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EXENDIN-4 IMPROVED RAT CORTICAL NEURON SURVIVAL UNDER OXYGEN/GLUCOSE DEPRIVATION THROUGH PKA PATHWAY

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Abstract—Previous studies demonstrated that exendin-4 (Ex-4) may possess neurotrophic and neuroprotective functions in ischemia insults, but its mechanism remained unknown. Here, by using real-time PCR and ELISA, we identified the distribution of active GLP-1Rs in the rat primary cortical neurons. After establishment of an in vitro ischemia model by oxygen/glucose deprivation (OGD), neurons were treated with various dosages of Ex-4. The MTT assay showed that the relative survival rate increased with the dosage of Ex-4 ranging from 0.2 to 0.8 μg/ml ($P < 0.001$, vs. OGD group). The apoptosis rate was reduced from (49.47 ± 2.70)% to (14.61 ± 0.81)% after Ex-4 treatment (0.4 μg/ml) 12 h after OGD ($P < 0.001$). Moreover, immunofluorescence staining indicated that Ex-4 increased glucose-regulated proteins 78 (GRP78) and reduced C/EBP-homologous protein (CHOP). Western blot analysis demonstrated that, after neurons were treated with Ex-4, GRP78 was up-regulated over time ($P < 0.01$, vs. OGD group), while CHOP levels rose to a peak 8 h after OGD and then decreased ($P < 0.05$, vs. OGD group). This effect was changed by both the protein kinase A (PKA) inhibitor H89 ($P < 0.01$, $P < 0.05$, respectively, vs. Ex-4 group) and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 ($P < 0.01$, $P < 0.01$, respectively, vs. Ex-4 group) but not by the mitogen-activated protein kinase (MAPK) inhibitor U0126. Our study also revealed that, compared with the Ex-4 group, inhibition of the PKA signaling pathway significantly decreased the survival rate of neurons, down-regulated the expression of B-cell lymphoma 2 (Bcl-2) and up-regulated the Bax expression 3 h after ODG ($P < 0.05$, $P < 0.01$, respectively), while neither PI3K nor MAPK inhibition exerted such effects. Furthermore, Western blotting exhibited that PKA expression was elevated in the presence or absence of OGD insults ($P < 0.05$). This study indicated that Ex-4 protected neurons against OGD by modulating the unfolded protein response (UPR) through the PKA pathway and may serve as a novel therapeutic agent for stroke.

Key words: exendin-4, oxygen/glucose deprivation, endoplasmic reticulum stress, unfolded protein response, neurons, rat.

INTRODUCTION

Stroke, the most common cause of death and disability, can be disastrous for the afflicted individual. As a generally accepted risk factor, type 2 diabetes (T2DM) is believed to be related with episodes, onset or relapse, of stroke (Hillen et al., 2003; Perry et al., 2003) suggesting that these two conditions have something in common in their pathogeneses. Recent findings revealed that the central nervous system shares some signal transduction pathways involved in the regulation of glucose metabolism in peripheral tissues (Perry et al., 2002).

As we know it, both neurons and pancreatic β cells are rich in developed endoplasmic reticulum (ER) (Lin et al., 2008). The ER is a key cellular organelle taking part in protein homoeostasis. Various conditions can affect ER functions, which in turn induces ER stress and activates the unfold protein response (UPR) to restore ER homeostasis (Boyce and Yuan, 2006; Schröder, 2008). As a main UPR target protein, glucose-regulated proteins 78 (GRP78) is induced and can improve cell survival due to its central actions on ER homeostasis regulation, such as effects on protein folding, ER calcium binding, and controlling of the activation of transmembrane ER stress sensors (Li and Lee, 2006; Luo et al., 2006; Chakrabarti et al., 2011). However, persistent or severe ER stress and the UPR activation are detrimental to the stressed cells, finally resulting in cellular damages and apoptosis (Boyce and Yuan, 2006; Schröder, 2008). One of the apoptosis pathways is C/EBP homologous protein (CHOP/GADD15),

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Abbreviations: ATF4, activating transcription factor-4; ATF6, activating transcription factor-6; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; CHOP, C/EBP-homologous protein; ER, endoplasmic reticulum; Ex-4, exendin-4; GLP-1, glucagon-like peptide; GLP-1R, glucagon-like peptide 1 receptor; GRP78, glucose-regulated proteins 78; IRE1, inhibitor resistant esterase 1; MAPK, mitogen-activated protein kinase; OGD, oxygen/glucose deprivation; PC12 cells, rat pheochromocytoma cells; PERK, double-stranded RNA-activated protein kinase-like endoplasmic reticulumkinase; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; T2DM, type 2 diabetes; UPR, unfolded protein response.

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which operates at the convergence of the three arms of the UPR (double-stranded RNA-activated protein kinase-like endoplasmic reticulumkinase (PERK), inhibitor resistant esterase 1 (IRE1), and activating transcription factor-6 (ATF6)) (Ma et al., 2002; Oyadomari and Mori, 2004). And its pro-apoptotic effect might be related to the modulation of the expression of B-cell lymphoma 2 (Bcl-2) family proteins, such as Bcl-2-associated X protein (Bax), Bcl-2, Bim and BH3-only protein (McCullough et al., 2001; Li et al., 2006; Puthalakath et al., 2007; Mecha et al., 2012). So far, the impact of ER stress and the UPR activation on both cerebral ischemia and T2DM has already been confirmed (Liu and Kaufman, 2003; Lin et al., 2008). This double-edged sword feature, a naturally occurring tactic directed at the modulation of ER stress and the UPR, may be used as a therapeutic strategy for the treatment of ischemia stroke.

Exendin-4 (Ex-4), an agonist of long-acting glucagon-like peptide 1 (GLP-1), is a 39-amino-acid peptide found in venom from the Gila monster Helicoderma suspectum (Christel and Denardo, 2007). It can effectively normalize insulin signals in T2DM by binding to specific receptors on the surface of pancreatic β cells in a cAMP/PKA-dependent manner (Drucker, 2003; De León et al., 2006). Intriguingly, GLP-1 receptors (GLP-1Rs) are extensively found in the brain and may possess neurotrophic and neuroprotective functions. Previous studies have shown that GLPs play important roles in the central nervous system, such as modulation of satiety, improvement of memory and learning and defense against insults (Szayna et al., 2000; Tibaduiza et al., 2001; Perry et al., 2002; McClean et al., 2010). Nevertheless, little is known about their role in brain ischemia. Whether Ex-4 can protect neurons against brain ischemia warrants further study, but it has been confirmed that Ex-4 treatment reduces infarct size and improves outcome in MCAO (Li et al., 2009). Recent work by Teramoto and fellow researchers also confirmed the neuroprotective action of Ex-4 against ischemic injury and the mechanism might be its ability to increase intracellular cAMP levels (Teramoto et al., 2011). In this study, we studied the effects of Ex-4 on ER-related cortical neuron apoptosis in an in vitro ischemia model [by oxygen/glucose deprivation (OGD), cultured rat cortical neurons], and evaluated the potential of using Ex-4 as a neuroprotective agent for the treatment of stroke.

EXPERIMENTAL PROCEDURES

Animals

This study was approved by the Ethics Committee on Animal Experimentation of Tongji Medical College, Huazhong University of Science and Technology, China. Neonatal Sprague–Dawley rats (< 24 h) were obtained from the Animal Department of Tongji Medical College, Huazhong University of Science and Technology. Animals were handled by strictly following the guidelines for ethical care of animals formulated by the Chinese National Institutes of Health.

Cell cultures and OGD

Rat pheochromocytoma cells (PC12 cells) were cultured in DMEM containing high glucose (GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (GIBCO). Primary cortical neurons were taken from neonatal Sprague–Dawley male rats (< 24 h) as described previously (Caughlan et al., 2004). Dissociated cortical neurons (10^6 cell/ml) were cultured in neuron-basal medium (GIBCO) supplemented with 2% (v/v) B27 (GIBCO), in a chamber (5% CO_2/95% O_2) at 37 °C for 7–10 days before experiments. Cultures contained more than 90% neurons as shown by neuronal-specific nuclear protein (NeuN) immunostaining (Zhongshan Glodenbridge Bio, Beijing, China). OGD on primary neuronal cultures was established as previously reported (Meloni et al., 2001). Briefly, cultured primary cortical neurons, which were washed three times with PBS, were treated with glucose-free Earle's medium and stored for 6 h in an anoxic chamber (5% CO_2/95% N_2). After that, neurons were removed from the anoxic chamber, and the culture medium was replaced by fresh neuron-basal medium supplemented with B27. Afterwards, cultures were stored under normoxic conditions (5% CO_2/95% O_2) at 37 °C to allow for reoxygenation. Control cells were incubated in Earle's medium with glucose under normoxic conditions (5% CO_2/95% O_2).

Cell treatment paradigm

Ex-4 (Sigma, St. Louis, MO, USA) was dissolved in ddH_2O and cultures were randomly divided into three groups. In the Ex-4 group, cells were treated with Ex-4, and the final concentration of Ex-4 was determined by the MTT assay during the phases of pre-OGD (2 h), OGD (6 h) and at different time points after OGD. 10 μM of H89, LY294002 and U0126 was added to the cell cultures for 2 h before OGD and remained in the culture throughout Ex-4 treatment. In the OGD group, cells were treated with an equivalent volume of ddH_2O. With the control group, cells did not receive any treatment.

Demonstration of GLP-1R presence in cortical neurons

Quantitative real-time PCR. To confirm the presence of GLP-1R on primary cortical neurons, the mRNA level of GLP-1R was determined by real-time PCR. In this experiment, we used PC12 cells as the positive control (Perry et al., 2002).

cAMP analyses. GLP-1R activation is reported to induce a rapid, transient increase in intracellular cAMP by stimulating adenylyl cyclase (Perry et al., 2002). Therefore, levels of cAMP were measured by employing a Rat cAMP Sandwich ELISA kit (Cusabio Biotech Co., Ltd., USA) in accordance with the manufacturer's instructions. Neurons were plated into 6-well plates at a density of 1×10^6 cells per well and incubated with Ex-4 (0.3 μg/ml), and the intracellular cAMP levels were measured at various time points (t = 0, 5, 10, 15, 20, 25, 30 min) (McCullough et al., 2001; Perry et al., 2003). The optical densities were measured by using a microplate reader (ThermoElectron Corporation Muhiskan MK3, USA) at 450 nm. The concentrations of cAMP in the samples are then determined by comparing the O.D. of the samples against a standard curve.

MTT assay

To study the effects of Ex-4 on the survival of primary cultured cortical neurons, the viability of cells was then evaluated by utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay. This colorimetric method