Neuroscience 339 (2016) 329–337

THE NA^+/HCO_3^- CO-TRANSPORTER IS PROTECTIVE DURING ISCHEMIA IN ASTROCYTES

HANG YAO,[a] PRITI AZAD,[a] HUIWEN W. ZHAO,[a] JUAN WANG,[a] ORIT POULSEN,[a] BEATRIZ C. FREITAS,[b] ALYSSON R. MUOTRI[b] AND GABRIEL G. HADDAD[c,d]*

[a] *Departments of Pediatrics, University of California San Diego, La Jolla, CA 92093, United States*

[b] *Department of Pediatrics/Rady Children's Hospital San Diego, Department of Cellular & Molecular Medicine, Stem Cell Program, La Jolla, CA 92093, United States*

[c] *Departments of Pediatrics & Neuroscience, University of California-San Diego, La Jolla, CA 92093, United States*

[d] *Rady Children's Hospital-San Diego, San Diego, CA 92123, United States*

Abstract—The sodium bicarbonate co-transporter (NBC) is the major bicarbonate-dependent acid–base transporter in mammalian astrocytes and has been implicated in ischemic brain injury. A malfunction of astrocytes could have great impact on the outcome of stroke due to their participation in the formation of blood-brain barrier, synaptic transmission, and electrolyte balance in the human brain. Nevertheless, the role of NBC in the ischemic astrocyte death has not been well understood. In this work, we obtained skin biopsies from healthy human subjects and had their fibroblasts grown in culture and reprogrammed into human-induced pluripotent stem cells (hiPSCs). These hiPSCs were further differentiated into neuroprogenitor cells (NPCs) and then into human astrocytes. These astrocytes express GFAP and S100β and readily propagate calcium waves upon mechanical stimulation. Using pH-sensitive dye BCECF [2′,7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein] and qPCR technique, we have confirmed that these astrocytes express functional NBC including electrogenic NBC (NBCe). In addition, astrocytes exposed to an ischemic solution (IS) that mimics the ischemic penumbral environment enhanced both mRNA and protein expression level of NBCe1 in astrocytes. Using IS and a generic NBC blocker S0859, we have studied the involvement of NBC in IS-induced human astrocytes death. Our results show that a 30 μM S0859 induced a 97.5 ± 1.6% (n = 10) cell death in IS-treated astrocytes, which is significantly higher than 43.6 ± 4.5%, (n = 10) in the control group treated with IS alone. In summary, a NBC blocker exaggerates IS-induced cell death, suggesting that NBC activity is essential for astrocyte survival when exposed to ischemic penumbral environment. Published by Elsevier Ltd on behalf of IBRO.

Key words: iPSCs, astrocytes, NBC, ischemia, penumbra.

INTRODUCTION

Ischemic penumbral tissue surrounds the infarct core and is at risk of injury due to the continued presence of ischemic environment (Fisher, 2004). Therefore, understanding the mechanisms of cell death in this region holds great promise for developing strategies for stroke therapy. Multiple cell types are involved in the ischemic penumbra and astrocytes are believed to have great impact on the prognosis of penumbral tissue, especially that astrocytes constitute the most numerous cell type in the mammalian brain. In addition, astrocytes play an important role in maintaining neuronal synaptic transmission, controlling cerebral blood flow, maintaining blood–brain barrier integrity and scavenging free radicals, among other roles. Hence, the fate of astrocytes in the ischemic penumbra is critical for the outcome of ischemic stroke.

The Na^+/HCO_3^- co-transporters (NBCs) are bicarbonate-dependent acid–base transporters on the cell membrane. NBCs are classified into either electrogenic (NBCe) or electroneutral (NBCn) transporters based on the stoichiometry of HCO_3^- and Na^+ transport (Boron et al., 2009). NBCs are expressed in the mammalian brain with NBCe predominately expressed in the astrocytes (Majumdar and Bevensee, 2010; Theparambil et al., 2014). NBC is involved not only in intracellular pH (pH_i) regulation in astrocytes but also plays a critical role in maintaining astrocytic ionic homeostasis and water balance between the intra- and extra-cellular space in the brain. Under pathological conditions, such as stroke, altered function and/or expression of NBC would have a profound impact on the fate of astrocytes, and therefore brain injury. In a gerbil middle cerebral artery occlusion (MCAO) model, for example, NBCe mRNA and protein expression is increased in the penumbral region and closely related to delayed cell death in the CA1 region of hippocampus. This delayed damage does not occur in CA3 region, where no alteration is detected in NBCe mRNA and protein expression (Sohn et al., 2011). In a permanent MCAO model, the expression of NBCe was found significantly elevated in the penumbra after three hrs of ischemia, suggesting that

*Correspondence to: G. G. Haddad, Department of Pediatrics, University of California San Diego, 9500 Gilman Drive, MC 0735, La Jolla, CA 92093-0735, United States. Fax: +1 858 534 6972.
E-mail address: ghaddad@ucsd.edu (G. G. Haddad).

Abbreviations: ACSF, Artificial cerebral spinal fluid; BCECF, 2′,7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein; hiPSCs, human-induced pluripotent stem cells; IS, ischemic solution; MCAO, middle cerebral artery occlusion; NBC, sodium bicarbonate co-transporter; NBCe, electrogenic sodium bicarbonate co-transporter; NBCn, electroneutral sodium bicarbonate co-transporter; NPCs, neural progenitor cells; PI, propidium iodide.

http://dx.doi.org/10.1016/j.neuroscience.2016.09.050
0306-4522/Published by Elsevier Ltd on behalf of IBRO.

NBCe could play a role in stroke pathophysiology (Jung et al., 2007).

Although earlier work has related enhanced expression and/or activity of NBC to brain injury, it is unclear as to whether this alteration is causal to injury or whether the alteration is beneficial. For example, previous work has demonstrated that hypoxia/ischemia induces astrocytic injury by changing the expression and function of NBC (Fujita et al., 1999; Khandoudi et al., 2001; Fantinelli et al., 2014). However, others have demonstrated that the alteration in NBC is tantamount to the activation of a protection mechanism for astrocytes (Kumar et al., 2007, 2011). In this work, we investigate the role of NBC in *human* astrocyte death after exposure to an ischemic solution (IS) that simulates the ischemic penumbral environment (Yao et al., 2007a). We demonstrate that human-induced pluripotent stem cells (hiPSCs)-derived astrocytes express a functional NBC and blocking NBC exaggerates ischemic injury in astrocytes. Therefore, our data indicate that the increased expression of NBC and their activity seen in the ischemic penumbra is a protective mechanism following stroke.

EXPERIMENTAL PROCEDURES

Reprogramming of human fibroblast cells and generation of iPSCs and neural progenitor cells (NPCs)

Detailed protocols for generating iPSCs and NPCs were described in our previous study (Zhao et al., 2015). In brief, fibroblast cells were generated from skin biopsies of two healthy adult subjects with an informed written consent and under approval by the University of California San Diego. Fibroblast cells were infected with retrovirus vectors containing OCT4, SOX2, KLF4 and c-MYC human cDNAs (Salk Institute Gene Transfer, Targeting and Therapeutics Core, La Jolla, CA, USA). The infected fibroblast cells were then plated onto the irradiated mouse embryonic fibroblast feeder cells incubated with human embryonic stem (ES) cell medium containing 20% knockout serum replacement, 1% non-essential amino acids, 0.2% beta-mercaptoethanol, and 30 ng/ml FGF2. Three weeks later, the iPS colonies were manually picked and maintained in the mTeSR™ medium (StemCell Technologies, Canada). To obtain NPCs, iPSCs were triturated into single cells and embryoid bodies (EBs) were formed using AggreWell plate (Stem Cell Technologies, Canada) with N2 medium containing 0.5 × N2, 0.5 × B27, 1% penicillin/streptomycin in DMEM/F12 medium plus 5 μM Y-27632, 1 μM dorsomorphin, 10 μM SB431542 (Tocris Bioscience, MN, USA). A day later, EBs were transferred to an ultra low attachment petri dish for a 24-h suspension culture. The next day, EBs were seeded on a matrigel-coated plate using N2 medium for 7–10 days. Rosette-bearing EBs were manually picked and dissociated into individual cells. These dissociated cells were then plated into a poly-L-ornithine/laminin-coated plate to generate a monolayer of NPC culture with N2 medium plus 20 ng/μl FGF2. NPC markers including Sox2 (1:100, Stemgent, MA) and Nestin (1:60, R&D systems, MN) were confirmed positive in these cells using immunocytochemistry.

Generation of astrocytes from NPCs

Astrocytes were differentiated from the NPCs following a protocol detailed elsewhere. Briefly, a confluent 100-mm diameter NPC plate was scraped forming neurospheres in a six-well plate by keeping at constant shaking (95 rpm). The medium was changed on the day after cells were suspended once the neurospheres were well formed using the NPC medium containing FGF. After efficient formation of spheres around 48 h post scrapping, the rock inhibitor was added to a final concentration of 5 μM for 48 h concomitant with the removal of FGF from the medium in the next medium change. Cells were kept in constant shaking with neuronal inducing medium for a week. Next, the astrocyte growth medium (Lonza, Allendale, NJ, USA) was added to the spheres for two weeks still under 95 rpm's. Following two weeks on the astrocyte medium, spheres are plated in laminin-coated plates and the astrocytes grow out of the sphere spreading on the plate to form a multilayer cell formation. After the first passage, cells surrounding the neurospheres were dissociated enzymatically using accutase (Cellgro) and plated. The neurospheres were removed manually by vacuum suction using a Pasteur pipet and the result was a confluent and homogeneous plate of GFAP- and S100β-positive astrocytes.

Experimental conditions

Artificial cerebral spinal fluid (ACSF) was balanced with 5% CO_2 + room air. ACSF contained (in mM): NaCl 129, KCl 5, $CaCl_2$ 1.3, $MgCl_2$ 1.5, $NaHCO_3$ 21, and glucose 10. The osmolarity was 315 mOsm and pH was 7.4. IS (Yao et al., 2007a) was balanced with 15% CO_2 + 1.5% O_2 + N_2. IS contained (in mM): NaCl 47, KCl 29, K-gluconate 35, $MgCl_2$ 1.5, $CaCl_2$ 0.13, $NaHCO_3$ 4, glucose 3, and glutamate 0.1. The osmolarity was adjusted to 315–320 mOsm with sucrose and pH was 6.4. All the above chemicals and S0859 were obtained from Sigma.

Intracellular pH measurement

A coverslip with cultured astrocytes was mounted on a thermostatically controlled holding chamber and incubated with 2 μM membrane-permeable ester 2′,7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)-AM for 20 min at room temperature. The chamber was then secured on the stage of the microscope and supplied with ACSF bubbled with 5% CO_2 balanced with air. Intracellular fluorescence was determined in individual cells using fluorescence microscopy and digital image processing. A 175-W Xenon lamp and an ultra high-speed wavelength switcher (Lambda DG-4, Sutter Instrument, Novato, CA, USA) provided an alternate 440/495-nm fluorescence excitation. The emission from BCECF loaded cells was detected at wavelengths of 535 nm using a F-Fluar 40X/1.3 NA oil immersion objective (Zeiss Axiovert 200 M microscope, Zeiss, Yena, Germany) and the attached 12-bit CCD camera. The light source, wavelength switcher,