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Sodium influx through cerebral sodium-glucose transporter type 1 exacerbates the development of cerebral ischemic neuronal damage

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ABSTRACT

We recently reported that cerebral sodium-glucose transporter type 1 (SGLT-1) plays a role in exacerbation of cerebral ischemia. However, the mechanism by which cerebral SGLT-1 acts remains unclear. Here we demonstrated that sodium influx through cerebral SGLT-1 exacerbates cerebral ischemic neuronal damage. SGLT-specific sodium ion influx was induced using α-methyl-D-glucopyranoside (α-MG). Intracellular sodium concentrations in primary cortical neurons were estimated using sodium-binding benzofuran isophthalate fluorescence. SGLT-1 knockdown in primary cortical neurons and mice was achieved using SGLT-1 siRNA. The survival rates of primary cultured cortical neurons were assessed using biochemical assays 1 day after treatment. Middle cerebral artery occlusion (MCAO) was used to generate a focal cerebral ischemic model in SGLT-1 knockdown mice. The change in fasting blood glucose levels, infarction development, and behavioral abnormalities were assessed 1 day after MCAO. Treatment with 200 mM α-MG induced a continuous increase in the intracellular sodium concentration, and this increase was normalized after α-MG removal. Neuronal SGLT-1 knockdown had no effect on 100 μM H_2O_2-induced neuronal cell death; however, the knockdown prevented the neuronal cell death induced by 17.5 mM glucose and the co-treatment of 100 μM H_2O_2/8.75 mM glucose. Neuronal SGLT-1 knockdown also suppressed the cell death induced by α-MG alone and the co-treatment of 100 μM H_2O_2/0.01 mM α-MG. Our *in vivo* results showed that the exacerbation of cerebral ischemic neuronal damage induced by the intracerebroventricular administration of 5.0 μg α-MG/mouse was ameliorated in cerebral SGLT-1 knockdown mice. Thus, sodium influx through cerebral SGLT-1 may exacerbate cerebral ischemia-induced neuronal damage.

1. Introduction

Cerebral ischemia remains a major global cause of death and can lead to permanent disability (Elgendy et al., 2016). Diabetes, diet, cardiovascular problems, hypertension, smoking, obesity, metabolic syndrome, depression, and traumatic brain injury are risk factors for cerebral ischemia (Doi et al., 2010; Sarwar et al., 2010; Vijayan and Reddy, 2016). Notably, cerebral ischemia is worse in patients with a history of hyperglycemia or diabetes (Doi et al., 2010; Olsson et al., 1990). In addition, cerebral ischemic stress can lead to a hyperglycemic condition in nondiabetic patients called post-ischemic hyperglycemia (Capes et al., 2001; Matz et al., 2006). Post-ischemic hyperglycemia confers an increased risk of cerebral ischemic mortality and poor functional recovery in nondiabetic cerebral ischemia survivors (Capes et al., 2001). The normalization of blood glucose levels appears to provide potent survival benefits for patients with cerebral ischemia (Gentile et al., 2006; Rizk et al., 2006). Similarly, our cerebral ischemic mouse model exhibits hyperglycemia at both 12 h and 1 day after cerebral ischemic stress (Harada et al., 2013b, 2011, 2010, 2009). Regulation of this post-ischemic hyperglycemia using antidiabetic agents, such as insulin or metformin, ameliorates the development of cerebral ischemic neuronal damage (Harada et al., 2010, 2009).

Abbreviations: SGLT, sodium-glucose transporter; DIV, Day *in vitro*; SBFI, sodium-binding benzofuran isophthalate; STD, standard; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MAP2, microtubule-associated protein 2; DAPI, 4′,6-diamidino-2-phenylindole; siRNA, small interfering RNA; SDS, sodium dodecyl sulfate; TBS, tris buffered saline; TBS-T, TBS containing 0.1% Tween 20; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; i.c.v., intracerebroventricular; MCAO, middle cerebral artery occlusion; FBG, Fasting blood glucose; TTC, 2,3,5-triphenyltetrazolium chloride; NDS, neurological deficit score; SEM, standard errors of the mean

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However, the detailed relationship between post-ischemic hyperglycemia and the development of cerebral ischemic neuronal damage remains undetermined. Thus, we focused on the sodium-glucose transporter (SGLT) to assess the mechanism of post-ischemic hyperglycemia-induced exacerbation of cerebral ischemic neuronal damage.

SGLTs are a large family of membrane proteins involved in the transport of glucose and sodium ions (Sabino-Silva et al., 2010; Wright et al., 2011). Six isoforms of SGLT have been identified, and SGLT-1, -3, -4, and 6 are expressed in the brain (Matsuoka et al., 1998; Poppe et al., 1997; Scheepers et al., 2004; Wright and Turk, 2004; Wright et al., 2011). Using the SGLT family specific inhibitor phlorizin and cerebral SGLT-1 knockdown mice, we previously showed that SGLT-1involved in post ischemic hyperglycemia-induced exacerbation of cerebral ischemia (Harada et al., 2013a; Yamazaki et al., 2015a, 2015b, 2012). However, the detailed mechanism behind this cerebral SGLT-1-mediated exacerbation remains unknown.

In cerebral ischemia, calcium ions mediate oxidative stress and excitotoxicity, and noxious stimuli-induced cellular calcium ion overload can cause cytotoxicity and cell death (Orrenius et al., 2003; Zheng et al., 2013). Membrane depolarizing currents evoked by cerebral ischemic stress can cause severe damage after cerebral ischemia (Dreier, 2011; Murphy et al., 2008; Wang et al., 2016). Notably, sodium influx through SGLT induces membrane depolarization and calcium ions overload (O'Malley et al., 2006; Wright et al., 2011). Therefore, we examined if an excessive influx of sodium ions through cerebral SGLT-1 during post-ischemic hyperglycemia exacerbates the development of cerebral ischemic neuronal damage.

2. Materials and methods

2.1. Animals

All experiments were performed on ddY mice (17-day pregnant females or 5 week-old males, 25–30 g) purchased from SLC (Shizuoka, Japan). All mice were housed at a temperature of 23–24 °C under a 12 h light-dark cycle with food and water *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. All of the experiments were approved by the Ethical Committee for Animals at Kobe Gakuin University (approval number: 16-10).

2.2. Primary cortical neuronal cultures

Primary neurons were obtained from the cortex of 17-day pregnant ddY mice fetuses as previously described (Yamazaki et al., 2016, 2015a). Briefly, dissociated primary cortical neurons were plated in 35 mm glass bottom culture dishes (Matsunami Glass Ind, Osaka, Japan) for the measurement of intracellular sodium ion concentrations and fluorescent immunostaining, 96-well plates for neuronal survival experiments, or 6-well plates for western blot analysis at a density of 1.5×10^5 cells/cm^2. Plates were coated with poly-D-ornithine (100 μg/ml; Sigma-Aldrich, Tokyo, Japan). Primary cortical neurons were cultured in Dulbecco's Modified Eagle's Medium/Ham's F-12 (L-glutamine, sodium pyruvate, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Nacalai Tesque, Kyoto, Japan) supplemented with 5% heat-inactivated bovine serum (Biowest, Nuaillé, France), 5% heat-inactivated horse serum (Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Invitrogen), and 0.1 mg/ml streptomycin (Invitrogen). Primary cortical neurons were maintained in a humidified incubator with 5% CO_2 at 37 °C. Cytosine β-D-arabinofuranoside (0.1 μM; Sigma-Aldrich) was added to cultures on the second day after seeding (second day *in vitro* [DIV 2]) to inhibit the proliferation of non-neuronal cells. In this culture preparation, > 90% of cells are neurons (Fujita-Hamabe and Tokuyama, 2012).

2.3. Measurement of intracellular sodium ion concentrations

SGLT-specific sodium influx was induced by α-methyl-D-glucopyranoside (α-MG), which is a SGLT-specific non-metabolic glucose analog (Gribble et al., 2003; Wright et al., 2011). Changes in intracellular sodium levels were measured using the fluorescent dye sodium-binding benzofuran isophthalate (SBFI) as a sodium indicator. Primary cortical neurons (DIV 3 or 4) were loaded with 10 μM SBFI and 0.02% pluronic F-127 (detergent) in a standard (STD) solution containing 140.0 mM NaCl, 5.0 mM KCl, 2.5 mM CaCl2, 1.0 mM MgCl2, 10.0 mM HEPES, and 5.56 mM glucose (pH 7.4) for 60 min at 35 °C in the dark. The neurons were then rinsed twice with STD solution. A culture dish was placed on the stage of an inverted microscope (DIAPHOTO 300; Nikon, Tokyo, Japan) and continuously perfused with 2 ml/min STD solution at 35 °C. Fluorescent images of primary cortical neurons were taken at 340 and 380 nm in 10 s intervals using an image processor (ARGUS-50; Hamamatsu Photonics, Hamamatsu, Japan). Culture dishes were perfused with glucose-free STD solution for 3 min until a constant fluorescence ratio was achieved (F340/F380). The glucose-free STD solution was replaced with the experimental solution containing 200 mM α-MG or 200 mM mannitol. The measurements of intracellular sodium ion concentrations in primary cortical neurons were completed in 2–3 experiments. For each experiment, the fluorescence was measured in 9–39 cells.

2.4. Fluorescent immunostaining of primary cortical neurons

Primary cortical neurons were cultured for 3 days (DIV 3) and then processed for immunofluorescence. Primary cortical neurons were incubated in 98% ethanol for 10 min at −20 °C, washed with phosphate-buffered saline (PBS; 5 min ×3 times), and incubated with blocking buffer [3% bovine serum albumin (BSA) in PBS] for 1 h at room temperature. Next, the cells were incubated in reaction buffer (1% BSA in PBS) overnight at 4 °C with rabbit polyclonal anti-SGLT-1 (1:50; Abcam, Cambridge, UK). The cells were then washed with PBS (5 min ×3 times) and incubated with Alexa Fluor 488-conjugated donkey polyclonal anti-rabbit IgG (1:200 in reaction buffer; Life Technologies, Carlsbad, CA, USA) for 2 h. Next, the cells were washed with PBS (5 min ×3 times), incubated with 3% BSA in PBS for 1 h at room temperature, and then incubated overnight at 4 °C with a microtubule-associated protein 2 (MAP2) antibody (1:1000, Abcam) and 1% BSA in PBS. The cells were then washed with PBS (5 min ×3 times) and incubated with Alexa Fluor 594-conjugated donkey polyclonal anti-rabbit IgG and goat polyclonal anti-mouse IgG (1:200 in reaction buffer; Life Technologies). A small volume of 4′,6-diamidino-2-phenylindole (DAPI; Boshide Biological Engineering Co., Wuhan, China) was added to the dish, and the cells were incubated for 10 min. Next, the cells were washed with PBS-T (5 min×3 min) and coverslipped with Fluoromount/Plus™ (Diagnostic BioSystems, CA, USA). Immunoreactivity was detected with a confocal fluorescence microscope (FV1000, Olympus, Tokyo, Japan).

2.5. Treatment with SGLT-1 small interfering RNA (siRNA) in primary cortical neurons

Primary cortical neurons (DIV 3) were treated with ON-TARGETplus SMART Mouse Slc5a1 small interfering RNA (SGLT-1 siRNA, 40 nM, Thermo Scientific, Kanagawa, Japan) or ON-TARGETplus Non-targeting Pool (control siRNA, 40 nM, Thermo Scientific) and Lipofectamine™ RNAiMAX transfection reagent (Thermo Scientific) for 48 h to knockdown SGLT-1.

2.6. Western blot analysis

Western blotting was performed as previously described with some modifications (Yamazaki et al., 2015b, 2012). Briefly, primary cortical