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SODIUM-GLUCOSE TRANSPORTER TYPE 3-MEDIATED NEUROPROTECTIVE EFFECT OF ACETYLCHOLINE SUPPRESSES THE DEVELOPMENT OF CEREBRAL ISCHEMIC NEURONAL DAMAGE ☆

Y. YAMAZAKI, S. HARADA AND S. TOKUYAMA *

Department of Clinical Pharmacy, School of Pharmaceutical Sciences, Kobe Gakuin University, Japan

Abstract—Cerebral ischemia can be exacerbated by post-ischemic hyperglycemia, which may involve the cerebral sodium-glucose transporter (SGLT). However, the contribution of each SGLT isoform in cerebral ischemia is still unclear. SGLT-1, -3, -4, and -6 have been reported to be expressed in various brain regions. Among these isoforms, only SGLT-3 does not transport glucose, but depolarizes the plasma membrane when glucose is bound, suggesting that SGLT-3 is a glucose sensor. Therefore, in this study, we investigated the involvement of cerebral SGLT-3 in the development of ischemia. The mouse model of focal ischemia was generated by middle cerebral artery occlusion (MCAO). Neuronal damage was assessed by histological and behavioral analyses. Fasting blood glucose levels on day 1 after MCAO were not affected in SGLT-3 siRNA-mediated knockdown of SGLT-3. The development of infarct volume and behavioral abnormalities on day 1 after MCAO were exacerbated in SGLT-3 knockdown mice (control group: $n = 7$, $94.2 \pm 21.8\ mm^3$, 2 (1.6–2.4), SGLT-3 knockdown group: $n = 6$, $1414.8 \pm 492.4\ mm^3$, 6 (5.8–6.3), $P < 0.05$). Moreover, SGLT-3 expression levels were significantly decreased in the striatum (65.0 ± 8.1%, $P < 0.05$) on day 1, and in the hippocampus (67.6 ± 7.2%, $P < 0.05$) and hypothalamus (47.5 ± 5.1%, $P < 0.01$) on day 3 after MCAO ($n = 12–13$). These effects were significantly inhibited by donepezil (DPZ) treatment (SGLT-3 knockdown group: $n = 6$, $1419.0 \pm 181.5\ mm^3$, 3.6 (3.4–3.7), SGLT-3 knockdown and 3 mg/kg DPZ-treated group: $n = 5$, $611.3 \pm 205.3\ mm^3$, 1.5 (1.4–1.8), $P < 0.05$). Immunofluorescence revealed that SGLT-3 and choline acetyltransferase were co-localized in the cortex. Our results indicated that cerebral SGLT-3 suppressed neuronal damage by the activation of cholinergic neurons, which are neuroprotective. In contrast, other cerebral SGLT isoforms may be involved in the development of ischemia.

Key words: glucose transporter, cerebral ischemia, hyperglycemia, SGLT, neuroprotection.

INTRODUCTION

Cerebral stroke is the leading cause of death and disability worldwide (Rezazadeh et al., 2013). Risk factors for cerebral stroke include cardiac diseases, hypertension, diabetes, smoking, alcohol consumption, an unhealthy diet, abdominal obesity, lack of exercise, psychosocial stress, and depression (Saito, 2012; He et al., 2013). Strokes are particularly exacerbated by diabetes or hyperglycemia (Fuentes et al., 2009; Harada et al., 2012). A clinical study has reported that disorders of glucose metabolism exist in a population of acute cerebral stroke patients. However, some patients with stroke do not have a prior history of diabetes (Matz et al., 2006). Moreover, insulin treatment in the acute phase of cerebral stroke suppresses the disability of stroke (Gentile et al., 2006; Rizk et al., 2006). Previous reports have also shown that post-ischemic hyperglycemia can develop in acute cerebral stroke resulting from impaired insulin sensitivity and enhanced gluconeogenesis. Moreover, we have reported that in a mouse model of focal cerebral ischemia, post-ischemic hyperglycemia exacerbates neuronal damage (Harada et al., 2009, 2011). However, the exact mechanism underlying this effect is unclear. Therefore, we focused on the sodium-glucose transporter (SGLT).

The SGLT family is a member of the solute carrier 5A families, consisting of six isoforms (SGLT-1–6) (Scheepers et al., 2004). The presence of SGLT-1, -3, -4, and -6 has been reported in various brain regions (Wright and Turk, 2004; O'Malley et al., 2006; Vemula et al., 2009). SGLT transports glucose together with Na^+ ions into cells, generating inward currents in the process (Wright and Turk, 2004; Scheepers et al., 2004). Cerebral stroke has been shown to be accompanied by glutamate-mediated excitotoxicity, release of reactive oxygen species (ROS), and the initiation of apoptosis

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*Corresponding author. Address: Department of Clinical Pharmacy, School of Pharmaceutical Sciences, Kobe Gakuin University, 1-1-3 Minatojima, Chuo-ku, Kobe 650-8586, Japan. Tel/fax: +81-78-974-4780.

E-mail address: stoku@pharm.kobegakuin.ac.jp (S. Tokuyama).

Abbreviations: Ach, acetylcholine; BSA, bovine serum albumin; ChAT, choline acetyltransferase; DPZ, donepezil; FBG, fasting blood glucose; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; i.c.v., intracerebroventricular; MCAO, middle cerebral artery occlusion; NDS, neurological deficit score; PBS, phosphate-buffered saline; PBST, PBS containing 0.1% Tween 20; rCBF, relative cerebral blood flow; ROS, reactive oxygen species; SGLT, sodium-glucose transporter; siRNA, small interfering RNA.

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(Rezazadeh et al., 2013). High-glucose conditions decrease cell viability and increase apoptosis (Benchoua et al., 2001). Therefore, post-ischemic hyperglycemia may activate the cerebral SGLT family and exacerbate ischemic neuronal damage through glucose and SGLT-induced depolarization. We have previously demonstrated that the cerebral SGLT family is involved in exacerbating cerebral ischemic neuronal damage (Yamazaki et al., 2012). However, the SGLT isoform that is involved in this damage remains unclear. Although SGLT transports glucose with Na^+ into the cell, the glucose sensor, SGLT-3 does not transport glucose, but plays a role in depolarizing the plasma membrane via the influx of sodium when glucose is bound (Wright and Turk, 2004; O'Malley et al., 2006). Moreover, SGLT-3 is expressed in the small intestine and skeletal muscle, regulating both the peristaltic action of the small intestine and muscle contraction, respectively (Diez-Sampedro et al., 2003). However, the physiological function of brain SGLT-3 has not been reported.

The aim of the present study was to investigate the involvement of cerebral SGLT-3 in the development of cerebral ischemic neuronal damage.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on male ddY mice (5 weeks old, 25–30 g), which were obtained from SLC (Shizuoka, Japan). The animals were housed between 23 and 24 °C with a 12-h light–dark cycle. Food and water were available *ad libitum*. The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, adopted by the Japanese Pharmacological Society. Additionally, all experiments were approved by the ethics committee for animals of the Kobe Gakuin University (approval number: A13–25).

Western immunoblot analysis

Western immunoblotting was performed as previously described (Yamazaki et al., 2012) but with some modifications. Briefly, each brain region was homogenized in homogenization buffer, and protein samples were loaded (20 or 40 μg/lane) and separated in 7.5% sodium dodecyl sulfate polyacrylamide gels and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). SGLT-3 or -1 was detected using (1:200) (Alpha Diagnostic Intl. Inc., San Antonio, TX, USA) or (1:1,000) (Millipore, Billerica, MA, USA), respectively. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (loading control) was detected with (1:20,000) (Chemicon, Temecula, CA, USA). Membranes were incubated with the primary antibodies in phosphate-buffered saline (PBS) containing 1% (v/v) Tween-20 and 5% (w/v) blocking agent (GE Healthcare, Toyko, Japan) at 4 °C, overnight. After washing, the membranes were incubated (1 h, at room temperature) with the secondary antibodies: horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:1000) (KPL, Guildford, UK) for SGLT-1 and 3, or HRP-conjugated anti-mouse IgG (1:10,000) (KPL) for GAPDH. Immunoreactive bands were visualized by the Light-Capture instrument (AE-6981) (ATTO, Tokyo, Japan) with the enhanced chemiluminescence western blotting analysis system (GE Healthcare, Toyko, Japan). The signal intensity of immunoreactive bands was analyzed using Cs-Analyzer software (Version 3.0) (ATTO).

Administration of SGLT-3 small interfering RNA (siRNA) in the brain

SGLT-3 was intracerebroventricularly (i.c.v.) administered to mice ON-TARGETplus SMART pool (SGLT-3 siRNA) (Thermo Scientific, Kanagawa, Japan), ON-TARGETplus Nontargeting Pool (control siRNA) (Thermo Scientific), and jetPEI + 5% glucose (cationic polymer transfection reagent; Polyplus Transfection, New York, NY, USA) was administered to knockdown SGLT-3 protein levels. I.c.v. was performed, as described previously (Haley and McCormick, 1957; Harada et al., 2011). In brief, a microsyringe with a 27-gauge stainless-steel needle was used for all experiments. Tubing covered all but the terminal 2.5–3.0 mm of the needle to make a track through the brain and into the lateral ventricle but not through the floor of the lateral ventricle. The needle was inserted unilaterally into the lateral ventricle of the brain (1.0 mm lateral and 1.0 mm posterior to bregma), as described previously (Harada et al., 2011). Verification of the needle position in the lateral cerebroventricular region was made by an i.c.v. injection of the dye and subsequent postmortem brain section verification of dye placement. SGLT-3 siRNA was administrated on day 3 before the operation.

Animal model of focal cerebral ischemia

The mouse model of transient focal ischemia was generated by middle cerebral artery occlusion (MCAO), as described previously (Harada et al., 2009). Briefly, mice were anesthetized with isoflurane and kept under a heating lamp to maintain the core body temperature at 37.0 ± 0.5 °C. The left middle cerebral artery was occluded for 2 h by the insertion of 8–0 nylon monofilament with a thin silicon coat through the common carotid artery followed by reperfusion. Sham-operated mice underwent the same surgical procedure but without suture insertion. A laser Doppler probe inserted into the acrylic sheath was positioned over the left skull 2 mm posterior to bregma and 6 mm to the left side of the midline. Baseline relative cerebral blood flow (rCBF) values measured before the occlusion were defined as 100%. MCAO was recorded as a decrease in rCBF to 40% of control values, and rCBF was recovered to about 100% by reperfusion (Harada et al., 2009). Physiological parameters were measured before, during and 30 m after MCAO by using the sphygmomanometer (TK-370C) (Brain Science idea, Osaka, Japan) and i-STAT (300F; FUSO Pharmaceutical Industries, Osaka, Japan), as described previously (Harada et al., 2009). We eliminated mice by pricking the brain on the silicon-coated 8–0 nylon monofilament and unsuccessful infarction on day 1 after MCAO.