Neurochemistry International 99 (2016) 52–61

Contents lists available at ScienceDirect

Neurochemistry International

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

Disruption of neuronal nitric oxide synthase dimerization contributes to the development of Alzheimer's disease: Involvement of cyclin-dependent kinase 5-mediated phosphorylation of neuronal nitric oxide synthase at Ser^{293}

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ARTICLE INFO

Article history:
Received 11 February 2016
Received in revised form
27 May 2016
Accepted 9 June 2016
Available online 11 June 2016

Keywords:
Alzheimer's disease
Neuronal nitric oxide synthase
Dimerization
Cyclin-dependent kinase 5
p25
Phosphorylation

ABSTRACT

Although previous studies have suggested that neuronal nitric oxide synthase (nNOS)-derived NO has neuroprotective effects on the development of Alzheimer's disease (AD), the underlying molecular mechanisms are not fully elucidated. Here, we investigated whether and how disruption of nNOS dimerization contributes to the development of AD. No differences in synaptic number or expression of synaptic markers, including synaptophysin and postsynaptic density 95, were found in the cortex of 5 × FAD mice, which possess 5 familial AD mutations, at 6 months of age compared with control littermates. nNOS dimerization was disrupted in the 5 × FAD cortex, accompanied by an increase in reactive oxygen species (ROS) production. The subcellular distribution of cyclin-dependent kinase 5 (CDK5) shifted more diffusely toward a cytosolic compartment, but there was no change in total expression. Furthermore, the levels of p25, a CDK5 activator, increased significantly and it colocalized with nNOS in the 5 × FAD cortex. *In silico* analysis revealed that a new nNOS-specific GSP (glycine-serine-proline) motif was well-conserved across species at nNOS-Ser^{293}, which is located ahead of the N-terminal hook. This motif was not present in the closely related isoform, endothelial NOS. Motif scan analysis also predicted that CDK5 can phosphorylate nNOS-Ser^{293} with a high likelihood. An *in vitro* phosphorylation assay clearly showed that CDK5/p25 does indeed phosphorylate nNOS-Ser^{293}. Finally, nNOS-S293D mutant, a phosphomimetic form of nNOS-Ser^{293}, and nNOS-S293A mutant, a neutral form of nNOS-Ser^{293}, significantly decreased nNOS dimerization and NO production. Taken together, our results demonstrate that nNOS dimers are disrupted in the 5 × FAD cortex, and nNOS-Ser^{293}, a potential site of CDK5 phosphorylation, may be involved in the decrease in nNOS dimerization and NO production, and the development of AD.

Abbreviations: AD, Alzheimer's disease; CDK5, cyclin-dependent kinase 5; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; 5 × FAD, 5 familial Alzheimer's disease mutations; PSD95, postsynaptic density 95; WT, wild-type; BAEC, bovine aortic endothelial cells; LT-PAGE, low-temperature SDS-PAGE; IHC, immunohistochemistry.

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http://dx.doi.org/10.1016/j.neuint.2016.06.005
0197-0186/

1. Introduction

The prevalence rate of dementia, including that caused by Alzheimer's disease (AD), is sharply increasing due to lengthened lifespans. It is associated with many socioeconomic problems in developed countries. The development of AD has been attributed to excessive production of amyloid β proteins and/or tau proteins causing the formation of senile plaques and neurofibrillary tangles, respectively. Although these molecular mechanisms are recognized as fundamental to almost all aspects of AD pathogenesis (Masters and Beyreuther, 2006), recently various intracellular signaling molecules, such as cyclin-dependent kinase 5 (CDK5), glycogen synthase 3β, and mammalian target of rapamycin, have also been implicated in the development of AD (Amin et al., 2015; Dhariwala and Rajadhyaksha, 2008; Tramutola et al., 2015). Among these, CDK5 has been identified as a key mediator of AD pathogenesis (Barnett and Bibb, 2011; Crews et al., 2011; Dhariwala and Rajadhyaksha, 2008).

Homozygous knockout of the CDK5 gene is perinatal lethal and these mice show defects in cortical lamination and neuronal development (Hirota et al., 2007; Ohshima et al., 1996), which demonstrates that CDK5 is an essential kinase for normal neuronal development during embryogenesis. CDK5 also plays an important role in adult neurogenesis and synaptic plasticity (Jessberger et al., 2008, 2009). Furthermore, aberrant activation of CDK5 is linked to the development of neurodegenerative diseases, including AD (Cruz and Tsai, 2004). To become active, CDK5 has to associate with activator protein such as p35 or p25, a truncated form of p35. While the CDK5/p35 complex is known to mediate normal physiological processes, an association with p25 aberrantly increases CDK5 activity and leads to neuronal cell death and neurodegeneration (Lee et al., 2000; Patrick et al., 1999).

Nitric oxide (NO) is a gaseous signaling molecule produced from one of the three isoforms of NO synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). NO derived from these NOS enzymes plays an important role in the maintenance of vascular tone, synaptic integrity, and inflammation, respectively (Forstermann and Sessa, 2012). Among these, nNOS-derived NO is reported to not only regulate synaptic plasticity and memory formation (Cserep et al., 2011; Zoubovsky et al., 2011), but also to inhibit AD pathogenesis (Harry et al., 2001; Puzzo et al., 2005). It is well established that NOS dimerization is necessary for NOS enzymatic activity (Forstermann and Sessa, 2012). When NOS dimers are disrupted, each monomer produces reactive oxygen species (ROS), including superoxide, instead of NO (Forstermann and Sessa, 2012). In nNOS, N-terminal motifs including N-terminal leader sequences, N-terminal hooks, and Zn-loops are reported to be critical for dimerization (Panda et al., 2003).

Although eNOS dimerization is largely disrupted in vessels of aged mice, which provides a molecular mechanism for endothelial dysfunction in aged vessels (Yang et al., 2009), there have been no reports on the implications of nNOS dimerization in neuronal degeneration. To address this issue, we investigated whether and how disruption of nNOS dimerization in the cortex of 5 × FAD (5 familial Alzheimer's disease mutations) mice contributes to the development of AD. We for the first time demonstrate that nNOS dimers are disrupted in the 5 × FAD cortex and nNOS-Ser^{293}, a potential site of CDK5 phosphorylation, may be involved in the decrease in nNOS dimerization and NO production, and the development of AD.

2. Materials and methods

2.1. Materials

Antibodies against FLAG and β-actin were purchased from Sigma-Aldrich Co. (St. Louis, MO), and antibodies against NeuN and postsynaptic density 95 (PSD95) were purchased from EMD Millipore (Darmstadt, Germany). Antibodies against nNOS and CDK5 were obtained from Novus Biologicals (Littleton, CO). Antibodies against p35/p25 and GFAP were purchased from Abcam (Cambridge, MA). Antibodies against synaptophysin and Iba-1 were purchased from BD biosciences (Franklin Lakes, NJ) and Wako chemicals (Cambridge, MA), respectively. Antibodies against ToPro-3 were obtained from ThermoFisher Sci., (Waltham, MA). [γ-^{32}P]-ATP was obtained from PerkinElmer Life Sciences (Boston, MA). The purified recombinant CDK5/p25 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Protein A agarose was obtained from Thermo Scientific Inc. (Rockford, IL). Collagenase (type 2) was purchased from Worthington Biochemical Corporation (Freehold, NJ). Minimal essential medium (MEM), Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), penicillin and streptomycin antibiotics, L-glutamine, trypsin−ethylenediaminetetraacetic acid (EDTA) solution, and plasticware for cell culture were purchased from Gibco-BRL (Gaithersburg, MD). All other chemicals were of the purest analytical grade.

2.2. Site-directed mutagenesis of nNOS

Rat FLAG-tagged wild-type (WT) nNOS (accession number X59949, UniProt ID: P29476) construct subcloned into mammalian expression vector pcDNA3 was a kind gift from Prof. Masatomo Watanabe (Laboratory of Molecular and Cellular Neuroscience, Kagawa School of Pharmaceutical Sciences, Tokushima Bunri University, Sanuki-city, Kagawa, Japan) (Watanabe and Itoh, 2011). Using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), we manufactured two mutants: a phosphomimetic nNOS-S293D (serine is replaced by aspartate at serine 293) and a neutral nNOS-S293A mutant (serine is replaced by alanine), and confirmed successful mutations for each construct by fully sequencing the mutants. The following PCR primer pairs were designed to amplify each mutant: nNOS-S293D-F, 5′-CCACCAAGAACGGCGACCCTTCCAGGTGCC-3′; nNOS-S293D-R, 5′-GGCACCTGGAAGGGTCGCCGTTCTTGGTGG-3′; nNOS-S293A-F, 5′-CCACCAAGAACGGCGCCCCTTCCAGGTGCC-3′; and nNOS-S293A-R, 5′-GGCACCTGGAAGGGGCGCCGTTCTTGGTGG-3′.

2.3. In silico analysis for comparative alignment of nNOS, eNOS, and iNOS, and motif scan

The nNOS, eNOS, and iNOS protein sequences in different species were obtained from the NCBI web site (http://www.ncbi.nlm.nih.gov/protein) and their accession numbers are as follows: nNOS *Homo sapiens* - UniProtKB/Swiss-Prot: P29475.2, nNOS *Bos taurus* - NCBI Reference Sequence: XP_002694631.2, nNOS *Rattus norvegicus* - UniProtKB/Swiss-Prot: P29476.1, nNOS *Mus musculus* - UniProtKB/Swiss-Prot: Q9Z0J4.1, eNOS *Rattus norvegicus* - UniProtKB/Swiss-Prot: Q62600.4, iNOS *Rattus norvegicus* - UniProtKB/Swiss-Prot: Q06518.2. These sequences were then subjected to comparative alignment analysis using NPS@: Network Protein Sequence Analysis (Combet et al., 2000). The sequence motifs of nNOS rattus norvegicus most likely to be phosphorylated by specific protein kinases were identified using the motif scanning program Scansite, available at http://scansite3.mit.edu.

2.4. Cell culture and transfection

Bovine aortic endothelial cells (BAEC) were isolated as described previously (Kim et al., 1999) and were maintained in MEM supplemented with 5% FBS at 37 °C under 5% CO_2 in air. The endothelial cells were confirmed by their typical cobblestone configuration