Immunobiology 221 (2016) 1355–1361

Contents lists available at ScienceDirect

Immunobiology

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

The role of carbohydrate component of recombinant α7 nicotinic acetylcholine receptor extracellular domain in its immunogenicity and functional effects of resulting antibodies

Olena Lykhmus[a], Lyudmyla Koval[a], Dar'ya Pastuhova[a], Marios Zouridakis[b], Socrates Tzartos[b], Sergiy Komisarenko[a], Maryna Skok[a,*]

[a] Palladin Institute of Biochemistry, 9, Leontovycha str., 01030 Kyiv, Ukraine
[b] Hellenic Pasteur Institute, 127 Vassilissis Sofias Avenue 115 21, Athens, Greece

ARTICLE INFO

Article history:
Received 24 May 2016
Received in revised form 20 July 2016
Accepted 22 July 2016
Available online 25 July 2016

Keywords:
α7 nicotinic acetylcholine receptor
Antibody
Carbohydrate component
Inflammation
Amyloid-beta
Alzheimer disease

ABSTRACT

Nicotinic acetylcholine receptors of α7 subtype (α7 nAChRs) attenuate the inflammatory cytokines production by macrophages and are involved in pathogenesis of Alzheimer disease by directly influencing the processing of amyloid-beta (Aβ) precursor protein in the brain. Previously we found that regular injections of bacterial lipopolysaccharide (LPS) decreased the level of α7 nAChRs and stimulated accumulation of Aβ peptide (1–42) in the brain of mice resulting in memory impairment. Similar effects were observed in mice immunized with recombinant extracellular domain (1–208) of α7 nAChR subunit. However, the mechanism of inflammation-like effect of α7-specific antibodies remained unclear. The aim of the present study was to reveal the impact of carbohydrate component of recombinant α7(1–208) produced in yeast in the functional effect of resulting antibodies. For this purpose, C57Bl/6 mice were immunized with either initial α7(1–208) or with that pre-treated with endoglycosidase. Control groups of mice obtained injections of either LPS or complete Freund's adjuvant. Mice were tested for memory performance, their blood sera were examined for the presence and fine specificity of α7(1–208)-specific antibodies and the brain preparations were studied for the levels of α7 nAChR, Aβ(1–42) and interleukin-6. It was found that the original α7(1–208) was more immunogenic than the deglycosylated one, and their epitopes were recognized with different efficiency. In contrast to LPS and original α7(1–208), deglycosylated α7(1–208) did not stimulate interleukin-6 elevation in the brain, i.e. had no pro-inflammatory effect. Nevertheless, immunizations with either the original or deglycosylated α7(1–208) resulted in similar decrease of α7 nAChRs, accumulation of Aβ(1–42) in the brain and significant episodic memory decline, comparable to those exerted by LPS injections. We conclude that the decrease of α7 nAChR density, caused by α7(1–208)-specific antibody, is critical for Aβ(1–42) accumulation and episodic memory impairment, while pro-inflammatory capacity of α7(1–208)-specific antibody plays a secondary role for the development of Alzheimer-like symptoms.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels mediating fast synaptic transmission in muscles and autonomic ganglia (Skok, 2002; Kalamida et al., 2007). Structurally, they are pentamers composed of either similar (homopentamers) or different (heteropentamers) subunits. The nAChRs expressed in the brain are located mainly extrasynaptically and regulate the release of various neurotransmitters, as well as the viability of brain cells (Gotti et al., 2009). Consequently, they are involved in many cognition and psycho-emotional processes and their decreased expression is observed upon multiple neurological and neurodegenerative disorders like schizophrenia, depression or Alzheimer disease (Vallés et al., 2014; Beinat et al., 2015).

Nicotinic acetylcholine receptors composed of α7 subunits (homomeric α7 nAChRs) are important components of cholinergic anti-inflammatory pathway: they mediate the inhibitory effect

Abbreviations: nAChR, nicotinic acetylcholine receptor; Aβ, amyloid-beta; ECD, extracellular domain; LCH, *Lens culinaris* hemagglutinin; LPS, lipopolysaccharide; NOR, novel object recognition test; WGA, wheat-germ agglutinin.

* Corresponding author.
E-mail addresses: elenalykhmus@yandex.ua (O. Lykhmus), lkoval@biochem.kiev.ua (L. Koval), darinka31@mail.ru (D. Pastuhova), mzouridakis@pasteur.gr (M. Zouridakis), stzartos@gmail.com (S. Tzartos), svk@biochem.kiev.ua (S. Komisarenko), skok@biochem.kiev.ua (M. Skok).

http://dx.doi.org/10.1016/j.imbio.2016.07.012
0171-2985/

of acetylcholine on pro-inflammatory cytokine production in various cell types including macrophages, neutrophils and glial cells in the brain (Báez-Pagán et al., 2015; Terrando et al., 2015). In addition, this nAChR subtype participates in the normal metabolism of amyloid-beta (Aβ) precursor protein and interacts directly with its peptide Aβ(1–42), which forms extracellular senile plaques upon Alzheimer disease (Parri and Dineley, 2010). Previously we found that antibodies formed *in vivo* against recombinant extracellular domain (1–208) of α7 nAChR subunit decreased the level of α7 nAChRs and stimulated accumulation of Aβ(1–42) in the brain of mice resulting in memory impairment. Similar effects were observed in mice regularly treated with bacterial lipopolysaccharide (LPS) indicating the critical role of inflammation in the pathogenesis of Alzheimer disease (Lykhmus et al., 2015). However, the mechanism of α7-specific antibody-triggered neuroinflammation and memory deficit remained unclear.

Like many other plasma membrane components, the nAChRs are glycoproteins containing at least one *N*-glycosylation site in the extracellular portion of each subunit (Gehle et al., 1997). The carbohydrate component was shown to be critical for proper nAChR subunits folding, assembly and plasma membrane expression (Chen et al., 1998). The recombinant α7(1–208) used in our experiments has been produced in yeast expression system and, therefore, contained carbohydrate residues, which, potentially, could influence the immune reaction to this antigen, in particular, its inflammatory component. The aim of the present study was to reveal the impact of carbohydrate moiety on the antigenic and immunogenic properties of α7(1–208) and on any functional effect of the resulting antibodies. We immunized mice with either original α7(1–208) produced in yeast ("glyc") or the one chemically deglycosylated with an endoglycosydase ("deglyc"). The data obtained indicate that carbohydrate component contributes to immunogenicity, epitope specificity and pro-inflammatory capacity of α7(1–208), but not to ability of resulting antibodies to affect the level of α7 nAChR and Aβ(1–42) in the brains of immunized mice and to impair memory.

2. Materials and methods

2.1. Mice, antibodies, reagents

Female 4 months-old C57Bl/6J mice were kept in the animal facility of Palladin Institute of Biochemistry, Kyiv. They were housed in a quiet, temperature-controlled room (22–23 °C) and were provided with water and dry food pellets *ad libitum*. All procedures conformed to the guidelines of Palladin Institute in accordance with the EU Directive 2010/63/EU for animal experiments.

All reagents were of chemical grade and were purchased from Sigma unless specially indicated. Recombinant α7(1–208) (ECD, extracellular domain) was produced in yeast, as described previously (Zouridakis et al., 2009) and was deglycosylated with EndoHf endoglycosidase (NEB), using 10 units of the enzyme per 1 μg of protein and incubating at 4 °C for 3 days, in 25 mM Tris, 150 mM NaCl, pH 7.5. All experiments with deglycosylated α7 ECD were performed after removal of EndoHf by gel filtration on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 25 mM Tris, 150 mM NaCl, pH 7.5, at a flow rate of 0.5 ml min^{-1}. α7 peptides (11–23), (91–106), (159–167/179–188), (171–193) and α7(179–190) were synthesized in Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia (Vol'pina et al., 2006) and were a kind gift of Prof. V. Tsetlin. Antibodies against α7(179–190) and α7(1–208) nAChR fragments were obtained and characterized by us previously (Skok et al., 1999; Lykhmus et al., 2010). The antibodies against amyloid-beta peptides Aβ(17–24), Aβ(1–40) and Aβ(1–42) were purchased from Covance, USA.

2.2. SDS-PAGE and western blot

SDS-PAGE of glycosylated (glyc) and deglycosylated (deglyc) forms of α7(1–208) was performed in 10% polyacrylamide gel during 1.5 h at 40 mA using the markers of molecular weight from Fermentas (Thermo Scientific, France). A half of the gel was fixed and stained with 0.25% Coomassie G250, another half was blotted to nitrocellulose (Hybond, Amersham) and the blot was stained with biotinylated α7(1–208)-specific antibody followed by Streptavidin-peroxidase and α-chloro-1-naphthol-containing substrate solution.

2.3. Lectin ELISA

The immunoplates (NUNC Maxisorp) were coated with either glyc or deglyc α7(1–208) (30 μg/ml, at 4 °C overnight) and blocked with 1% BSA. The carbohydrate residues were revealed with biotinylated *N*-acetylglucosamine-specific wheat germ agglutinin (WGA) and mannose-specific lectin from *Lens culinaris* (LCH). In addition, the adsorbed glyc/deglyc α7(1–208) were tested with biotinylated α7(1–208)-specific or α7(179–190)-specific antibodies. The bound lectins or antibodies were revealed with Streptavidin-peroxidase conjugate and *o*-phenylendiamine-containing substrate solution, and the optical density was determined at 490 nm.

2.4. Immunization schedule; sera and brain lysates preparation

Two groups of mice, 8 animals in each, were immunized intraperitoneally with either glyc or deglyc α7(1–208) (50 μg per mouse) in complete Freund's adjuvant and were reimmunized in three weeks with the same dose of antigens in incomplete Freund's adjuvant. Two control groups (5 animals in each) were injected with either the adjuvant emulsified in PBS or LPS (60 μg per mouse) instead of the antigen. The third control group of mice was intact. The blood from the tail vein was taken every 5 days. After the end of immunization cycle, mice were examined in behavioral tests and then sacrificed by cervical dislocation. The brains were removed, homogenized with the glass homogenizer, and the detergent lysates for further examination were prepared as described previously (Lykhmus et al., 2015). The protein content was measured with the BCA kit (Thermo Scientific, France).

2.5. ELISA for the blood sera and brain preparations

To determine the presence and epitope specificity of α7(1–208)-specific antibodies in the mouse blood sera the immunoplates were coated with 30 μg/ml of either glyc/deglyc α7(1–208) or BSA-conjugated α7 peptides: (11–23), (91–106), (159–167/179–188), (171–193) or α7(179–190). The plates were blocked with 1% BSA for 1 h at 37 °C and the immune sera were applied in 0.05% Tween 20-containing PBS for overnight at 4 °C. The bound antibodies were detected with peroxidase-conjugated goat anti-mouse IgM- or IgG-specific antibody (Sigma, USA) and *o*-phenylendiamine-containing substrate solution; the optical density was read at 490 nm.

To determine the level of nAChR subunits within the brain lysates, the immunoplates were coated with rabbit α7(1–208)-specific antibody (20 μg/ml), blocked with 1% BSA, and the brain detergent lysates were applied into the wells (1 μg of protein per 0.05 ml per well) for 2 h at 37 °C. The plates were washed with water and the second biotinylated α7(179–190) specific antibody was applied for additional 2 h being revealed