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Research report

Fasudil hydrochloride ameliorates memory deficits in rat model of streptozotocin-induced Alzheimer's disease: Involvement of PI3-kinase, eNOS and NFκB

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ABSTRACT

Restoration of PI3-kinase signaling portrays therapeutic potential in Alzheimer's disease (AD). Hyperactive Rho-kinase in AD negatively modulates PI3-kinase pathway, thereby cause cognitive decline. Fasudil is a Rho-kinase inhibitor that has shown therapeutic benefits in brain disorders. The present study is aimed to decipher the role of PI3-kinase pathway in neuroprotective activity of fasudil using STZ-ICV model of AD. MWM and NORT showed that fasudil (300 µg/kg, ICV) averted the STZ-ICV (3 mg/kg) induced memory dysfunctions in rats. Wortmannin (5 µg/rat) or L-NAME (20 mg/kg) attenuated the memory restorative function of fasudil in STZ treated rats. However, L-Arginine (50 mg/kg) group exhibited marked improvement in memory functions. Markers of oxidative stress (TBARS, GSH, SOD, CAT), nitrite, AChE, TNF-α, eNOS and NFκB were measured in whole brain of rats. STZ-ICV group exhibited significant elevation in brain oxidative stress, AChE activity, TNF-α, NFκB expression and decrease in eNOS level. These effects of STZ were effectively ameliorated by administration of fasudil for 21 days. Wortmannin (PI3-kinase inhibitor) or L-NAME (NOS blocker) attenuated the antioxidative, anti-inflammatory and cholinergic activities of fasudil. Although brain nitrite content was decreased by L-NAME and wortmannin, the L-NAME group depicted rise in eNOS content (not activity) and NFκB expression, whereas, decrease in same was observed in wortmannin group. L-Arginine lowered the brain oxidative stress, inflammation, AChE activity, eNOS expression (not activity), NFκB levels and elevated nitrite content. In STZ-ICV rat model of AD, fasudil (Rho-kinase inhibitor) ameliorated the AD symptoms by reinstating PI3-kinase mediated upregulation of eNOS and control over brain NFκB activity.

1. Introduction

Alzheimer's disease (AD) is most prevalent form of dementia associated with hastened brain ageing and progressive neurodegeneration. Chronic oxidative stress, neuro-inflammation and insulin resistance in brain hasten formation of several neurotoxic products in early stages of AD [1,2]. Acetylcholine plays role in acquisition, consolidation and retrieval of memory, and loss of cholinergic neurons (*e.g.* in basal forebrain) conjure cognitive decline in AD. Intracellular neurofibrillary tangles (NFTs) and deposition of Aβ plaques in brain are histopathological hallmarks of AD. At present the palliative therapeutic strategy of AD includes some nootropics (*e.g.* piracetam), NMDA antagonist (*e.g.* memantine) and cholinesterase inhibitors (*e.g.* galantamine, rivastigmine) with limited therapeutic benefit in longer perspective that prompted search of new pharmacological targets in pathology of AD [1].

Rho-kinase (Rho-associated coiled-coil-containing protein kinase) is a serine/threonine kinase, abundantly present in brain (ROCK 1/2) and is downstream effecter of small GTPase RhoA. Rho-kinase has been associated with Aβ plaques and release of pro-inflammatory cytokines (*e.g.* TNF-α) by microglia [3,4]. The decrease in neuron survival signals by Rho/ROCK led to development of Rho-kinase inhibitors (*e.g.* fasudil, ripasudil, RKI-1447, Y-27632) that afforded therapeutic relief in neurodegenerative disorders [5,6]. Rho/ROCK mediated impairment of brain insulin signaling [7], and induction of tau phosphorylation, amyloidogenic processing of APP and neurodegenerative signals (*e.g.* caspase) [6] have been associated with decreased PI3-kinase signaling that expedites AD pathogenesis [4,7]. Rho-kinase directly activates PTEN (phosphate tensin homologue) that negatively regulates the PI3-kinase mediated control over downstream targets through dephosphorylation of phosphtidylinositol-3,4,5-triphosphate (PIP_3) at 3′ position [4]. The significant contribution of phosphatidylinositols (PIs) in

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many brain functions (*e.g.* neuron survival, glucose metabolism, synaptic plasticity, LTP) rationalizes exploration of PI3-kinase in pathogenesis of AD. Downstream of many cell surface receptors (*e.g.* RTKs, GPCRs, TLRs), PI3-kinase transduces signals in response to many growth factors (*e.g.* BDNF), insulin and cytokines (*e.g.* TNF-α) through PIP_3 recognized by proteins (*e.g.* Akt) having pleckstrin homology domains (PH-domain) entailed for modulation of several downstream targets (*e.g.* BACE-1, p53, BCL-2, eNOS, NFκB, GSK-3) [8]. The modulation of cognitive functions by endothelial nitric oxide synthase (eNOS) is attributed to involvement of nitric oxide (NO) in synaptic plasticity and long-term potentiation (LTP) in retrograde fashion [9,10]. Mice gene mutation studies revealed that eNOS and nNOS have compensatory functions [11], however, Kantor et al. [12] demonstrated that LTP is a distinctive feature of eNOS only not nNOS. Induction of AD type alterations in brain through direct suppression of eNOS activity (*e.g.* L-NAME, L-NIO) or through inhibition of brain PI3-kinase also substantiates these findings [13,14]. Hence, it can be inferred that restoration of PI3-kinase signaling through inhibition of Rho/ROCK activity is a useful therapeutic strategy in the management of AD.

The streptozotocin (ICV) induced increase in expression of brain Rho/ROCK is associated with oxidative stress, inflammatory cascade (*e.g.* TNF-α, IL-1), cholinesterase activity, endothelial dysfunction and cognitive debility [15,16]. Decrease in brain insulin sensitivity constitutes the common basis of pathogenesis in sporadic and familial forms of AD [2]. The inhibition of IRS-1 tyrosine phosphorylation and PI3-kinase activation by Rho-kinase [7] is correlated with STZ-ICV induced insulin resistance in brain [18]. Furthermore, several CNS studies endorse that STZ-ICV induced impairment of PI3-kinase signaling initiate AD related abnormalities such as decreased expression of brain insulin receptors (IRs), cerebral ischemia, formation of $A\beta_{42}$ plaques, NFTs and neurodegeneration [17,18]. Agrawal et al. [19] reported that suppression of brain PI3-kinase activity by LY294002 aggravated AD symptoms in STZ-ICV treated rats. Muller et al., [20] showed that decline of brain insulin sensitivity in response to STZ-ICV treatment alters phospholipid metabolism, neuron membrane structure and cognitive functions [21]. In preclinical studies PI3-kinase signaling conferred therapeutic benefits in AD symptoms in brain of STZ treated rats were comprehended [22]. In light of above findings we used STZ-ICV as a model of AD in rats to comprehend the PI3-kinase mediated neuroprotective activity of fasudil (Rho-kinase inhibitor).

Fasudil (hexahydro-1-(5-isoquinolylsulfonyl)-1H-1,4-di-azepine) is a isoquinoline sulphonamide derivative initially developed as an intracellular Ca^{2+}-antagonist for pharmacotherapy of cerebral vasospasm after subarachnoid hemorrhage (SAH). Fasudil has hydrophilic structure, short half-life ($t_{1/2}$ 30 min), and good peripheral tissue distribution. However, the limited blood brain barrier penetration ability prompted development of liposomal formulations of fasudil [23,24]. The potent inhibition of Rho-kinase (K_i 330 nM) and enhancement of eNOS activity by fasudil evinced worth in numerous cardiovascular (*e.g.* angina, hypertension, atherosclerosis), cerebrovascular (*e.g.* ischemic, inflammatory and demyelinating diseases) and neurodegenerative disorders (*e.g.* AD) that prompted to associate these activities of fasudil with PI3-kinase pathway *via* inhibition of Rho-kinase in light of experimental evidences. Subsequently, a number of studies reported improvement of memory in transgenic (*e.g.* APP/PS1 transgenic mice) and non-transgenic animal models (*e.g.* Aβ, STZ-ICV) of AD in response to inhibition of Rho-kinase by fasudil [23]. Chen et al. [25] reported that suppression of PI3-kinase activity by Rho/ROCK ensue GSK-3 mediated hyperphosphorylation of tau that expedites AD pathology. In a previous study fasudil increased the hippocampus synaptic plasticity and conferred neuroprotection through Rho-kinase-p-LIMK2/p-cofilin pathway in streptozotocin (STZ-ICV) model of AD [26]. However, the role of PI3-kinase pathway in neuroprotective effect of fasudil has not been appreciated so far. Hence, the present study is aimed to comprehend the role of PI3-kinase and its downstream targets such as eNOS and NFκB in neuroprotective action of fasudil (Rho-kinase inhibitor).

2. Materials and methods

2.1. Drugs

Fasudil hydrochloride (HA-1077; fasudil) was procured from Adooq Biosciences, California (USA). Streptozotocin (SRL Pvt. Ltd., Mumbai, India), wortmannin (Sigma–Aldrich), $N^{(G)}$-nitro-L-arginine methyl ester (Sigma–Aldrich) and L-Arginine (Himedia Laboratories, Mumbai, India) were used. A 5% dimethylsulfoxide (DMSO) in artificial cerebrospinal fluid (147 mM NaCl, 2.9 mM KCl, 1.6 mM $MgCl_2$, 1.7 mM $CaCl_2$, 2.2 mM dextrose in 10 ml of water for injection; pH 7.3) solution was used as vehicle for intracerebroventricle (ICV-vehicle) administrations. All the drug solutions were freshly prepared before administration.

2.2. Subjects

The subjects used were Wistar rats (180–220 g, either sex), procured from Central Animal Facility, All India Institute of Medical Sciences, New Delhi and reared at Central Animal Facility of the institute under standard laboratory conditions (temperature, 23 ± 2 °C; humidity, 40 ± 10%; natural light-dark cycle, 12 h each). The experimental protocol of this study was approved by Institutional Animal Ethics Committee of the institute (No. ASCB/IAEC/08/15/107) and all procedures followed were as per guidelines of CPCSEA, Ministry of Forests and Environment, Government of India and Indian National Science Academy. The animals were housed in polyacrylic cages in group of five per cage ($44 \times 29 \times 16$ cm^3) prior to surgery, one per cage ($30 \times 23 \times 14$ cm^3) during the entire surgical procedure including the healing period (7 days), and afterwards, three rats were housed per cage ($44 \times 29 \times 16$ cm^3) till completion of the study, and nurtured with standard rodent pellet diet (Ashirwad Industries, Mohali) and water *ad libitum*.

2.3. Surgical implantation of cannula

The animals were acclimatized to laboratory conditions two weeks prior to the surgical procedure. The rats were rendered unresponsive using general anesthetic agent chloral hydrate (350 mg/kg, *i.p.*). During the surgery a heating pad was used to maintain the body temperature at 37 ± 0.5 °C. The body of anesthetized rat was placed on a warm pad and head was positioned in the frame of stereotaxic apparatus (INCO, Ambala, India). The partially shaved scalp was wiped with 70% ethyl alcohol swab before incision. A middle sagittal incision was made in the scalp, skin was retracted and skull was exposed. A local anesthetic (1:1 mixture of lidocaine and bupivacaine) was applied over the periosteum. Burr hole was drilled through the parietal bone of skull by using stereotaxic coordinates: antero-posterior from bregma = −0.8 mm, mediolateral from mid-sagittal suture = ± 1.5 mm, dorso-ventral from the skull = ± 3.6 mm; in order to access a randomly selected lateral cerebral ventricle [27]. The heparinized intracerebroventricular guide-cannula (22 gauge, stainless steel) was implanted 3.6 mm beneath the skull surface and fastened firmly by applying dental cement. A stainless steel stylet (28 gauges) was inserted to block the lumen of cannula, to prevent blood clogging and keep the cannula track patent. The stylet was removed only for ICV injections. The wound was infiltrated with local anesthetic (1:1 mixture of lidocaine and bupivacaine) and skin was sutured. Neosporin® (GlaxoSmithKline Pharmaceuticals Ltd, Mumbai) was applied over the wound for next few days and Reflin® (cephazolin sodium, Ranbaxy) was injected (30 mg/kg, *i.p.*) once postoperatively to prevent sepsis. The rats received meloxicam (2 mg/kg, *p.o.*) for three consecutive days and lactated Ringers saline (0.9%) (5 ml, *s.c.*) to prevent dehydration. Due postoperative care was taken of rats for 4 days after cannula implantation. Moistened rodent pellet diet was semi-grinded and fed at the bottom of cage. The rats were kept warm after surgery to prevent hypothermia due to their large surface area to body weight ratio.