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CHANGES IN HIPPOCAMPAL SYNAPSES AND LEARNING-MEMORY ABILITIES IN A STREPTOZOTOCIN-TREATED RAT MODEL AND INTERVENTION BY USING FASUDIL HYDROCHLORIDE

Y. HOU, L. ZHOU, QD. YANG,* XP. DU, M. LI, M. YUAN AND ZW. ZHOU

Department of Neurology, Xiangya Hospital, Central South University, Changsha, Hunan, PR China

Abstract—Fasudil hydrochloride (FH), a Rho kinase inhibitor, is used to treat neurological diseases. This study aims to elucidate the anti-dementia role of FH in Alzheimer's disease. Twenty-four Sprague–Dawley rats were randomly divided into four groups: (1) sham-operated group (control), (2) sham-operated followed by FH administration group (sham+FH), (3) streptozotocin (STZ)-treated group (STZ), and (4) STZ treatment followed by FH administration group (STZ+FH). Rats in the STZ and STZ+FH groups received two divided doses of STZ (1.5 mg/kg) intracerebroventricularly on days 1 and 3, whereas control and sham+FH group rats were given citric acid/sodium citrate buffer. Rats in the sham+FH and STZ+FH groups were then treated intraperitoneally with FH (10 mg/kg) for 4 weeks, and rats in the STZ and control groups were treated with saline. Learning and memory were measured using the Morris water maze test. The synaptic ultrastructure in the CA1 region of the hippocampus was observed using electronic microscopy. The expression of synaptophysin (SYP) was measured using real-time polymerase chain reaction and western blot analyses; the expression of p-LIMK2 and p-cofilin were also detected using western blot analysis. The results indicate that STZ induced deficit in learning/memory, decrease in SYP expression, degeneration in synaptic structures, and increase in the expressions of p-LIMK2 and p-cofilin. These changes were reversed by the administration of FH, suggesting that FH has anti-dementia properties that protect synaptic structure and function. FH induced dephosphorylation (inactivation) of LIMK2 and subsequent dephosphorylation (activation) of cofilin, which may be responsible for the amelioration of neuronal synaptic structure and function. Published by Elsevier Ltd on behalf of IBRO.

Key words: streptozotocin, synapses, fasudil hydrochloride, Alzheimer's disease, LIMK2, cofilin.

The progressive impairment of learning/memory that typifies Alzheimer's disease (AD) is thought to result from synaptic dysfunction in the hippocampus (Scheff et al., 2007). Synaptic loss occurs early in AD (Heinonen et al., 1995), appearing regionally in the brain and concentrating in the hippocampus of AD patients (Honer et al., 1992). Synaptic dysfunction occurs at an early stage in neurodegenerative diseases such as AD, and this process is evident long before synaptic loss. Thus, dysfunction and abnormal synaptic signal transduction may be key indicators in the early stages of neurodegenerative diseases (Wong et al., 2000). Synaptic dysfunction or loss can be considered an important target for treatments aimed at improving cognitive deficits and preventing the development of AD (Wang et al., 2007; Garcia et al., 2010).

Streptozotocin (2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose; STZ), a glucosamine-nitrosourea compound, was found to cause decreases in glucose uptake, excess tissue oxidative stress, cholinergic deafferentation, and improvements in cognitive performance in learning and memory (Blokland and Jolles, 1993, 1994; Duelli et al., 1994; Prickaerts et al., 1999) when rats were i.c.v. injected with STZ in subdiabetogenic doses. Collectively, these effects resemble those seen in humans with AD, and rats administered with i.c.v. STZ injections have been proposed as a simple animal model of sporadic AD (Hoyer et al., 2000).

Rho GTPase (Rho) regulates actin cytoskeleton organization, cell adhesion, motility, and neuron morphology by signaling its effector Rho kinase (ROCK) (Fukata et al., 2001; Luo, 2002). The activities of cofilin/actin-depolymerizing factors (hereafter referred to as cofilin) are reversibly regulated by phosphorylation (inactivation) and dephosphorylation (activation) at Ser3 (Agnew et al., 1995). Lin-11, Isl-1, and Mec-3 kinases (LIMKs), composed of LIMK1 and LIMK2, phosphorylate and thereby inhibit cofilin specifically at Ser3 (Arber et al., 1998). ROCK directly phosphorylates and activates LIMK2 (but not LIMK1) at Thr505 downstream of RhoA, in turn phosphorylating and inactivating cofilin (Sumi et al., 2001) and inducing actin cytoskeletal reorganization (Yang et al., 1998). Neuron function requires proper regulation of Rho activity, and abnormal activation of the Rho-ROCK pathway antagonizes dendrite stability (Nakayama et al., 2000; Sfakianos et al., 2007). Increased Rho activity has been reported in AD rat brains and has been hypothesized to contribute to AD pathogenesis (Petratos et al., 2008); thus, ROCK inhibitors are considered a potential target for the prevention of synaptic degeneration and the slowing of AD progression (Mueller et al., 2005).

Fasudil, a typical ROCK inhibitor, has been reported to improve cognitive deficits in aged rats (Huentelman et al., 2009) and in rats with cerebral ischemia (Huang et al., 2008). However, whether fasudil exerts anti-dementia effects in AD models remains unclear, and few studies have focused on the mechanism(s) of how it exerts anti-dementia properties.

*Corresponding author. Tel: +86-13808415736; fax: +86-731-84327936.
E-mail address: hyhyhy1017@gmail.com (QD. Yang).

Abbreviations: AD, Alzheimer's disease; cofilin, cofilin/actin-depolymerizing factors; FH, fasudil hydrochloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KIBRA, kidney and brain expressed protein; LIMKs, Lin-11, Isl-1, and Mec-3 kinases; mRNA, messenger RNA; MWM, Morris water maze; PCR, polymerase chain reaction; PSD, postsynaptic density; Rho, Rho GTPase; ROCK, Rho kinase; STZ, streptozotocin; SYP, synaptophysin; TBS-T, Tris-buffered saline with Tween 20; Tris, Tris(hydroxymethyl)aminomethane.

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In this study, an i.c.v.-injected STZ *in vivo* model was used to observe the effect of fasudil hydrochloride (FH), an active metabolite of fasudil, on synaptic changes, the expression of synaptophysin (SYP), p-LIMK2, p-cofilin, and learning/memory. We sought to determine whether FH improves learning/memory in STZ-induced dementia, and if it does, by what mechanism.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats weighing 250–300 g were obtained from the Experimental Animal Centre, Central South University (Hunan, China). We randomly divided the rats into four groups (n=6 in each group): (1) sham-operated group (control); (2) sham-operated followed by FH administration group (sham+FH); (3) STZ-treated group (STZ); and (4) STZ treatment followed by FH administration group (STZ+FH). After i.c.v. injection, rats were housed in groups of four per cage. They were given free access to food and water in a laboratory with an ambient temperature of 22±0.5 °C and a 12-h dark/light cycle. The animals were handled in accordance with the Chinese Council on Animal Care's Guide for the Care and Use of Laboratory Animals. The Ethics Committee of Central South University on Animal Care and Use approved all protocols, and efforts were made to minimize the number of animals used and their suffering.

I.c.v. administration of STZ

Rats were anesthetized with an i.p. injection of 1% pentobarbital sodium (60 mg/kg; Department of Pharmacy, Xiangya hospital, Changsha, Hunan, China). STZ (Sigma, USA) was dissolved in 0.1 mol/L citric acid/sodium citrate buffer (pH=4.2–4.4). Two divided doses of STZ (1.5 mg/kg) were injected into the bilateral ventricles of the brain in STZ and STZ+FH rats on days 1 and 3. The concentration of STZ in the citrate buffer was adjusted to deliver 10 μl of the solution. The stereotaxic coordinates for the i.c.v. injection were as follows: 1.0 mm posterior to the bregma, 1.5 mm lateral to the sagittal suture, and 3.8 mm beneath the surface of the brain. The same volume (10 μl) of citric acid/sodium citrate buffer alone was injected into control and sham+FH rats on days 1 and 3.

Drug administration

FH (Tianjing Chase Sun Pharmaceutical Company, China) administration started after the STZ or citric acid/sodium citrate buffer i.c.v. injections. Sham+FH rats and STZ+FH rats received FH injections intraperitoneally (10 mg/kg) once each day for 4 weeks. FH was dissolved in saline, and the concentration was adjusted to deliver 1 ml of the solution. This dosage was based on previously published intraperitoneal injection dosages (Toshima et al., 2000). The same volume (1 ml) of saline was administrated intraperitoneally to the control and STZ rats.

The Morris water maze (MWM) test

After 4 weeks of drug application, the MWM test was used to assess the learning and memory of the rats. The experimental apparatus consisted of a black circular water pool (diameter, 120 cm; height, 50 cm) filled with tap water at 24 °C to a depth of 30 cm. The water was made opaque with a black non-toxic dye. The pool was divided into four quadrants (Q1, Q2, Q3, and Q4) by two imaginary lines crossing the center of the pool. A black hidden platform (10×10 cm^2; height, 28 cm) was submerged 2 cm below the water surface at the midpoint of Q4, where it was maintained throughout the test. A USB camera was mounted above the center of the maze to record the movements of the rats and send the feed to a computer. A tracking system (Image J 1.40 g, National Institutes of Health, USA) was used to measure the escape latency, swimming speed, time spent in the target quadrant, and number of times a rat crossed the spot where the platform was located.

Rats received one session of two training trials per day for five subsequent days. In each trial, the animals were released facing the wall from one of two predetermined and equally spaced starting points around the water maze in a random order. Each trial was terminated when the rat found the platform or wandered around for a maximum of 90 s. The time for the animals to climb the hidden platform was recorded as escape latency. If the rat failed to find the platform in the allocated time, it was gently placed on the platform, and the escape latency was recorded as 90 s. In either case, the rat was allowed to stay on the platform for 15 s and was then taken off from the platform. The next trial was started after 20 s. An average latency per day was determined for each animal.

On day 6, the platform was removed, and each rat was allowed to explore the pool for 90 s; mean time spent in all four quadrants and the number of times a rat crossed the former location of the platform were recorded.

Real-time polymerase chain reaction

Total RNA was extracted from hippocampal tissue at −70 °C by using a Trizol kit (Molecular Research Center, USA). Complementary DNA was synthesized from 1 μg of total RNA according to the manufacturer's instructions and was used as the template in a 25-μl reaction mixture. Polymerase chain reaction (PCR) was performed as follows: (1) 95 °C for 5 min; (2) 40 cycles at 94 °C for 20 s, annealing temperature for 20 s, 72 °C for 20 s; (3) 72 °C for 5 min and 55 °C for 10 s. The annealing temperatures were as follows: SYP, 58 °C and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 58 °C. Primers were designed using Primer 5.0 software and synthesized with ProMab. The sequences (5′ to 3′) of PCR primers used for amplification were as follows: GAPDH, CTC ATG ACC ACA GTC CAT GC and TTC AGC TCT GGG ATG ACC TT; and SYP, TAC CAA GAT CTT CCT GGT TG and CTG TAG CCA GAA AGT CCA TC. The resulting SYP and GAPDH products were 167 bp and 155 bp, respectively. The cycle threshold (the cycle number at which the fluorescence crossed the threshold) was determined, and the formula 2^ (delta-delta cycle threshold) was used to determine the relative quantity of the amplified fragment. Every sample was tested in triplicate, and the mean value was used.

Western blotting

Hippocampal tissues were homogenized in lysis buffer (NaCl, tris(hydroxymethyl)aminomethane [Tris]–HCl, ethylenediaminetetraacetic acid, aprotinin, and phenylmethylsulfonyl fluoride), and the homogenate was centrifuged at 0 °C for 5 min at 5000 rpm. The supernatants were collected; placed in sample buffer containing 1M Tris-Cl (pH 6.8), 50% glycerol, 10% sodium dodecyl sulfate, 2-mercaptoethanol, and 1% bromophenol blue; and denatured by boiling at 100 °C for 3 min. Proteins were separated on 10% polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene fluoride membranes. Membranes were blocked for 1 h at room temperature with 5% non-fat milk dissolved in Tris-buffered saline with Tween 20 (TBS-T) buffer (100-mM Tris-Cl [pH 7.5], 0.9% [W/V] NaCl, and 0.1% Tween 20) and then incubated for 2 h at room temperature with either one of the following primary antibodies: rabbit SYP antibody (1:300; Bioworld, USA), rabbit LIMK2 antibody (1:500; Santa Cruz, USA), rabbit p-LIMK2 antibody (1:500; Abcam, USA), rabbit cofilin antibody (1:400; CST, USA), rabbit p-cofilin antibody (1:400; CST), and mouse GAPDH antibody (1:1000; ProMab, USA). After washing four times with TBS-T, membranes of SYP, LIMK2, p-LIMK2, cofilin, and p-cofilin