Experimental Neurology 288 (2017) 176–186

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research Paper

Novel GLP-1R/GIPR co-agonist "twincretin" is neuroprotective in cell and rodent models of mild traumatic brain injury

Ian A. Tamargo [a,*], Miaad Bader [b], Yazhou Li [a], Seong-Jin Yu [c], Yun Wang [c], Konrad Talbot [d], Richard D. DiMarchi [e], Chaim G. Pick [b,f], Nigel H. Greig [a,*]

[a] Translational Gerontology Branch, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224, USA
[b] Department of Anatomy and Anthropology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel
[c] Center for Neuropsychiatric Research, National Health Research Institutes, Miaoli, Taiwan
[d] Department of Neurology, UCLA, Los Angeles, CA, USA
[e] Department of Chemistry, Indiana University, Bloomington, IN 47405, USA
[f] Sagol School of Neuroscience, Tel-Aviv University, Tel-Aviv, Israel

ARTICLE INFO

Article history:
Received 2 September 2016
Received in revised form 8 November 2016
Accepted 10 November 2016
Available online 11 November 2016

Keywords:
Traumatic brain injury
Neurodegeneration
Incretin mimetic
Incretin
Glucagon-like peptide-1
Glucose-dependent insulinotropic peptide

ABSTRACT

Several single incretin receptor agonists that are approved for the treatment of type 2 diabetes mellitus (T2DM) have been shown to be neuroprotective in cell and animal models of neurodegeneration. Recently, a synthetic dual incretin receptor agonist, nicknamed "twincretin," was shown to improve upon the metabolic benefits of single receptor agonists in mouse and monkey models of T2DM. In the current study, the neuroprotective effects of twincretin are probed in cell and mouse models of mild traumatic brain injury (mTBI), a prevalent cause of neurodegeneration in toddlers, teenagers and the elderly. Twincretin is herein shown to have activity at two different receptors, dose-dependently increase levels of intermediates in the neurotrophic CREB pathway and enhance viability of human neuroblastoma cells exposed to toxic concentrations of glutamate and hydrogen peroxide, insults mimicking the inflammatory conditions in the brain post-mTBI. Additionally, twincretin is shown to improve upon the neurotrophic effects of single incretin receptor agonists in these same cells. Finally, a clinically translatable dose of twincretin, when administered post-mTBI, is shown to fully restore the visual and spatial memory deficits induced by mTBI, as evaluated in a mouse model of weight drop close head injury. These results establish twincretin as a novel neuroprotective agent and suggest that it may improve upon the effects of the single incretin receptor agonists via dual agonism.

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1. Introduction

The CDC estimates that 1.7 million Americans suffer a traumatic brain injury (TBI) each year (Faul et al., 2010), while 3.2 (Zaloshnja et al., 2008) to 5.3 (Thurman et al., 1999) million Americans are estimated to be living with TBI-related disabilities. These injuries, which primarily affect toddlers (0–4), young adults (15–19) and the elderly (older than 65) (Faul et al., 2010), are particularly alarming as they may cause a host of long-term cognitive, behavioral and physical impairments. There is a growing body of evidence suggesting that TBIs initiate various biochemical cascades and processes in the brain that potentiate neuropsychiatric disorders (Chen et al., 2014), as well as neurodegenerative diseases such as early-onset dementia (Barnes et al., 2014; Gardner et al., 2014), Parkinson's disease (Gardner et al., 2015), and Alzheimer's disease (Tweedie et al., 2013a; Tweedie et al. 2013b; Tweedie et al. 2016).

The type of TBI may be categorized as either "open," if the skull and dura mater are perforated, or "closed," when they are not, and the severity of the of injury may be denoted as either "mild," "moderate," or "severe." These three classifications of TBI severity differ in regards to the structural imaging in the brain, the duration of lost consciousness and amnesia, and the patient's scores on the Glasgow Coma and Abbreviated Injury Scales - broadly accepted scoring evaluations to reproducibly classify the severity of TBI based on clinical/anatomical observations

Abbreviations: BCA, bicinchoninic acid; cAMP, cyclic AMP; CDC, Centers for Disease Control and Prevention; CREB, cyclic AMP response element-binding protein; Ex-4, exendin-4; Ex 9–39, exendin fragment 9–39; GIP, glucose-dependent insulinotropic peptide; GIPR, glucose-dependent insulinotropic peptide receptor; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; mTBI, mild traumatic brain injury; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; pCREB, phosphorylated cyclic AMP response element-binding protein; ROS, reactive oxygen species; T2DM, type 2 diabetes mellitus; TBI, traumatic brain injury; TBST, tris-buffered saline and Tween 20; TCT, twincretin; TH, tyrosine hydroxylase; VM, ventral mesencephalon.

* Corresponding authors at: Drug Design and Development Section, Translational Gerontology Branch, National Institute on Aging, 251 Bayview Blvd., Baltimore, MD 21224, USA.
E-mail addresses: iatamargo@gmail.com (I.A. Tamargo), Greign@mail.nih.gov (N.H. Greig).

http://dx.doi.org/10.1016/j.expneurol.2016.11.005
0014-4886/Published by Elsevier Inc.

(Orman et al., 2011). Mild TBIs (mTBIs), which are primarily closed-head, and which are most readily characterized by normal brain imaging, <30 min of lost consciousness, and less than one day of post-traumatic amnesia, account for approximately 70–90% of all reported cases (Cassidy et al., 2004).

At the cellular level, TBI damages accumulate in a two-phase process. During the acute primary phase, which occurs at the moment of injury, brain cells undergo immediate necrotic cell death due to contusions and lacerations of brain tissue, as well as intracranial hemorrhage and diffuse axonal injury (LaPlaca et al., 2007). In the extended secondary phase, neurodegenerative processes initiated in the primary phase, such as neuroinflammation, oxidative stress and glutamate excitotoxicity, lead to progressive neuronal loss via apoptosis in mild and moderate TBI (Morganti-Kossmann et al., 2002; Schmidt et al., 2005; Greve and Zink, 2009; Bales et al., 2010; Barkhoudarian et al., 2011; Mehta et al., 2013; Rachmany et al., 2013a).

Despite the high incidence of TBI throughout the world, the capacity of these injuries to initiate debilitating neurodegenerative disorders, and the fairly sophisticated understanding of the cellular processes that underpin the extensive brain damages that occur during a TBI, no effective treatments have been developed to mitigate the deleterious effects of these injuries. To this end, incretins and incretin mimetics have been investigated in regard to their anti-apoptotic, neuroprotective and neurotrophic effects in neurons expressing incretin receptors: namely the glucagon-like peptide 1 (GLP-1) receptor (GLP-1R) and the glucose-dependent insulinotropic peptide (GIP) receptor (GIPR) (Rachmany et al., 2013b; Tweedie et al., 2013a; Li et al., 2015; Tweedie et al., 2016; Yu et al., 2016).

These peptide hormones, which were originally identified in the gut, where they are released by intestinal enteroendocrine cells in response to elevated levels of dietary glucose in the intestinal lumen, were first investigated for their use in the treatment of type 2 diabetes mellitus (T2DM). Via their receptors on pancreatic β- and α-cells, GLP-1 and GIP stimulate insulin secretion and inhibit that of glucagon in order to induce glucose metabolism (Campbell and Drucker, 2013; Wu et al., 2016). As such, GLP-1 mimetics such as exendin-4 (Ex-4) and liraglutide have been developed and approved for the treatment of T2DM.

Importantly, GLP-1 and GIP have trophic and anti-apoptotic properties (Salcedo et al., 2012) mediated through the cAMP-dependent CREB pathway (Perry and Greig, 2003; Kim et al., 2008; Shao et al., 2013). The discovery of the anti-apoptotic activity of GLP-1 and GIP, in addition to the realizations that incretin mimetics pass the blood-brain barrier (Kastin et al., 2002), and that GLP-1Rs and GIPRs are expressed on central nervous system (CNS) neurons (Alvarez et al., 2005; Nyberg et al., 2007), led to the investigation of GLP-1, GIP and their mimetics in the treatment of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and now TBI (Salcedo et al., 2012; Greig et al., 2014; Bassil et al., 2014; Holscher, 2014; Athauda and Foltynie, 2016).

Recently, a novel, synthetic incretin mimetic was shown to maximize the metabolic benefits of these peptides in rodent and monkey models of T2DM, as well as in humans via clinical trials (Finan et al., 2013). This peptide, nicknamed “twincretin”, which features sections of both the Ex-4 and GIP sequences, was shown to bind both the GLP-1R and GIPR, and was reported to improve upon the performance of the single receptor agonists via the combined effect of its dual receptor agonism (Finan et al., 2013).

In the current study, we investigate the neurotrophic and neuroprotective effects of this promising new peptide in cell and rodent models of mTBI. Using human neuroblastoma cells, we show that twincretin has activity at both the GLP-1R and GIPR, and that it significantly reduces cell death in response to toxic doses of glutamate and hydrogen peroxide, mimicking in part the neuroinflammatory conditions present in the secondary phase of mTBI (Morganti-Kossmann et al., 2002; Barkhoudarian et al., 2011; Baratz et al., 2011; Walker and Tesco, 2013; Greig et al., 2014; Barkhoudarian et al., 2016). In light of the vulnerability of dopaminergic neurons to TBI (Shahaduzzaman et al., 2013; Acosta et al., 2015; Impellizzeri et al., 2016), this neuroprotective effect is recapitulated in a dopaminergic neuronal cell group; specifically, primary cultures of rat ventral mesencephalon (VM) neurons. Twincretin is also shown to improve upon the effects of the single-receptor agonists, Ex-4 and GIP. Finally, we show that twincretin protects mice against mTBI-induced deficits in spatial and visual memory.

2. Methods

2.1. Materials

Twincretin was obtained from the Richard DiMarchi Research Group at Indiana University Bloomington. Ex-4 and GIP were purchased from AnaSpec Inc. (Fremont, CA, USA). Human Pro3GIP was purchased from Abgent (San Diego, CA, USA). Exendin Fragment 9–39 (Ex 9–39), L-Glutamic acid monosodium salt hydrate (glutamate), and hydrogen peroxide solution 30% (w/w) in H_2O (H_2O_2) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Hydrochloric acid was purchased from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA) and Triton X-100 was purchased from Fisher Scientific (Waltham, MA, USA).

2.2. Cell culture

Two immortal cell lines were used: SH-SY5Y cells, a human neuroblastoma cell line purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA), and SH-hGLP-1R#9 cells, which are SH-SY5Y cells that stably overexpress the human GLP-1R (Li et al., 2010b). All cultures were grown in a mixture of 44.5% Eagle's Minimum Essential Medium (EMEM), 44.5% Ham's F12-K (Kaighn's) Medium, 10% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin by volume (normal medium). Cultures were maintained in a humidified, 37 °C incubator comprising 5% CO_2 and 95% air. Medium was replaced every other day, and the cells were split in a 1:3 ratio every week using a 0.25% trypsin, 0.53 mM ethylenediaminetetraacetic acid (EDTA) solution.

Primary cultures were prepared from embryonic (E14–15) VM tissue samples obtained from fetuses of timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), according to published procedures with some modification (Lotharius et al., 1999). The whole brain was removed aseptically, and a small piece of tissue comprising the VM was dissected. After removal of blood vessels and the meninges, pooled VM tissues were trypsinized (0.25%; Invitrogen, Carlsbad, CA) with gentle mixing for 15 min at 37 °C. After rinsing with pre-warmed DMEM/F-12 (Invitrogen) to remove any remaining trypsin, cells were dissociated by trituration, counted and then plated into 96-well (6.0×10^4/well) cell culture plates pre-coated with poly-D-lysine (Sigma-Aldrich, St Louis, MO). The culture plating medium comprised of Dulbecco's modified Eagle medium/F12, supplemented with 10% heat-inactivated fetal bovine serum, 1 mM L-glutamine and 2% B27 (Invitrogen). Cultures were then maintained at 37 °C in a humidified atmosphere of 5% CO2 and 95% air, and were fed by exchanging 50% of media with feed media (Neurobasal medium, Invitrogen) with 0.5 mM L-glutamate and 2% B27 with antioxidants supplement on DIV (days in vitro) 3 and 5.

2.3. cAMP Assays

All cAMP assays were performed using 24-well plates containing 500 μL of cells in normal medium at a concentration of 280×10^4 cells/mL, as determined by hemocytometer cell count. Cells were treated with 500 μL of the appropriate peptide solutions for 0, 5, 10, 15, 30 or 60 min and then lysed using a solution of 0.5 M HCl with 0.5% Triton X-100. After allowing the cells to lyse for 20 min, the lysates were centrifuged at 1000 rpm for 10 min. The supernatants were extracted and