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NEUROPEPTIDE Y Y2 AND Y5 RECEPTORS AS PROMISING TARGETS FOR NEUROPROTECTION IN PRIMARY NEURONS EXPOSED TO OXYGEN-GLUCOSE DEPRIVATION AND IN TRANSIENT FOCAL CEREBRAL ISCHEMIA IN RATS

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Abstract—It was postulated that neuropeptide Y (NPY)-ergic system could be involved in the ischemic pathophysiology, however, the role of particular subtypes of NPY receptors (YRs) in neuroprotection against ischemia is still not well known. Therefore, we investigated the effect of NPY and YR ligands using *in vitro* and *in vivo* experimental ischemic stroke models. Our *in vitro* findings showed that NPY (0.5–1 μM) and specific agonists of Y2R (0.1–1 μM) and Y5R (0.5–1 μM) but not that of Y1R produced neuroprotective effects against oxygen-glucose deprivation (OGD)-induced neuronal cell death, being also effective when given 30 min after the end of OGD. The neuroprotective effects of Y2R and Y5R agonists were reversed by appropriate antagonists. Neuroprotection mediated by NPY, Y2R and Y5R agonists was accompanied by the inhibition of both OGD-induced calpain activation and glutamate release. Data from *in vivo* studies demonstrated that Y2R agonist (10 μg/6 μl; i.c.v.) not only diminished the infarct volume in rats subjected to transient middle cerebral artery occlusion (MCAO) but also improved selected gait parameters in CatWalk behavioral test, being also effective after delayed treatment. Moreover, we found that a Y5R agonist (10 μg/6 μl; i.c.v.) did not reduce MCAO-evoked brain damage but improved stride length, when it was given 30 min after starting the occlusion. In conclusion, our studies indicate that Y5 and especially Y2 receptors may be promising targets for neuroprotection against ischemic damage.

Key words: neuroprotection, NPY, Y receptors, OGD, MCAO, CatWalk.

INTRODUCTION

Ischemic stroke accounts for ~85% of all stroke cases and it is caused by occlusion of a major cerebral artery by a thrombus or an embolism, which leads to cessation or critical reduction of cerebral blood flow (CBF) that results in oxygen and energy deprivation and then tissue damage in the affected area (Gibson, 2013; Pedata et al., 2016). The sequence of biochemical events, termed the ischemic cascade that occurs during ischemia, includes: glutamate-mediated excitotoxicity and calcium overload, free radical formation, nitric oxide (NO) production, membrane degradation, mitochondrial damage, inflammation, activation of various enzymes: caspases, calpains, liposomal proteases, and endonucleases, oxidative stress, DNA deamination and consequently cell death by necrosis and/or apoptosis (Lipton, 1999; Broussalis et al., 2012; Sutherland et al., 2012; Wang et al., 2015b). Neuroprotection, which is the principal therapeutic strategy to treat ischemic stroke that antagonizes, interrupts or slows down the sequence of events within the ischemic cascade has been extensively explored for many years (Liu et al., 2012; Fluri et al.,

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Abbreviations: BIIE0246, *N*-[(1*S*)-4-[(Aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl] butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b*,*e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide; BOS, base of support; Ca^{2+}, calcium ion; cAMP, cyclic adenosine monophosphate; CBF, cerebral blood flow; CGP 71683, *N*-[[*trans*-4-[[(4-amino-2-quinazolinyl) amino]methyl]cyclohexyl]methyl]-1-naphthalenesulfonamide hydrochloride; DIV, day *in vitro*; EBBS, Earle's balanced salt solution; FP, front paws; GPCRs, G-protein-coupled receptors; HP, hind paws; i.c.v., intracerebroventricular; K^+, potassium ion; LDF, cerebrocortical microflow; LDH, lactate dehydrogenase; LF, left front paw; LH, left hind paw; MCAO, middle cerebral artery occlusion; MCAO/R, middle cerebral artery occlusion/reperfusion; MTT, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide; NPY 13-36, Y2 receptor agonist; NPY, neuropeptide Y; NPY-ir, NPY-immunoreactivity; OFT, open field test; OGD, oxygen-glucose deprivation; PI, propidium iodide; PSS, physiological saline solution; RF, right front paw; RH, right hind paw; TDM, total distance moved; TTC, 2,3,5-triphenyltetrazolium chloride; Y1R agonist, [Leu^{31},Pro^{34}]-NPY; Y5 receptor agonist, [cPP1-7,NPY19-23,Ala31, Aib32,Gln34]-hPP; YRs, NPY receptors.

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2015). However, despite promising neuroprotective effects of compounds in animal stroke models, clinical trials have still been unsuccessful (Wang et al., 2015b; Chamorro et al., 2016).

It has been postulated that neuropeptide Y (NPY)-ergic system can be involved in the pathophysiology of ischemia. NPY is a 36-amino acid peptide, widely distributed in the mammalian central nervous system, where it acts as a neurotransmitter and neuromodulator (Chronwall et al., 1985; Gray and Morley, 1986; Dumont et al., 1992). This peptide was shown to activate the specific membrane bound G-protein-coupled receptors (GPCRs) denoted as Y1, Y2, Y3, Y4, Y5, and y6 (Michel et al., 1998; Bettio and Beck-Sickinger, 2001). They are negatively coupled to adenylyl cyclase through G_i/G_o proteins and their activation leads to the inhibition of cyclic adenosine monophosphate (cAMP) formation, and to the modulation of calcium (Ca^{2+}) and potassium (K^+) channels (Michel, 1991; Cabrele and Beck-Sickinger, 2000).

It was observed that NPY-immunoreactivity (-ir) increased locally in the cerebral cortex around the site of infarct following middle cerebral artery occlusion (MCAO) in rats (Allen et al., 1995; Cheung and Cechetto, 1997), in addition NPY-ir increased in the gerbil's hippocampus after hypoxic and ischemic preconditioning (Duszczyk et al., 2009). However, the neuroprotective role of NPY in ischemia remains controversial. Previously, we showed that the Y2R agonist, NPY 13-36 injected intracerebroventricularly (i.c.v.) 30 min after the start of occlusion significantly diminished the infarct volume following MCAO in rats (Smialowska et al., 2009). Moreover, it was found that elimination of NPY activity by inhibition of Y1R significantly increased the infarct volume after MCAO in rats (Cheung and Cechetto, 2000). However, there are also studies showing that peripheral and central administration of NPY in a rat MCAO model not only did not diminish the brain damage but also worsened the outcome of the ischemia (Chen and Cheung, 2002). In other experiments, the same authors reported that Y1R activation enhanced ischemic injuries, but inhibition of Y1R reduced the size of the damage both in the rat MCAO model (Chen and Cheung, 2003) and in human neuroblastoma SK-N-MC cell line exposed to OGD (Chen and Cheung, 2004).

In order to shed more light on the neuroprotective role of NPY and its receptors in ischemia and to explain some controversies, in the present study we examined the effects of NPY and YR ligands using *in vitro* and *in vivo* experimental ischemic stroke models. Primary cortical cell cultures exposed to OGD were used as a simple experimental *in vitro* model, in which we examined the effects of NPY and its Y2R, Y5R and Y1R ligands against OGD-induced neuronal cell death. Moreover, we investigated the cellular and molecular mechanisms of neuroprotective effects of these compounds. Since a wider therapeutic time window is recommended to be used in preclinical studies (Fisher et al., 2009; Sutherland et al., 2012), therefore, we examined the possibility of neuroprotective action of the studied compounds at different time points, especially after delayed treatment.

In vivo transient MCAO was used to study whether the Y2R and Y5R agonists have also neuroprotective effects in ischemic rats, particularly when given during reperfusion and whether these drugs can ameliorate behavioral deficits induced by ischemia. The functional outcome was evaluated by the CatWalk gait analysis, which has been employed to quantify motor deficits following experimental ischemic stroke (Wang et al., 2008; Encarnacion et al., 2011; Parkkinen et al., 2013).

EXPERIMENTAL PROCEDURES

In vitro study

Chemicals. Neuropeptide Y (NPY), NPY 13-36, [cP P1-7,NPY19-23,Ala31,Aib32,Gln34]-hPP, [Leu^{31},Pro^{34}]-NPY, MK-801, *N*-[(1*S*)-4-[(Aminoiminomethyl)amino]-1-[[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5*H*-dibenz[*b*,*e*]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentaneacetamide (BIIE 0246), *N*-[[*trans*-4-[[(4-Amino-2-quinazolinyl)amino]methyl]cyclohexyl]methyl]-1-naphthalenesulfonamide hydrochloride (CGP 71683) and MDL28170 were purchased from Tocris Bioscience (Bristol, UK). Neurobasal A medium and supplement B27 were from Gibco (Invitrogen, Poisley, UK). The Cytotoxicity Detection Kit and BM Chemiluminescence Western Blotting Kit were obtained from Roche Diagnostic (Mannheim, Germany). Amplex Red Glutamic Acid/Glutamate Oxidase assay kit was from Molecular Probes (Eugene, OR, USA). Primary antibodies: anti-spectrin α II (sc-48382) and anti-β-actin (sc-47778), protein markers and appropriate secondary antibody were from Santa Cruz Biotechnology Inc. (CA, USA). All other reagents were from Sigma–Aldrich (St. Louis, MO, USA).

Primary mouse cortical neuronal culture. The experiments were carried out on primary cultures of mouse cortical neurons. All the procedures were done in accordance with the guidelines of the European Community Council (Directive 86/609/EEC) and approved by the local Ethics Committee. Cortical tissues were obtained from brains of Swiss mouse fetuses (Charles River, Germany) on embryonic day 15/16 and were cultured as described previously (Domin et al., 2015). In short, pregnant mice were anesthetized with CO_2 vapor, killed by cervical dislocation, and embryos were taken out. Next, cortical tissue of the fetal brains was dissected, minced into small pieces, digested with trypsin and finally the cortical cells were suspended in Neurobasal medium containing penicillin (0.06 μg/ml) and streptomycin (0.1 μg/ml), supplement B27 without antioxidants and plated at a density of 1.5 x 10^5 cells/cm^2 onto poly-ornithine (0.01 mg/ml)-coated multi-well plates. Such a procedure leads to obtaining the cultures containing more than 90% of neurons and the remaining were glial cells, which was checked by immunocytochemistry (not shown). The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 8 days

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