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HYPERGLYCEMIA REDUCES FUNCTIONAL EXPRESSION OF ASTROCYTIC Kir4.1 CHANNELS AND GLIAL GLUTAMATE UPTAKE

D. E. RIVERA-APONTE, [a]* M. P. MÉNDEZ-GONZÁLEZ, [a] A. F. RIVERA-PAGÁN, [a] Y. V. KUCHERYAVYKH, [a] L. Y. KUCHERYAVYKH, [a] S. N. SKATCHKOV [a,b]* AND M. J. EATON [a]

[a] *Department of Biochemistry, Universidad Central del Caribe, Bayamón, PR, USA*

[b] *Department of Physiology, Universidad Central del Caribe, Bayamón, PR, USA*

Abstract—Diabetics are at risk for a number of serious health complications including an increased incidence of epilepsy and poorer recovery after ischemic stroke. Astrocytes play a critical role in protecting neurons by maintaining extracellular homeostasis and preventing neurotoxicity through glutamate uptake and potassium buffering. These functions are aided by the presence of potassium channels, such as Kir4.1 inwardly rectifying potassium channels, in the membranes of astrocytic glial cells. The purpose of the present study was to determine if hyperglycemia alters Kir4.1 potassium channel expression and homeostatic functions of astrocytes. We used q-PCR, Western blot, patch-clamp electrophysiology studying voltage and potassium step responses and a colorimetric glutamate clearance assay to assess Kir4.1 channel levels and homeostatic functions of rat astrocytes grown in normal and high glucose conditions. We found that astrocytes grown in high glucose (25 mM) had an approximately 50% reduction in Kir4.1 mRNA and protein expression as compared with those grown in normal glucose (5 mM). These reductions occurred within 4–7 days of exposure to hyperglycemia, whereas reversal occurred between 7 and 14 days after return to normal glucose. The decrease in functional Kir channels in the astrocytic membrane was confirmed using barium to block Kir channels. In the presence of 100-μM barium, the currents recorded from astrocytes in response to voltage steps were reduced by 45%. Furthermore, inward currents induced by stepping extracellular $[K^+]_o$ from 3 to 10 mM (reflecting potassium uptake) were 50% reduced in astrocytes grown in high glucose. In addition, glutamate clearance by astrocytes grown in high glucose was significantly impaired. Taken together, our results suggest that down-regulation of astrocytic Kir4.1 channels by elevated glucose may contribute to the underlying pathophysiology of diabetes-induced CNS disorders and contribute to the poor prognosis after stroke. **© 2015 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: astrocytes, hyperglycemia, Kir4.1 potassium channels, glutamate clearance, diabetes.

INTRODUCTION

Diabetes mellitus is a metabolic disorder that also affects the central nervous system (CNS) by raising brain glucose levels. These higher levels of glucose in the CNS result in a glucose neurotoxicity that can lead to abnormal brain function and trauma (Tomlinson and Gardiner, 2008). It is known that both type I and II diabetes increase seizure susceptibility especially in non-ketogenic hyperglycemic diabetic patients due to metabolic disturbances such as mild hyperosmolality (Lee et al., 2014). Moreover, it is well documented that people with diabetes are at a greater risk for stroke when compared to non-diabetics and elevated blood glucose concentrations during stroke are associated with poor outcome (Desilles et al., 2013). High glucose concentrations within the CNS environment also affect astrocytes by increasing the levels of reactive oxygen species (ROS) which lead to oxidative stress (Hsieh et al., 2013) as well as an increase in the production of inflammatory cytokines (Shin et al., 2014).

Astrocytes are the most abundant cells in the CNS and their function goes beyond being supportive cells for neurons (Jing et al., 2013). In normal conditions, astrocytes play a major role in the CNS by maintaining extracellular homeostasis of neuroactive substances such as K^+, H^+, GABA and glutamate. A more hyperpolarized membrane potential compared to neurons can be found in astrocytes which provides the necessary driving force for K^+ spatial buffering and glutamate transport (Kucheryavykh et al., 2007, 2009; Olsen, 2012).

A major ion channel expressed by astrocytes is the inward rectifying potassium channel Kir4.1 (encoded by the gene KCNJ10) (Steinhauser and Seifert, 2002; Seifert et al., 2009). This potassium channel is not only a key player in efficient uptake of K^+ released by neurons during axon potential propagation (Neusch et al., 2006; Djukic et al., 2007; Chever et al., 2010), but these

*Corresponding authors. Address: Department of Physiology, School of Medicine, Universidad Central del Caribe, P.O. Box 60327, Bayamón, PR 00960-6032, USA (S.N. Skatchkov). Tel: +1-787-798-3001x2101/2057; fax: +1-787-786-6285.

E-mail addresses: david.rivera11@upr.edu (D. E. Rivera-Aponte), miguel.mendez3@upr.edu (M. P. Méndez-González), aixafernanda@gmail.com (A. F. Rivera-Pagán), k_yurii@hotmail.com (Y. V. Kucheryavykh), kuc-lilia@mail.ru (L. Y. Kucheryavykh), sergueis64@gmail.com (S. N. Skatchkov), misty.eaton@uccaribe.edu (M. J. Eaton).

Abbreviations: BBSS, bicarbonate balanced salt solution; CNS, central nervous system; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ICS, intracellular solution; ROS, reactive oxygen species.

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channels also influence the ability of glial glutamate transporters to clear glutamate from the synaptic space (Djukic et al., 2007; Kucheryavykh et al., 2007).

Dysfunction of normal astrocytes can compromise their ability to maintain extracellular homeostasis and can disrupt the normal function of neurons (Anderson et al., 2003). In addition, astrocyte dysfunction can lead to greater damage in response to an ischemic insult such as the one produced in stroke (Muranyi et al., 2006; Raghubir, 2007).

Studies aimed at determining the causes of diabetic retinopathy have shown reduction of connexin 26 and 43 gene and protein expression and corresponding functional deficits in Müller glial cells after 6 weeks of diabetes (Ly et al., 2011). It has also been demonstrated that protein levels of Kir4.1 and K^+ inward current are decreased leading to a higher osmotic stress to the Müller glial cells in streptozotocin-treated diabetic rats (Pannicke et al., 2006). In cultured Müller cells, hyperglycemia causes a reduction in both Kir4.1 and GLAST protein expression and these reductions could be reversed by administration of pigment epithelium-derived factor (Xie et al., 2012).

Although there have been conflicting reports about high glucose and diabetes altering glial fibrillary acidic protein (GFAP) levels in astrocytes (Coleman et al., 2004; Guven et al., 2009; Nagayach et al., 2014), there is not much information about the consequences of high glucose levels on the function of brain astrocytes. The purpose of the current study was to determine the effect of high glucose on the expression and function of Kir4.1 potassium channels in astrocytes thereby affecting potassium and glutamate uptake by astrocytes.

EXPERIMENTAL PROCEDURES

Astrocyte primary cultures

Primary cultures of astrocytes were prepared from the neocortex of 1–2-day-old rats as previously described (Kucheryavykh et al., 2007) and in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC). Briefly, brains were removed after decapitation and the meninges stripped away to minimize fibroblast contamination. The forebrain cortices were collected and dissociated using the stomacher blender method. The cell suspension was then allowed to filter by gravity through a #60 sieve and then through a #100 sieve. After centrifugation, the cells were suspended in two groups. One group was plated in normal glucose DMEM (containing 5 mM glucose, 2 mM glutamine, 1 mM pyruvate, 10% fetal bovine serum and 100 iU/ml penicillin/100 μg/ml streptomycin). The second group was plated in high glucose DMEM where the glucose concentration was 25 mM. Both groups were plated in uncoated 75 cm^2 flasks at a density of 300,000 cells/cm^2. The medium was exchanged with the appropriate fresh culture medium about every 4 days. At confluence (about 12 days), the mixed glial cultures were treated with 50 mM leucine methylester (pH 7.4 in PBS) for 60 min to kill microglia (Simmons and Murphy, 1992). Cultures were then allowed to recover for at least one day in growth medium prior to experimentation. Astrocytes were dissociated by trypsinization and reseeded onto appropriate plates and cover glasses for the glutamate uptake study and for the patch clamp experiments.

Time course experiment

Two groups were studied according to the experimental design: (1) normal glucose to high glucose to see how rapidly Kir4.1 was downregulated and (2) high glucose to normal glucose to analyze when or if Kir4.1 protein levels recover. The medium changes were done over a period of 14 days using the following time points: 0, 7, 10, 12, 13 or 14 days and all cells were harvested at the same time. Therefore, the days of 0, 1, 2, 4, 7 or 14 after medium change were examined. As a control, astrocytes were cultured in 5 mM or 25 mM glucose and on days 0, 7, 10, 12, 13 or 14, the medium was exchanged for fresh medium containing the same initial 5 mM or 25 mM glucose. After 14 days the cells were lysed and prepared for protein level analysis by Western blot. The results were expressed as percent of control and compared to astrocytes for which medium was changed, but the glucose concentration was kept the same (i.e., normal to normal or high to high glucose).

SDS–PAGE and Western blotting analysis

Astrocytes were harvested, pelleted and resuspended in homogenization buffer (pH 7.5) containing: (in mM) Tris–HCl 20, NaCl 150, EDTA 1.0, EGTA 1.0, Phenylmethylsulfonyl Fluoride (PMSF) 1.0, and 1% Triton X-100 and an additional mixture of peptide inhibitors (leupeptin, bestatin, pepstatin, and aprotinin). Lysates were mixed with Urea sample buffer (plus dithiothreitol), boiled, spun briefly to pellet debris, and immediately run on 10% SDS–polyacrylamide gels. Protein concentration of cell homogenates was determined with the DC protein assay (Bio-Rad), followed by addition of an appropriate volume of Urea sample buffer (62 mM Tris/HCl pH 6.8, 4% SDS, 8 M Urea, 20 mM EDTA, 5% β-Mercaptoethanol, 0.015% Bromophenol Blue) for a final concentration of 0.5–1.5 μg protein/μl, and incubation in a water bath at 94 °C for 10 min. Western blotting was performed as previously described (Kucheryavykh et al., 2007) using a rabbit polyclonal antibody against Kir4.1 (1:400, Alomone, cat. # APC-035). Final detection was performed with enhanced chemiluminescence methodology (SuperSignal® West Dura Extended Duration Substrate; Pierce, Rockford, IL, USA) as described by the manufacturer, and the intensity of the signal measured in a gel documentation system (Versa Doc Model 1000, Bio Rad). In all cases, intensity of the chemiluminescence signal was corrected for minor differences in protein content after densitometry analysis of the India ink stained membrane.

Real-time RT-PCR

Gene expression levels were determined by RT-PCR analysis for the KCNJ10 gene encoding Kir4.1. Total

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