SUMO1 promotes A β production via the modulation of autophagy

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Abbreviations: AD, Alzheimer disease; ATG, autophagy-related; AV, autophagic vacuole; MAP1LC3/LC3, microtubule-associated protein 1 light chain 3; MDC, monodansylcadaverine; shRNA, small hairpin RNA; SUMO1, small ubiquitin-like modifier 1; A β , amyloid- β ; TEM, transmission electron microscopy; Tg, transgenic.

Autophagy is one of the main mechanisms in the pathophysiology of neurodegenerative disease. The accumulation of autophagic vacuoles (AVs) in affected neurons is responsible for amyloid- β (A β) production. Previously, we reported that SUMO1 (small ubiquitin-like modifier 1) increases A β levels. In this study, we explored the mechanisms underlying this. We investigated whether AV formation is necessary for A β production by SUMO1. Overexpression of SUMO1 increased autophagic activation, inducing the formation of LC3-II-positive AVs in neuroglioma H4 cells. Consistently, autophagic activation was decreased by the depletion of SUMO1 with small hairpin RNA (shRNA) in H4 cells. The SUMO1-mediated increase in A β was reduced by the autophagy inhibitors (3-methyladenine or wortmannin) or genetic inhibitors (siRNA targeting *ATG5*, *ATG7*, *ATG12*, or *HIF1A*), respectively. Accumulation of SUMO1, ATG12, and LC3 was seen in amyloid precursor protein transgenic mice. Our results suggest that SUMO1 accelerates the accumulation of AVs and promotes A β production, which is a key mechanism for understanding the AV-mediated pathophysiology of Alzheimer disease.

Introduction

Autophagy is a primary mechanism for maintaining cellular homeostasis. It is involved in the degradation and recycling of cellular proteins and aids cell survival on exposure to internal or external cellular stress.¹ The autophagic process starts with the entrapment of material within a double-membrane vesicle called the autophagosome.² MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3) is converted from unconjugated LC3 (LC3-I) to phosphatidylethanolamine-conjugated LC3 (LC3-II), which is associated with the phagophore (i.e., the autophagosome precursor) and autophagosome membrane.² Recently, autophagy has been implicated in a number of diseases, including neurodegenerative diseases such as Alzheimer disease (AD) and Parkinson disease, as well as lysosomal storage diseases and cancer. Numerous autophagic vacuoles accumulate within dystrophic neurites in the human brains affected by AD and mouse models of AD.^{3,4}

The small ubiquitin-like modifier (SUMO) is conjugated to lysine residues in target proteins and regulates numerous cellular processes.⁵ In mammalian cells, 3 SUMO isoforms are expressed.

SUMO1 shares 50% sequence identity with SUMO2 and SUMO3. An important function of SUMO is to stabilize its target protein.⁶ Several recent studies have shown that SUMO modification of proteins follows potentially harmful conditions, including ischemia, hypoxia, or oxidative stress.⁷ Hypoxia upregulates the expression of SUMO1, which participates in HIF1A (hypoxia inducible factor 1, α subunit [basic helix-loop-helix transcription factor]) sumoylation.⁸ HIF1A is subjected to sumoylation activated by transient cerebral ischemia.⁹ Because HIF1A levels are correlated with BECN1/Beclin1 and LC3 expression,¹⁰ increased HIF1A levels might serve to promote autophagy. Because SUMO modification stabilizes HIF1A,^{11,12} it has been suggested that the regulation of HIF1A by SUMO influences autophagy. However, there is no direct evidence of a relationship between SUMO and autophagy.

AD is the most common form of dementia in the elderly. It is a brain disease characterized by amyloid plaque and neurofibrillary tangles. A β ₁₋₄₀ and A β ₁₋₄₂, the main components of amyloid plaque, are peptides generated via proteolytic processing of APP [amyloid β (A4) precursor protein] by BACE1/ β -secretase

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Figure 1. SUMO proteins upregulate autophagy induction in neuroglioma H4 cells. ((**A**) and **B**) H4 and H4 cells stably expressing SUMO1 (HGS1 cells) were incubated in full-serum medium. Western blotting was performed to compare the levels of LC3-I and LC3-II protein using anti-LC3. TUBA4A (TUBA) was used as a loading control. LC3-II/I band intensity ratios were quantified using NIH image software and expressed as percentage of the control level. * indicates $P < 0.05$. ((**C**) and **D**) H4 and HGS1 cells were treated with 10 nM bafilomycin A₁ (BafA) for 4 h. The levels of LC3-II protein were compared by protein gel blotting using anti-LC3. LC3-II band intensity was quantified using NIH image software and expressed as percentage of the control level. * indicates $P < 0.05$. This is a representative image of triplicate experiments. Values represent the mean $\pm$ SEM of 3 experiments.

(β -site APP-cleaving enzyme 1) and the γ -secretase complex.¹³ An unusual accumulation of toxic proteins is evident in AD brains and studies have suggested that autophagy is involved in A β production and the pathogenesis of AD. Recent studies have shown that autophagic vacuoles (AVs) accumulate in cells in the brains of early AD patients and are associated with a variety of brain-degenerative pathologies.¹⁴ The early accumulation of pathological AVs has been reported in the hippocampus of a young mouse model of AD, in transmission electron microscopy images of plaque-associated aberrant neuritis.¹⁵ It has been suggested that AVs, within the axonal dystrophies, are a possible compartment for A β oligomerization.¹⁵ AVs are very condensed complexes of any kind of organelle components and could be a reservoir for many other cellular molecules, including APP, γ -secretase complexes, and BACE1-derived C-terminal fragments.^{4,16} Recently, we reported that SUMO1 increases BACE1 accumulation and A β production.¹⁷ In the present study, to

further explore the mechanisms underlying the SUMO1-mediated increase in A β production, we investigated whether autophagosome formation is responsive to SUMO1.

We hypothesized that autophagic activation is associated with A β production by SUMO1 overexpression. Our data provide evidence for a role of SUMO1 in autophagy. We also showed that SUMO1-induced A β production was blocked remarkably by autophagy inhibition. In addition, SUMO1 immunoreactivity was colocalized strongly with LC3 in the APP transgenic mice. Our results suggest that SUMO1 positively accelerates the accumulation of AVs and contributes to AV-mediated AD pathogenesis.

Results

SUMO1 proteins induce autophagy in H4 cells

To investigate the role of SUMO1 protein in inducing autophagy, we quantified the level of LC3-II protein in H4 cells and H4 cells stably expressing SUMO1 (HGS1 cells) (Fig. 1A). The LC3-II/I ratio increased by 600% in HGS1 cells ($P < 0.05$; Fig. 1B). We treated H4 and HGS1 cells with the lysosomal inhibitor bafilomycin A₁ (BafA) to block the fusion between autophagosomes and lysosomes, and trap LC3-II in autophagosomes. HGS1 cells, as compared to H4 cells, show more LC3-II accumulation upon BafA treatment, suggesting that autophagic flux is increased in HGS1 cells (Fig. 1C and D). We also examined autophagosome formation by transfection with GFP-tagged LC3, which appears as puncta.¹⁸ To verify whether autophagosome formation is activated by SUMO1 in H4 neuroglioma cells, H4 cells were transiently transfected with GFP-tagged LC3 and then incubated without or with 100 mM trehalose, which enhances autophagy,¹⁹ as a positive control. Autophagic markers, observed as LC3-II-positive dots, which represent autophagic vacuoles, were dispersed as small puncta throughout the cytosol of H4 cells. When the H4 cells were treated with 100 mM trehalose after transient transfection with GFP-tagged LC3, more LC3-II-positive vesicular puncta were observed throughout the entire H4 cell (Fig. 2A). After transient cotransfection of H4 cells with GFP-tagged LC3 and myc-tagged SUMO1, LC3-II positive autophagosomes were increased compared to control cells.

Cytoplasmic acidic vacuolation is a typical metabolic feature of autophagosome formation and autofluorescent monodansylcadaverine (MDC) has been used to stain AVs.²⁰ MDC staining appeared mainly as small dispersed puncta in the cytosol and was weakly enriched in the soma in H4 cells. As a positive control, H4 cells were treated with 100 mM trehalose and showed increased AVs with MDC staining (Fig. 2B). HGS1 cells also increased remarkably with MDC-positive AVs in the cytosol with markedly stronger intensity throughout the entire cell body (Fig. 2B). To monitor the process of autophagy maturation, we used H4 cells stably expressing tandem mRFP-GFP-tagged LC3.^{21,22} The results showed that overexpression of SUMO1 increased the expression of LC3 and the colocalization of both mRFP and GFP fluorescence, indicating autophagosome formation was increased (Fig. 2C). Since GFP fluorescence decays in the acidic condition of autolysosomes, vacuoles with the red fluorescence of mRFP represent autolysosomes. SUMO1-overexpressed cells had some vacuoles with the red fluorescence of mRFP, indicating that autolysosomes were also formed (Fig. 2C). As a positive control, trehalose treatment dramatically increased the red fluorescence of mRFP representing autolysosomes, suggesting that trehalose increase autophagic flux (Fig. 2C). Together, these results implicate that overexpression of SUMO1 may increase autophagosome formation.

Consistent with the results of GFP-LC3 dots or MDC-positive AVs, electron microscopy analysis (Fig. 3A, C and E;

inserts Fig. 3B, D and F) revealed that there were abundant autophagosomes in the cytosol of SUMO1-transfected H4 cells (HGS1) (Fig. 3C, D and G). As a positive control, treatment of trehalose also increased autophagosome formation in H4 cells (Fig. 3E, F and G). Collectively, these results indicate that SUMO1 increases autophagosome formation in H4 neuroglioma cells.

Figure 2. For figure legend, see page 103.

Figure 3. Ultrastructural features of SUMO1-transfected H4 cells. ((A)to F) Control H4 cells, HGS1 cells or H4 cells treated with 100 mM trehalose for 24 h were harvested and fixed, and the electron microscopic observation was performed. (B, D, F) High magnification images of boxed areas. Scale bars = 2 μ m (A, C, E) and 500 nm (B, D, F). Numerous vesicles are accumulated in the cytoplasm (white arrowheads) together with typical double-membrane-bound autophagosomes (enlarged inset). (G) Number of autophagosomes or autolysosome per cell was calculated by counting the number of double-membrane organelles in 10 or more individual cells. * indicates $P < 0.001$.

Depletion of SUMO1 decreases LC3-II levels in H4 cells

Next, we investigated whether SUMO1 depletion inhibits autophagy induction in H4 cells. After H4 cells were transfected with *SUMO1* shRNA for 60 h, the cells were harvested and subjected to western blot analysis. When the SUMO1 levels were knocked down in H4 cells using shRNA, a 40% decrease in SUMO1 protein levels was observed compared with endogenous SUMO1 (Fig. 4A). Interestingly, SUMO1 depletion reduced the LC3-II levels in H4 cells ($P < 0.05$; Fig. 4B and C). The BECN1 levels were not changed significantly in H4 cells (data not shown). To further investigate autophagic flux, we treated cells with BafA for 1 h under SUMO1

depletion conditions. As expected, BafA treatment increased LC3-II level in H4 cells transfected with shControl. We found that depletion of SUMO1 decreased accumulation of LC3-II by BafA treatment compared with shControl-transfected cells treated with BafA (Fig. 4B and C).

SUMO1 increases A β production via autophagy

In a previous study, macroautophagy-induced A β generation is suppressed by treatment with the autophagy inhibitor 3-methyladenine (3-MA).^{16,23} To verify whether autophagy inhibition reduces A β secretion, we used the autophagy inhibitor 3-MA in H4 cells. The A β_{1-40} levels in conditioned medium were

Figure 2 (See previous page). Autophagy is activated by SUMO1 in H4 cells. (A) H4 cells were transiently transfected with GFP-tagged LC3 and then incubated with or without 100 mM trehalose for 24 h. H4 cells treated with trehalose showed increased LC3-II-positive vesicular puncta. H4 cells transiently cotransfected with GFP-tagged LC3 and myc-tagged SUMO1 also contained markedly more LC3-II-positive autophagosomes. GFP-LC3 puncta number per cell in 20 cells chosen in random was quantified using the ImageJ program. The results shown are mean $\pm$ SEM; * $P < 0.05$. (B) H4 cells were incubated with or without 100 mM trehalose for 24 h. H4 and HGS1 cells were stained with the autophagic marker MDC. H4 cells treated with 100 mM trehalose contained more autophagic vacuoles. HGS1 cells also showed remarkably more MDC-positive vacuoles. MDC puncta number per cell in 20 cells chosen in random was quantified using the ImageJ program. The results shown are mean $\pm$ SEM; * $P < 0.05$. (C) H4 cells stably expressing tandem mRFP-GFP-tagged LC3 were transfected with mock vector and myc-SUMO1. Twenty-4 h after transfection, the cells were fixed and analyzed under fluorescence microscopy. After H4 cells stably expressing tandem mRFP-GFP-tagged LC3 were treated with 100 mM trehalose, the cells were fixed and analyzed under fluorescence microscopy. Yellow or red puncta number per cell in 20 cells chosen in random was quantified using the ImageJ program. The results shown are mean $\pm$ SEM; * $P < 0.05$. This is a representative image of triplicate experiments.

Figure 4. SUMO1 depletion decreases LC3-II protein levels in H4 cells. **(A)** H4 cells were transiently transfected with shSUMO1 or shControl and collected after 60 h. After SUMO1 depletion using shRNA, western blotting was performed with anti-LC3, anti-BECN1, and anti-SUMO1. Anti-TUBA4A (TUBA) was used as a loading control. **(B)** After H4 cells were transiently transfected with shSUMO1 or shControl for 72 h, the cells were treated with 1 nM BafA for 1 h. Western blotting was performed with anti-LC3 antibody. **(C)** The LC3-II/actin band intensities were quantified using NIH image software and expressed as percentage of the control level. This is a representative image of triplicate experiments. Values represent the mean $\pm$ SEM of 3 experiments. * indicates $P < 0.05$.

measured using a sensitive enzyme-linked immunosorbent assay (ELISA). It was blocked by about 20% compared to the control in H4 cells stably expressing human wild-type APP695 (HAMg cells) after treatment with 5 mM 3-MA ($P < 0.05$; Fig. 5A). To confirm this inhibitory effect of 3-MA on autophagosome formation, protein gel blot analysis was used to determine LC3-II levels. As expected, LC3-II levels were decreased by treatment with 3-MA (Fig. 5B). Cotreatment of 3-MA and BafA reduced accumulation of LC3-II, indicating 3-MA inhibit autophagic flux in HAMg cells. Previously, we reported that overexpression of SUMO1 increases A β generation.¹⁷ Hence, we investigated whether SUMO1-induced A β production is due to autophagosome formation. We determined the A β levels following SUMO1 overexpression in the presence or absence of 3-MA in H4 cells stably expressing Swedish mutant-type APP695 (APP_{sw}) (HSW cells). As in HAMg cells, the level of secreted A β_{1-40} in conditioned medium in HSW cells was also blocked by about 30% compared to the control in HSW cells after treatment with 5 mM 3-MA for 8 h ($P < 0.05$; Fig. 5C). The production of A β_{1-40} in conditioned medium with SUMO1-transient transfection was increased by about 370% compared to the control. When cells were treated with 5 mM 3-MA for 8 h, A β_{1-40} production was 30% less ($P < 0.05$; Fig. 5C). Moreover, when HSW cells were treated with 5 mM 3-MA for 16 h, 3-MA markedly inhibited SUMO1-mediated A β production in HSW cells (Fig. 5D).

Next, we also investigated the effect of wortmannin (an inhibitor of autophagosome initiation) on SUMO1-mediated A β production (Fig. 5E and F). As expected, the accumulation of LC3-II was decreased in cells treated with wortmannin (Fig. 5E). When we treated HSW cells with wortmannin for 8 h, the level of secreted A β_{1-40} in conditioned medium was also blocked by about 30% compared to the control in HSW cells after treatment with wortmannin (Fig. 5F). Furthermore, wortmannin markedly reduced A β production from SUMO1-transfected HSW cells

(Fig. 5F). It is known that the BECN1-PIK3C3 complex is a core components of class III phosphatidylinositol 3-kinase, whose catalytic unit is PIK3C3.²⁴ Since the recruitment of the BECN1-PIK3C3 complex is essential to initiate phosphatidylinositol-3-phosphate generation and autophagosome formation, we investigated whether overexpression of SUMO1 affects BECN1-PIK3C3 complex formation (Fig. 6). Immunoprecipitation was carried out with an anti-PIK3C3 antibody in H4 cells and HGS1 cells. BECN1 could be detected in PIK3C3 immune complexes in H4 cells by western blotting using anti-BECN1 antibody. Furthermore, BECN1-PIK3C3 complex was increased in HGS1 cells compared to H4 cells (Fig. 6). These results suggest that overexpression of SUMO1 increases BECN1-PIK3C3 complex and autophagosome formation.

Depletion of ATG5, ATG7, or ATG12 inhibits SUMO1-mediated A β production

We examined whether there is autophagy-related (ATG) gene involvement in SUMO1-mediated A β production. ATG5 and ATG7 are essential for macroautophagy. ATG12 is conjugated to ATG5 to form the ATG12-ATG5 complex that acts as an E3 enzyme in the LC3 lipidation for autophagosome formation.^{25,26} Thus, we inhibited the autophagy pathway by transfecting ATG7, ATG5 or ATG12 siRNA into HSW cells (Fig. 7). As seen in Fig. 7A, C and E, the protein level of ATG5, ATG7 or ATG12 was downregulated by each siRNA, respectively. The level of secreted A β_{1-40} in conditioned medium was slightly reduced by transfection of ATG7 or ATG5 siRNA (Fig. 7B and D). We found a weak but significant decrease of A β production by ATG5 or ATG7 depletion in SUMO1-transfected HSW cells (Fig. 7B and D). When HSW cells were transfected with ATG12 siRNA, we observed a ~30% decrease in A β levels in HSW cells (Fig. 7F). Furthermore, depletion of ATG12 markedly reduced A β production from SUMO1-transfected HSW cells (Fig. 7F).

Figure 5. A β production was reduced by autophagy inhibitor 3-MA. **(A)** HAMg cells were incubated with or without the autophagy inhibitor 3-MA (5 mM) for 24 h. The yield of secreted A β_{1-40} in conditioned medium was measured by ELISA. A β_{1-40} production was blocked by 3-MA in HAMg cells. **(B)** After HAMg cells were incubated with 5 mM 3-MA for 18 h, cells were treated with 10 nM BafA for 3 h. Western blotting was performed with anti-LC3. Actin was used as a loading control. **(C and D)** HSW cells were transiently transfected with control or SUMO1 for 48 h. After treatment with or without 5 mM 3-MA for 8 h **(C)** or 16 h **(D)**, the secreted A β_{1-40} levels in conditioned medium were measured using ELISA. **(E)** After cells were treated with 1 μ M wortmannin for 3 h, cells were treated with 10 nM BafA for 3 h. The levels of LC3-II protein were compared by protein gel blotting using anti-LC3 antibody. LC3-II band intensity was quantified using NIH image software and expressed as percentage of the control level. * indicates $P < 0.05$. **(F)** After treatment with or without 1 μ M wortmannin for 8 h, the level of secreted A β_{1-40} in conditioned medium was measured using ELISA. * indicates $P < 0.05$. This is a representative image of triplicate experiments. Values represent the mean $\pm$ SEM of 3 experiments.

Together, these results support the finding that autophagosome formation contributes to SUMO1-mediated A β production.

SUMO1 increases A β production via HIF1A

Since overexpression of SUMO1 can induce autophagic activation by HIF1A stabilization,^{8,11,12} we next investigated the effect of HIF1A depletion on SUMO1-induced A β production. After HSW cells were transfected with siRNA against *HIF1A*, the knockdown of HIF1A was confirmed by protein gel blotting (Fig. 7G). Interestingly, the level of secreted A β_{1-40} in conditioned medium was reduced by about 28% compared to the HSW cell transfected with scrambled RNA as a control (Fig. 7H). Furthermore, the level of secreted A β_{1-40} in conditioned medium from HSW cells cotransfected with GFP-tagged SUMO1 together with siRNA of *HIF1A* was reduced by approximately 40% compared with the HSW cells transfected with

GFP-tagged SUMO1 (Fig. 7H). These results suggest that HIF1A also contributes to the SUMO1-mediated A β production.

Depletion of SUMO1 decreases the ATG12-ATG5 complex

Since depletion of HIF1A was decreased A β production, we investigated the level of BECN1. It has been reported that the levels of BECN1 are not decreased by depletion of *HIF1A* by siRNA in normoxia condition.²⁷ In our study, the levels of BECN1 were not altered by depletion of HIF1A in H4 cells (Fig. 8A). Interestingly, we found that depletion of HIF1A decreased the ATG12-ATG5 complex. Next, we wondered whether depletion of SUMO1 could affect the level of the ATG12-ATG5 complex. Although depletion of SUMO1 could not change the level of BECN1 (Fig. 4A), the levels of the ATG12-ATG5 complex were decreased after depletion of

Figure 6. SUMO1 increases BECN1-PIK3C3 complex formation. H4 and HGS1 cell lysates were immunoprecipitated (IP) with anti-PIK3C3, and then the BECN1-PIK3C3 complex formation was analyzed by immunoblotting using anti-BECN1 and anti-PIK3C3 antibodies. This is a representative experiment of 2 performed.

SUMO1 (Fig. 8B). Our results suggest that depletion of SUMO1 or HIF1A may decrease the ATG12–ATG5 complex and this may lead to the reduction of LC3-II.

Both SUMO1 and LC3 localize to the amyloid plaques in APP transgenic mouse brain

Several lines of investigation demonstrate a marked accumulation of the LC3 proteins in murine models of AD.^{15,28} Previously,

we have reported that increased SUMO1 immunoreactivity is found in brains of mouse models of AD.¹⁷ To further investigate the relationship between SUMO1 and LC3 elevation in brain of a murine model of AD, we first determined whether autophagosome formation was induced in APP Swedish/PS1dE9 transgenic (APP Tg) mice. The LC3-II levels in the brain of APP Tg mice were determined by western blot (Fig. 9A). Our data clearly demonstrated the existence of a marked accumulation of LC3-II in the brain of 18-month-old APP Tg mice (Fig. 9A). These observations were further confirmed by immunostaining of brain sections from 16-month-old wild type and Tg mice. Diaminobenzidine-stained images of the cortex are shown in Fig. 9B. The immunoreactivities of LC3 and SUMO1 were increased in the brain of APP Tg mice (Fig. 9B). Consistent with the previous observation,²⁹ we also found that some of the amyloid plaques stained by Congo Red in the brain of APP Tg mice were surrounded by LC3 immunopositive cells (Fig. 9B). When the cortex of the mouse model of AD was investigated by double-label immunofluorescence confocal microscopy, colocalization of both LC3 and SUMO1 was increased in APP Tg mice compared to wild-type mice (Fig. 9C). Furthermore, colocalization of both ATG12 and SUMO1 was also increased in APP Tg mice compared to wild-type mice (Fig. 9D). Our data suggests that SUMO1 may be involved in autophagosome accumulation in AD.

Figure 7. Inhibition of autophagy decreases SUMO1-mediated A β production. HSW cells were transiently transfected with siControl (siNON), siRNA of ATG5 (A and B), siRNA of ATG7 (C and D), siRNA of ATG12 (E and F) or siRNA of HIF1A (G and H) for 48 h. And then cells were transfected with mock vector or SUMO1 for 24 h, the secreted A β ₁₋₄₀ levels in conditioned medium were measured using ELISA (B, D, F, or H). Total cell extracts were analyzed by western blotting using anti-ATG5 (A), anti-ATG7 (C), anti-ATG12 (E) or anti-HIF1A antibody (G). This is a representative image of triplicate experiments. Values represent the mean $\pm$ SEM of 3 experiments. * indicates $P < 0.05$.

Figure 8. Depletion of SUMO1 decreases the ATG12-ATG5 complex. (A) H4 cells were transiently transfected with siControl or siRNA of *HIF1A* for 72 h. Western blotting was performed with anti-ATG12 or anti-BECN1. (B) H4 cells were transiently transfected with sh*SUMO1* or shControl and collected after 72 h. Western blotting was performed with anti-ATG12. Anti-actin was used as a loading control. This is a representative experiment of 2 performed.

Discussion

Our previous studies revealed that enhanced A β levels are upregulated by SUMO1 in neuronal cells. Furthermore, we had found that the overexpression of SUMO1 resulted in a highly significant accumulation of BACE1 and A β production. Here, we demonstrated a novel function of SUMO1 in regulating autophagy-dependent A β production. We found that the overexpression of SUMO1 stimulated autophagosome formation, and that A β production by SUMO1 was blocked by the autophagy inhibition using inhibitors (3-MA, wortmannin) or genetic depletion (siRNA targeting *ATG12*, *ATG5* and *ATG7*).

Post-translational modifications of proteins by ubiquitin and ubiquitin-like proteins can change the activity and stability of proteins. Like ubiquitin, SUMO also frequently appears to alter protein function. In the case of the protein HTT/huntingtin, ubiquitin promotes protein degradation, whereas SUMO exerts a stabilizing effect. Shao et al. report a significant increase in *SUMO1* mRNA and protein after hypoxic stimulation *in vivo*.⁸ They demonstrate that the increased levels of SUMO1 participate in the modulation of HIF1A function in the brain. HIF1A is stabilized by SUMO modification and promotes autophagy.¹⁰ Furthermore, cerebral ischemia induces an increase in sumoylated proteins in the hippocampus³⁰ and increases autophagic activity.^{31,32} Here, we showed that the overexpression of SUMO1 induces autophagosome formation. We also observed that overexpression of SUMO2 or SUMO3 induced autophagy (data not shown). Our previous report shows that SUMO1 level is increased in a mouse model of AD, whereas SUMO2/3 levels are not changed in this model.¹⁷ A β production was decreased by the depletion of SUMO1, but not that of SUMO2/3.¹⁷ Furthermore, the tau protein is modified by SUMO1,³³ which also supports our conclusion. Taken together, these results implicate that increased autophagosome formation in AD due to the accumulation of SUMO1 may play an important role in AD pathogenesis.

Unwanted aggregates or accumulations of waste proteins could result from the dysregulation of autophagic degradation. Autophagic marker proteins such as LC3 are associated with amyloid

plaque in AD.³⁴ Several reports have revealed that A β , APP, BACE1-derived C-terminal fragment, and γ -secretase are enriched in LC3-positive autophagosomes in mouse models of AD,^{16,35,36} suggesting that these autophagic vacuoles are a key source of A β production in AD. Regarding the hypoxia-mediated pathogenesis of AD, it has also been reported that repeated hypoxia increases A β generation and neuritic plaque formation and that autophagy is involved in the hypoxia-induced increase in A β production.²³ Although it is speculated that hypoxia or ischemia may contribute to AD pathogenesis, molecular links between those have not well established. It is noteworthy that the depletion

of HIF1A attenuated A β production in HSW cells, which was more dramatic in the cells transfected with SUMO1 (Fig. 7H). Given that the sumoylation of HIF1A increases its own stability, HIF1A may contribute to A β production via autophagy. Consistent with this hypothesis, in H4 cells stably expressing APP, treatment with the autophagy inhibitors 3-MA or wortmannin (Fig. 5) decreased A β production, suggesting that autophagosome formation increases A β production. It has also been demonstrated that autophagy is involved in the A β production caused by hypoxia.²³ We also demonstrated that A β production by SUMO1 overexpression was attenuated by treatment with the autophagy inhibitors 3-MA and wortmannin and by genetic inhibition (siRNA targeting *ATG12*, *ATG5* and *ATG7*), suggesting that the elevation of SUMO1 by various stress conditions, including A β accumulation and hypoxia, precedes the formation of autophagic vesicles and leads to A β production and the progression of AD. Although mammalian macroautophagy can occur through an *Atg5* and *Atg7*-independent alternative pathway,³⁷ it is believed that *Atg5* and *Atg7* are important genes for autophagy. Given that knockdown of ATG5 or ATG7 reduced SUMO1-mediated A β production, our observation indicates that ATG5 and ATG7-dependent conventional autophagy pathway may contribute to SUMO1-mediated A β production. It is worthy of note that autophagic marker proteins such as ATG12 and LC3 are associated with amyloid plaque in human AD.³⁴ Colocalization of ATG12 with SUMO1 has been observed in APP Tg mice (Fig. 9D). Furthermore, depletion of SUMO1 or HIF1A decreased levels of the ATG12-ATG5 complex (Fig. 8). Given that depletion of ATG12 reduced A β production (Fig. 7F), our observation indicates that ATG12 is important for autophagosome-mediated A β production during AD pathogenesis.

Increasing evidence suggests that autophagy is activated by various stress, such as ischemia and inflammation.³⁸ It is also implicated in many diseases, such as cancer and neurodegenerative diseases.³⁹ Under hypoxic or ischemic conditions, autophagy degrades unwanted proteins, while the abnormal activation of autophagy might promote cell death. Wang et al. show that A β induces autophagic cell death via reactive oxygen species in

Figure 9. For figure legend, see page 109.

neuronal cells.⁴⁰ Autophagy is regulated by the MTOR (mechanistic target of rapamycin) kinase pathway, which is controlled by the AKT pathway or AMPK.⁴¹ It has been reported that the formation of AVs is mediated by the activation of AMPK in the brains of APP PS1 double-transgenic mice.⁴² Previously, we have demonstrated that SUMO1 levels are increased in the mouse model of AD and that A β increased SUMO1 levels in neuronal cells and vice versa. We also note that SUMO1 and AVs formation are increased in the brain of APP Tg mice. These data implicate a novel relationship between A β and the induction of autophagosome via SUMO1. Here, SUMO1 increased autophagic flux (Figs. 1 and 3), but is likely to make autolysosomes be partially defective because the number of red AVs was relatively lowered compared to trehalose (Fig. 2C). Furthermore, we also observed that the cellular levels of SQSTM1/p62, which is degraded in autolysosomes, were increased in HGS1 cells (unpublished data). Taken together, it might explain why AVs are enriched in AD.

Much evidence suggests that the existence of autophagosomes in the brain of mouse models of AD is evidenced by increased LC3 immunoreactivity.²⁹ In this study, we also found that LC3 immunoreactivity was increased in the murine model of AD. Because autophagosomes can be detected in human brains and the mouse model of AD, and accumulate in swollen dystrophic neurites,^{3,16} they are thought to be a sign of impaired autophagosomal degradation. It is noteworthy that increased SUMO1 immunoreactivity was colocalized with LC3 in the mouse model of AD (Fig. 9). Pathological inclusions containing misfolded proteins are a frequent feature of age-related neurodegenerative diseases, including AD, Parkinson disease, Huntington disease, spinocerebellar ataxias, and amyotrophic lateral sclerosis.⁴³ There is growing evidence that proteins involved in neurodegeneration, such as ATXN1/ataxin 1,⁴⁴ HTT,⁴⁵ MAPT/tau, and SNCA/ α -synuclein,³³ are sumoylated. Taken together, SUMO1 might activate the formation of autophagosomes, implicating increased SUMO1 in neurodegenerative diseases including AD via the unwanted accumulation of proteins and autophagosome formation. Further studies must be conducted to determine whether manipulating the autophagic pathway has therapeutic potential in early- and late-onset AD.

In conclusion, we have provided evidence of SUMO1-mediated autophagic activation and A β production. SUMO1-mediated autophagosome formation might be a key step in A β production by SUMO1. Our results help to elucidate the molecular mechanism of AV accumulation in AD pathologies.

Materials and Methods

Materials

Three-methyladenine (3-MA) (M9281), and trehalose (T5251), bafilomycin A₁ (B1793), wortmannin (W1628) and monodansylcadaverine (MDC) (30432) were obtained from Sigma. The following primary antibodies were used: anti-SUMO1 (Cell Signaling Technology, 4930), anti-GFP (Molecular Probes, A11122), anti-LC3 (MBL, PD014), anti-BECN1 (Cell Signaling Technology, 3495), anti-ATG5 (Cell Signaling Technology, 8540), anti-ATG7 (Cell Signaling Technology, 2631), anti-ATG12 (Cell Signaling Technology, 4180), anti-PIK3C3 (Thermo, PA1-46456), anti-PIK3C3 for IP (Echelon, Z-R015), anti-HIF1A (Cell Signaling Technology, 3716), anti-Myc (Cell Signaling Technology, 2272), anti-ACTB/ β -actin (Sigma, A3853) and anti-TUBA4A/ α -tubulin (Sigma, T6074).

Animals

APP transgenic mice were used in the present study, as previously reported.¹⁷ All studies were conducted with a protocol approved by the local Institutional Animal Care Use Committee in compliance with Korea National Institute of Health guidelines for the care and use of experimental animals.

Cell culture and stable cell lines

Human neuroglioma H4 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, 11995-065) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, 15140). H4 cells stably expressing wild-type human APP with MYC tag (HAMg), mRFP-GFP-LC3 or SUMO1 with EGFP tag (HGS1) were established by G418 selection. H4 cells stably expressing the Swedish FAD mutant APP695 (HSW) have been reported previously.⁴⁶

Plasmids

SUMO1 was subcloned into pEGFP-C1 (Clontech, 6084-1) or pCS2-MT with a Myc tag.¹⁷ GFP-LC3 plasmid and mRFP-GFP-LC3 was a gift from Dr. Tamotsu Yoshimori (Osaka University, Japan).²

siRNA and shRNA

The target sequence of shSUMO1, has been already reported, and was synthesized by Bioneer (Daejeon, Korea).¹⁷ The siRNA against *HIF1A* (L-004018-00-0005) was purchased from Dharmacon RNA Technology. The siRNA against *ATG5* (6345) and

Figure 9 (See previous page). Immunostaining for SUMO1 and LC3 in the brain of APP Tg mice. **(A)** The LC3-II/I ratio in the brain of 18-month-old wild type and amyloid precursor protein (APP) transgenic mice was determined by protein gel blot. Actin was used as a loading control. LC3-II/I band intensity ratios were quantified using NIH ImageJ software and expressed as percentage of the WT. *indicates $P < 0.01$. Values represent the mean $\pm$ SEM of 3 experiments. **(B)** Brain sections from 16-month-old wild type and APP transgenic mice were immunostained with anti-LC3 or anti-SUMO1, and counterstained with Congo Red for amyloid plaques. Congophilic plaque was indicated by an asterisk. **(C)** Sections from the cortex of wild type and APP Tg mice were immunolabeled with anti-LC3, and colabeled with anti-SUMO1, and imaged with a laser scanning confocal microscope. Immunoreactivity of SUMO1 and LC3 are visualized by double staining with Alexa 488 and Alexa 555, respectively. **(D)** Sections from the cortex of 21-month-old wild type and APP Tg mice were immunolabeled with anti-ATG12, and colabeled with anti-SUMO1, and imaged with a laser scanning confocal microscope. Immunoreactivity of SUMO1 and ATG12 are visualized by double staining with Alexa 488 and Alexa 555, respectively.

siRNA against *ATG7* (6604) were purchased from Cell Signaling Technology. The siRNA against *ATG12* (sc-72578) was purchased from Santa Cruz Biotechnology. The negative control siRNA (SN-1013) was purchased from Bioneer.

Immunoprecipitation

Cells were washed with phosphate-buffered saline (PBS; Sigma, P3813) and lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100 [Sigma, T8532]) supplemented with protease inhibitor (Sigma, P8340). The lysates from each sample set were incubated with protein A/G-agarose (Santa Cruz Biotechnology, sc-2003) for 2 h at 4 °C for preclearing. The pre-cleared lysates were incubated with the primary antibody overnight at 4 °C. After the incubation with A/G agarose for 2 h, the resin was washed, and the pellets were resuspended in SDS sample buffer.

Western blotting

Cells were washed with PBS and lysed with 1×RIPA (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na₃VO₄, 10 mM NaF, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid [EGTA; Sigma, E0396], 0.2 mM phenylmethylsulfonyl fluoride (Sigma, P7626), 1% Triton X-100, 0.1% SDS [Sigma, L5750], 0.5% deoxycholate [Sigma, D6750]). Total proteins were separated by NuPage 4–12% BisTris-polyacrylamide gel electrophoresis (Invitrogen, NP0335) using MES SDS running buffer (Invitrogen, NP0002–02). For quantification, blots were scanned, and the signal densities were measured using NIH ImageJ software.

Immunohistochemistry

Brains from 16-mo-old APP Swedish/PS1dE9 Tg mice together with their wild-type controls were fixed in 4% (w/v) paraformaldehyde. Cryostat sagittal sections were cut on a sliding microtome into 10 µm slices at –20 °C and placed on a microslide for immunostaining. The sections were immunostained with mouse monoclonal antibodies against LC3 (1:200, Abgent, AM1800a), SUMO1 (1:100, Invitrogen, 33–2400), and rabbit polyclonal antibodies against SUMO1 (1:100; Cell Signaling Technology, 4930), ATG12 (1:100, Cell Signaling Technology, 4180). Sections were then incubated for 1 h at room temperature with secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen, A11001, A11008) and 568 (Invitrogen, A11004, A11011). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) using ProLong[®] Gold antifade reagent with DAPI mounting solution (Invitrogen, P36931). When required, immunolabeled sections were then incubated for 3 min in a solution of 20% Congo Red (Sigma, C6277). AxioLab Pol Polarizing microscope (Carl Zeiss, Jena, Germany) with Axio Vision Release 4.8 software was used for analysis of 3,3'-diaminobenzidine photomicrographs. Confocal images were captured with an Olympus FluoView 1000 microscope (Tokyo, Japan).

Labeling autophagic vacuoles with monodansylcadaverine

Monodansylcadaverine is a spontaneously fluorescent dye that is incorporated into autophagosomes and autolysosomes. Cells

were washed twice with PBS and incubated with 50 µM MDC in PBS at 37 °C for 30 min. After incubation, the cells were washed 3 times with PBS and analyzed immediately by fluorescence microscopy (excitation wavelengths 380 to 420, barrier filter 450 nm) using an Olympus IX71 microscope (Tokyo, Japan).

ELISA

Secreted Aβ_{1–40} was measured in conditioned medium using SoftMax Pro 5.0 software. Concentrations of Aβ_{1–40} were quantified using commercial human Aβ ELISA kits (Invitrogen, KHB3482) according to the manufacturer's instructions.

Electron microscopy (EM)

Human neuroglioma H4 cells and HGS1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, 15140). For positive control, H4 cells were treated with 100 mM of trehalose for 24 h. Cells were fixed overnight in a mixture of cold 2.5% glutaraldehyde (EMS, 16020) in 0.1 M phosphate buffer (pH 7.2) and 2% paraformaldehyde (Merck, 1.04005.1000) in 0.1 M phosphate buffer (pH 7.2) and embedded with epoxy resin (EMS, 14120). The epoxy resin-mixed samples were loaded into capsules and polymerized at 60 °C for 48 h. Thin sections were sliced on an ultramicrotome (Leica, UC7, Wetzlar, Germany) and collected on a copper grid. Appropriate areas for thin sectioning were cut at 70 nm and stained with saturated 2% uranyl acetate (EMS, 22400) and lead citrate (EMS, 17800) before examination on a transmission electron microscope (Carl Zeiss, LIBRA 120, Oberkochen, Germany) at 120 kV.

Statistical analyses

Data were expressed as mean ± SEM. Statistical analyses were performed using the Student *t* test and ANOVA followed by the Duncan *post-hoc* test using an SPSS program (SPSS, Inc.) to study the relationships among variables with NIH Image 5.0 software. All assays were repeated at least 3 times, and each experiment was performed at least in triplicate. *P* values < 0.05 were considered to indicate statistical significance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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