IBRO Reports 2 (2017) 31–40

Contents lists available at ScienceDirect

IBRO Reports

journal homepage: http://www.journals.elsevier.com/ibro-reports

Expression of prosaposin and its receptors in the rat cerebellum after kainic acid injection

Xuan Li[a], Hiroaki Nabeka[a,*], Shouichiro Saito[b,**], Tetsuya Shimokawa[a], Md. Sakirul Islam Khan[a], Kimiko Yamamiya[a], Fengping Shan[c], Huiling Gao[d], Cheng Li[c], Seiji Matsuda[a]

[a] Department of Anatomy and Embryology, Ehime University Graduate School of Medicine, Toon, Ehime, Japan
[b] Laboratory of Veterinary Anatomy, Faculty of Applied Biological Sciences, Gifu University, Yanagido, Gifu, Japan
[c] Department of Immunology, School of Basic Medical Science, China Medical University, Shenyang, PR China
[d] College of Life and Health Science, Northeastern University, Shenyang, PR China

ARTICLE INFO

Article history:
Received 2 December 2016
Received in revised form 2 February 2017
Accepted 21 February 2017

Keywords:
Prosaposin
GPR37
GPR37L1
Kainic acid
Cerebellum
Neurodegeneration

ABSTRACT

Prosaposin (PSAP), a highly conserved glycoprotein, is a precursor of saposins A–D. Accumulating evidence suggests that PSAP is a neurotrophic factor that induces differentiation and prevents death in a variety of neuronal cells through the active region within the saposin C domain both *in vivo* and *in vitro*. Recently, GPR37 and GPR37L1 were recognized as PSAP receptors. In this study, we examined the alteration in expression of PSAP and its receptors in the cerebellum using rats injected with kainic acid (KA). The results show that PSAP was strongly expressed in the cytoplasm of Purkinje cells and interneurons in the molecular layer, and that PSAP expression in both types of neurons was markedly enhanced following KA treatment. Immunoblotting revealed that the expression of GPR37 was diminished significantly three days after KA injection compared with control rats; however, no changes were observed through immunostaining. No discernable changes were found in GPR37L1. These findings may help us to understand the role of PSAP and the GPR37 and GPR37L1 receptors in alleviating the neural damage caused by KA.

1. Introduction

Prosaposin (PSAP) is the precursor of four small non-enzymatic glycoproteins, termed saposins A, B, C, and D. Each saposin acts as sphingolipid activator protein and coenzyme, and is necessary for enzymatic hydrolysis of certain sphingolipids in lysosomes (Sano et al., 1989; O'Brien and Kishimoto, 1991; Schulze et al., 2009). In addition, intact PSAP is widely expressed in various tissues, including the Purkinje cell layer of the cerebellum, the spinal cord, testes, ovaries and kidneys (Qi and Grabowski, 2001; Li et al., 2013; Saito et al., 2014), and is secreted into various body fluids including bile, pancreatic juice, breast milk, cerebrospinal fluid, and seminal plasma (Hineno et al., 1991; Kondoh et al., 1991; Hiraiwa et al., 1993; Koochekpour et al., 2012). In the past two decades, PSAP has been identified as a potent neurotrophic factor, protecting neural cells against cellular damage (O'Brien et al., 1995; Kotani et al., 1996; Morita et al., 2001; Ochiai et al., 2008; Gao et al., 2013c) through its active region within the saposin C domain (O'Brien et al., 1994; O'Brien et al., 1995). The PSAP gene contains at least 15 exons, and generates alternatively spliced forms by inclusion or exclusion of 9-bp exon 8 (Pro+9 and Pro+0, respectively) (Lamontagne and Potier, 1994). Pro+9 is secreted preferentially from cells, whereas Pro+0 is trafficked mainly to the lysosomes (Madar-Shapiro et al., 1999).

GPR37 and GPR37-like 1 (GPR37L1) are two orphan G-protein-coupled receptors (GPCRs) that have enhanced expression in the rat brain. GPR37, also known as parkin-associated endothelin-like receptor (Peal-R), is a substrate of the E3 ubiquitin ligase parkin (Imai et al., 2001). GPR37L1 shares 42% identity with GPR37. Phylogenetic analysis indicated that the receptors closest to the endothelin receptors were the bombesin receptors. However, neither the endothelin nor bombesin peptides act as the endogenous ligands for GPR37 and GPR37L1 (Leng et al., 1999). Recent reports

Abbreviations: BSA, bovine serum albumin; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; H-E staining, hematoxylin-eosin staining; IF, immunofluorescence; IHC, immunohistochemistry; ISH, *in situ* hybridization; KA, kainic acid; PSAP, prosaposin; SSC, standard saline citrate.

* Corresponding author.
** Corresponding author.
E-mail addresses: nabeka@m.ehime-u.ac.jp (H. Nabeka), shouichi@gifu-u.ac.jp (S. Saito).

http://dx.doi.org/10.1016/j.ibror.2017.02.002

demonstrate that these two related receptors could be stimulated by prosaposin and its active peptide fragment prosaptide (Leng et al., 1999; Meyer et al., 2013a).

Kainic acid (KA) (2-carboxy-4-isopropenyl-pyrrolidin-3-ylacetic acid), a glutamate analog, is a powerful neurotoxic agent (Olney and de Gubareff, 1978) that stimulates excitatory neurotransmitter release (Ferkany et al., 1982). Excitotoxicity is believed to contribute to the pathogenic process of acute and chronic neurodegenerative disease (Doble, 1999). Systemic injection of KA has been widely used as a tool to explore the mechanism involved in excitotoxicity (Wang et al., 2005; Nabeka et al., 2014; Mohd Sairazi et al., 2015).

In a previous study, we established a rat model using systemic injection of KA, and investigated the expression and function of PSAP in the brain (Nabeka et al., 2014, 2015). However, we did not determine what happened to the cerebellum using this model. Therefore, we examined the expression of PSAP and its two receptors, GPR37 and GPR37L1, in rat cerebellum using the same KA-injected rat model in this study.

2. Experimental procedures

2.1. Animals

Ten-week-old, 220–260-g male Wistar rats (Clea Japan Inc., Tokyo, Japan) were housed at a constant temperature (22 °C) under a 12/12-h light/dark cycle and given food and water *ad libitum*. The experiments were conducted in accordance with the Guide for Animal Experimentation of the Ehime University School of Medicine, Japan. The protocol was approved by the Animal Care Committee of Ehime University (Permit Number: 05A261). All surgeries were performed under chloral hydrate anesthesia (10 mg/kg), and all efforts were made to minimize suffering in accordance with ARRIVE guidelines.

2.2. Specific antibodies for PASP, GPR37 and GPR37L1

Specific polyclonal antibodies against rat PSAP (PSAP-Ab) and its two receptors were generated by Eurofins Genomics Co., Ltd. (Tokyo, Japan), and all the procedures were performed as described elsewhere (Gao et al., 2013a; Shimokawa et al., 2013; Nabeka et al., 2014). Briefly, specific antibodies were created by immunizing rabbits with synthetic oligopeptides based on the rat amino acid protein sequences specific to PSAP (M19936(Collard et al., 1988)), GPR37 (NP_476549.1(Dutta et al., 2014)), or GPR37L1 (NP_665727.2(Leng et al., 1999)). The sequences used were:

PSAP: 409-PKEPAPPKQPEEPKQSALRAHVPPQK-434,
GPR37: 134-REPTDSQLFRQTSE-147 (#12795V),
GPR37L1: 286-CIMKPSADLPESLYS-300 (#12796V), and 34-RAKVQEQQSRPRRG-47 (#13493VP).

The PSAP sequence did not encode any saposins and was acquired from a PSAP amino acid sequence analysis that included protein secondary structure predictions, analyses of accessibility to solvents, flexibility, surface probability, antigenicity, hydrophilicity, and dipole analyses. All the antibodies were tested for specificity using immunoblotting.

Two commercial antibodies for GPR37 (PAB16206, Abnova, Taipei, Taiwan) and GPR37L1 (A-405, LifeSpan BioSciences, Int. Seattle, WA, U.S.A) were also utilized in our study.

2.3. Preliminary study to determine the optimal KA dose

According to previous studies, the optimal dose of KA for administration in the hippocampus is 5 mg/kg in the rat model (Nabeka et al., 2014). Accordingly, we first applied several does of KA (0, 5, 8, 10, and 12 mg/kg) to determine the optimum dose for the rat cerebellum. Briefly, rats were anesthetized with diethyl ether, and clonazepam (an anticonvulsant) was injected intraperitoneally (0.2 mg/kg). After 10 min, rats were anesthetized again with diethyl ether and injected subcutaneously with KA dissolved in normal saline at various doses (5, 8, 10, and 12 mg/kg) or with saline as a control. Seven days after KA injection, each rat was anesthetized and perfused transcardially. The cerebellums were dissected, fixed, dehydrated, and embedded in paraffin for microtome serial coronary sectioning (7-μm thickness). Notably, the rats injected with 12 mg/kg KA were not available because two-thirds of them died during the experimental period.

Sections were stained with hematoxylin-eosin (H-E) using standard procedures. In brief, sections were deparaffinized, rehydrated and stained with hematoxylin for 6 min. Sections were then stained with eosin for 30 s and rinsed with ethanol. The slides were subsequently dehydrated and mounted with coverslips.

Microscopically, the Purkinje cells of rats injected with 5 mg/kg KA exhibited normal morphologic structures, whereas some shrunken and condensed Purkinje cells were observed in rats injected with 8 mg/kg KA, and even greater damage was observed in those injected with 10 mg/kg KA (Fig. 1a–d). These data indicated that 5 mg/kg KA was the optimum dose that could stimulate neurons but not kill them at the ordinal light-microscopic level. Rats younger than 9 weeks injected with 5 mg/kg KA occasionally suffered some neuronal damage in the cerebellum, which was similar to that observed in 10-week-old rats injected with 8 mg/kg KA.

2.4. KA injection and tissue preparation

After determining the appropriate dose of KA (5 mg/kg), rats were injected with normal saline or KA as described above. Under these conditions, no animal experienced status epilepticus, even with KA injection. Rats were sacrificed on days 1 and 3 after injection.

In one group of rats, the cerebellums were freshly stored at −80 °C immediately after extraction. The tissues were then homogenized for immunoblotting. In another group of rats, deep anesthetic was given and the rats were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). The cerebellums were dissected and fixed with the same solution overnight at 4 °C. The samples were then dehydrated and embedded in paraffin for microtome serial coronary sectioning (7-μm thickness), and used for immunohistochemistry (IHC) and immunofluorescence (IF).

For *in situ* hybridization, the rats were anesthetized on the indicated day and their cerebellums were removed quickly and frozen immediately on dry ice. Coronary sections (20-μm thickness) were cut on a cryostat, thaw-mounted onto silane-coated slides, and then stored at −80 °C until use.

2.5. Immunoblotting

Cerebellums were sonicated (1:5 w/v) in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.25% sodium deoxycholate, 1% NP-40, pH 7.4) for 2 min, $NaVO_3$ (0.5%), protease inhibitor cocktail (1%, Nacalai Tesque, Inc., Kyoto, Japan) and phosphatase inhibitor cocktail (1%, Nacalai Tesque, Inc., Kyoto, Japan) were included in the lysis buffer. All procedures were performed on ice. Homogenates were centrifuged for 30 min at 12,000 × *g* and 4 °C and the supernatants were collected. Protein concentration was examined by DC protein assay (Bio-Rad, Hercules, CA, U.S.A.), with bovine serum albumin (BSA) as the standard using a FlexStation 3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). Equal amounts (21 μg) of total protein