Efficient Transduction of 11 Poly-arginine Peptide in an Ischemic Lesion of Mouse Brain

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Direct intracellular delivery of intact proteins has been successfully achieved by tagging cell-penetrating peptide (CPP), which consists of short positively charged amino acids, such as 11 poly-arginine (11R); however, in vivo delivery of the proteins to the brain has remained challenging because it is unclear whether CPP would enable proteins to cross the blood–brain barrier (BBB). In this study, we conducted an in vivo kinetic study to investigate the efficiency of 11R-mediated peptide delivery in the normal and ischemic brain. The 11R was observed in the microvessels and neurons surrounding the microvessels throughout the brain 1 hour after systemic administration, but the signal of the peptide was faint after 2 hours. In a transient middle cerebral artery occlusion mouse model, 11R was markedly enhanced and remained detectable in the cells on the ipsilateral side for as long as 8 hours after administration compared with the contralateral side. These results suggest that 11R is capable of in vivo delivery to the brain by passing through the BBB. Furthermore, 11R-mediated protein transduction could be used for the delivery of therapeutic molecules in cerebral ischemia. **Key Words:** Protein transduction—poly-arginine—brain—ischemia.

Introduction

Stroke is a leading cause of death and the principal cause of adult disability worldwide.[1] To treat an ischemic stroke, current therapies use thrombolytic approaches to dissolve blood clots.[2] These treatments restore blood flow and prevent further brain damage; however, there is no medicine that can treat brain damage and prevent neuronal cell death by directly targeting neuronal cells. One reason is the difficulty of crossing the blood–brain barrier (BBB), which is composed of a layer of specialized endothelial cells that segregate the brain from the circulating blood.[3] Extensive efforts have been made to engineer and deliver biologically active molecules to the brain across the BBB.[4]

Among the delivery systems, a technology using cell-penetrating peptide (CPP) has been emerging as a potential tool to pass through biological membranes.[5-7] CPP consists of short peptides containing positively charged amino acids, such as lysine and arginine. Protein transduction domains (PTDs) in the transactivator of transcription (TAT) protein of HIV and VP22 in herpes simplex virus 1 enable efficient intracellular transduction of peptides and proteins through a macropinocytosis-dependent mechanism.[8] Subsequent studies have aimed to apply TAT-derived PTD for in vivo delivery across the BBB, and it has been found that TAT-derived PTD is capable of

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transduction into the intact brain[9,10]; however, several groups have reported that TAT protein could not enter the intact brain using the same construct.[11,12] Currently, whether TAT-derived PTD is capable of transduction to the intact brain is still controversial, and improvement of the delivery system is required to achieve high efficiency. We previously reported that 11 poly-arginine (11R) was capable of intracellular delivery both in vitro and in vivo, such as to the mouse kidney and heart, more efficiently than TAT.[13] Taken together, these studies suggest that 11R may be used as a potential tool to deliver biologically active molecules to the brain across the BBB.

In this study, we investigated whether 11R could enter intact and ischemic brains across the BBB after systemic administration.

Materials and Methods

Peptide Synthesis

Eleven arginine peptides (11R) composed of basic amino acids were chemically synthesized by Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis on Rink amide resin, as described previously.[14] Briefly, deprotection of the peptide and cleavage from the resin were achieved by treatment with a trifluoroacetic acid/ethanedithiol mixture (95:5) at room temperature for 3 hours followed by reversed-phase high-performance liquid chromatography purification. Fluorescent labeling at the amino-terminus of the peptides was conducted by treatment with fluorescein isothiocyanate (FITC; Invitrogen, Carlsbad, CA) in a dimethylformamide/methanol mixture (1:1) for 1.5 hours followed by HPLC purification. The structure of the products was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. As a control, we synthesized FITC-conjugated 11 polyglutamate (11E-FITC), which consists of 11 glutamates, acidic amino acids, and FITC.

The structure of each peptide was as follows:

1. FITC-conjugated 11 poly-arginine (11R-FITC):

FITC-RRRRRRRRRRR-COOH

2. 11E-FITC

FITC-EEEEEEEEEEE-COOH.

Animal Studies

All animal procedures were approved by the Animal Ethics Committee of Kurume University and followed the Guide for the Care and Use of Experimental Animals of the university. C57BL/6 male mice, aged 8-9 weeks (20-24 g), were assigned to transient focal cerebral ischemia followed by systemic injection of peptides.

Systemic Injection of Peptides

Mice were anesthetized by inhaling 1.2% concentration of isoflurane in air under spontaneous breathing. 11R-FITC (.2 μmol) or 11E-FITC (.2 μmol) was dissolved in 20-μL saline and then injected into the right femoral vein.

Transient Focal Cerebral Ischemia

Mice were anesthetized by inhaling 1.2% concentration of isoflurane in air under spontaneous breathing. The rectal temperature was controlled at 37.0 ± .5°C via a temperature-regulated heating pad during surgery. Focal cerebral ischemia was induced by occluding the middle cerebral artery (MCA) using the intraluminal filament technique. The right common carotid artery (CCA) was exposed via a midline pretracheal incision. The external carotid artery was ligated. The CCA was then ligated permanently, and a small incision was made 1 mm distal to the ligation. A 6-0 nylon monofilament coated with silicone (Doccol Corporation, Sharon, MA) was introduced through a small incision in CCA and advanced 9 mm distal to the carotid bifurcation. The wound was sutured and the animal returned to its cage. After a 120-minute occlusion period, the mouse was reanesthetized and reperfusion was accomplished by withdrawing the intraluminal filament from the CCA. Immediately after reperfusion, .2 μmol 11R-FITC or .2 μmol 11E-FITC dissolved in 20-μL saline was injected into the right femoral vein.

Assessment of Cerebral Blood Flow

To monitor regional cerebral blood flow continuously, a laser Doppler flowmetry probe was fixed to the intact skull (2 mm posterior and 5 mm lateral to the bregma). Only mice whose regional cerebral blood flow showed a drop of more than 70% from the baseline just after MCAO were included.

Immunohistochemistry

At 1, 2, 4, and 8 hours after peptide injection, mice were deeply anesthetized with an overdose of pentobarbital and perfused transcardially with heparinized physiological saline followed by 4% paraformaldehyde in .1 M phosphate-buffered saline (PBS). The brains and livers were removed, cut into 2-mm coronal sections, fixed in 4% paraformaldehyde in .1 M PBS for 4 hours, and immersed in 30% sucrose in .1 M PBS overnight. Brains were embedded in optimum cutting temperature compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen. Brain and liver sections at a thickness of 10 μm were prepared using a cryostat. The sections were washed 3 times in .1 M PBS and then immersed for 30 minutes in 1% vH_2O_2 and preblocked with .1% Triton X-100 and 1% bovine serum albumin in PBS for 1 hour. The brain sections were then incubated with goat anti-FITC polyclonal antibody (1:200; GeneTex, Irvine, CA)