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Research Paper

Neurotrophic and neuroprotective effects of oxyntomodulin in neuronal cells and a rat model of stroke

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ABSTRACT

Proglucagon-derived peptides, especially glucagon-like peptide-1 (GLP-1) and its long-acting mimetics, have exhibited neuroprotective effects in animal models of stroke. Several of these peptides are in clinical trials for stroke. Oxyntomodulin (OXM) is a proglucagon-derived peptide that co-activates the GLP-1 receptor (GLP-1R) and the glucagon receptor (GCGR). The neuroprotective action of OXM, however, has not been thoroughly investigated. In this study, the neuroprotective effect of OXM was first examined in human neuroblastoma (SH-SY5Y) cells and rat primary cortical neurons. GLP-1R and GCGR antagonists, and inhibitors of various signaling pathways were used in cell culture to characterize the mechanisms of action of OXM. To evaluate translation in vivo, OXM-mediated neuroprotection was assessed in a 60-min, transient middle cerebral artery occlusion (MCAo) rat model of stroke. We found that OXM dose- and time-dependently increased cell viability and protected cells from glutamate toxicity and oxidative stress. These neuroprotective actions of OXM were mainly mediated through the GLP-1R. OXM induced intracellular cAMP production and activated cAMP-response element-binding protein (CREB). Furthermore, inhibition of the PKA and MAPK pathways, but not inhibition of the PI3K pathway, significantly attenuated the OXM neuroprotective actions. Intracerebroventricular administration of OXM significantly reduced cerebral infarct size and improved locomotor activities in MCAo stroke rats. Therefore, we conclude that OXM is neuroprotective against ischemic brain injury. The mechanisms of action involve induction of intracellular cAMP, activation of PKA and MAPK pathways and phosphorylation of CREB.

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1. Introduction

Oxyntomodulin (OXM), an endogenous proglucagon-derived intestinal peptide, was isolated from porcine jejunoileum extract in 1981. OXM contains a 29-amino acid sequence of glucagon followed by 8 amino acids at its carboxyl-terminus. It was mainly produced in enteroendocrine L-cells and released together with glucagon-like peptide-1 (GLP-1) in response to food intake. Since OXM modulated gastric acid secretion in gastric oxyntic glands, it was named "oxyntomodulin" (Bataille et al., 1981). OXM is a dual agonist for the GLP-1 receptor (GLP-1R) and the glucagon receptor (GCGR) (Pocai et al., 2009). Limited reports have indicated that OXM activities control nutrition and metabolism in the periphery (Estall and Drucker, 2006; Tan and Bloom, 2013), and reduce pancreatic β-cell apoptosis (Maida et al., 2008). OXM improved glucose tolerance, inhibited food intake, increased energy expenditure, and reduced body weight in both rodents and humans (Dakin et al., 2004 and Wynne and Bloom, 2006). Several long-acting OXM analogs, which avoid rapid inactivation of OXM by dipeptidyl peptidase-4 (DPP4), are currently under investigation in clinical trials for the treatment of type 2 diabetes mellitus (T2DM) and obesity (Santoprete et al., 2011, Bagger et al., 2015 and Muppidi et al., 2016).

OXM can cross the blood brain barrier (BBB) through mechanisms similar to those of GLP-1 (Kastin et al., 2002). We previously demonstrated that the GLP-1R agonists exenatide and liraglutide had neuroprotective and neurotrophic effects in cell and animal models of ischemic stroke, traumatic brain injury, Parkinson's disease (PD), Alzheimer's disease (AD) and ALS (Li et al., 2009; Li et al., 2010a, 2012; Salcedo et al., 2012; Rachmany et al., 2013; Eakin et al., 2013; Greig et al., 2014). These data, together with findings by other groups (Bassil et al., 2014; Holscher, 2014; Darsalia et al., 2015; Athauda and Foltynie, 2016 and Kuroki et al., 2016), suggest that incretin-based anti-diabetic drugs are neuroprotective and hold promise for repurposing as a treatment strategy for neurodegeneration. Positive pre-clinical data on these compounds has led to several ongoing clinical trials, such as a recent successful open label clinical trial of exenatide in Parkinson's disease patients (Aviles-Olmos et al., 2013). Unlike GLP-1 or glucagon, the CNS effect of OXM has not been well examined (Bataille and Dalle, 2014). One recent study has indicated that D-Ser2-oxytomodulin, a protease-resistant OXM analog, improved locomotor activities and protected dopaminergic neurons in a 1-methyl-4-

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phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Liu et al., 2015). The neuroprotective actions of OXM, however, have yet to be fully characterized in other neurodegenerative conditions, including stroke that involves both necrotic and apoptotic cell death where the actions of OXM remain unknown.

We hypothesize that OXM is neuroprotective in ischemic stroke based on its structural similarity with other glucagon-derived peptides, its anti-apoptotic actions in pancreatic β-cells, and its features of inducing intracellular cAMP and glucoregulatory effects. Elevations in intracellular cAMP levels, whether the consequence of activation of membrane receptors by neuropeptides, classical neurotransmitters or other protein-ligand interactions, have long been associated with plasticity and cytoprotection within the nervous system (Silveira and Linden, 2006; Sakamoto et al., 2011). Of note, obesity and T2DM, for which OXM analogs are being evaluated as treatment strategies, are major risk factors for stroke (Najarian et al., 2006; Chen et al., 2016). Based on these factors, it is feasible that OXM may be a potentially useful therapy, which targets stroke and metabolism (Shankar et al., 2013). In this study, we characterize the neuroprotective effects of OXM peptide in cellular and animal models of stroke. Our data suggest that OXM is, indeed, a potentially neuroprotective agent against ischemic stroke and warrants further evaluation.

2. Materials and methods

2.1. Materials

Oxyntomodulin was purchased from the Phoenix Pharmaceuticals (Burkingama, CA). GLP-1R antagonist Exendin 9–39 was obtained from Anaspec (Fremond, CA). GCGR antagonist des-His1-[Glu9]-Glucagon (1–29) amide was purchased from Tocris (Minneapolis, MN). The kinase inhibitors, H89, LY294002 and U0126 were purchased from Calbiochem (Gibbstown, NJ). All other reagents were from Sigma (St. Louis, MO), unless otherwise stated.

2.2. Cell culture

SH-SY5Y cells, obtained from American Type Culture Collection (Manassa, VA), and SH-hGLP-1R#9 cells, a human GLP-1R over-expressing cell line derived from SH-SY5Y cells were maintained as described previously (Li et al., 2010b). Primary cultures of cortical neurons (PCN) were prepared from embryonic day 15 Sprague-Dawley rats in accordance with approved procedures by the Animal Care and Usage Committee of the National Health Research Institutes, Taiwan, and were cultured as previously described (Howard et al., 2008; Yu et al., 2016a, 2016b). They were maintained and evaluated from 10 day in vitro (DIV) onward. Briefly, brain cortices from E15 embryos were pooled and digested for 20 min in 0.05% trypsin-ETDA (0.2% (Invitrogen, Carlsbad, CA), 37 °C, 1 ml/embryo). Cortices were triturated and then diluted into plating media (Neurobasal media (Invitrogen) containing 2% heat-inactivated fetal bovine serum (Sigma-Aldrich, St Louis, MO), 2% B27 supplement (Invitrogen), 0.5 mM L-glutamate with and without antioxidants (AO) supplement (on DIV 3 and 5 (with AO) and DIV 7 and 10 (without AO)) at 2 ml/ embryo. Following evaluation of viability, cells were then plated at 5×10^4 viable cells/well in 0.2 ml plating media into 96 well plates pre-coated with 0.15–0.2% polyethyleneimine in 150 mM sodium borate, pH 8.5 (Sigma-Aldrich). Plated cells were maintained in a 37 °C humidified incubator with 5.5% CO_2, and fed by 50% media exchange on DIV3, 5, 7 and 10 with feed media.

2.3. Cell viability assays

Cell viability was assessed via 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay performed in 96-well plates. Cells were serum-starved (with 0.5% of serum) overnight before pretreatment with various concentrations of OXM for 1 h. Cells were then exposed to different concentrations of glutamate or H_2O_2 for either 24 h or 48 h, time points selected from prior studies. Glutamate concentrations were selected from concentration-dependent pilot studies focused to provide a statistically significant but submaximal loss of viability. A CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) was utilized to measure a formazan product, which is directly proportional to the cell viability.

2.4. cAMP assay

Cells grown in 24-well plates were first serum-starved overnight and then treated with various concentrations of OXM (10^{-9}, 10^{-8}, 10^{-7} M) in serum-free media for 15 min at 37 °C. Cells were then lysed with 0.1 M HCl containing 0.5% Triton X-100 for 10 min at room temperature. Cell lysates were collected and centrifuged at 600*g* at room temperature to remove cell debris. Supernatants were directly used for cAMP measurement. Intracellular cAMP content was determined using the Direct cAMP ELISA kit (Enzo Life Sciences, Inc., Farmingdale, NY), as per the manufacturer's protocol for the acetylated version.

2.5. Immunocytochemistry

At 48 h after drug treatment, PCN cells were fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature. After removing the 4% PFA solution, cells were washed with phosphate-buffered saline (PBS), and the fixed cells were treated with blocking solution (2% BSA, 0.1% Triton X-100, and 5% goat serum in PBS) for 1 h. The cells were then incubated with a mouse monoclonal antibody against MAP2 (1:500; Millipore, Billerica, MA) for 1 day at 4 °C. The cells were next rinsed three times in PBS and treated with a secondary antibody (AlexaFluor 488 goat anti-mouse Ab, 1:500, Invitrogen, Carslbad, CA). Images were acquired using a monochrome camera Qil-mc (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a NIKON TE2000 inverted microscope (Nikon, Melville, NY). Data were analyzed using NIS Elements AR 3.2 Software (Nikon).

2.6. Western blotting

Cells grown in 100 mm-plates at a density of approximately 5×10^6 cells were used to extract total protein. Standard Western blotting procedure was used with approximately 50 μg of protein extracts for all samples. cAMP-response element-binding protein (CREB) and phospo-CREB (pCREB) antibodies from Cell Signaling (Danvers, MA) were used at a dilution of 1:1000. Glucagon receptor antibody from Novus Biologicals (Littleton, CO) was used at a dilution of 1:500. α-Tubulin antibody from Sigma was used at a dilution of 1:5000. Densitometric quantification of the protein bands was performed using a PC version of NIH IMAGE (ImageJ software).

2.7. Animals and drug administration

Adult male Sprague-Dawley rats were used and treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) on protocols approved by the Animal Care and Usage Committee of the National Health Research Institutes, Taiwan. Briefly, animals were anesthetized with chloral hydrate (0.4 g/kg, i.p.). As a proof of concept, following randomization of animals into groups, either OXM (20 μg in 20 μl sterile physiological saline per rat) or vehicle (20 μl sterile physiological saline per rat) was given intracerebroventricularly through a 25 μl Hamilton syringe 15 min before a 60 min MCAo. The coordinates for intracerebroventricular injections were: 0.8 mm posterior to the bregma; 1.5 mm lateral to the midline; 3.5 mm below the dura surface. The speed of injection was controlled by a syringe pump at a rate of 2.5 μl