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Technical note

Diazotization of kynurenine by acidified nitrite secreted from indoleamine 2,3-dioxygenase-expressing myeloid dendritic cells

Toshiaki Hara [a], Fumiyuki Yamakura [b], Osamu Takikawa [c], Rie Hiramatsu [a], Tsutomu Kawabe [a], Ken-ichi Isobe [d], Fumihiko Nagase [a,*]

[a] *Department of Medical Technology, Nagoya University School of Health Sciences, Nagoya, Aichi, Japan*
[b] *Department of Chemistry, Juntendo University School of Medicine, Inba, Chiba, Japan*
[c] *National Institute of Longevity Science, National Center for Geriatrics and Gerontology, Obu, Aichi, Japan*
[d] *Department of Immunology, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan*

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Abstract

Indoleamine 2,3-dioxygenase (IDO)-initiated tryptophan metabolism along the kynurenine (Kyn) pathway regulates T-cell responses in some dendritic cells (DC) such as plasmacytoid DC. A Kyn assay using HPLC showed that samples were frequently deproteinized with trichloroacetic acid (TCA). In the present study, bone marrow-derived myeloid DC (BMDC) were differentiated from mouse bone marrow cells with GM-CSF. CpG oligodeoxynucleotides (CpG) induced the expression of IDO protein with NO production in BMDC cultured for 24 h. The concentrations of Kyn in the culture supernatants were not increased by stimulation with CpG but rather decreased by based on the Kyn assay after deproteinization with TCA. The level of Kyn exogenously added into the cell-free culture supernatant of BMDC stimulated with CpG was severely decreased by deproteinization with TCA but not methanol, and the decrease was prevented when BMDC was stimulated with CpG in the presence of a NOS inhibitor. Under acidic conditions, Kyn reacted with nitrite produced by BMDC, and generated a new compound that was not detected by Ehrlich reagent reacting with the aromatic amino residue of Kyn. An analysis by mass spectrometry showed the new compound to be a diazotization form of Kyn. In conclusion, the deproteinization of samples by acidic treatment should be avoided for the Kyn assay when NO is produced.

Keywords: Bone marrow-derived myeloid dendritic cells; Indoleamine 2,3-dioxygenase; Tryptophan; Kynurenine; Nitric oxide; Diazotization

1. Introduction

The indoleamine 2,3-dioxygenase (IDO)-initiated tryptophan (Trp) metabolism along the kynurenine (Kyn) pathway regulates the T-cell responses in some dendritic cells (DC) such as plasmacytoid DC or $CD8^-$ DC in mouse spleen cells (Mellor and Munn, 2004). IDO is induced by inflammation or immune responses such as infections or tumor immunity. IDO expression is induced in DC by various stimuli such as IFN-γ,

Abbreviations: BMDC, bone marrow-derived myeloid dendritic cells; CpG, CpG oligodeoxynucleotides; DC, dendritic cells; IDO, indoleamine 2,3-dioxygenase; Kyn, kynurenine; NMA, N^G-monomethyl-L-arginine acetate; NO, nitric oxide; NOS, NO synthase; TCA, trichloroacetic acid; Trp, tryptophan.

* Corresponding author. Department of Medical Technology, Nagoya University School of Health Sciences, 1-20 Daikominami-1-chome, Higashi-ku, Nagoya, Aichi, 461-8673, Japan. Tel./fax: +81 52 719 1189.
E-mail address: nagase@met.nagoya-u.ac.jp (F. Nagase).

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toll-like receptor (TLR)-ligation by LPS or CpG oligodeoxynucleotides (CpG) or CD80/CD86-ligation by CTLA-4 expressed on the regulatory T cells (Mellor and Munn, 2004). The induction of IDO activity was observed in IFN-γ-activated murine peritoneal macrophages when nitric oxide synthase (NOS) was inhibited (Thomas et al., 1994). Incorporation of the heme prosthetic group into the active site is required for IDO activity, and the inhibition of IDO activity by NO generators was abrogated by co-addition of oxyhemoglobin, an antagonist of NO function (Thomas et al., 1994). NO also led to an accelerated degradation of IDO protein in the proteasome (Hucke et al., 2004).

To determine IDO activity, three assay methods are commonly used. They are based on the conversion of L-Trp to *N*-formyl-kynurenine followed by hydrolysis to produce Kyn. The amount of Kyn produced can be quantified directly by HPLC or through radiometric methods. Alternatively, a colorimetric method is used to measure Kyn indirectly. This technique relies on absorption of the imine produced by the reaction of the aromatic amino group of Kyn with *p*-dimethylaminobenzaldehyde (Ehrlich reagent) at λ max 480 nm (Takikawa et al., 1988). Formerly, Kyn was measured by diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine (Mason and Berg, 1952). For diazotization, Kyn was treated with sodium nitrite in trichroloacetic acid (TCA) solution. IDO activity has been frequently estimated by the Kyn assay using samples deproteinized by acidic treatment before HPLC assay (Takikawa et al., 1988; Fujigaki et al., 2002; López et al., 2006). Nitrite is a stable derivative of NO. It has therefore been suggested that acidic treatment induces an underestimation of Kyn when NO is produced.

In the present study, Kyn was degraded by treatment with TCA for deproteinization through the reaction with nitrite produced by BMDC stimulated with CpG. The errors were avoided by the Kyn assay using methanol for deproteinization.

2. Materials and methods

2.1. Reagents

The phosphorothioate CpG1826 (5-TCC ATG ACG TTC CTG ACG TT-3), N^G-monomethyl-L-arginine acetate salt (NMA), and L-Kyn were purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse IDO polyclonal antibody was prepared as described previously (Suzuki et al., 2001). Anti-inducible NOS (iNOS) polyclonal antibody was purchased from BD Bioscience (San Diego, CA). Human serum was purchased from Gemini Bio-Product (Woodland, CA).

2.2. Preparation of BMDC

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All experiments were done according to the Guidelines for Animal Experimentation Nagoya University. BMDC were generated as described previously (Inaba et al., 1992). Briefly, bone marrow cells were cultured in RPMI1640 medium (10% fetal calf serum, 300 μg/ml glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol) containing 0.3% GM-CSF supernatant (from murine GM-CSF producing Chinese hamster ovary cells, a gift from T. Sudo, Toray Silicon, Tokyo, Japan). The DC culture medium was changed every 2 days to remove any nonadherent cells. Loosely adherent clustering cells were collected on day 6 and then were used as immature DC. BMDC were activated by stimulation with CpG (5 μg/ml) alone or together with NMA (100 μM) for 24 h.

2.3. Characterization BMDC by flow cytometry, Western blot and NO production

For the detection of cell surface markers, cells were incubated with PE-conjugated anti-CD11c and FITC-conjugated anti-CD11b antibodies at 4 °C for 30 min. These cells were analyzed by an EPICS XL flow cytometer (Beckman Coulter).

A Western blot analysis was carried out as described previously (Du et al., 2000). BMDC were stimulated with CpG (5 μg/ml) with or without NMA (100 μM) for 24 h. The cell lysates were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels by electrophoresis and then transferred to a nitrocellulose membrane. The membrane was incubated with anti-IDO, anti-iNOS or anti-actin antibody at 4 °C overnight, then with horseradish peroxidase-conjugated anti-rabbit IgG antibody for 1 h at room temperature, and finally, developed with a Western lightning chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA).

The amount of NO production in the medium was estimated by the assay of nitrite using Griess reagent (Ding et al., 1988). Fifty microliter of each supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water). The absorbance of the mixture at 590 nm was determined by a plate reader, and nitrite concentration

was determined using standard solutions of sodium nitrite.

2.4. Assay for Kyn

Concentrations of Kyn were determined by HPLC as previously described (Takikawa et al., 1988; Widner et al., 1997), with minor modifications. Before HPLC assay, the culture medium was deproteinized by the treatment with 5% TCA or 86% methanol (1:6, vol/vol). Twenty-five microliters of sample was injected into a 5 μm endcapped Purospher RP-18 column (Merck, Darmstadt, Germany) and analyses were carried out at a flow rate 1.0 ml/min. The mobile phase was 10 mM acetic ammonium (pH 6.5) containing 10% methanol (Takikawa et al., 1988) or 15 mM acetic acid–sodium acetate (pH 4.0) containing 27 ml/l acetonitrile (Widner et al., 1997). Kyn was detected by UV-detector at a wavelength of 360 nm.

Kyn was also assayed indirectly by using Ehrlich reagent (0.4% *p*-dimethylaminobenzaldehyde in acetic acid; Takikawa et al., 1988). Samples treated or untreated with 5% TCA were centrifuged, and 100 μl supernatant was mixed with 100 μl Ehrlich reagent. The absorbance at 490 nm was determined.

2.5. UV visible spectroscopy and mass spectrometry

The spectra were recorded at room temperature on a UV-vis-NIR recording spectrophotometer (Shimadzu, Kyoto, Japan).

Mass spectroscopy for Kyn and its derivatives was carried out using a LCQ Deca XP (Thermo Fisher Scientific, CA, USA), which consisted of an electrospray and a 3D ion trap detector, by direct infusion of the samples. The conditions for the MS analysis were as follows: ion spray voltage, 2.5 kV; ion transfer tube, 250 °C; scan range, 150–500 m/z; picotip, 15 μm IDCE; collision energy, 35%.

3. Results

3.1. NO-linked secretion of TCA-triggered Kyn-degrading substances from BMDC stimulated with CpG

BMDC were differentiated with GM-CSF for 6 days. Most (67.1%) of the BMDC were $CD11c^+$ $CD11b^+$ myeloid DC (Fig. 1A). The expression of IDO and iNOS proteins was induced in BMDC stimulated with CpG for 24 h (Fig. 1B). NO production was also induced in BMDC stimulated with CpG (Fig. 1C). These results show that the expression of IDO protein and iNOS activity is induced in BMDC by stimulation with CpG.

The production of Kyn by the IDO^+ BMDC was examined in the culture supernatants by HPLC after deproteinization with TCA. The level of Kyn in the culture supernatant of IDO^+ BMDC was not increased even after the exogenous addition of Trp and rather decreased by the stimulation with CpG (data not shown). The concentration of exogenously added Kyn to the culture of BMDC markedly decreased by CpG stimulation and this decrease of Kyn concentration was partially inhibited by NMA (data not shown). These results suggest the possibility that NO-related products of BMDC degrade Kyn. The Kyn-degrading activity of the culture supernatants of BMDC was tested. Kyn (50 μM) was added for 2 h into the cell-free culture supernatant of BMDC stimulated with CpG in the presence or absence of NMA for 24 h. Because TCA and methanol have been used for deproteinization, both methods were tested comparatively. The Kyn concentration (50 μM) in the TCA-treated culture supernatants of BMDC stimulated with CpG was decreased by 50% and this decrease was prevented by NMA (Fig. 1D). In contrast, the Kyn concentration of the culture supernatant of BMDC stimulated with CpG was not decreased by methanol treatment. These results indicate that methanol treatment for deproteinization is suitable for the measurement of Kyn in order to avoid TCA-triggered Kyn degradation. The effects of pH of elution buffer in the HPLC assay on Kyn degradation were also tested. The Kyn concentrations estimated by HPLC using an elution buffer at pH 4.0 and pH 6.5 were almost identical. These results indicate that elution buffer at higher pH than pH 4.0 is suitable for avoiding Kyn degradation by acidic treatment. The HPLC elution profile of the absorption at 360 nm was shown in Fig. 1E. The treatment of the culture supernatant of BMDC stimulated with CpG with TCA yielded a new absorption peak at a shorter retention time (3.9 min), corresponding to the decrease of the absorption peak of Kyn at a retention time (6.3 min).

3.2. Reaction of Kyn with nitrite under acidic conditions detected by HPLC, Ehrlich reagent and absorption spectra

The amount of Kyn-degrading substance secreted from BMDC was correlated with NO production. Therefore, the effects of nitrite, a stable NO derivative, on the Kyn treated with TCA were tested by HPLC. The absorption of 50 μM Kyn in PBS at 360 nm was

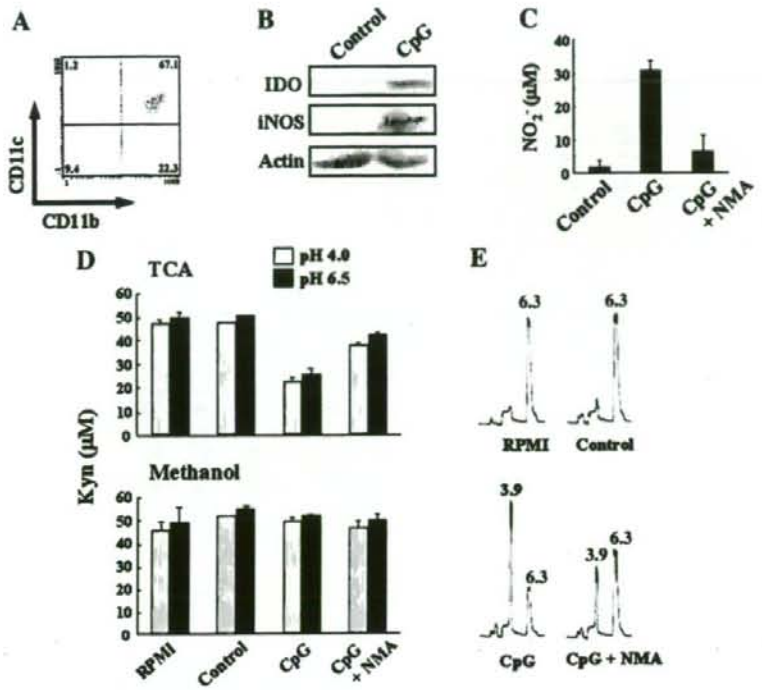

Fig. 1. NO-linked secretion of TCA-triggered Kyn-degrading substance from BMDC stimulated with CpG. (A) Surface expression of CD11c and CD11b on BMDC was analyzed by flow cytometry. The percentage of cells in each quadrant is presented. (B) IDO and iNOS expressions were assessed by a Western blot analysis. (C) Nitrite accumulation in the culture supernatant was measured using Griess reagent. Means ± SD of triplicate cultures are presented. (D, E) After BMDC (3×10^5 cells/0.2 ml) were stimulated with CpG alone or together with NMA for 24 h, Kyn (50 μM) were added to the cell-free culture supernatants and incubated at 37 °C for 2 h. Then, culture supernatants were deproteinized with 5% TCA or 86% methanol, and concentrations of Kyn were measured by HPLC using acetic acid–sodium acetate buffer (pH 4.0) or acetic ammonium buffer (pH 6.5). (D) The means ± SD of triplicate cultures are presented. (E) HPLC elution (pH 4.0) profiles of the absorption at 360 nm with the retention time (min) of the culture supernatants treated with 5% TCA for deproteinization were presented.

completely lost by TCA treatment in the presence but not absence of 50 μM sodium nitrite (Fig. 2A). Corresponding to the disappearance of the absorption peak of Kyn, a new absorption peak appeared at a shorter retention time (Fig. 2B). This change of the absorption of Kyn was the same with that observed by TCA treatment of the mixture of Kyn and the cell-free culture supernatant of BMDC stimulated with CpG (Fig. 1E). These results indicate that in response to the TCA treatment, Kyn reacts with nitrite secreted from BMDC stimulated with CpG. The extent to which TCA treatment influences Kyn measurement was further studied in human serum. As shown in Fig. 2C, the concentration of Kyn (50 μM) exogenously added into human serum was decreased by more than 20% by treatment with 0.5% TCA in the presence of 50 μM $NaNO_2$. This concentration of TCA was 10 times lower than that required for deproteinization.

The concentration of Kyn was also indirectly assayed using Ehrlich reagent which reacts with the amino residue of the benzoic ring of Kyn (Takikawa et al., 1988). Ehrlich reagent could not detect any compounds generated through the TCA-triggered reaction of Kyn with nitrite, however, reacted with TCA-treated Kyn (Fig. 2D). These results suggest that the amino residue of the benzoic ring of Kyn is used for the TCA-triggered generation of a new compound, and Ehrlich reagent does not react with the altered Kyn.

The pH-dependence and kinetics of the reaction of Kyn with nitrite in PBS were assayed by absorption spectroscopy. The absorption peak of Kyn at 360 nm was completely lost by the TCA treatment in the presence of 50 μM $NaNO_2$, and a new absorption peak at 340 nm appeared (Fig. 2E). This change in the absorption was induced by treatment with nitrite at pH 1–4 (Fig. 2F). The absorption peak of Kyn at 360 nm

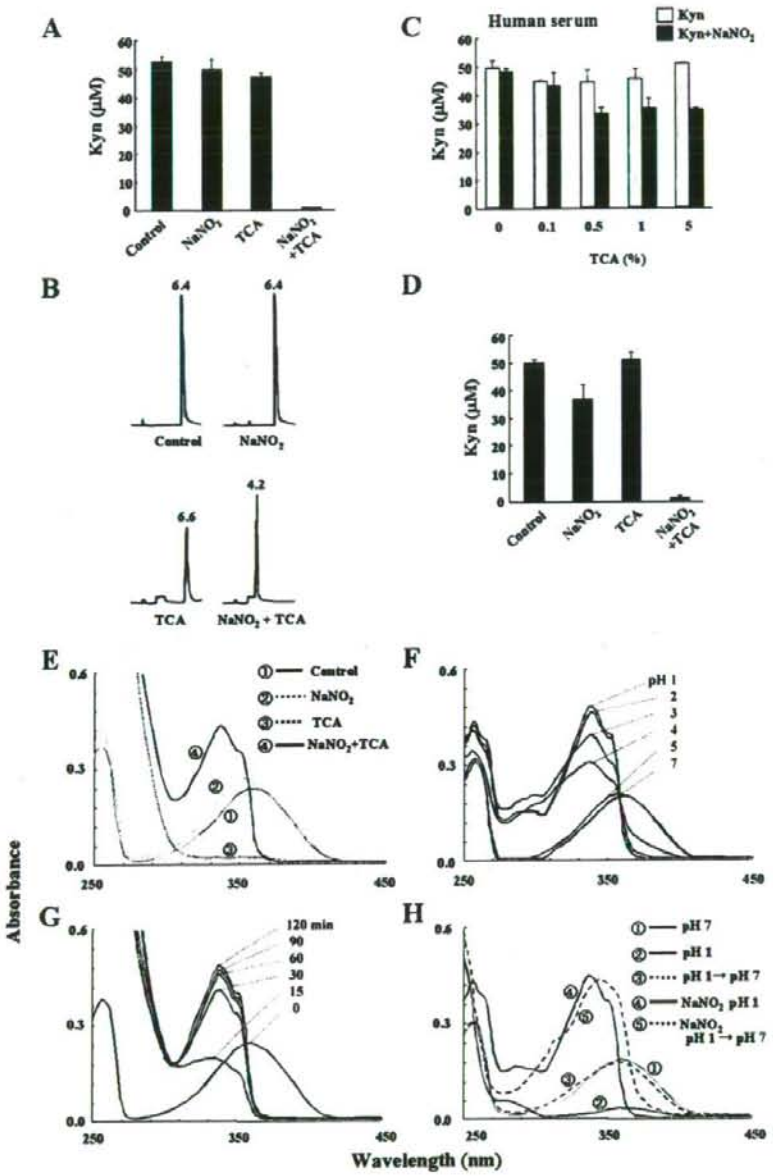

Fig. 2. The reaction of Kyn with nitrite under acidic conditions detected by HPLC, Ehrlich reagent and absorption spectra. (A–D) Kyn (50 μM) were added with or without $NaNO_2$ (50 μM) in (A, B, D) PBS or (C) human serum. (A, B, D) These solutions or (C) human serum 2-fold diluted with PBS was incubated with or without (A, B, D) 5% or (C) the indicated % of TCA at 37 °C for 2 h. Concentrations of Kyn in the reaction solution were assayed by HPLC with elution buffer at (A, B) pH 4.0 or (C) pH 6.5 after deproteinization with 86% methanol at neural pH, or (D) using Ehrlich reagent. (A, C, D) Means ± SD of triplicate cultures and (B) HPLC elution profiles of the absorption at 360 nm with retention time (min) are presented. (E, F) Kyn (50 μM) in PBS was incubated with 50 μM $NaNO_2$ together with or without (E) 5% TCA or (F) HCl at the indicated pH at 37 °C for 2 h. (G) Kyn was incubated with $NaNO_2$ and TCA at 37 °C for 0–2 h. (H) Kyn was incubated with HCl alone at pH 1 or together with $NaNO_2$ at 37 °C for 2 h. Thereafter, the pH of these reaction solutions was adjusted to pH 7, and the volume of reaction solution was adjusted. The absorption spectra of these samples are presented.

was rapidly lost by the treatment with TCA in the presence of $NaNO_2$, whereas the new absorption peak at 340 nm increased gradually after 60 min (Fig. 2G). On the other hand, the absorption peak of Kyn at 360 nm was decreased by 90% without the appearance of a new absorption peak by the treatment with TCA alone (Fig. 2E). The reversibility of the reaction of Kyn with nitrite by acidic treatment was tested (Fig. 2H). The absorption spectrum of a new compound generated through the reaction of Kyn with nitrite by treatment with HCl at pH 1 was not reversed by treatment at neutral pH. However, neutral pH treatment completely recovered the acid-triggered loss of the absorption spectrum of Kyn within a second. These results indicate that the reaction of Kyn with nitrite by acidic treatment is irreversible, whereas the change in the absorption of Kyn induced by acidic treatment is reversible.

3.3. Mass spectrometric analysis of the diazo compound of Kyn through the reaction with $NaNO_2$ under acidic conditions

The new compound generated by the reaction of Kyn with nitrite under acidic conditions was analyzed by mass spectrometry. A proton adduct of Kyn $[M+H]^+$ appeared at 209 m/z (Fig. 3A). This peak was lost by the treatment of Kyn with TCA (Fig. 3B) or HCl at pH 1 (Fig. 3C) in the presence of $NaNO_2$, and new peaks at 220 m/z and 182 m/z appeared as main products. The product with $[M]^+ = 220$ m/z shows an increase mass of 12 Da from mass of Kyn (208 Da). This difference in molecular mass corresponds to the generation of a new compound of Kyn through the addition of nitrogen atom of $NaNO_2$ (+N:+14) to an amino group at the aromatic ring of Kyn with releasing 2 hydrogen atoms (−2H:−2). This suggests that a diazo ($-N^+ \equiv N$) compound from an amino residue of the benzoic ring of Kyn was derived by the reaction of Kyn with $NaNO_2$ under acidic conditions (Fig. 3D). The possibility of the formation of a diazocompound at α-amino group is excluded, since the diazo group formed from primary amine is very unstable. Furthermore, lack of reactivity of Ehrlich reagent with the generated compound (Fig. 2C) also supports formation of the speculated compound. The product at 182 m/z was strongly retained in C18 column of HPLC, thus showing a different polarity from Kyn derivatives (data not shown). Furthermore, the depletion of molecular group corresponding to 26 m/z from Kyn is hardly estimated. Although it is difficult to speculate on the structure of the product at present, it might not have originated from Kyn.

4. Discussion

TCA treatment for deproteinization has been frequently done before the Kyn assay by HPLC. The current study showed that TCA treatment induced errors in the estimation of the Kyn assay by acid-triggered reaction of Kyn with nitrite produced by BMDC stimulated with CpG. This error could be avoided by deproteinization with methanol. It has been shown that NO production inhibits IDO at the post-transcriptional

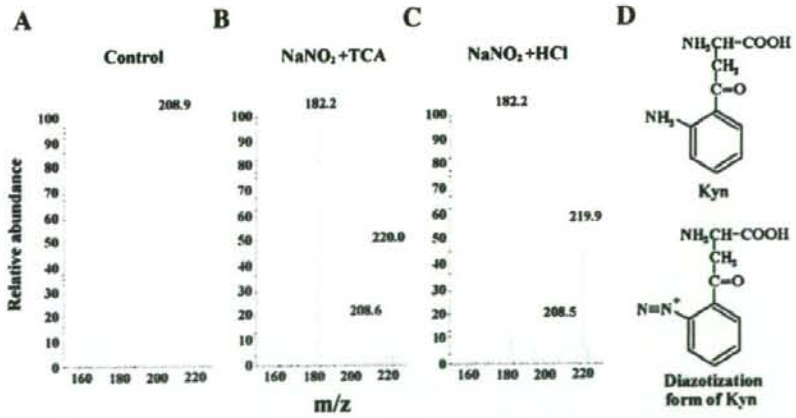

Fig. 3. The analysis of compounds of Kyn generated through the reaction with nitrite under acidic conditions by mass spectrometry. (A) Kyn (50 μM) in water was incubated with 50 μM $NaNO_2$ together with (B) 5% TCA or (C) HCl at pH 1 at 37 °C for 2 h, and dried with methanol. These samples were analyzed by mass spectrometry. (D) The predictable structure of a diazo compound generated by the reaction of Kyn with $NaNO_2$ under acidic conditions.

(Thomas et al., 1994; Hucke et al., 2004) or transcriptional (Alberati-Giani et al., 1997) levels. The present study suggests that the IDO activity is underestimated unless acidic treatment is avoided in the Kyn assay, when NO is produced.

We demonstrated the reaction of Kyn with nitrite in TCA solution by the assay of HPLC, absorption spectra and mass spectrometry. The data of the absorption spectra shows that the reaction of Kyn with acidified nitrite is induced irreversibly and gradually for 1 h at a lower pH than pH 4. These results are consistent with the report that the diazonium salt of Kyn was decomposed, apparently completely, by incubation for an hour at 36 °C (Mason and Berg, 1952). The loss of the absorption spectrum of Kyn by TCA treatment alone is very rapid and reversible so this change of Kyn absorption is not detected by HPLC assay using elution buffer at pH 4.0–6.5.

The product generated by the reaction of Kyn with acidified nitrite could not be detected by Ehrlich reagent that reacts with the amino residue of the aromatic ring (Takikawa et al., 1988), thus suggesting the modification of the aromatic amine of Kyn by acidified nitrite. The data of mass spectrometry suggest the generation of a new compound of Kyn through the reaction of the nitrogen of $NaNO_2$ with one of 2 amino residues of Kyn. Based on the above findings, we conclude that the diazotization of Kyn is generated through the reaction with acidified nitrite secreted from BMDC stimulated with CpG. Under acidic conditions, protonation of nitrite ions yield nitrous acid (HNO_2), which can be protonated a second time. Water leaves the molecule forming the nitrosonium ion (+NO), which acts as an excellent nucleophile to modify a number of amino acids and secondary amines. Griess described the diazotization of aryl amine by nitrite and coupling of the product to form an azochrome in 1879 (Griess, 1879). The diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine was applied for the Kyn measurement (Mason and Berg, 1952). However, these reports were published when the biological action of NO production was not known. The current study showed for the first time the errors in the estimation of the Kyn assay caused by the acid-initiated diazotization of Kyn in the biological system producing NO.

Some reports show which concentrations of Kyn produced in the NO-generating systems have been assayed by HPLC after deproteinization by acidic treatment (Fujigaki et al., 2002; López et al., 2006). *Toxoplasma gondi* infection increase the levels of both Kyn and NO in the plasma of mouse, and a NOS inhibitor enhance the Kyn concentration (Fujigaki et al., 2002). IFN-γ-induced increase of the concentration of Kyn in the culture supernatant of human monocytic U-937 cells is inhibited by a NO donor (López et al., 2006). The present study suggests the possibility that the IDO activities in these reports may be underestimated by the diazotization of Kyn induced by acidified nitrite.

In conclusion, the deproteinization of samples by acidic treatment should therefore be avoided for the Kyn assay when NO is produced.

Acknowledgements

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Novel Role of Presenilins in Maturation and Transport of Integrin $\beta1$[†]

Kun Zou,[*,‡,§,∥] Takashi Hosono,[‡] Toshiyuki Nakamura,[‡] Hirohisa Shiraishi,[‡] Tomoji Maeda,[∥] Hiroto Komano,[‡,∥] Katsuhiko Yanagisawa,[‡] and Makoto Michikawa[*,‡]

Department of Alzheimer's Disease Research, National Institute for Longevity Sciences, NCGG, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan, Japan Society for the Promotion of Science (JSPS), Tokyo 102-8471, Japan, and Department of Neuroscience, Faculty of Pharmaceutical Sciences, Iwate Medical University, 2-1-1 Nishitokuda, Yahaba, Iwate 028-3694, Japan

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ABSTRACT: Presenilins (PSs) play important roles in modulating the trafficking and maturation of several membrane proteins. However, the target membrane proteins whose trafficking and maturation are regulated by PS are largely unknown. By characterizing PS-deficient fibroblasts, we found that integrin $\beta1$ maturation is promoted markedly in PS1 and PS2 double-deficient fibroblasts and moderately in PS1- or PS2-deficient fibroblasts; in contrast, nicastrin maturation is completely inhibited in PS1 and PS2 double-deficient fibroblasts. Subcellular fractionation analysis demonstrated that integrin $\beta1$ maturation is promoted in the Golgi apparatus. The mature integrin $\beta1$ with an increased expression level was delivered to the cell surface, which resulted in an increased cell surface expression level of mature integrin $\beta1$ in PS1 and PS2 double-deficient fibroblasts. PS1 and PS2 double-deficient fibroblasts exhibited an enhanced ability to adhere to culture dishes coated with integrin $\beta1$ ligands, namely, fibronectin and laminin. The inhibition of γ-secretase activity enhances neither integrin $\beta1$ maturation nor the adhesion of wild-type cells. Moreover, PS deficiency also promoted the maturation of integrins $\alpha3$ and $\alpha5$ and the cell surface expression of integrin $\alpha3$. Integrins $\alpha3$ and $\alpha5$ were coimmunoprecipitated with integrin $\beta1$, suggesting the formation of the functional heterodimers integrins $\alpha3\beta1$ and $\alpha5\beta1$. Note that integrin $\beta1$ exhibited features opposite those of nicastrin in terms of maturation and trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus in PS1 and PS2 double-deficient fibroblasts. Our results therefore suggest that PS regulates the maturation of membrane proteins in opposite directions and cell adhesion by modulating integrin maturation.

Mutations in the genes encoding presenilin-1 (PS1[1]) and PS2 account for most cases of familial early onset Alzheimer's disease (FAD) (*1, 2*). PS1 and PS2 most likely provide the catalytic subunit of the γ-secretase complex (*3*). FAD-linked mutant PS proteins increase the level of highly amyloidogenic Aβ42, which is generated by the proteolytic processing of the amyloid precursor protein (APP) and deposited early as senile plaques in the brains of aged individuals and AD patients (*4–6*). PS-mediated cleavage occurs within the transmembrane domain of several type I membrane proteins such as Notch, APP, the APP homologues APLP1 and APLP2, ErbB-4, CD44, N- and E-cadherins, the low-density lipoprotein receptor-related protein (LRP), Syndecan, Delta, Jagged, and Nectin1α (*7*).

PS1 and PS2 may also have other functions, in addition to their central role as catalytic subunits of the γ-secretase complex. Previous studies have shown their involvement in β-catenin turnover, apoptosis, Ca^{2+} homeostasis, and protein trafficking (*8, 9*). PS proteins have also been shown to function as endoplasmic-reticulum (ER)-resident chaperones affecting the maturation of nicastrin (*10–12*), APP (*13–15*), TrkB (*16*), N-cadherin (*17*), and the neurotrophin receptor-like death domain (NRADD) protein (*18*). Nicastrin maturation and cell-surface delivery are completely inhibited in the absence of PS1 and PS2 (*10–12*). PS1 aspartic acid mutants expressed in a PS-null background restore nicastrin maturation but not γ-secretase activity, suggesting a γ-secretase-independent function of PS in the maturation and trafficking of nicastrin (*19*). PS1-null neurons exhibit compromised TrkB maturation (*16*). The transfection of dominant-negative PS1 D385A in SH-SY5Y cells leads to disrupted maturation and a decreased cell-surface expression level of N-cadherin (*17*). In addition, the absence of PS1 and PS2 results in the intracellular retention of caveolin 1, the loss of caveolae (*20*),

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* To whom correspondence should be addressed. Tel: +81 562 46 2311. Fax: +81 562 46 8569. E-mail: kunzou@iwate-med.ac.jp (K.Z.); michi@nils.go.jp (M.M.).

[‡] National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology.

[§] Japan Society for the Promotion of Science (JSPS).

[∥] Iwate Medical University.

[1] Abbreviations: PS, presenilin; PS1, presenilin-1; PS2, presenilin-2; wt, wild-type; PS-ko, presenilin-1 and -2 double knockout; Aβ, amyloid β-protein; APP, amyloid precursor protein; PNGase F, peptide: *N*-glycosidase F; ER, endoplasmic reticulum; NTF, N-terminal fragment; CTF, C-terminal fragment.

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and an abnormal accumulation of telencephalin/ICAM in intracellular compartments (*21*). These suggest that PS deficiency disrupts the ER-to-Golgi apparatus trafficking of a set of membrane proteins. In contrast to these membrane proteins, APP exhibits enhanced expression and cell surface accumulation in PS1- and PS2-deficient cells. The expression of dominant-negative PS1 D385A or treatment with a γ-secretase inhibitor, DAPT, also leads to an enhanced cell surface accumulation of APP, via the acceleration of APP trafficking (*13*) or the delay of APP endocytosis (*14*).

We determined whether PS deficiency affects the maturation of other membrane proteins and whether ER-to-Golgi apparatus trafficking is generally disrupted in PS-deficient cells. We examined several type I membrane proteins in PS1 and PS2 double-deficient cells and found that the loss of PS1 and PS2 results in an enhanced maturation of integrin β1 and an enhanced cell-surface delivery of mature integrin β1.

MATERIALS AND METHODS

Cell Culture and Antibodies. Wild-type (wt), PS1- and PS2 double-knockout (PS-ko), PS1-deficient (PS1 −/−), and PS2-deficient (PS2 −/−) mouse embryonic fibroblast (MEF) cell lines were kindly provided by Dr. Bart De Strooper (*22*). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS). The cells were lysed in RIPA buffer [10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 0.2% sodium deoxycholate, containing a protease inhibitor cocktail (Roche, Mannheim, Germany)] at a point before or after confluence. Monoclonal antibodies against integrins β1, α3, αV, syntaxin 6, BiP/GRP78, and calnexin were obtained from BD Biosciences (San Jose, CA). Polyclonal antibodies against integrins β1, α1, α2, α4, α5, α6, α7, αL, and the N-terminus of PS1 (H-70) were from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody against the loop of PS1 (MAB5232), which recognizes the C-terminal fragment of PS1, was purchased from Chemicon (Temecula, CA). A polyclonal antinicastrin antibody raised against the C-terminus of nicastrin (amino acids 693–709) was purchased from Sigma (Saint Louis, MO).

Electrophoresis, Immunoblotting, Deglycosylation, and Immunoprecipitation. Total protein (50 μg) from cell lysates was dissolved in SDS sample buffer, separated on 4–20% gradient gels, and transferred to nitrocellulose membranes (equal loading was confirmed by Western blotting for Bip/GRP78 or α-tubulin). The target proteins were visualized using SuperSignal (Pierce, Rockford, IL) with antibodies to integrins, nicastrin, Bip/GRP78, calnexin, syntaxin 6 and PS1. To assess integrin β1 maturation and nicastrin glycosylation, lysates from the wt and PS-ko cells were treated with PNGase F, *O*-glycanase, or sialidase A using an enzymatic deglycosylation kit according to the manufacturer's instructions (PROzyme, San Leandro, CA). For immunoprecipitation, the cells were homogenized in a solution of 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, and 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) containing a protease inhibitor cocktail, and the homogenate was centrifuged at 10,000*g* and 4 °C for 10 min. The supernatant was immunoprecipitated with a polyclonal antibody to integrin β1 or PS1 and protein G sepharose (Amersham Biosciences, Uppsala, Sweden). Coimmunoprecipitated PS1, nicastrin, integrin β1, and integrin α subunits were detected by Western blotting.

Subcellular Fractionation on Iodixanol Gradient. The wt and PS-ko cells were grown in eight 10-cm tissue culture dishes, and subcellular fractionation was performed as previously described (*23*). They were homogenized in an ice-cold homogenization buffer [10 mM HEPES (pH 7.4), 1 mM EDTA, and 0.25 M sucrose containing a protease inhibitor cocktail]. The postnuclear supernatant was centrifuged for 1 h at 4 °C and 65,000*g*. The resultant vesicle pellets were rehomogenized in 0.8 mL of the homogenization buffer and layered on a step gradient consisting of 1 mL of 2.5%, 2 mL of 5%, 2 mL of 7.5%, 2 mL of 10%, 0.5 mL of 12.5%, 2 mL of 15%, 0.5 mL of 17.5%, 0.5 mL of 20%, and 0.3 mL of 30% (v/v) iodixanol (GIBCO). After centrifugation at 90,000*g* (SW41 rotor, Beckman) for 2.5 h at 4 °C, 11 fractions were collected from the top of the gradient.

Transfection, γ-Secretase Inhibitors Treatment and Aβ ELISA. The retrovirus-mediated gene expression of human APP695, PS1, PS2, PS1D257A, PS1D385A, PS1ΔE 9, PS1I143F, PS1R278K, and PS1L392V was carried out as previously described (*24*). The fibroblasts were transfected at 10% confluence and maintained in DMEM containing 10% fetal calf serum. The transfection efficiency was nearly 100% in this study, as estimated by the control transfection of the pMX-green fluorescent protein (pMX-GFP). γ-secretase inhibitors, namely, DAPT and L-685,458, were added to the wt cells stably expressing hAPP695 immediately after passage. The culture medium was collected two days after confluence, and the level of Aβ1-40 secreted was measured using an Aβ ELISA kit (Wako Pure Chemical, Osaka, Japan).

Cell Surface Biotinylation and Cell Surface Uptake of Integrin β1. Cell surface biotinylation was carried out using a Pinpoint cell surface protein isolation kit (Pierce). The wt and PS-ko cells were grown in four 10-cm tissue culture dishes, and washed twice with ice-cold PBS (GIBCO). The cells were incubated in 10 mL of ice-cold 0.25 mg/mL sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (Sulfo-NHS-SS-Biotin) (Pierce) in ice-cold PBS for 30 min at 4 °C. Then, 500 μL of the quenching solution was added to each dish to quench the reaction. The cells were scraped and washed twice with Tris-buffered saline (TBS) [10 mM Tris/HCl (pH 7.5) and 150 mM NaCl] and lysed in the lysis buffer containing protease inhibitors. Each lysate was incubated with streptavidin-agarose beads (Pierce) at 4 °C for 60 min, and captured proteins were eluted with 50 mM DTT in Laemmli's SDS sample buffer. To assess cell surface integrin β1 internalization, immunostaining was performed as previously reported (*14*). The cells plated on a fibronectin-coated culture slide were washed in ice-cold PBS, and incubated on ice with a monoclonal antibody against integrin β1 at 1:200 dilution in PBS containing 0.1% BSA. After 20 min, the cells were washed with ice-cold PBS, and then incubated in prewarmed culture medium for various durations at 37 °C. After the indicated durations, the cells in the culture slides were fixed with 4% paraformadehyde in PBS for 20 min. After rinsing three times in PBS, permeabilization was achieved in 0.1% Triton X-100/PBS for 5 min, and the slides were incubated with rhodamine-coupled goat antimouse IgG

(Chemicon) for 20 min. Confocal images were taken with a Zeiss LSM 510 confocal system (Carl Zeiss, Jena, Germany).

Cell Attachment Assay. Ninety-six- and 6-well plates (Corning Inc., Corning, NY) were coated with 10 μg/mL fibronectin or laminin (Sigma) for 8 h at 4 °C. After aspirating the coating reagent, 0.2 or 1.5 mL of 10 mg/mL filtered, heat-denatured bovine serum albumin (BSA) (Sigma) was dispensed into the wells, and the plates were incubated at 4 °C for 16 h. The cell attachment assay using single resuspended cells was carried out in 96-well plates as previously reported (*25*). Subconfluent cells were washed with HEPES-buffered saline [HBS, 150 mM NaCl, and 25 mM HEPES (pH 7.5)] and resuspended at a density of 0.2 to 1 × 10^6 cells/mL. A 50-μL aliquot of the cell suspension was then added to each well. The plates were incubated for 30 min at 37 °C in 5% (v/v) CO_2. Unbound or loosely bound cells were removed by aspiration and gentle washing with HBS. To assess the total number of cells added, 100%, 75%, 50%, 25%, and 0% cells were added to the wells and fixed by adding 1/10 vol of 50% (v/v) glutaraldehyde. To assess the attachment strength of the wt and PS-ko cells after the cells reached confluence, the cell attachment assay was performed in 6-well plates. After aspirating the conditioning medium, the cells were incubated in 1 mM EDTA for 30 min at room temperature. Detached cells were washed out with HBS. The cells were fixed in the wells by adding 5% (v/v) glutaraldehyde in HBS and stained with 0.1% (w/v) crystal violet in 200 mM MES (pH 6.0) for 60 min. The solution in the wells was then aspirated, and the wells were washed with water. Acetic acid [10% (v/v)] was dispensed into the wells of the 96-well plates, and the absorbance at 570 nm of each well was measured with a multiscan plate reader. Images of the 6-well plates were taken when the wells were dried, and the area of the wells occupied by adherent cells was measured using Image J 1.36b software (NIH, Bethesda, MD).

RESULTS

Integrin β1 is synthesized as an 87-kDa polypeptide that undergoes glycosylation in ER and the Golgi apparatus. In ER, the most prevalent, incompletely glycosylated immature integrin β1 has a mass of 105 kDa. The mature form of this 105 kDa integrin β1 has a mass of 125 kD (*26, 27*). The immature form is not found on the cell surface and has no role in cell adhesion or cell signaling (*26, 28*). These features of integrin β1 in terms of maturation are similar to those of nicastrin, a member of the γ-secretase complex. To determine how the absence of PS affects integrin β1 maturation, we performed a Western blotting of cell lysates prepared from wt, PS-ko, PS1(−/−), and PS2(−/−) fibroblasts. In the wt cells, integrin β1 was detected as a nonglycosylated core protein (87 kDa), a poorly glycosylated immature protein (105 kDa), and a highly glycosylated mature protein (125 kDa). Most of these integrin β1 isoforms are of the immature form. Interestingly, a marked increase in the expression level of mature integrin β1 and a decrease in that of ER-localized immature integrin β1 were observed in the PS-ko cells, suggesting that PS proteins exert an inhibitory effect on the post-translational maturation of integrin β1 (Figure 1A). The PS1(−/−) or PS2(−/−) cells exhibited an intermediate increase in the expression level of mature integrin β1 and a decrease in that of immature integrin β1, suggesting that the regulation of the post-translational maturation of integrin β1 is PS-dependent, not cell-line-dependent (Figure 1A). The PS-ko cells showed a level ratio of mature integrin β1 to immature integrin β1 18-fold higher than that of the wt cells. The PS1(−/−) cells showed a 4-fold increase in the level ratio of mature integrin β1 to immature integrin β1 (Figure 1C). In contrast to integrin β1, the 125-kDa mature nicastrin species showed a significant decrease in expression level (Figure 1B), in agreement with previous reports (*10–12*). These results suggest that PS regulates the maturation of membrane proteins in opposite directions.

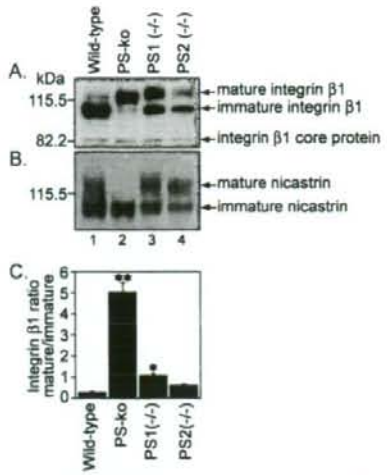

FIGURE 1: Presenilin deficiency promotes integrin β1 maturation and inhibits nicastrin maturation. The wt, PS-ko, PS1(−/−), and PS2(−/−) fibroblasts were lysed in RIPA buffer 2 days after reaching confluence. Western blots of 50 μg of total protein from the cells were probed with an anti-integrin β1 monoclonal antibody (A) or antinicastrin polyclonal antibody (B). Three isoforms of integrin β1 were observed in the cells: the ~87-kDa core protein, ~105-kDa immature glycosylated form, and ~125-kDa mature glycosylated form (indicated by arrows). Note that the predominant isoform of integrin β1 in the wt fibroblasts was the immature form and that in the PS-ko fibroblasts was the mature form. For nicastrin, the immature form of ~105 kDa and the mature form of ~125 kDa were observed in the wt fibroblasts. The mature form of nicastrin was absent in PS-ko fibroblasts. The expression level ratio of mature integrin β1 to immature integrin β1 was determined by densitometry (C). Data represent the means ± SEM; $n = 3$, $^*p < 0.05$, $^{**}p < 0.001$, PS-ko or PS1(−/−) vs wt, Bonferroni/Dunn test.

To determine whether the mature integrin β1 with an increased expression level is glycosylated in PS-ko cells as it is in wt cells, we experimentally examined the glycosylation of mature integrin β1. Similar to nicastrin, both immature and mature integrin β1s were sensitive to PNGase F. Digestion with PNGase F decreased the apparent size of mature and immature integrin β1s to 95 kDa as a major species in the wt cells (Figure 2A, lane 5). Integrin β1 in the PS-ko cells was partially resistant to PNGase F. In addition to the 95-kDa species, a 105-kDa species was detected after digestion with PNGase F. This PNGase F-resistant 105-kDa integrin β1 was likely generated from the 125-kDa mature integrin β1 because of its abundance in the PS-ko cells and scarcity in the wt cells (Figure 2A, lane 6). However, the wt cells showed a 105-kDa nicastrin PNGase F-resistant species in addition to the 70-kDa

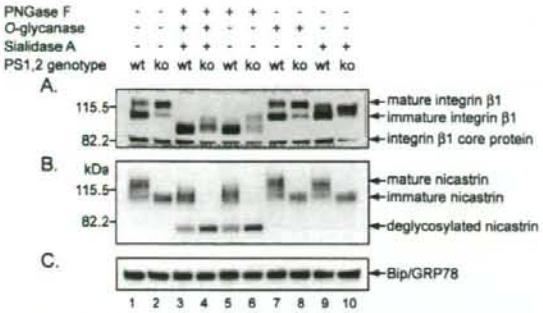

FIGURE 2: Deglycosylation of integrin β1 and nicastrin. Total protein (50 μg) from the wt or PS-ko fibroblast lysate was digested with PNGase F (0.1 U/mL), *O*-glycanase (0.025 U/mL), or sialidase A (0.1 U/mL) and analyzed by SDS−PAGE and immunoblotting. Western blots were probed with an anti-integrin β1 monoclonal (A), antinicastrin polyclonal (B), or anti-Bip/GRP78 (C) antibody. Lanes 1 and 2, no treatment; lanes 3 and 4, PNGase F, *O*-glycanase, and sialidase A treatment; lanes 5 and 6, PNGase F treatment; lanes 7 and 8, *O*-glycanase treatment; and lanes 9 and 10, sialidase A treatment. The mature form of integrin β1 in the PS-ko cells and the mature form of nicastrin in the wt cells are partially PNGase F-resistant. Equal amounts of protein loaded are shown by the Western blot of Bip/GRP78.

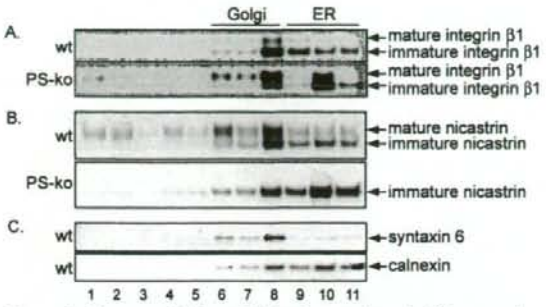

FIGURE 3: Absence of PS1 and PS2 enhances integrin β1 maturation in the Golgi apparatus and has no effect on the intracellular distribution of integrin β1. The wt and PS-ko fibroblasts were harvested and fractionated on iodixanol gradients. Fractions rich in ER (lanes 9–11) are at the bottom, and 11 fractions were collected from top to bottom. Golgi-apparatus-rich fractions are shown in lanes 6–8. The fractions were analyzed by immublotting with an anti-integrin β1 antibody (A), an antinicastrin antibody (B), and antibodies to the Golgi apparatus marker protein syntaxin-6 and ER marker protein calnexin (C). Note that nicastrin was retained in the ER fractions of the PS-ko cells, but not integrin β1. The maturation of integrin β1 in PS-ko fibroblasts in the Golgi apparatus was markedly accelerated compared with that in wt fibroblasts.

deglycosylated species; the PS-ko cells showed that the apparent size of the 105-kDa immature nicastrin as a single species decreased to 70 kDa (Figure 2B, lanes 1, 2, 5, and 6). Treatment with *O*-glycanase had no effect on the SDS−PAGE mobility of either integrin β1 or nicastrin, indicating the absence of an *O*-linked glycosylation of these two proteins (Figure 2A and B, lanes 7 and 8). The mature forms, not the immature forms of integrin β1 and nicastrin were sensitive to sialidase A digestion, indicating the sialylation of the mature forms of both proteins (Figure 2A and B, lanes 9 and 10). The ER protein Bip/GRP78 served as the internal control protein, which indicated the same amount of protein loaded in each lane (Figure 2C). These results show that (i) in contrast to that of nicastrin, whose mature form is absent in PS-ko cells, the maturation of integrin β1 in PS-ko cells is enhanced compared with that in wt cells and that (ii) mature integrin β1 in PS-ko cells is normally glycosylated by *N*-glycans, the characteristics of which are similar to those of *N*-glycans in mature nicastrin in wt cells.

The results described above demonstrate that the absence of PS1 and PS2 promotes integrin β1 maturation. We also confirmed that the absence of PS1 and PS2 inhibits nicastrin maturation, as shown by previous studies. To investigate how the absence of PS1 and PS2 disrupts the processing and intracellular distribution of integrin β1 and nicastrin, we carried out iodixanol gradient fractionation to separate the Golgi apparatus and ER-derived membranes (*23*). Syntaxin 6 and calnexin in the wt cells served as the Golgi apparatus and ER markers, respectively (Figure 3C). The distributions of syntaxin 6 and calnexin in PS-ko cells did not differ from those in wt cells (data not shown). In the wt cells, integrin β1 and nicastrin underwent normal maturation and ER-to-Golgi apparatus trafficking, with most of their immature forms localizing in the ER, and most of their mature forms localizing in the Golgi apparatus. The mature and immature forms of nicastrin showed patterns similar to those of the mature and immature forms of integrin β1. In contrast, in the PS-ko cells, mature forms of nicastrin were absent, and most of the immature forms of nicastrin were restricted in the ER fractions, suggesting the disrupted exit of nicastrin from ER. Interestingly, in the PS-ko cells, the expression level of mature integrin β1 increased predominantly in the Golgi apparatus, whereas that of immature integrin β1 significantly decreased in the Golgi apparatus, as compared with those in the wt cells, indicating that the maturation of integrin β1 in the Golgi apparatus is enhanced. In addition, there was no selective retention of integrin β1 in the ER in the PS-ko cells. These results suggest that the trafficking of immature integrin β1 from ER to the Golgi apparatus is accelerated in PS-ko cells (Figure 3A and B).

Because PS1 and PS2 can regulate cell signaling pathways via γ-secretase activity (*29*), we determined whether the blockade of γ-cleavage leads to an enhanced maturation of integrin β1. We treated hAPP-transfected wt cells with two major γ-secretase-specific inhibitors, namely, DAPT and L-685,458, to inhibit γ-secretase activity and monitored the level of Aβ1-40 secreted in the culture medium to evaluate γ-secretase activity (Figure 4). γ-secretase inhibitors at a concentration higher than 2.5 μM completely inhibited γ-secretase activity but did not facilitate integrin β1 maturation, indicating that the inhibition of γ-secretase activity is not sufficient to facilitate integrin β1 maturation (Figure 4 A and B). In Figure 1, we show that the expression level of mature integrin β1 was enhanced in three independent PS-deficient cell lines, namely, PS-ko, PS1(−/−), and PS2(−/−). The expression level of mature integrin β1 inversely correlated with the expression level of PS in these three independent cell lines. The transfection of PS-ko cells with human PS1 and PS2 restored the normal expression of mature nicastrin and inhibited the maturation of integrin β1, suggesting that PS is essential for post-translational maturation of nicastrin and for inhibiting the maturation of integrin β1 (Figure 4C). The transfection with PS1 aspartate mutants lacking γ-secretase activity, namely, PS1 D257A and PS1 D385A, did not restore the expression of mature integrin β1, whereas it restored the normal expression of mature

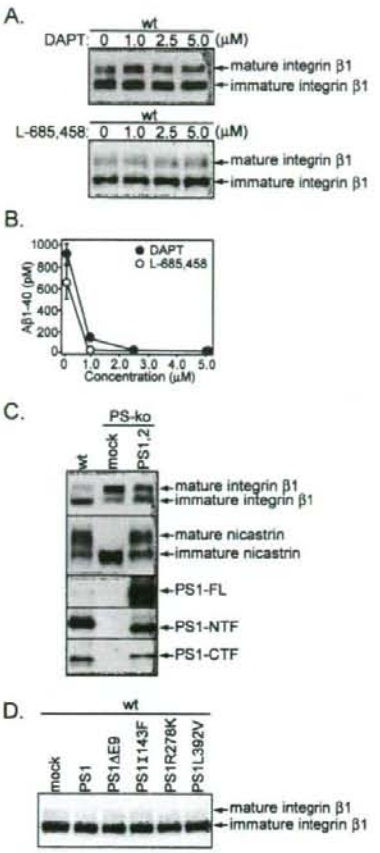

FIGURE 4: Effects of γ-secretase inhibitors and FAD PS mutants on integrin β1 maturation and restoration of integrin β1 and nicastrin maturation by transfection with PS1 and PS2. The wt fibroblasts stably expressing human APP695 were treated with or without DAPT or L-685,458 immediately after passage. The cells were lysed after reaching confluence, and the lysate was analyzed by immunoblotting with an anti-integrin β1 antibody (A). The level of Aβ1-40 secreted to the culture medium was measured using an Aβ1-40 ELISA kit (Wako) (B). γ-secretase inhibitors at a concentration greater than 2.5 μM completely inhibited γ-secretase activity, which was monitored by analyzing Aβ1-40 secretion; however, integrin β1 maturation remained unchanged. The PS-ko fibroblasts were transfected with human PS1 and PS2. Western blots of 50 μg of total protein from the transfected PS-ko fibroblasts were probed with an anti-integrin β1 antibody, an antinicastrin antibody, and anti-PS1 antibodies (C). Double transfection of PS-ko fibroblasts with human PS1 and PS2 restored the maturation of integrin β1 and nicastrin. The wt fibroblasts were transfected with human PS1 and FAD PS1 mutants, and integrin β1 maturation remained unchanged (D). FL, full-length; NTF, N-terminal fragment; CTF, C-terminal fragment.

nicastrin (data not shown). Because PS1 aspartate mutants do not form the mature, high molecular weight PS complexes (*30*), these results suggest that the formation of the high molecular weight PS complex may be required to inhibit integrin β1 maturation. The expression of transfected PS1 was confirmed by Western blotting. The exogenous human PS1 in PS-ko cells was maintained in larger amounts of the full-length form and smaller amounts of the N-terminal fragment (NTF) and C-terminal fragment (CTF) than those of endogenous mouse PS1 in wt cells. Interestingly, the human PS1-NTF in the PS-ko cells showed a lower molecular weight than the mouse PS1-NTF in the wt cells, whereas the full-length human and mouse PS1s showed the same molecular weight. In agreement with this result, the human PS1-CTF in the PS-ko cells showed a higher molecular weight than the mouse PS1-CTF in the wt cells (Figure 4C, bottom three panels). These results suggest that mouse and human PS1s may undergo principal endoproteolytic cleavage at different sites or that presenilinase cleaves PS1 at different sites in wt and PS-ko cells. We also examined whether the overexpression of the PS1 mutants of familial Alzheimer's disease (FAD) alters integrin β1 maturation in wt cells. The overexpression of human wt PS1 and FAD PS1 mutants, namely, PS1ΔE 9, PS1I143F, PS1R278K, and PS1L392V, did not affect integrin β1 maturation in the wt cells, suggesting that the loss of PS function, probably the loss of both the γ-secretase and the chaperone protein functions of PS, may facilitate integrin β1 maturation (Figure 4D).

Integrin β1 associates with multiple integrin α-subunits to form transmembrane receptors of extracellular matrix proteins, including fibronectin, collagen, and laminin (*31–33*). To determine whether mature integrin β1 with an increased expression level in PS-ko cells is delivered to the cell surface, we determined the expression level of integrin β1 on the cell surface by surface biotinylation. Neither immature integrin β1 nor immature nicastrin was biotinylated in the wt or PS-ko cells, indicating that no immature forms of the two proteins localize on the cell surface (Figure 5A and B). The expression level of surface-biotinylated mature integrin β1 in the PS-ko cells significantly increased compared with that in the wt cells, indicating that the cell-surface delivery of integrin β1 is enhanced in PS-ko cells (Figure 5A, lanes 3 and 6). The expression level of integrin β1 on the surface of the PS-ko cells was 2.5-fold that on the surface of the wt cells (Figure 5C). In contrast to integrin β1, surface-biotinylated nicastrin was detected in the wt cells; however, no apparent signal of this protein was detected in the PS-ko cells, indicating that the cell-surface delivery of nicastrin is impaired in PS-ko cells (Figure 5B, lanes 3 and 6). Because the increased surface expression level of mature integrin β1 can be induced by delayed internalization and accelerated trafficking to the cell surface, we investigated the internalization of mature integrin β1 in living wt and PS-ko cells. The cells were labeled on ice with an antibody that recognizes integrin β1, washed, and incubated at 37 °C to initiate internalization. At the indicated time points, the cells were fixed, permeabilized and processed for immunofluorescence staining. The PS-ko cells had a large amount of surface-labeled integrin β1 in the absence of incubation at 37 °C (Figure 5D), which was consistent with the results of biotinylation. After 10 min of incubation at 37 °C, surface integrin β1 was partially internalized, and after 30 min, surface integrin β1 disappeared and was completely internalized in both the wt and PS-ko cells, indicating that PS deficiency has no effect on integrin β1 internalization. Combined with the results of the iodixanol gradient fractionation, these results suggest that the increased cell-surface expression level of mature integrin β1 is induced by the accelerated trafficking of integrin β1 from ER to the Golgi apparatus and then to the cell surface.

To determine whether an increased cell-surface expression level of mature integrin β1 has the same effect in PS-ko cells as in wt cells, we performed a cell attachment assay to

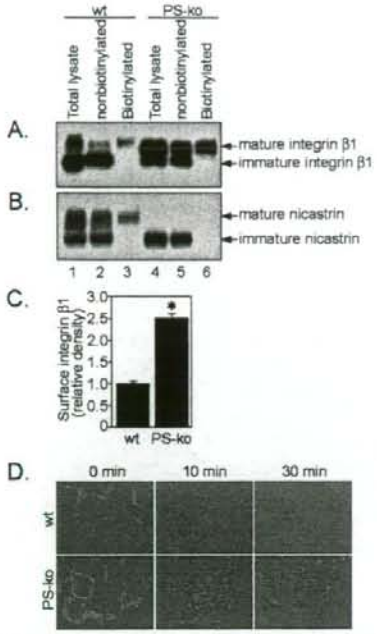

FIGURE 5: Presenilin deficiency promotes integrin β1 cell surface delivery and has no effect on integrin β1 internalization. Lysates of surface-biotinylated wt and PS-ko cells were incubated with streptavidin-agarose. Total lysate (lanes 1 and 4), nonbiotinylated (streptavidin-agarose nonbound, lanes 2 and 5), and biotinylated proteins (streptavidin-agarose bound, lanes 3 and 6) were analyzed by immunoblotting with antibodies against integrin β1 (A) and nicastrin (B). Only mature integrin β1 and nicastrin were delivered to the cell surface. The cell surface expression level of integrin β1 significantly increased in the PS-ko cells, and the relative density of integrin β1 on the cell surface was calculated (C). Data represent the means ± SEM; $n = 3$, $^*p < 0.001$, Bonferroni/Dunn test. Living wt and PS-ko cells were labeled on ice with an antibody to integrin β1, washed, and incubated at 37 °C to initiate internalization. After 0, 10, or 30 min of incubation at 37 °C, the cells were permeabilized and stained with rhodamine-coupled goat antimouse IgG. Confocal images were taken with a Zeiss LSM 510 confocal system (D).

measure the ability of cell adhesion to integrin-β1-ligand-coated dishes. Resuspended wt and PS-ko single cells prepared from subconfluent cultures showed similar attachment strengths to fibronectin-coated dishes (Figure 6A). In their subconfluent states, the PS-ko cells showed a moderate increase in the expression level of mature integrin β1 compared with the wt cells. However, in their confluent states, the new PS-ko cells showed a significantly increased expression level of mature integrin β1, with the expression level ratio of mature integrin β1/immature integrin β1 increasing 2-fold that in the subconfluent PS-ko cells (Figure 6B). We then examined whether PS-ko cells exhibit stronger attachment in their confluent states. The wt and PS-ko cells were plated on fibronectin- or laminin-coated 6-well dishes and cultured until the cells reached confluence. After treatment with EDTA, detached cells were washed out, and adhering cells were stained with crystal violet. The PS-ko cells exhibited a significantly stronger attachment to the fibronectin- or laminin-coated dishes than the wt cells. DAPT, a γ-secretase inhibitor, did not enhance the adhesion of the wt cells, indicating that the stronger attachment of the PS-ko cells to fibronectin- and laminin-coated dishes was

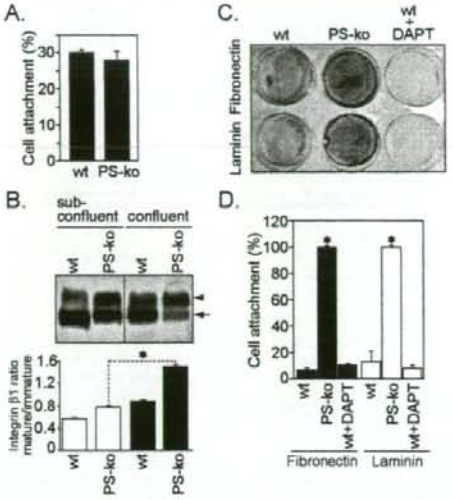

FIGURE 6: Enhanced cell adhesion to integrin β1 ligands, fibronectin and laminin in confluent PS-ko cells. The single wt and PS-ko fibroblasts prepared from subconfluent cultures were plated on fibronectin-coated 96-well plates. The plates were incubated for 30 min at 37 °C in 5% (v/v) CO_2. Unbound or loosely bound cells were removed by aspiration and gentle washing in HBS. The bound cells were fixed and stained. The percentage of bound cells was measured and calculated (A). Lysates of the subconfluent or confluent wt and PS-ko fibroblasts were subjected to SDS−PAGE and Western blotting. The confluent PS-ko fibroblasts showed enhanced integrin β1 maturation compared with the subconfluent PS-ko fibroblasts. Arrowhead, mature integrin β1; arrow, immature integrin β1; □, subconfluent fibroblasts; ■, confluent fibroblasts. Data represent the means ± SEM; $n = 3$, $^*p < 0.001$, confluent PS-ko cells vs subconfluent PS-ko cells, Bonferroni/Dunn test (B). The wt and PS-ko fibroblasts were plated on fibronectin- or laminin-coated 6-well plates with or without 5 μM DAPT after reaching confluence, and adhesion ability was estimated. The cells were incubated with 1 mM EDTA for 30 min at room temperature. Detached cells were washed out with HBS buffer, and attached cells were stained with 0.1% (w/v) crystal violet (C). The percentage area occupied by the attached cells was measured. ■, fibronectin-coated; □, laminin-coated. Data represent the means ± SEM; $n = 3$, $^*p < 0.001$, PS-ko cells vs wt or wt + DAPT cells, Bonferroni/Dunn test (D).

a γ-secretase-independent effect (Figure 6C and D). These results suggest that cell-surface-mature integrin β1 with an increased expression level in PS-ko cells works as a receptor of fibronectin and laminin and that this increased expression level induces a strong adhesion of the cells to the ligands of integrin β1.

Because the increased expression level of integrin β1 on the surface of PS-ko cells seems to generate multiple functional integrin heterodimers, which enhance cell adhesion, we determined whether PS regulates the maturation of integrins α1-α7, αV, and αL and whether PS1 associates with integrin β1. No integrin α1, α4, α6, α7, or αL was detected in these cells by Western blotting (data not shown). The total expression level of integrin α2 was downregulated by PS deficiency (Figure 7A). Integrin αV expression level remained unchanged in the PS-ko cells and increased in the PS1(−/−) and PS2(−/−) cells; however, PS deficiency had no effect on the maturation of integrins α2 and αV (Figure 7A). Interestingly, similar to integrin β1, integrins α3 and α5 in the PS-ko, PS1(−/−), and PS2(−/−) cells showed higher molecular weights than those in the wt cells, indicating

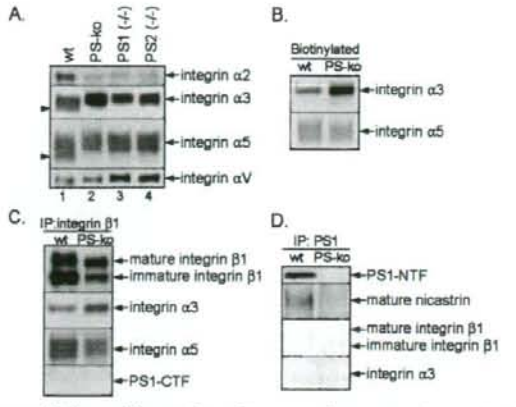

FIGURE 7: Presenilin regulates the expression, maturation and cell surface delivery of integrin α subunits. The wt, PS-ko, PS1(−/−), and PS2(−/−) cells were lysed in RIPA buffer after reaching confluence. Western blots of 50 μg of total protein from the cells were probed with anti-α2, anti-α3, anti-α5, and anti-αV integrin antibodies. Arrowheads indicate immature integrins α3 and α5; arrows indicate mature integrins α2, α3, α5, and αV (A). Biotinylated cell surface proteins of the wt and PS-ko cells were probed with anti-integrin α3 and α5 antibodies. Arrows indicate cell surface mature integrins α3 and α5 (B). Total cell lysates of wt and PS-ko cells were immunoprecipitated with a polyclonal anti-integrin β1 antibody and probed with monoclonal antibodies to integrin β1, α3, and PS1 and with a polyclonal antibody to integrin α5. Mature integrins α3 and α5 were coimmunoprecipitated with integrin β1 (C). Total cell lysates of wt and PS-ko cells were immunoprecipitated with a polyclonal anti-PS1 antibody and probed with polyclonal antibodies to PS1 and nicastrin and with monoclonal antibodies to integrin β1 and α3 (D). Mature nicastrin was coimmunoprecipitated with PS1, whereas integrins β1 and α3 were not.

that their maturation was enhanced (Figure 7A). We used cell-surface biotinylation to examine the cell-surface delivery of integrins α3 and α5. As expected, only mature integrins α3 and α5 with high molecular weights were delivered to the cell surface; moreover, the expression level of integrin α3 on the cell surface increased in the PS-ko cells, whereas that of integrin α5 remained unchanged (Figure 7B). We determined whether integrin β1 forms heterodimers with integrins α3 and α5 using immunoprecipitation analysis. Integrins α3 and α5 were coimmunoprecipitated with integrin β1, indicating the formation of functional heterodimers of integrins α3β1 and α5β1 (Figure 7C). We also found that the level of coimmunoprecipitated integrin α3 in the PS-ko cells increased compared with that in the wt cells, suggesting the enhanced maturation and cell-surface delivery, and the heterodimer formation of integrins β1 and α3 in PS-ko cells (Figure 7C). PS1 was not coimmunoprecipitated by the anti-integrin β1 antibody, indicating that PS1 was not associated with integrin β1 (Figure 7C). This was also confirmed by immunoprecipitation using the anti-PS1 antibody; mature nicastrin was coimmunoprecipitated with PS1, whereas neither integrin β1 nor integrin α3 was coimmunoprecipitated with PS1 (Figure 7D). These results suggest that PS may exert an inhibitory effect on the maturation of integrins β1 and α3 via a less direct pathway.

DISCUSSION

In this study, we demonstrated that PS deficiency leads to the enhanced maturation and cell-surface delivery of integrin β1. A marked decrease in the expression level of immature integrin β1, which is partially glycosylated and localized in ER, and an increase in that of mature integrin β1 in the PS-ko cells indicate the accelerated trafficking of integrin β1 from the ER to the Golgi apparatus. These data suggest that PS proteins also exert an inhibitory effect on the trafficking of a membrane protein from the ER to the Golgi apparatus in addition to serving as an ER-resident chaperone, which was consistent with the results of previous studies showing that the loss of function of PS results in a disrupted maturation or an enhanced intracellular retardation of nicastrin, TrkB, N-cadherin, caveolin 1, and telencephalin/ICAM (*12, 16, 17, 20, 21*). Therefore, we conclude that PS regulates the trafficking of membrane proteins from the ER to the Golgi apparatus in opposite directions, that is, PS promotes or inhibits the trafficking of membrane proteins in a protein-specific manner. In agreement with our results, results of previous studies showed that APP maturation is enhanced in PS-ko cells (*15*) and that PS1 deficiency or the loss of the PS function enhances APP maturation or causes cell-surface APP accumulation (*13, 34*). Moreover, a PS1 deletion mutant (ΔM1,2) exhibits an increased cell-surface expression level of nicotinic acetylcholine receptors (AChRs) (*15*). The post-translational maturation of integrin β1 is strictly regulated by the expression of PS. The PS-ko cells showed a marked increase in the expression level of mature integrin β1, whereas the PS1(−/−) or PS2(−/−) cells exhibited an intermediate increase in that of mature integrin β1 (Figure 1).

It is reasonable to speculate that the cell surface accumulation of integrin β1 is a result of attenuated internalization or an increased expression level of integrin β1 because a previous study showed that the loss of PS1 or PS2 function induced by the mutation of one of the critical aspartate residues or by γ-secretase inhibitors results in delayed APP reinternalization and APP accumulation on the cell surface (*14*). Another study showed that an enhanced cell-surface expression level of mature APP is not accompanied by a decrease in the expression level of immature APP in PS-ko or ΔM1,2 cells, suggesting that the total expression level of APP increases in these cells (*15*). We also found that the expression levels of both mature and immature APPs are higher in PS-ko cells than in wt cells (data not shown), in agreement with the finding of a previous study. This is also the case of acetylcholine receptors (AChRs), that is, the cell-surface and total expression levels of AChRs increase in PS1-ΔM1,2 cells (*15*). In our study, the total expression level of integrin β1 in the PS-ko cells was not altered compared with that in the wt cells because the increase in the expression level of mature integrin β1 was always accompanied by a decrease in that of immature integrin β1. The localization or trafficking of integrin β1 strictly depends on the glycosylation state of integrin β1, and partially glycosylated immature integrin β1 forms a stable pool within the ER (*26, 35, 36*). Subcellular fractionation studies showed an enhanced maturation of integrin β1 in Golgi apparatus fractions (Figure 3). Moreover, mature integrin β1 was modified by sialic acid (Figure 2). Note that the modification of complex N-linked oligosaccharides by sialic acid occurs within the Golgi apparatus (*37*). Finally, we demonstrated that the internalization of mature integrin β1 in PS-ko cells is unchanged compared with that in wt cells. Thus, these

lines of evidence suggest that an increase in the expression level of mature integrin β1 in PS-ko cells is specifically caused by the accelerated trafficking of integrin β1 from ER to the Golgi apparatus, not by an increase in integrin β1 total expression level or the delayed internalization of mature integrin β1.

PS can regulate cell adhesion via its γ-secretase substrates, such as E-cadherin, N-cadherin, CD44, and the voltage-gated sodium channel β2-subunit, and via the regulation of β-catenin and telencephalin turnover (*9, 29, 38*). Here, we demonstrated that PS regulates cell adhesion to integrin β1 ligands by modulating integrin β1 maturation and cell-surface delivery, although whether integrin β1 is a substrate of PS remains to be elucidated. We also provided evidence that the inhibition of γ-secretase activity does not enhance the expression level of mature integrin β1 or the adhesion of wt cells to fibronectin or laminin. The results of our study and previous studies show that PS1 D257A or PS1 D385A lacking γ-secretase activity expressed in a PS-null background restores nicastrin maturation; however, these mutants did not restore the expression of mature integrin β1. Because PS1 aspartate mutants do not form the mature, high molecular weight PS complexes (*30*), these results suggest that the formation of a high molecular weight PS complex is necessary for its inhibitory effect on integrin β1 maturation. Another possibility is that the loss of both the γ-secretase activity and the chaperone protein function of PS may be required to facilitate integrin β1 maturation because enhanced integrin β1 maturation was only found in PS-deficient cells.

Integrin β1 associates with multiple α-subunits to form integrin heterodimers that show cell adhesion activity (*31–33*). Our results suggest that an increase in the expression level of integrin β1 results in enhanced cell adhesion to integrin β1 ligands, fibronectin and laminin. We postulated that some integrin α-subunits are also regulated by PS deficiency. We estimated the maturation of some integrin α-subunits and observed increases in the cell-surface expression level of mature α3 subunits in PS-ko cells. Mature integrin α3 coimmunoprecipitated with integrin β1 also showed an increase in expression level in the PS-ko cells. However, previous studies have consistently shown that integrin α3β1 is a strong receptor for laminin-5, laminin-10, and laminin-11 but that it mediates cell adhesion to fibronectin or laminin-1 either very poorly or not at all (*39, 40*). Some studies showed that integrin α3β1 inhibits the activities of receptors for these ligands through transdominant inhibition (*41, 42*). Therefore, although the integrin α3β1 heterodimer showed an increase in expression level in the PS-ko cells, the stronger adhesion of the PS-ko cells may have been due to changes in the expression levels of other integrin heterodimers. In agreement with the results of previous studies, it was found that mature nicastrin associates with PS1 and that immature nicastrin is impeded in the ER of PS-ko cells, suggesting that nicastrin maturation is directly mediated by PS. We did not find a physical interaction between PS1 and integrins β1 or α3. These results suggest that the maturation of integrins β1 and α3 is inhibited by PS via a less direct pathway, which may be activated by the absence of PS. The maturation of integrins β1 and α3 is likely regulated via the same pathway through their interaction.

PS mutants play an important role in the neurodegeneration of familial Alzheimer's disease; however, the mechanism of such neurodegeneration is not yet fully understood. Although the overexpression of human FAD PS1 mutants did not affect integrin β1 maturation in the wt cells, the chronic effects of PS1 mutants on the maturation of integrins need to be investigated in vivo. Recently, some roles of integrin β1 in the nervous system have been identified. Conditional integrin β1 gene deletion in neural crest cells leads to a delayed migration of Schwann cells and induces multiple defects in spinal nerve arborization and morphology (*43*). Interestingly, a postnatal forebrain and excitatory neuron-specific knockout of the integrin β1 mouse model shows impaired hippocampal AMPA receptor-dependent synaptic transmission, synaptic plasticity, and working memory (*44*). These studies suggest that integrin β1 is important for nervous system development and serves as a regulator of synaptic glutamate receptor functions and working memory. Thus, our results suggest that modulation of integrin β1 maturation by PS plays a role in nervous system development and memory.

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Angiotensin-Converting Enzyme as a Potential Target for Treatment of Alzheimer's Disease: Inhibition or Activation?

Kun Zou[1] and Makoto Michikawa[2]

[1]*Department of Neuroscience, Faculty of Pharmaceutical Sciences, Iwate Medical University, Iwate and*
[2]*Department of Alzheimer's Disease Research, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Aichi, Japan*

SYNOPSIS

The accumulation of the amyloid β-protein (Aβ), the main constituent of the 'amyloid plaque', is widely considered to be the key pathological event in Alzheimer's disease (AD). In particular, the accumulation of $A\beta_{42}$ is the central event triggering neurodegeneration. Reduction of Aβ is now a major therapeutic strategy. However, only a few patients show evidence of increased Aβ production. Thus, defects in proteases that degrade Aβ could underlie some or many cases of familial and sporadic AD. Among the Aβ degrading enzymes, namely, neprilysin (NEP), insulin-degrading enzyme (IDE), endothelin-converting enzyme (ECE) and angiotensin-converting enzyme (ACE), ACE is the most commonly targeted enzyme by inhibitors in elderly populations because it plays a central role in the regulation of blood pressure and hypertension. Genetic, pathological and biochemical studies have associated ACE with AD. This review discusses genetic, molecular and clinical studies that might help explain the relationship between ACE, hypertension, Aβ degradation and AD.

KEY WORDS

angiotensin-converting enzyme (ACE), Alzheimer's disease, amyloid β-protein (Aβ), Aβ deposition, Aβ degradation, ACE inhibitor

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Reprint address:
Kun Zou, M.D., Ph.D.
Department of Neuroscience
Faculty of Pharmaceutical Sciences
Iwate Medical University
Yahaba, Iwate 028-3694, Japan
e-mail: kunzou@iwate-med.ac.jp

ANGIOTENSIN-CONVERTING ENZYME POLYMORPHISM AND ALZHEIMER'S DISEASE

More than half a century ago, ACE was first isolated as a 'hypertensin-converting enzyme' /83/. It plays an important role in the renin-angiotensin system (RAS), which is involved in the long-term regulation of blood pressure and blood volume in the human body. The classic view of RAS is that renin acts on angiotensinogen to yield angiotensin I, which in turn is cleaved by ACE to form the active angiotensin II. Angiotensin II is a potent vasoconstrictor and exerts its hypertensive effects by its action on two receptors /12,89/. However, ACE also degrades other functional peptides, such as the vasodilator and inflammatory peptide bradykinin, substance P, cholecystokinin, and luteinizing hormone-releasing hormone (LHRH) /86,90/. ACE is both a membrane-bound zinc metallopeptidase and a dipeptidyl carboxypeptidase, and is expressed on the surface of endothelial cells and several types of epithelial and neuroepithelial cells. The active site of ACE is found in the extracellular space, and the unbound form of ACE circulates in biological fluids, such as plasma and cerebrospinal fluid (CSF), and both types of ACE have enzymatic activity /68,82,95/. It is now well established that the brain has its own intrinsic RAS with all its components present in the central nervous system /71,87/.

Plasma ACE levels are stable when measured repeatedly in the same individual, whereas large inter-individual differences are observed /1/. In 1990, Rigat and coworkers /68/ found a polymorphism involving the insertion (I) or deletion (D)