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Alzheimer's Disease-Linked Mutation of Presenilin 2 (N141I-PS2) Drastically Lowers APPα Secretion: Control by the Proteasome

P. Marambaud, C. Alves da Costa, K. Ancolio, and F. Checler[1]
Institut de Pharmacologie Moléculaire et Cellulaire du CNRS, UPR411, 660 Route des Lucioles, Sophia Antipolis, 06560 Valbonne, France

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Most of early onset familial forms of Alzheimer's disease (FAD) are due to inherited mutations located on two homologous proteins, presenilins 1 and 2 (PS1 and PS2) encoded by chromosomes 14 and 1, respectively. Here we show that the expression of wild type (wt)-PS2 in human HEK293 cells increases the production of the physiological α-secretase-derived product, APPα. By contrast, APPα secretion is drastically reduced in cells expressing the FAD-linked N141I-PS2. We establish that wt-PS2, N141I-PS2 and their C-terminal maturation fragment are degraded by the enzymatic multicatalytic complex, proteasome. Interestingly, two selective proteasome inhibitors, Z-IE(Ot-Bu)A-Leucinal and lactacystin potentiate the APPα secretion observed in wtPS2-expressing cells and further amplify the N141I-PS2-induced decrease in APPα production. By contrast, a series of pharmacological agents unable to affect the proteasome do not modify PS2 immunoreactivities and APPα recoveries. Altogether, our data indicate that: 1) wtPS2 positively modulates the α-secretase physiological pathway of βAPP maturation in human cells; 2) N141I mutation on PS2 drastically lowers the secretion of APPα; 3) Proteasome inhibitors prevent the degradation of wtPS2, N141I-PS2 and their C-terminal maturation product. This protection against proteasomal degradation directly modulates the APPα secretion response elicited by wt- and FAD-linked PS2 expression in human HEK293 cells.

Alzheimer's Disease (AD) is a devastating age-related neurodegenerative syndrome that generally occurs in the late 60s. However, genetic forms of the disease can appear much earlier and are due to inherited autosomal dominant mutations located on βAPP, presenilin 1 and presenilin 2, three gene products encoded by chromosomes 21, 14 and 1, respectively (for reviews see 1-5). These agressive and early onset forms of AD are thought to derive from the exacerbation of the production of a poorly soluble peptide called Aβ, the main constituent of the senile plaques invading cortical and subcortical areas in the AD affected brains. Thus several lines of evidence derived from neuropathological, cell biology and transgenesis approaches (for review see 6-8) have indicated that presenilins 1 and 2 mutations particularly favor the overproduction of the more readily aggregable 42 amino-acids long Aβ species (9).

We recently demonstrated that in human HEK293 cells, missense PS1 mutations not only elicited a drastic increase of the Aβ42 over total Aβ ratio (10) but also triggered an important lowering of secreted APPα, the α-secretase-derived physiological product of βAPP maturation (11). More recently, we established that PS1 and its FAD-linked counterparts were degraded by the multicatalytic complex proteasome and that accordingly, proteasome inhibitors drastically exacerbated the mutated PS1-related increase in the Aβ42/Aβ total ratio (12).

Here we show that overexpression of wild type PS2 in human cells leads to increased secretion of APPα while the FAD-linked N141I mutation on PS2 drastically lowers APPα recovery. In addition, we establish that the proteasome inhibitors drastically enhance the immunoreactivities of wtPS2, N141I-PS2 and their common C-terminal fragment (CTF-PS2)-like in stably transfected HEK293 cells overexpressing these three proteins. Interestingly, proteasome inhibitors also potentiate the wtPS2-related increase in APPα secretion while they further exacerbate the N141I-PS2-induced APPα decrease. This reinforces the demonstration that PS2 exerts a positive control on the α-secretase pathway and that the N141I FAD-linked mutation of PS2,

[1] To whom correspondence should be addressed. Fax: (33) 4 93 95 77 08/04. E-mail: checler@ipmc.cnrs.fr.

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besides the exacerbation of the pathogenic production of Aβ42, concomittantly decrease the physiological production of APPα.

MATERIALS AND METHODS

N141I-PS2 and CTF-PS2 mutagenesis. The N141I-PS2 was obtained by oligonucleotide-directed mutagenesis from PS2 cDNA (obtained from Dr. S.S. Sisodia) according to the uracylated single strands strategy described by Kunkel et al. (13). The CTF-PS2 construction was engineered by introducing the Kozak sequence of PS2 upstream of the ATG codon encoding the methionine in position 298. An additional Kpn restriction site was also added, adjacent to the Kozak sequence for further subcloning of the construction in pcDNA3.

Stable transfections in HEK293 cells. HEK293 cells were grown as previously described (14). Cells were stably transfected by calcium phosphate precipitation with 1μg of pcDNA3-containing either wild type PS2, N141I-PS2 or CTF-PS2. Transfectants were identified after western blot analysis of electrophoresed proteins by means of the αPS2Loop antibody (15).

Western blot analysis of PS2, N141I-PS2, and CTF-PS2 immunoreactivities. Stably transfected cells were maintained in F12/DMEM (vol/vol) supplemented with 10% fetal calf serum containing penicillin (100U/ml), streptomycin (50μg/ml) and geneticin (1mg/ml). Cells were incubated for 15 hours at 37°C, in absence or in the presence of lactacystin (5μM) or Z-IE(Ot-Bu)A-Leucinal (5μM) then cells were washed, lysed and proteins were analysed for PS2- or CTF-PS2 immunoreactivities with αPS2Loop as described above.

Immunoprecipitation and detection of APPα. Stably transfected cells were maintained in the above F12 /DMEM supplemented medium then washed, and secretion of APPα was initiated for 5 hours at 37°C in the absence or with proteasome inhibitors. Media were recovered, diluted in an equal volume of RIPA buffer and incubated overnight with a 3000-fold dilution of 207 antibody (16) in the presence of pansorbin (20μl, Calbiochem). Samples are centrifuged, and the pellets washed three times with RIPA 1X containing NaCl (350mM), rinsed with TBS buffer then resuspended in the loading buffer, electrophoresed on a 8% SDS-PAGE and western blotted for 3 hours. Nitrocellulose sheets were capped with skim milk (5% in TBS buffer) and exposed overnight to a 200 fold dilution of mAb2H3 antibodies. Nitrocelluloses were rinsed with TBS buffer then incubated with adequate anti-IgGs, revealed and quantified as previously described (14).

Western blot analysis of βAPP immunoreactivity. Stably transfected cells were treated as above then lysed and analysed for βAPP content by means of BR188 antibody as described previously (11).

Antibodies. FCA3340 and FCA3542 specifically interact with Aβ40 and Aβ42 respectively (10). The 207 antibody (donated by Drs. M. Savage and B. Greenberg, Cephalon, West Chester) recognizes the N-terminus of βAPP and APPα. 2H3 (given by Dr. D. Schenk, Athena Neurosciences) specifically recognizes the C-terminus of APPα. αPS2Loop (provided by Drs. G. Thinakaran and S.S. Sisodia, John Hopkins, Baltimore) specifically interacts with the hydrophilic loop of PS2 located between its predicted sixth and seventh transmembrane domain. BR188 (supplied by Dr. M. Goedert, Cambridge, England) recognizes the C-terminus of mature and immature βAPP.

RESULTS AND DISCUSSION

wtPS2 and the FAD-linked N141I-PS2 modulate APPα secretion in human HEK293 cells. It has been extensively documented that the presenilin mutations responsible for Alzheimer's disease trigger an increased formation of Aβ, and particularly increase the ratio of Aβ42 over total Aβ (for review see 6-8). We recently demonstrated that wtPS1 overexpression in HEK293 cells also leads to an augmented secretion of APPα, while FAD-linked mutated PS1 expression drastically lowers APPα recovery (11). Previous works indicate that FAD-linked PS2 also augment the ratio of Aβ42 over total Aβ production (17,18). We have selected several clones of stably transfected HEK293 cells overexpressing wtPS2 or N141I-PS2 and displaying similar levels of endogenous βAPP (Fig. 1A) to examine their APPα secretion. The overexpression of wild type PS2 leads to a statistically significant increased secretion of APPα when compared to mock transfected cells (Fig. 1B,C). By contrast, it appears clearly that two independent clones expressing N141I-PS2 secrete drastically lower ($p<0,01$ or $p<0,001$) amounts of APPα than mock- and wtPS2-transfected

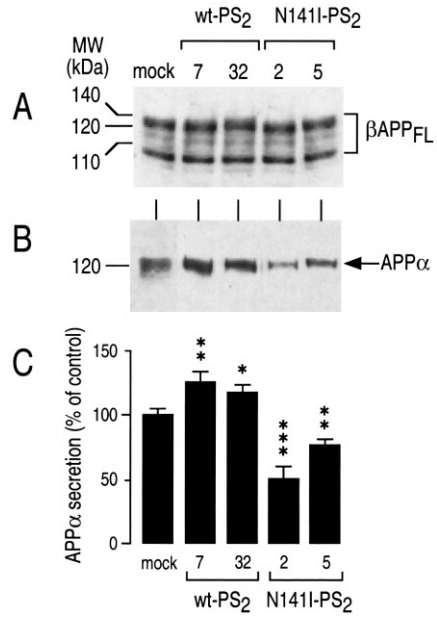

FIG. 1. Influence of wtPS2, and N141I-PS2 overexpression on APPα secretion. Mock-transfected HEK293 cells or transfectants overexpressing wtPS2 or N141I-PS2 were grown as described in the Methods. APPα was immunoprecipitated after 5 hours of secretion by means of 207 antibody, and quantified after gel electrophoresis, western blot, and revelation of immunological complexes with mAb2H3 (B) that recognizes the C-terminus of APPα (absent in APPβ). Intracellular content of mature, and immature forms of βAPP (A) was established after cell lysis by means of the BR188 antibody as described in the methods. In panel C, bars represent the densitometric analysis of APPα secretion expressed as the percent of control corresponding to APPα secretion by mock transfected cells. Values are the means, +/− SEM of 4 independent experiments carried out on the indicated numbered independent clones. Statistical analysis of wt, and N141I-PS2 when compared to mock transfected cells: * $p<0,02$; ** $p<0,01$; *** $p<0,001$.

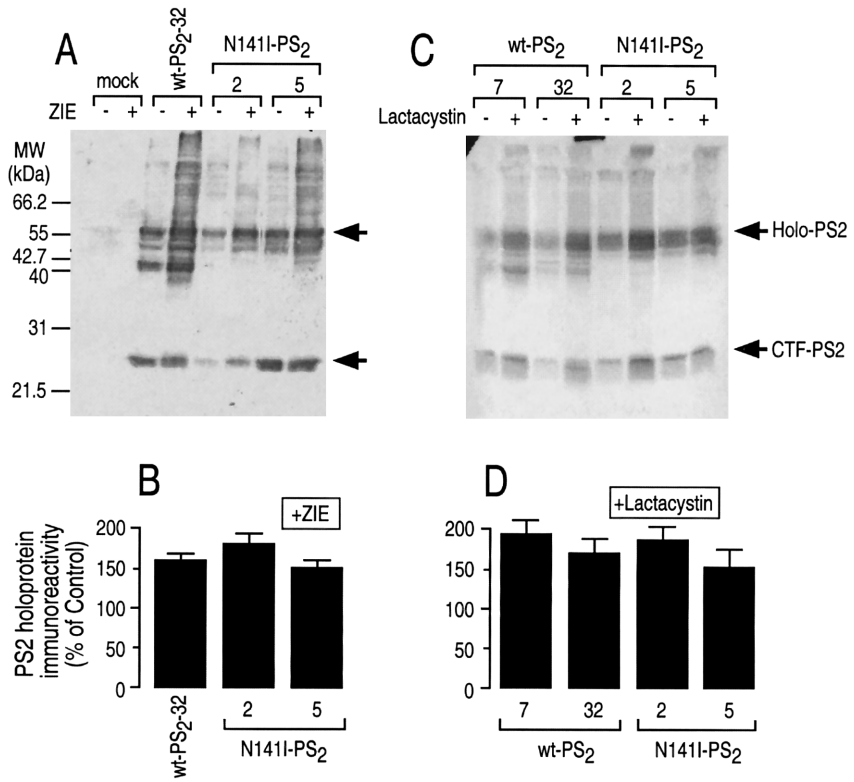

FIG. 2. Effect of proteasome inhibitors on wtPS2, and N141I-PS2 immunoreactivities in HEK293 cells. Mock transfected HEK293 cells, and transfectants expressing wtPS2, and N141I-PS2 were incubated for 15 hours with a final concentration of 5μM of Z-IE(Ot-Bu)A-leucinal (ZIE, panels A, and B) or lactacystin (C,D) then cells were lysed, and immunoreactivities of PS2 holoprotein (Holo-PS2), and its C-terminal fragment (CTF-PS2) were revealed with αPS2Loop antibody. Histograms in B, and D represent the densitometric analysis of PS2 holoprotein obtained in presence of Z-IE(Ot-Bu)A-leucinal (B), and lactacystin (D), and are expressed as the percent of control (PS2 immunoreactivity in untreated cells). Bars correspond to the means +/− of 3-4 independent experiments performed on the indicated numbered clones.

cells (Fig. 1B,C). Therefore, we demonstrate for the first time that wtPS2 expression increases APPα secretion while the FAD-linked N141I-PS2, besides favoring the production of β/γ-secretases-derived Aβ42, also controls the α-secretase pathway by lowering secreted APPα.

wtPS2, N141I-PS2 and CTF-PS2 are degraded by the proteasome in human HEK293 cells. The αPS2Loop antibody directed towards the hydrophilic loop of PS2 reveals an immunoreactive band of about 55kDa in wtPS2-expressing cells that likely corresponds to the PS2 holoprotein as well as a 22-25kDa band (Fig. 2A,C) previously characterized as the C-terminal fragment derived from PS2 maturation (14). The same immunoreactivity pattern is observed in various HEK293 clones expressing N141I-PS2 (Fig. 2A,C). Two proteasome inhibitors, Z-IE(Ot-Bu)A-Leucinal (Fig. 2A) and lactacystin (Fig. 2C) potentiate the recovery of the wtPS2 holoprotein (150 to 200% of control Fig. 2B,D). It should be noted here that the treatment of the cells with both proteasome blocking agents reveal a immunoreactive smir of high molecular proteins. This likely corresponds to immunoreactive ubiquitinated PS2, the degradation of which has been prevented by proteasome inhibitors. This agrees well with the study by Kim et al. (19) showing that PS2 undergoes ubiquitination and is degraded by the proteasome. Interestingly, we further establish that N141I-PS2 immunoreactivities are enhanced to the same extent (150 to 200% of control Fig. 2B,D) in two distinct transfected clones Fig. 2A,C). Therefore, it appears that the proteasome does not discriminate between wild type and mutated PS2 as we also showed for wild type and mutated PS1 (12). Other inhibitors unable to affect the proteasome activity appear unable to modify the wtPS2-related immunoreactivity (Fig. 3A). Thus E64 (thiol and serine protease inhibitor), AEBSF (serine protease inhibitor), phosphoramidon (endopeptidase 3.4.24.11 inhibitor), pepstatin (acid protease inhibitor), and Z-L-Leucinal (calpain and cathepsin B inhibitor) do not protect wtPS2 from degradation in HEK293 cells (Fig. 3A).

Interestingly, proteasome inhibitors also enhance the CTF-PS2 immunoreactivities in wt- and N141I-PS2-expressing cells (Fig. 2A,C). This could be due to the direct blockade of CTF-PS2 degradation by the proteasome. Alternatively, it could not be excluded that this increase only reflects the maturation of higher amounts of wt and N141I-PS2, the degradation of which would have been slowed down by proteasome inhibitors. We have examined the effect of proteasome inhibitors on CTF-PS2 immunoreactivity in stably transfected human HEK293 cells. Clearly, the Fig. 3B shows that Z-IE(Ot-Bu)A-Leucinal and lactacystin drastically enhance CTF-PS2 immunoreactivity (Fig. 3B), indicating that CTF-PS2 also behaves as a proteasomal substrate. Other inhibitors unable to affect the proteasome activity do not affect CTF-PS2 immunoreactivity (Fig. 3B).

Proteasome inhibitors modulate the formation of APPα in PS2 and N141I-PS2-expressing HEK293 cells. If wtPS2 and N141I-PS2 degradation by the proteasome is physiologically relevant, one would expect to potentiate the effect of wtPS2 and mutated PS2 on APPα secretion upon proteasome inhibition. Indeed, the two proteasome inhibitors statistically significantly ($p<0,001$) potentiate the increase in APPα secretion triggered by overexpression of wtPS2 (Fig. 4B,D-E). It is interesting to note that these inhibitors also increase the APPα secretion by mock transfected cells ($p<0,01$), as expected from the protection of endogenous wild type PS1/PS2 (Fig. 4B,E) indicating that the involvement of the proteasome was not artifactually related with overexpresssion of the PS. Proteasome inhibitors also potentiate the altered phenotype of N141I-PS2 expressing cells (Fig. 4B,D,E). Thus, lactacystin significantly ($p<0,01$) amplifies the N141I-PS2-dependent decrease in APPα secretion (Fig. 4D,E). It should be noted here that proteasome inhibitors do not affect the βAPP immunoreactivity indicating that βAPP does not undergo proteasomal degradation in HEK293 cells (Fig. 4A,C). Therefore, the modification of APPα secretion can not be accounted for an altered catabolism of its precursor, βAPP.

Our work clearly establishes that wtPS2 is positively coupled to APPα secretion and that FAD-linked mutations trigger drastic diminishment of APPα secretion. This influence on the α-secretase pathway is under the control of the proteasome, the inhibitors of which potentiate the various phenotypic alterations of APPα secretion observed with wtPS2 and mutated PS2. It could therefore be hypothesized that the pathogenic phenotype of mutated PS2 is not only due to the increase in Aβ42 formation but also derives from the lowering of APPα secretion since several works have indicated that APPα could be neurotrophic and could protect cells from Aβ cytotoxicity (for review see 20).

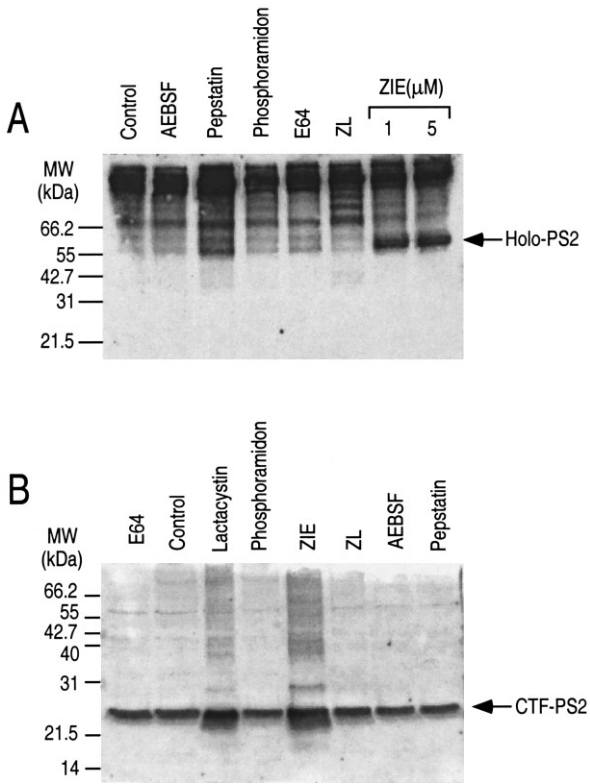

FIG. 3. Effect of various inhibitors on wtPS2-, and CTF-PS2 immunoreactivities in HEK293 transfected cells. Stably transfected HEK293 cells expressing wtPS2 (A) or CTF-PS2 (B) were incubated for 5 hours without (control) or in the presence of trans-epoxy-succinyl-L-leucylamido-(4-guanidino) butane (E64, 10^{-4}M), 4-(2-amido-ethyl) benzenesulfonyl fluoride (AEBSF, 10^{-4}M), phosphoramidon (10^{-5}M), pepstatin A (10^{-5}M), Z-L-Leucinal (ZL, 2,5 10^{-5}M), Z-IE(Ot-Bu)A-leucinal (ZIE, 10^{-6}M or 5 10^{-6}M), and lactacystin (5 10^{-6}M). Cells were lysed then immuno-reactivities of PS2 holoprotein (A) or CTF-PS2 (B) were revealed with αPS2Loop antibody as described in the Methods.

Therefore a strategy consisting in the design of proteasome activators increasing mutated PS2 degradation and, thereby, lowering its pathogenic effect on βAPP processing could be envisioned.

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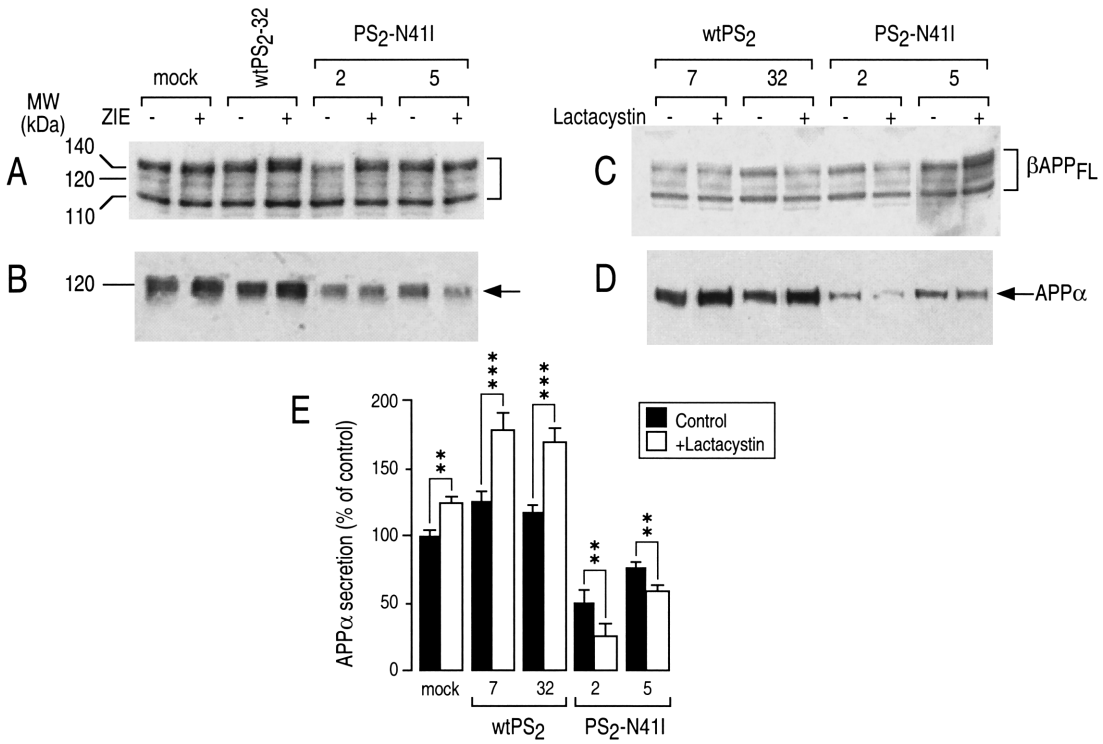

FIG. 4. Effect of proteasome inhibitors on APPα secretion by wtPS2, and N141I-PS2-expressing cells. Mock-transfected HEK293 cells or transfectants overexpressing wtPS2 or N141I-PS2 were grown as described in the Methods then secretion was initiated in the absence (−) or in the presence (+) of Z-IE(Ot-Bu)A-leucinal (A,B) or lactacystin (C,D). APPα was immunoprecipitated after 5 hours by means of 207 antibody, and revealed with mAb2H3 (B,D) as in the Figure 1. Intracellular contents of mature, and immature forms of βAPP (A,C) were established after cell lysis by means of the BR188 antibody as described in the methods. In panel E, bars represent the densitometric analysis of APPα secretion (in arbitrary units) expressed as the percent of control corresponding to APPα secretion obtained with mock transfected cells in absence of inhibitor. Values are the means, +/− SEM of 3 to 4 independent experiments carried out on the indicated numbered independent clones. Statistical analysis between bracketed bars are ** $p<0,01$; *** $p<0,001$.

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