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Alteration of global protein SUMOylation in neurons and astrocytes in response to Alzheimer's disease-associated insults

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ABSTRACT

SUMOylation, a post-translational modification of lysine residues by small ubiquitin-like modifier (SUMO) proteins, has been implicated in the pathogenesis of neurodegenerative disorders including Alzheimer's disease (AD), and in neuron- and astrocyte-specific physiological functions. Global SUMOylation is increased in the AD mouse brain in the pre-plaque-forming stage but returns to wild-type levels in the plaque-bearing stage. To clarify the reason for the transient change in SUMOylation, we analyzed the alteration of global SUMOylation induced by AD-associated cytotoxic stimuli in neurons and astrocytes individually. In neurons, amyloid β42 oligomers induced some but not significant increase in levels of SUMO1-modified proteins. Both hydrogen peroxide and glutamate significantly reduced SUMO1-modified protein levels. These changes were more prominent in neurons than in astrocytes. The opposite effect of Aβ and oxidative/excitotoxic stimuli on SUMO1 modification may cause the pathological stage-associated change in the level of SUMO-modified proteins in the AD mouse brain.

1. Introduction

SUMOylation is a post-translational modification that significantly impacts cell physiology. During SUMOylation, a small ubiquitin-like modifier (SUMO) protein covalently binds to the target substrate protein at a lysine residue [1]. In humans, three SUMO proteins, SUMO1, 2, and 3, are ubiquitously expressed [2,3]. The SUMO1 has 50% homology with SUMO2 in protein level [1]. SUMO2 and SUMO3 are different only in three amino acids in the N-terminal region; therefore they are referred to as SUMO2/3. SUMOylation is a reversible modification. The conjugation process is mediated by E1, E2, and E3 enzymes, and deconjugation is mediated by deSUMOylation enzymes such as sentrin/SUMO-specific proteases (SENP) family proteins [4]. Unlike ubiquitination, the consequence of SUMOylation varies depending on the target proteins and conjugated SUMO paralogs [1]. In the central nervous system, several synaptic regulator proteins, plasma membrane receptors, channels, and transporters are SUMO substrates [1,5]. Thus, SUMOylation has emerged as a key post-translational modification in neuronal functions [2].

Alzheimer's disease (AD) is the major cause of dementia and is characterized by senile plaques and neurofibrillary tangles in the brain. Amyloid precursor protein (APP) and tau are responsible for the formation of these pathological hallmarks, and both APP and tau are targets of SUMOylation [6,7]. The amyloid hypothesis proposes that oligomeric forms of amyloid β (Aβ), which is produced from the proteolysis of APP, play a principal role in the pathogenesis of AD. Aβ oligomers evoke multiple toxic events in neurons, including the enhancement of excitotoxicity and oxidative stress [8,9]. Aβ oligomers increase glutamate release from astrocytes [10], and excess amounts of the extracellular excitatory neurotransmitter glutamate induce excitotoxicity to neurons. Aβ oligomers increase the production of reactive oxygen species in both neurons and astrocytes [11,12]. The imbalance between the production of reactive oxygen species and activities of antioxidants causes oxidative stress and induces the malfunction of biological molecules due to oxidation [13]. In addition, excitotoxicity and oxidative stress are induced by other factors including aging-related molecular impairments in metabolism and the stress response [14]. Thus, the combined effects of AD-specific (or Aβ-specific) and non-specific insults likely induce molecular pathological changes in AD brains.

Global changes in protein SUMOylation in the brains of AD

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patients and AD model mice have been reported. The levels of SUMO1-modified proteins increase in the cortex of AD patients compared with age-matched control individuals [15]. In the brains of Tg2576 AD model mice that overexpress Aβ, the levels of SUMO1-modified proteins are higher than in wild-type (wt) mice up to 6 months of age, whereas no significant difference is present between these two groups over 9 months of age [16,17]. How this transient increase in SUMOylation is associated with the onset or progression of AD pathology is unclear. To clarify the relationship between AD-related insults and protein SUMOylation, we analyzed the effect of cytotoxic insults on protein SUMOylation separately in neurons and astrocytes.

2. Materials and methods

2.1. Primary cortical neuron and cortical astrocyte culture

Cortical neurons were obtained from the embryonic day 16 Slc:ICR mouse embryos (Japan SLC, Shizuoka, Japan). The cortical neurons were plated at a density of 2.5×10^5 cells/well into 24-well poly-D-lysine-coated plates (BD Biosciences, Bedford, MA, USA) in Neurobasal Medium with 2%(v/v) B27 supplement, 1%(v/v) GlutaMAX, (Thermo Fisher Scientific, Waltham, MA, USA), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Wako Pure Chemical Industries, Osaka, Japan). Cortical astrocytes were obtained from postnatal 1–3 days newborn Slc:ICR mice, and plated at a density of 1.0×10^5 cells/well into 24-well poly-D-lysine-coated plates in Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (FBS), and antibiotics. The culture medium was replaced with DMEM with 10% FBS and antibiotics at 7 days *in vitro* (DIV) and with DMEM with 5% FBS and antibiotics at 14 DIV. All experimental procedures with animals were approved by the Institutional Animal Experiment Committee at Sophia University.

2.2. Chemical preparation and treatment of cells

L-glutamic acid (glutamate) and hydrogen peroxide (Wako Pure Chemical Industries) were dissolved in phosphate buffered saline (PBS). Oligomers of human amyloid β (1–42) (Peptide Institute Inc., Osaka, Japan) was prepared according to a previous protocol [18]. Cortical neurons and astrocytes were treated with glutamate or hydrogen peroxide at a concentration of either 10 μM or 100 μM for 3 h or with oligomer Aβ42 at 1 μM for 1 h or 24 h. Cells were washed with PBS twice and directly lysed with 1× SDS-PAGE sample buffer (62.5 mM Tris-HCl, 3 w/v% SDS, 7.5% glycerol, 0.005 w/v% bromophenol blue, 50 mM dithiothreitol) to inhibit the activity of protein modification enzymes, including deSUMOylation enzymes.

2.3. Animals and brain sample preparation

C57BL6/JJcl (CLEA Japan, Inc. Tokyo, Japan) and AD model mice overexpressing Aβ42, B6-Cg-Tg (APPSwFlLon, PSEN1:M146L*L286V) and 6799Vas (5xFAD, Mutant Mouse Resource and Research Center) were used [15]. The mice were housed in polycarbonate cages (3–4 animals per cage) at 22–24 °C under a 12-h light/12-h dark cycle with food and water *ad libitum*. Whole brains were obtained from wild-type and 5xFAD mice at 24 weeks of age. Brain homogenates were prepared in Tris buffer (20 mM Tris-HCl pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, protease inhibitors [Complete, Sigma, St. Louis, MO, USA], 1 mM Na_3VO_4, and 8 mM NaF) and mixed with 2× sample buffer (125 mM Tris-HCl, 6 w/v% SDS, 15% glycerol, 0.01 w/v% bromophenol blue, 100 mM dithiothreitol) at a 1:1 ratio. All animal care and experimental procedures complied with the Guidelines for the Care and Use of Laboratory Animals of Keio University and were approved by the Keio University Animal Ethics Committee (09084-8). All studies that involved animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals.

2.4. Immunoblot analysis

Protein samples were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST). To detect SUMOylated proteins, membranes were incubated with a rabbit anti-SUMO1 antibody (1:2000, #4972, Cell Signaling Technology, Danvers, MA, USA) or a rabbit anti-SUMO2/3 antibody (1:2000, #4974, Cell Signaling Technology) followed by a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Jackson Immuno Research, West Grove, PA, USA). β-actin was detected by a HRP-conjugated anti-β-action antibody (1:20000, A3854, Sigma). Protein bands were visualized with Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA), detected with an image analyzer (Pxi, Syngene, Cambridge, UK), and quantified using Gene Tools (Syngene). For the quantitation, visually prominent bands that were detected in all four independent samples on the same membrane were designated A-Q (mouse brain), A-N (neuron), and a-k (astrocyte), and the intensity was measured. For the global analysis, the total intensities of all immunostained bands with a molecular weight greater than 28 kDa (global), 75 kDa (over 75 kDa), and 63 kDa (over 63 kDa) were also measured. Each value was normalized with that of β-actin in the same lane, and the normalized values were used for statistical analysis.

2.5. Statistical analysis

All data are shown as the mean ± S.D. (standard deviation). Four to six independent samples in each experiment were statistically analyzed. The number of samples in each experiment is shown in the table legend. Comparisons between two groups were performed by *t*-test.

3. Results

We first investigated whether the increase in global SUMO1 modification observed in Tg2576 AD model mice [16,17] was also present in another widely used AD model, 5xFAD mice, at 6 months (24 weeks) of age. With immunoblotting using the anti-SUMO1 antibody, multiple bands were observed in both 5xFAD and wt mice (Fig. 1A). Quantitative analysis showed no significant difference in global SUMOylation by SUMO1 between the two groups, although a significant decrease in the intensity of bands A and B was detected in 5xFAD mice compared with wt mice (Fig. 1A and Table S1). These results were inconsistent with the findings in Tg2576 mice. On the other hand, consistent with previous observations in Tg2576 mice, the levels of protein modified by SUMO2/3 in wt and 5xFAD mice were similar, and no significant difference was detected with quantitative analysis (Fig. 1B and Table S1). At this age, the level of Aβ in the brain is much higher in 5xFAD mice than in Tg2576 mice [19]. The discrepancy in the results between these model mice may be due to differences in the amount of soluble and/or insoluble Aβ.

In these experiments, we prepared the cerebral homogenate using a buffer without N-ethylmaleimide (NEM), an inhibitor of deSUMOylating enzymes. We thus compared samples with and without NEM (Fig. S1). Stronger band intensities were detected by anti-SUMO1 and anti-SUMO2/3 antibodies across all molecular weights in the samples without NEM (Tris buffer) than that with

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