed by ANOVA. When a statistically significant effect was found, post-hoc analysis was performed to detect the difference between the groups. A value of P<0.05 was considered statistically significant.
References


