Neurobiology of Disease 66 (2014) 43–52

Contents lists available at ScienceDirect

Neurobiology of Disease

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

PRAS40 plays a pivotal role in protecting against stroke by linking the Akt and mTOR pathways

Xiaoxing Xiong [a,1], Rong Xie [a,c,1], Hongfei Zhang [a], Lijuan Gu [a], Weiying Xie [a], Michelle Cheng [a], Zhihong Jian [a], Kristina Kovacina [b], Heng Zhao [a,*]

[a] Department of Neurosurgery, Stanford University, Stanford, CA 94305, USA
[b] Department of Chemical and Systems Biology, Stanford University, Stanford, CA 94305, USA
[c] Department of Neurosurgery, Huashan Hospital, Fudan University, Shanghai 200040, China

ARTICLE INFO

Article history:
Received 17 September 2013
Revised 8 January 2014
Accepted 19 February 2014
Available online 27 February 2014

Keywords:
Focal cerebral ischemia
Akt
Stroke
mTOR
PRAS40
PTEN

ABSTRACT

The proline-rich Akt substrate of 40 kDa (PRAS40) protein is not only a substrate of the protein kinase Akt but also a component of the mTOR complex 1 (mTORC1), thus it links the Akt and the mTOR pathways. We investigated the potential protective role of PRAS40 in cerebral ischemia and its underlying mechanisms by using rats with lentiviral over-expression of PRAS40 and mice with PRAS40 gene knockout (PRAS40 KO). Our results show that gene transfer of PRAS40 reduced infarction size in rats by promoting phosphorylation of Akt, FKHR (FOXO1), PRAS40, and mTOR. In contrast, PRAS40 KO increased infarction size. Although the PRAS40 KO under normal condition did not alter baseline levels of phosphorylated proteins in the Akt and mTOR pathways, PRAS40 KO that underwent stroke exhibited reduced protein levels of p-S6K and p-S6 in the mTOR pathway but not p-Akt, or p-PTEN in the Akt pathway. Furthermore, co-immunoprecipitation suggests that there were less interactive effects between Akt and mTOR in the PRAS40 KO. In conclusion, PRAS40 appears to reduce brain injury by converting cell signaling from Akt to mTOR.

Introduction

The proline-rich Akt substrate of 40-kDa (PRAS40) protein was first identified as a 14-3-3 binding protein and substrate of Akt in the Roth laboratory 10 years ago (Kovacina et al., 2003). Later it was found that PRAS40 is also an important component and a substrate of the mammalian target of rapamycin complex 1 (mTORC1) (Wang et al., 2007). Thus, PRAS40 is an interactive linker between the Akt and mTOR pathways.

Both the Akt (Zhao et al., 2006) and mTOR pathways (Guertin and Sabatini, 2005, 2007) play protective roles against brain injury induced by stroke. Akt has long been known to contribute to neuronal survival after stroke (Gao et al., 2008; Noshita et al., 2003; Ouyang et al., 1999; Zhao et al., 2005). Recently, the involvement of the mTOR pathway has also been reported, as several neuroprotectants have been shown to inhibit infarction by upregulating mTOR activity (Koh, 2008; Koh et al., 2008; Wu et al., 2012). In alignment with the roles of Akt and mTOR, PRAS40 also appears to attenuate cerebral ischemic injury after stroke (Saito et al., 2004, 2006). Previous studies from the Chan laboratory have found that stroke reduces phosphorylated PRAS40 (p-PRAS40), and decreases binding of p-PRAS40 to phosphorylated Akt (p-Akt) and the 14-3-3 protein (Saito et al., 2004, 2006), suggesting that PRAS40 is involved in brain injury induced by stroke. More importantly, liposome-mediated p-PRAS40 cDNA transfection inhibits apoptotic neuronal cell death, suggesting that PRAS40 overexpression is neuroprotective (Saito et al., 2004).

Most recently, we reported that lentiviral vector gene transfer of the Akt isoforms Akt1 and Akt3 attenuated brain injury by promoting both p-PRAS40 and p-mTOR protein levels, and mTOR inhibition by rapamycin abolished the protective effects Akt1 and Akt3 gene transfer (Xie et al., 2013a). Our results suggest that a close relationship exists between Akt and mTOR in their protection against stroke, and that PRAS40 may serve a pivotal role (Xie et al., 2013a). Nevertheless, many questions remain about how PRAS40 functions in cerebral ischemia. First, the underlying protective mechanisms of PRAS40 against stroke remain elusive, such as how PRAS40 affects Akt and mTOR activity and how they interact. Second, previously reported protective effects of PRAS40 against stroke were demonstrated using liposome-mediated PRAS40 gene transfer, which is considered to have insufficient gene transfer efficacy for neurons in vivo, and this protective effect has not been confirmed with more efficient gene transfer techniques, such as lentiviral vector systems. Third, previous studies have suggested that overexpression of PRAS40 inhibits, while knockdown of PRAS40 promotes, mTOR activity

* Corresponding author at: Department of Neurosurgery, Stanford University School of Medicine, MSLS Bldg., P306, 1201 Welch Rd., Rm. P306, Stanford, CA 94305-5327, USA. Fax: +1 650 498 4134.
E-mail address: hzhao@stanford.edu (H. Zhao).
Available online on ScienceDirect (www.sciencedirect.com).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.nbd.2014.02.006
0969-9961/

(Vander Haar et al., 2007; Wang et al., 2008). As mTOR is neuroprotective, these previous studies suggest that overexpression of PRAS40 might promote neuronal death, which contradicts with the finding that PRAS40 overexpression attenuates infarction after stroke. Whether PRAS40 is truly neuroprotective or not requires further investigation. Fourth, previous studies on the cellular functions of PRAS40 were mostly performed in cell lines; the function of PRAS40 has not been studied in rodent gene knockout animal models.

In this study we constructed the lentiviral vector to express the PRAS40 gene and investigated how PRAS40 gene expression affects infarct sizes and protein expression in the Akt and mTOR pathways after stroke. We also studied the pathological outcomes of stroke in PRAS40 gene knockout (PRAS40 KO) mice and the effects of PRAS40 KO on Akt and mTOR activity, as well as their interactive effects.

Materials and methods

Animal experiments were conducted according to the protocols approved by the Stanford Institutional Animal Care and Use Committee and the NIH Guidelines for Care and Use of Laboratory Animals. Animals were housed under a 12:12 hour light:dark cycle with food and water available ad libitum.

Construction of lentiviral vectors

We constructed lentiviral vectors containing genes of constitutively-active PRAS40. Target genes were cloned from PRAS40 (pCMV5) plasmids kindly provided by Dr. Richard Roth, Department of Chemical and Systems Biology, Stanford University. Genes were cloned into the lentiviral backbone plasmid, pHR′tripCMV–IRES–eGFP, which contains a CMV promoter and an IRES sequence between its multiple cloning sites (MCS) and eGFP. The IRES sequence enables independent expression of both the target gene and eGFP simultaneously. SacII/PstI was the restriction enzyme used for cloning. The control vector was the lentiviral plasmid backbone with only eGFP inserted.

Lentiviral vector generation and titration

We used a 3 plasmid system for *Lentivirus* packaging: the lentiviral transfer vector (pHR′tripCMV–IRES–eGFP) that contains the coding region of various targeted genes as described above; the packaging plasmid (p-delta) that provides all vector proteins driven by the trip CMV promoter, except the envelope protein; and the envelope-encoding plasmid (p-VSVG) that encodes the heterologous vesicular stomatitis virus envelope protein (VSVG) (Hu et al., 2011). Briefly, 293T cells were grown in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). A mixture of 45 μg of transfer vectors, 30 μg of packaging plasmids and 15 μg of envelope-encoding plasmids was transiently transfected into 3 separate T175 flasks containing 1.5×10^7 HEK-293T cells using the calcium phosphate precipitation (CPP) method. Supernatants were collected 72 h post-transfection and viral particles were concentrated by ultracentrifugation. Viruses were resuspended in phosphate-buffered saline (PBS) and kept at −80 °C until use. The virus particles were titered with the TCID50 method as described previously (Apolonia et al., 2007; Breckpot et al., 2003). Virus titers ranged from 1×10^8–5×10^8 TU/ml and were diluted in PBS to the final concentration of 1×10^8 TU/ml before gene transfer was conducted.

Creation of PRAS40 KO mice and genotyping

PRAS40 KO mice were kindly provided by Dr. Richard Roth, Department of Chemical and Systems Biology, Stanford University. C57BL/6 PRAS40 KO mice were generated using standard homologous recombination methods. loxP sequences were inserted between exons 1 and 2 and after exon 5. A phosphoglycerine kinase (PGK) Neo cassette flanked by FLP recombinase target sequences was used to confer resistance to C57BL/6 ES cells that had successfully integrated the targeting vector. This procedure produces ES cells with exons 2 through 5 flanked by loxP sites. ES cells were microinjected and the chimeric mice were bred to generate heterozygous F1 mice. These floxed mice were crossed with Cre-deleter C57BL/6 mice, leading to the removal of the entire coding region of the PRAS40 gene. Founder animals were backcrossed with C57BL/6 mice for more than 12 generations.

Presence and copy numbers of the transgene in the offspring were identified by polymerase chain reaction (PCR) analysis. In brief, genomic DNA was extracted from the tail of mice using a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA), and genomic PCR was performed with Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) under the following conditions: 94 °C for 60 s; 30 cycles at 94 °C for 60 s, 55 °C for 45 s, and 72 °C for 45 s; and 72 °C for 6 min. One primer pair (forward PCR primer: GGGGCGCT CTGAGATTAAAG, reverse PCR primer: GGTGACAGTCCTCTAGCCC) amplifies a fragment of 225 bp in mice homozygous or heterozygous for endogenous gene (no band will be generated by this oligo pair in a cre-recombinant homozygous mice). Another set of primers (forward PCR primer: GTGGTGTGCATGTGTGACTTG, reverse PCR primer: GGTGACAGTCCTCTAGCCC) generates a product of 300 bp in a cre recombined homozygous and heterozygous mice but not in non-recombined mice.

In vitro oxygen glucose deprivation (OGD) model, gene transfer and cell viability assay

Primary neuronal cultures were prepared using timed-pregnant Sprague–Dawley rats (E18, Charles River Laboratories International, Wilmington, MA). Briefly, rats were anesthetized with isoflurane and the E18 embryos were removed. The cortical region of the fetal brains were dissected in warm media and pooled together. The cortices were triturated and incubated in papain for 20 min at 37 °C, then centrifuged at 1500 rpm for 5 min at room temperature (RT). Cells were resuspended in MEM (minimal essential medium) (Gibco, Grand Island, NY, USA) containing 10% fetal horse serum (Hyclone, Logan, UT, USA), 2 mmol/l glutamine (Gibco), 25 mM glucose, and 1% penicillin/streptomycin (Gibco). Cells were plated onto poly-D-lysine-coated tissue culture plates at 7.5×10^5 cells/ml. Media were completely changed after 24 h. One-half medium changes were performed at day 4. Cultures were incubated at 37 °C in a 5% CO_2 incubator and experiments were performed after days 9–11.

The lentiviral vectors, diluted with PBS to 5 μl for 24-well plates and to 10 μl for 6-well plates, were directly added into the medium of 9 day primary mixed neuron cultures with the multiplicity of infection (MOI) at 5. The same amount of PBS was also added to the medium as an experimental control (Cheng et al., 2009). Then cells were incubated at 37 °C in a 5% CO_2 incubator for another 2 days before OGD was conducted. Primary mixed neuron cultures were washed twice with glucose-free balanced salt solution (BSS0, pH 7.4) and the plates were transferred to a modular hypoxic chamber filled with mixed gases of 5% CO_2 and 95% N_2. Oxygen level was maintained at <0.02% at 37 °C. The cells were kept in the hypoxic chamber for 6 h. Cultures were then restored with glucose at a final concentration of 5.5 mM (BSS5.5, pH 7.4) and recovered at normoxic conditions (37 °C, 5% CO_2) for ~18 h (OGD restoration). The control groups without OGD were washed twice with 5.5 mM glucose in balanced salt solution (BSS5.5, pH 7.4) with no oxygen deprivation.

Cell viability was quantified by measuring lactate dehydrogenase (LDH) release that is always used to evaluate both apoptosis and necrosis at 18 h after OGD restoration using a previously described colorimetric assay (Dugan et al., 1995). Briefly, 100 μl of cell-free supernatant was transferred to 96-well plates. The supernatant was incubated with 150 μl of NADH/phosphate buffer (0.15 mg/ml)