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Neuroprotection against cerebral ischemia/reperfusion injury by intravenous administration of liposomal fasudil

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ABSTRACT

Fasudil, a Rho-kinase inhibitor, is a promising neuroprotectant against ischemic stroke; however, its low bioavailability is an obstacle to be overcome. Our previous study revealed that the liposomal drug delivery system is a hopeful strategy to increase the therapeutic efficacy of neuroprotectants. In the present study, the usefulness of intravenously administered liposomal fasudil for cerebral ischemia/reperfusion (I/R) injury treatment was examined in transient middle cerebral artery occlusion (t-MCAO) rats. The results showed that PEGylated liposomes of approximately 100 nm in diameter accumulated more extensively in the I/R region compared with those of over 200 nm. Confocal images showed that fluorescence-labeled liposomal fasudil was widely distributed in the I/R region, and was not noticeably taken up by microglia, which are well-known resident macrophages in the brain, and neuronal cells. These data indicated that liposomal fasudil mainly exerted its pharmacological activity by releasing fasudil from the liposomes in the I/R region. Moreover, liposomal fasudil effectively suppressed neutrophil invasion and brain cell damage in the t-MCAO rats, resulting in amelioration of their motor function deficits. These findings demonstrated both the importance of particle size for neuroprotectant delivery and the effectiveness of liposomal fasudil for the treatment of cerebral I/R injury.

1. Introduction

The only therapeutic agent available worldwide for the acute phase of ischemic stroke is recombinant tissue plasminogen activator (t-PA), although a lot of candidate neuroprotective agents have been studied for many years (Ginsberg, 2009). Fasudil, a Rho-kinase inhibitor (1-(5-isoquinolinylsulfonyl)homopiperazine hydrochloride), is an approved drug for cerebral vasospasm in patients after subarachnoid hemorrhage. Fasudil is also one of the candidate drugs effective for the treatment of ischemic stroke (Rikitake et al., 2005; Satoh et al., 2008). It was reported that Rho-kinase activation is induced by an ischemic insult, and is involved in the progression of brain injury through the restriction of neurons, inflammation, oxidative damage, and endothelial cell damage (Mueller et al., 2005). Thus, Rho-kinase is considered to be a promising therapeutic target for stroke therapy (Satoh et al., 2011). However, although phase III clinical trials for fasudil illustrated the usefulness and safety of it for acute ischemic stroke patients (Shibuya et al., 2005), the trial was terminated because of the less than anticipated clinical efficacy of the drug. In addition, it was reported that its short half-life in the circulation and poor ability to penetrate the blood–brain barrier (BBB) are problems to be addressed (Mueller et al., 2005). Therefore, the development of a technology for improving the therapeutic outcome with neuroprotectants is required to overcome the insufficiency in the clinical setting.

As an approach to enhance the therapeutic efficacy of cerebroprotective agents, we previously demonstrated the usefulness of a liposomal drug delivery system (DDS) for the treatment of cerebral ischemia/reperfusion (I/R) injury (Ishii et al., 2012b). This therapeutic strategy is based on enhanced permeability of blood vessels caused by a disintegrated BBB after I/R. Intravenous administration of liposomal drugs such as asialo-erythropoietin-modified liposomes and FK506 encapsulated in the lipid membrane of PEGylated liposomes (PEG-liposomes) was shown to be therapeutically effective for secondary ischemic brain injury in transient middle cerebral artery occlusion (t-MCAO) rats, an ischemic stroke model (Ishii et al., 2012a, 2013a). Considering

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these findings, if the intravenous treatment with liposomes encapsulating neuroprotective agents in their internal water phase is effective for cerebral I/R injury, it would be expected that a therapeutic strategy using liposomal DDS could be applied to many neuroprotectants including drugs that failed in clinical trials (Ginsberg, 2008). On the other hand, it was reported that intrathecal injection of liposomal fasudil would be effective against cerebral vasospasm and ischemia (Ishida, 2004) and that intratracheal administration of liposomal fasudil could be useful as an anti-pulmonary arterial hypertension therapy (Gupta et al., 2013). These reports suggest that liposomalization of fasudil should be a promising way to enhance its therapeutic benefits. Therefore, the intravenous delivery of fasudil via liposomes would be expected to be efficacious for the treatment of cerebral I/R injury by passage of the liposomes through the disintegrated BBB.

To achieve efficient delivery of neuroprotective agents to the brain parenchyma through the disintegrated BBB, the particle size of liposomes is one of the important factors to be considered. Although our previous study confirmed that liposomes of approximately 100-nm diameter can accumulate in the ischemic region (Fukuta et al., 2014; Ishii et al., 2012b), the influence of the particle size on liposomal distribution in the brain has not been sufficiently elucidated. Also, the intracerebral distribution of the liposomes has not yet been adequately examined. Therefore, in the present study, we focused on the intracerebral distribution of liposomes and evaluated the usefulness of intravenously administered liposomal fasudil for the treatment of cerebral I/R injury by examining its pharmacological activity and therapeutic effects.

2. Methods

2.1. Animals

Eight-week-old male Wistar rats (180–210 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were cared for according to the Animal Facility Guidelines of the University of Shizuoka. All animal procedures were approved by the Animal and Ethics Review Committee of the University of Shizuoka.

2.2. Preparation of liposomes

Distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC), cholesterol, and distearoylphosphatidylethanolamine (DSPE)-PEG2000 were gifts from Nippon Fine Chemical (Hyogo, Japan). PEG-liposomes composed of DSPC/cholesterol/DSPE-PEG2000 (20/10/1 molar ratio) were prepared by the thin-film method as described below. The lipids dissolved in chloroform were evaporated to form a thin lipid film, and then the lipid film was dried for at least 1 h under reduced pressure. For fluorescence labeling, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate ($DiIC_{18}$, Molecular Probes Eugene, OR, USA) was added to the initial chloroform solution at a concentration of 2.5 mol% of DSPC. Then, liposomes were formed by hydration with PBS (pH 7.4). The PEGylated liposome solution was freeze-thawed for 3 cycles by use of liquid nitrogen and then extruded through polycarbonate membrane filters having 100, 200, or 800-nm pores (Nuclepore, Cambridge, MA, USA). The particle size and ζ-potential of the PEG-liposomes were measured with a Zetasizer Nano ZS (MALVERN, Worcestershire UK, USA).

For preparing liposomal fasudil, a thin lipid film composed of DPPC/cholesterol/DSPE-PEG2000 (10/5/1 molar ratio) was hydrated with 250 mM ammonium sulfate (pH 3.0). After 3 freeze-thaw cycles, the liposomal solution was extruded through a polycarbonate membrane filter with 100-nm pores. For removal of external ammonium sulfate, the liposomal solution was then passed through a PD-10 column (GE Healthcare Japan, Tokyo, Japan) equilibrated with PBS, and the liposomal fraction was subsequently ultracentrifuged at 453,000*g* for 15 min at 4 °C (HITACHI, Tokyo, Japan). After the pellet had been resuspended in PBS, the liposome solution was incubated with fasudil dissolved in PBS at 65 °C for 10 min for loading the drug into the liposomes. For removing unencapsulated fasudil, the solution was ultracentrifuged at 453,000*g* for 15 min at 4 °C, and then the pellet was resuspended in PBS. The particle size and ζ-potential of liposomal fasudil were measured with the Zetasizer Nano ZS. The amount of fasudil encapsulated into the liposomes was determined by HPLC (HITACHI). Liposomal fasudil was dissolved in tetrahydrofuran, and 20 μL of the solution was then injected onto an octadecylsilane column (TSK gel ODS-80TS, 4.6 × 250 mm, Tosoh, Tokyo, Japan). The column was operated with an isocratic mobile phase (55% acetonitrile in water, 0.1% formic acid), and the HPLC conditions were as follow: column oven, 60 °C; flow rate, 0.5 mL/min; UV detection, 320 nm. For preparing fluorescently labeled liposomal fasudil, $DiIC_{18}$ was added to the initial chloroform solution at a concentration of 2.5 mol% of DPPC.

2.3. Preparation of t-MCAO rats

t-MCAO rats were prepared as described previously (Nagasawa and Kogure, 1989). In brief, anesthesia was induced with 3% isoflurane (Escain®, Pfizer, NY, USA) and maintained with 1.5% isoflurane during surgery with the animals immobilized in a small-animal anesthesia apparatus (Model TK-4, Bio Machinery, Chiba, Japan). Rectal temperature was maintained at 37 °C with a heating pad (Unique Medical Co., Ltd., Tokyo, Japan). Cervical skin was incised, and then right internal carotid artery (ICA) was exposed. Next, a 4.0 silicon-coated nylon filament cut into an 18-mm length (Keisei Medical Industrial Co., Ltd., Niigata, Japan) was inserted into the ICA and gently advanced to the origin of the MCA. The incision in the neck was sutured, and the animal was allowed to recover from the anesthesia. The success of occlusion was judged by both the appearance of hemiparesis and an increase in rectal temperature (to 37.8–38.8 °C) before reperfusion. Our previous study confirmed that the MCAO operation induced an obvious reduction in cerebral blood flow in the ipsilateral hemisphere when both of the above phenomena were observed (Ishii et al., 2013b). Reperfusion was induced by withdrawing the filament from the origin of the MCA after a 1-h occlusion under isoflurane anesthesia.

2.4. Preparation of liposomal fasudil composed of different internal water phases

A thin lipid film composed of DPPC/cholesterol/DSPE-PEG2000 (10/5/1 molar ratio) was prepared as described above and hydrated with 250 mM ammonium sulfate (pH 3.0 or 7.0), 250 mM ammonium acetate (pH 7.0), or 250 mM sodium sulfate (pH 3.0 or 7.0) to form liposomes having different internal water phases. After the hydration and extrusion, the external phase of the liposomes was changed to PBS by ultracentrifugation (453,000*g* for 15 min at 4 °C). The concentration of fasudil encapsulated into liposomes was analyzed by using HPLC.

2.5. Leakage of fasudil from various liposomal fasudil preparations

Liposomal fasudil containing 250 mM ammonium sulfate (pH 3.0) in the internal phase was prepared as described above. The liposomes were ultracentrifuged at 453,000*g* for 15 min at 4 °C for substituting the external water phase with 0 (distilled water), 1, 10 or 100 mM ammonium sulfate (pH 7.0), ammonium acetate (pH 7.0) or sodium acetate (pH 7.0). After incubation at 37 °C for 1, 6 or