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EPIGENETIC MECHANISMS OF AMYLOID-β PRODUCTION IN ANISOMYCIN-TREATED SH-SY5Y CELLS

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Abstract—Oxidative stress and inflammation as the pathological components of Alzheimer's disease (AD) have been well understood. Among a diversity of mitogen-activated protein kinase (MAPK) family members, JNK and p38 MAPK subfamilies are relevant to the response of environmental stress, inflammatory stimuli, or other insults. Recent studies have demonstrated that epigenetic mechanisms may play a pivotal role in AD pathogenesis and development. In the present study, we have investigated epigenetic mechanisms such as DNA methylation and histone acetylation involved in the activation of stress-related signaling pathways for amyloid-β (Aβ) production. Human neuroblastoma SH-SY5Y cells were treated by anisomycin, an activator of stress-related MAPKs (JNK and p38 MAPK). A significant increase of intracellular Aβ level in anisomycin-treated SH-SY5Y cells was observed. The expression of amyloid-β precursor protein (APP), β-site APP-cleaving enzyme 1 (BACE1), and presenilin 1 (PS1) was upregulated by demethylation in three gene promoters associated with the reduction of methyltransferases (DNMTs). Meanwhile, an enhanced level of global histone H3 acetylation accompanied with upregulation of histone acetyltransferases p300/CREB-binding protein (CBP) and downregulation of histone deacetylases (HDACs) was also observed. These findings indicated that the activation of stress-related signaling pathways could result in the increased transcription of *APP, BACE1,* and *PS1* genes through DNMT-dependent hypomethylation and histone H3 hyperacetylation, thus leading to Aβ overproduction. Moreover, our findings provided a novel insight into epigenetic mechanisms by which oxidative stress contributes to the pathogenesis of AD.

Key words: Alzheimer's disease, amyloid-β, DNA methylation, histone acetylation, anisomycin, SH-SY5Y cell.

Accumulation of β-amyloid plaques and neurofibrillary tangles is the pathological hallmark of Alzheimer's disease (AD) (Selkoe, 2001; Tanzi and Bertram, 2005). It is recognized that increased production, oligomerization, and aggregation of amyloid-β (Aβ) peptides are the crucial factors in the onset of AD. The toxic Aβ1-40 and Aβ1-42 are generated from the cleavage of amyloid-β precursor protein (APP) by β-site APP-cleaving enzyme 1 (BACE1, β-secretase) and γ-secretase complex comprising of presenilin 1 or 2 (PS1 or PS2), nicastrin, anterior pharynx-defective 1 (APH-1), and presenilin enhancer 2 (PEN-2) (LaFerla et al., 2007; Thinakaran and Koo, 2008). Most AD cases are sporadic and have no defined causes. Factors contributing to AD development are numerous and complex. In the last decade, increasing evidence suggests that oxidative stress and inflammation is an early event in AD pathogenesis (Nunomura et al., 2001; de la Monte and Wands, 2006), since AD brains exhibit the evidences of reactive oxygen species (ROS)-mediated injury, inflammatory responses, and free radical oxidative damage in critical intracellular targets responsible for neuronal cell death related to AD.

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that transmit extracellular signals to the nucleus and control cellular processes. In mammalian cells, three principle MAPK pathways, including ERK, JNK, and p38 MAPK, have been identified. The JNK and p38 MAPK pathways are especially relevant to the response of environmental stress and inflammatory stimuli (Saklatvala, 2004; Kumar et al., 2003). In AD brain, increased levels of activated p38 MAPK and JNK are detected and associated with neuritic plaques, neuropil threads, and neurofibrillary tangle-bearing neurons (Hensley et al., 1999; Sun et al., 2003). In AD transgenic mice, p38 MAPK is significantly activated in microglia, astrocytes and neurons, around and distant from the plaques, which indicates the possible involvement of stress-related signaling pathways during the pathogenesis of AD (Hwang et al., 2004, 2005; Giovannini et al., 2008).

To date, the pathophysiology of AD is not fully understood yet. Recent studies have suggested that epigenetic mechanisms may play an important role in the initiation and development of AD (Chouliaras et al., 2010; Zawia et al., 2009). Various studies on human postmortem brain samples and peripheral leukocytes, as well as transgenic animal models and cell culture have revealed that aging and AD are associated with epigenetic dysregulation of stress-related signaling pathways at various levels. Moreover, previous studies have also described that epigenetic mechanisms can modulate the risk for AD (Barrachina and Ferrer, 2009; Drake et al., 2004). Conversely, it is still not fully clear whether the observed epigenetic changes actually represent a cause or a consequence of the disease. In order to explore epigenetic mechanisms involved in the activation of stress-related signaling pathways in Aβ production, we treated human neuroblastoma SH-SY5Y cells by using an activator of stress-related MAPKs (JNK and p38 MAPK), anisomycin, to activate stress-related MAPKs

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Abbreviations: AD, Alzheimer's disease; APP, amyloid-β precursor protein; Aβ, amyloid-β; BACE1, β-site APP-cleaving enzyme 1; CBP, CREB-binding protein; DNMTs, methyltransferases; HDACs, histone deacetylases; MAPK, mitogen-activated protein kinase; NEP, neprilysin; PS1, presenilin 1; SAM, S-adenosyl methionine.

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(JNK and p38 MAPK). The activated stress-related signaling pathways resulted in the enhanced transcription of *APP, BACE1,* and *PS1* genes through DNMT-dependent hypomethylation and histone H3 hperacetylation, which correspondingly led to the overproduction of Aβ. The data will provide a novel insight into epigenetic mechanism by which oxidative stress or inflammation contributes to the pathogenesis of AD.

EXPERIMENTAL PROCEDURES

Cell culture

Human neuroblastoma SH-SY5Y cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, CA, USA), 100 μg/ml streptomycin, and 100 U/ml penicillin (Gibco, CA, USA) at 37 °C in a humidified incubator with 95% air and 5% CO_2. Cells were treated with anisomycin in DMSO (Alomone Laboratories Ltd., Jerusalem, Israel) at the dose of 2 ng/ml for 1 or 72 h, while the cells treated with same volume of DMSO as the experimental groups were used as control. The viability of SH-SY5Y cells was evaluated by MTT assay.

Quantification of Aβ level by ELISA

Aβ level was quantified in cell lysates by ELISA using Biosource kit (CA, USA) according to the manufacturer's protocol. Aβ1-40 and Aβ1-42 were evaluated by using standard curves generated in duplicate. The quantity of Aβ in each sample was measured in triplicate. Recombinant C99 was added to the assay to rule out the cross reaction of the Aβ ELISA assays with C99.

Quantitative real-time PCR

Cell RNA was extracted by using SV Total RNA Isolation System (Promega, WI, USA) according to the manufacturer's instructions. First-strand cDNA was obtained by reverse transcription through Reverse Transcription System (Promega, WI, USA) according to the manufacturer's instructions. The cDNAs were stored at −20 °C until use. Quantitative PCR (Q-PCR) was used to evaluate the cDNAs in cells treated with anisomycin and control samples without treatment (LightCycler1.5, Roche Diagnostics GmbH, Mannheim, Germany). Q-PCR reactions were performed in a final volume of 20 μl containing 1 μl of cDNA and the SYBR Green PCR Master Mix (Tiangen Biotech Corporation Ltd, Beijing, China). The PCR conditions were set up as follows: initial DNA denaturation at 95 °C for 7 min and 40 cycles of denaturation at 95 °C for 45 s, annealing at 62 °C for 35 s and extension at 72 °C for 60 s. Each Q-PCR sample was conducted in triplicate. The specificity of the Q-PCR reactions was examined by determining melting points. All cDNAs were normalized to 18S, and the relative transcription level was assessed by using the 2-ΔΔCt method (LightCycler® Software 4.1, Roche Diagnostics GmbH, Mannheim, Germany). Sequences of each primer were designed as follows: BACE1, forward primer: GTCGGAGGGAGCATGATCA and reverse primer: CCGCCGGATGGGTGTATAC; APP, forward primer: GAACTACATCCCGCTCTGC and reverse primer: CGCGGACATACTTCTTTAGC; PS1, forward primer: GGTCGTGGCTACCATTAAGTC and reverse primer: GCCCACAGTCTCGGTATCTT; 18S, forward primer: GTAACCCGTTGAACCCCATT and reverse primer: CCATCCAATCGGTAGTAGCG.

Western blot analysis

Cells were collected and washed with phosphate-buffered saline (PBS). After centrifugation, cell lysates were prepared by sonication for 5 min in lysis buffer (50 mmol/L Tris–HCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L DTT, 0.1% sodium deoxycholate, 0.5% NP-40, 1 μmol/L sodium orthovanadate, 5 mmol/L sodium pyrophosphate, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml chymostatin, 50 nmol/L okadic acid, and pH 7.4). The supernatant was harvested after 10 min centrifugation at 13000 g and stored at −20 °C until use. The protein concentration was determined by using BCA protein assay kit (Pierce, IL, USA). Samples (50 μg/lane for anti-phospho-p38 MAPK, anti-APP, anti-PS1, anti-BACE1, anti-DNMT1, anti-DNMT3a, and anti-TACE; 60 μg/lane for anti-histone deacetylase [HDAC]3 and anti-p300/CREB-binding protein [CBP]; 100 μg/lane for anti-neprilysin [NEP] and anti-ADAM10) were loaded in 8%, 10%, or 15% SDS-PAGE for the separation, and then transferred onto nitrocellulose membrane. After blocking with 5% milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T, pH 7.5) at room temperature for 1 h, the blots were incubated with primary antibody in TBS-T at 4 °C overnight. The primary antibodies used in this study included rabbit anti-phospho-p38 MAPK monoclonal antibody (1:1000, Cell Signaling, MA, USA), rabbit anti-APP polyclonal antibody (1:1000, Cell Signaling, MA, USA), rabbit anti-PS1 polyclonal antibody (1:500, Cell Signaling, MA, USA), rabbit anti-BACE1 polyclonal antibody (1:1500, Abcam, London, UK), rabbit anti-DNMT1 monoclonal antibody (1:1000, Cell Signaling, MA, USA), rabbit anti-DNMT3a polyclonal antibody (1:1000, Cell Signaling, MA, USA), mouse anti-p300/CBP monoclonal antibody (1:250, Abcam, London, UK), rabbit anti-HDAC3 monoclonal antibody (1:1000, Cell Signaling, MA, USA), mouse anti-β-actin monoclonal antibody (1:5000, Sigma, MO, USA), mouse anti-GAPDH (1:1000, Sigma, MO, USA), rabbit anti-TACE polyclonal antibody (1:200, Santa Cruz, CA, USA), mouse anti-ADAM10 monoclonal antibody (1:50, Santa Cruz, CA, USA), and rabbit anti-NEP polyclonal antibody (1:12000, Chemicon, MA, USA). After washing three times in TBS-T, the blots were incubated with HRP-conjugated secondary antibody at room temperature for 2 h. The antibody binding was visualized using Western blotting luminol reagent (Santa Cruz, CA, USA) according to the manufacturer's instructions. The quantification of the blots was carried out by using a Gel-Doc Image Scanner (Bio-Rad, CA, USA) and Quantity One software program (Bio-Rad, CA, USA). The data in accordance with normal distribution were analyzed by independent *T*-test (SPSS 11.5 system, SPSS, Chicago, USA). A significant difference was considered at $P<0.05$.

DNA methylation analysis

Genomic DNA was extracted from cultured cells using Wizard® SV Genomic DNA Purification System (Promega, WI, USA) according to manufacturer's instructions. The concentration and purity of the DNA were determined by absorbance at 260 and 280 nm. A total of 200 ng genomic DNA from each sample was bisulfite-treated with the Methylamp DNA Modification Kit (Epigentek, NY, USA). The quality of bisulfite conversion was evaluated by using PCR products without methyl groups as the control. The Sequenom MassARRAY platform (CapitalBio, Beijing, China) that was composed of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE) was used to quantitatively analyze the methylation of APP (Gen-Bank accession number NM_201414), PS1 (Gen-Bank accession number NM_007318), and BACE1 (Gen-Bank accession number NM_012104). PCR primers were designed with Methprimer (http://epidesigner.com). For each reverse primer, an additional T7 promoter tag for *in vivo* transcription was added, as well as a 10-mer tag on the forward primer for adjusting melting temperature. All primers used in this study were listed in Table 1. The methylation ratios were generated by Epityper software version 1.0 (Sequenom, San Diego, CA, USA). The generated data were put into the EPI 3.1 Database (EpiData Association, Odense, Denmark) and