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AP-1 mediates β-amyloid-induced iNOS expression in PC12 cells via the ERK2 and p38 MAPK signaling pathways

Jung-Hee Jang, Young-Joon Surh *

National Research Laboratory, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

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Abstract

Nitrosative stress with subsequent inflammatory cell death has been implicated in some neurodegenerative disorders such as Alzheimer's disease (AD). Expression of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO) have been frequently elevated in AD. In this study, we have investigated the molecular mechanisms underlying nitrosative stress induced by β-amyloid (Aβ), a neurotoxic peptide associated with senile plaques formed in the brains of patients with AD. Exposure of rat pheochromocytoma (PC12) cells to the Aβ resulted in increased mRNA and protein expression of iNOS and generation of NO. NO can rapidly interact with superoxide anion, forming more reactive peroxynitrite. Treatment of PC12 cells with Aβ led to increased peroxynitrite production and nitrotyrosine formation. Aβ induced activation of redox sensitive transcription factor activator protein-1 (AP-1), and AP-1 antisense oligonucleotide abolished the Aβ-induced iNOS expression. Moreover, Aβ transiently activated extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK) via phosphorylation. Pharmacologic inhibition of both enzymes or dominant-negative mutation of ERK2 or p38 MAPK effectively down-regulated DNA binding as well as transcriptional activity of AP-1 and subsequent iNOS expression and NO production. The above findings suggest that Aβ induces iNOS expression in PC12 cells through activation of AP-1 which is regulated by upstream kinases, such as ERK and p38 MAPK.

Keywords: AP-1; β-Amyloid; ERK; iNOS; Nitrosative stress; p38 MAPK; PC12 cells

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive degeneration and loss of neurons in the brain, which correlate with the appearance of neurofibrillary tangles and senile plaques, the two neuropathological hallmarks of the disease [1]. β-Amyloid (Aβ) peptide is the major component of senile plaques and considered to have a causal role in the development and progress of AD [2]. Experimental data from both in vitro and in vivo studies indicate that different molecular forms of Aβ affect a wide array of neuronal and glial functions, thereby leading to neuronal cell death [3]. There is compelling evidence supporting that enhanced pro-inflammatory activities provoked by Aβ are associated with the pathogenesis and/or progression of AD, and that some anti-inflammatory agents protect against Aβ-induced neurotoxicity [4,5]. One of the principal enzymes that play a pivotal role in mediating inflammatory response is inducible nitric oxide synthase (iNOS). iNOS is mainly localized in astrocytes and microglia, and catalyzes the oxidative deamination of L-arginine to produce nitric oxide (NO), a potent pro-inflammatory mediator. In Alzheimer tissue, pro-inflammatory iNOS is notably up-regulated and colocalized in Aβ plaques [6,7]. Several studies have demonstrated that Aβ stimulates microglial and astrocytic iNOS induction and subsequent NO production [8–10]. In addition, Aβ has been shown to exert synergistic action with cytokines to induce neuronal damage via

* Corresponding author. Fax: +82 2 874 9775.
E-mail address: surh@plaza.snu.ac.kr (Y.-J. Surh).

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an NO-dependent pathway [11–13]. However, little is known about the molecular mechanisms mediating Aβ-induced iNOS expression.

Previous studies have shown that Aβ induces iNOS expression in astrocytes through NF-κB-dependent mechanisms [9,10], but the possible involvement of other transcription factors in Aβ-induced iNOS activation cannot be ruled out. One alternative candidate transcription factor is activator protein-1 (AP-1) which, as a redox-sensitive transcription factor, regulates expression of genes that are involved in cellular differentiation, proliferation, apoptosis, oxidative response, inflammation, and immune response [14,15]. AP-1 exists as an either homo- or heterodimer of proteins that belong to c-Jun (c-Jun, JunB and JunD) or c-Fos (c-Fos, FosB, Fra-1, and Fra-2) families, Jun dimerization partners (JDP1 and JDP2), and the closely related activating factors (ATF2, LRF1/ATF3, and B-ATF) which interact via the heptad repeats and hydrophobic residues located in a leucine zipper domain. iNOS promoter harbors at least two AP-1 response elements [16,17], and activated AP-1 plays a crucial role in iNOS gene expression in response to pro-inflammatory signals or cellular stress, including lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) [18–22]. AP-1 activation was evident in postmortem AD brains [23], and Aβ treatment resulted in AP-1 activation in an in vitro cell culture system [24].

The activation of AP-1 through dimerization by means of leucine zippers is known to be mediated by a variety of upstream protein kinases. Mitogen-activated protein kinases (MAPKs) have been considered to be an important group of regulators of a broad range of genes involved in cellular responses to pro-inflammatory and other stress signals. Three distinct groups of well-characterized major MAPK subfamily members include extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK which are serine/threonine protein kinases. In an attempt to clarify the intracellular signaling events leading to iNOS expression mediating inflammatory cell death in Aβ-stimulated PC12 cells, we examined the role of AP-1 and representative MAPKs and their functional interrelation in iNOS induction.

Experimental procedures

Chemical and biochemical reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, horse serum, F-12, and N-2 supplement were provided from Gibco (Grand Island, NY, USA). $A\beta_{25-35}$ was supplied from Bachem (Torrance, CA, USA). $A\beta_{25-35}$ was dissolved in deionized distilled water at a concentration of 1 mM and stored at −20 °C. The stock solution was diluted to desired concentrations immediately before use and added to culture medium without the aging procedure. We note that both fresh and aged preparations of $A\beta_{25-35}$ have similar cytotoxic effects as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay in PC12 cells. Dihydrorhodamine123 (DHR123) was obtained from Molecular Probe (Eugene, OR, USA). The AP-1-promoter luciferase construct was kindly provided by Dr. Taeg Kyu Kwon (Keimyung University, Taegu, South Korea). U0126 and SB203580 were the products of Calbiochem (San Diego, CA, USA). Poly-D-lysine and other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA).

Cell culture. PC12 cells were maintained in DMEM supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum at 37 °C in a humidified atmosphere of 10% CO_2 and 90% air. All cells were cultured in poly-D-lysine-coated culture dishes. The medium was changed every other day, and cells were plated at an appropriate density according to each experimental scale. After 24 h subculture, cells were switched to serum-free N-2 defined medium for treatment.

Western blot analysis. $A\beta_{25-35}$-treated cells (1×10^6 cells/3 ml in 60 mm dish) were collected and washed with phosphate-buffered saline (PBS). After centrifugation, cell lysis was carried out at 4 °C by vigorous shaking for 15 min in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, and protease inhibitors]. After centrifugation at 23,000*g* for 15 min, supernatant was separated and stored at −70 °C until use. The protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). After addition of sample loading buffer, protein samples were electrophoresed on a 12.5% SDS–polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride blots at 300 mA for 3 h. The blots were blocked for 1 h at room temperature in fresh blocking buffer [0.1% Tween 20 in Tris-buffered saline (TBST), pH 7.4, containing 5% non-fat dried milk]. Dilutions (1:1000) of primary anti-iNOS (BD Transduction Laboratories, Chicago, IL, USA), anti-c-Jun, anti-phospho-c-Jun, anti-c-Fos, anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38 (all products of Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-actin (Sigma Chemical) antibodies were made in PBS with 3% non-fat dried milk, and blots were incubated for 3 h at room temperature. Following three washes with PBST (0.1% Tween 20 in PBS), the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in PBS with 3% non-fat dried milk for 1 h at room temperature. The blots were washed again three times in PBST buffer, and transferred proteins were incubated with ECL substrate solution (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 min according to the manufacturer's instructions and visualized with X-ray film.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from PC12 cells using TRIzol (Invitrogen, Carlslab, CA, USA) and converted to cDNA by M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions. Specific DNA sequences were amplified with a PCR mixture (HyMed, Seoul, Korea). Each PCR primer used in this study was as follows: iNOS, 5′-AAA TCC AGA TAA GTG ACA-3′ (sense) and 5′-TGA ACG TCC AGG TTT AGA-3′ (antisense); nNOS, 5′-GGC ACT GGC ATC GCA CCC TT-3′ (sense) and 5′-CTT TGG CCT GTC CGG TTC CC-3′ (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-AGT GTA GCC CAG GAT GCC CTT-3′ (sense) and 5′-GCC AAG GTC ATC CAT GAC AAC-3′ (antisense). Amplification products were resolved by 1.0% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light. All primers were purchased from Bionics (Seoul, South Korea).

Measurement of NO production. NO production was calculated from the amount of nitrite detected by the Griess reaction. This assay relies on a simple colorimetric reaction between nitrite and Griess reagent [0.1% *N*-(naphthyl)ethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid] to produce a pink azo product. Briefly, a 100 μl aliquot of media was mixed with an equal volume of Griess reagent and incubated for 20 min at room temperature.