Neuroscience Letters 525 (2012) 49–53

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

The γ-secretase inhibitor DAPT increases the levels of gangliosides at neuritic terminals of differentiating PC12 cells

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HIGHLIGHTS

- The γ-secretase inhibitor DAPT perturbed Aβ production like clinical presenilin mutations.
- Lipidomics protocol for neuritic terminals of PC12 cells was established.
- The γ-secretase inhibitor DAPT increased the gangliosides at neuritic terminals.

ARTICLE INFO

Article history:
Received 11 May 2012
Received in revised form 13 June 2012
Accepted 12 July 2012

Key words:
Alzheimer disease
γ-Secretase
Amyloid β-protein
Ganglioside
Liquid chromatography/electrospray ionization tandem mass spectrometry
Lipidomics

ABSTRACT

Mutations in presenilins are the major cause of early onset familial Alzheimer disease. It has recently been argued that clinical presenilin mutations work as loss-of-function but not toxic gain-of-function. To investigate whether presenilins are involved in the regulation of the distribution of neuronal membrane lipids, we treated neuronally differentiated PC12 cells with DAPT, an inhibitor of presenilin-dependent γ-secretase, and performed lipid analyses of neuritic terminals, which is an initial site of Aβ deposition in brains, using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) in combination with multiple reaction monitoring (MRM). With DAPT treatment, levels of sphingomyelin, phosphatidylcholine, and cholesterol remained unchanged. However, DAPT treatment increased the ganglioside levels in PC12 neuritic terminals. Together with a previous finding that accumulation of gangliosides at neuritic terminals facilitates Aβ assembly and deposition, the present data suggest that the loss-of-function of presenilins, i.e., a decrease in γ-secretase activity, has an impact on neuronal membrane architecture in a way that eventually exacerbates Alzheimer pathology.

1. Introduction

Mutations in presenilins were identified as causative factors for early onset familial Alzheimer disease (AD); later, presenilins were found to be the major components of the γ-secretase complex, which is responsible for the intramembranous cleavage of type-I membrane proteins, including the amyloid precursor protein (APP) and Notch. The cleavage of APP by γ-secretase liberates the intracellular domains of APP (AICD) and amyloid β-protein (Aβ), a major proteinaceous constituent of senile plaques in AD brains. Previous studies suggested that the familial AD-linked presenilin mutations induce a shift in the APP cleavage, resulting in the increase in the ratio of the level of Aβ with 42 residues (Aβ42) (the longer and more hydrophobic isoform) to that of Aβ with 40 residues (Aβ40) [22]. However, the presenilin mutations identified in familial AD do not necessarily increase but rather decrease Aβ production level [15]; furthermore, the vast majority of the mutations decrease the generation levels of AICD [29] and the cleaved product of Notch (NICD) [24]. Strikingly, a recently identified mutation in a familial AD pedigree causes a nearly complete suppression of γ-secretase activity despite the significant development of AD pathology [10]. Alternatively, although the mechanism remains to be determined, treatment with γ-secretase inhibitors at low concentrations in a culture or cell-free system can paradoxically enhance Aβ42 production, mimicking the effect of a part of clinical presenilin mutations [2,34]. Along this line of evidence, it has been argued that presenilin mutations cause AD through loss-of-function, i.e., a decrease in γ-secretase activity [4,23].

Abbreviations: Aβ, amyloidβ-peptide; AD, Alzheimer disease; APP, β-amyloid precursor protein; ESI, electrospray ionization; GAβ, ganglioside-bound Aβ; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; nanoESI, nanospray ESI; NGF, nerve growth factor; NT, neuritic terminal plasma membrane; PS, presenilin; UPLC, ultraperformance liquid chromatography.

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http://dx.doi.org/10.1016/j.neulet.2012.07.027

If loss-of-function accounts for the pathological significance of clinical presenilin mutations, one of the intriguing issues that need to be addressed is how accelerated Aβ assembly and deposition, which is an invariable and initial pathological feature of AD, occur under the condition of suppressed Aβ production. A possible explanation for this phenomenon is alterations in the levels of neuronal membrane lipids because of the following. First, the composition of cellular membrane lipids is readily changed through transgenic expression or deficiency of presenilins (wild type and mutants), although the alteration patterns observed were diverse probably owing to differences in materials and experimental procedures [5–7]. Second, evidence exists that Aβ starts to assemble into amyloid fibrils in the brain through interaction with one of the neuronal membrane lipids, GM1 ganglioside, at neuritic terminals, leading to the generation of GM1-ganglioside-bound Aβ (GAβ), which works as an endogenous seed for AD amyloid (see [32] for review).

In this study, we examined whether the suppression of γ-secretase activity through pharmacological treatment alters the levels and distribution of neuronal membrane lipids, particularly gangliosides, in NGF-induced differentiating PC12 cells (PC12N cells).

2. Materials and methods

2.1. Cell culture

Maintenance and NGF-induced differentiation of PC12 cells were performed as previously reported [33] except the density of cells at 0.5×10^4 cells/cm^2. For the analysis of Aβ production, PC12N cells were treated with a γ-secretase inhibitor, DAPT, at various concentrations, for 1 day from the 6th to 7th day of NGF-induced differentiation. For lipid analyses, PC12N cells were treated with 1 μM DAPT for 6 days from the initial day of NGF-induced differentiation.

2.2. Collection of neuritic terminals of PC12N cells

Neuritic terminals of PC12N cells were collected by sucrose-density-gradient fractionation as previously reported [21]. PC12N cells (twenty 150-mm dishes) were homogenized on ice in Tris buffer (10 mM Tris–HCl, 0.25 M sucrose, pH 7.4) with Dounce homogenizer. Post nuclear supernatant was adjusted to 40% (wt/vol) sucrose, and layered on 50% sucrose solution. Subsequently, a discontinuous sucrose gradient was prepared by pouring 35, 25, 15, and 0% sucrose solutions sequentially on top of the 40% sucrose fraction. The gradient was ultracentrifuged at 85,000 × *g* (SW41 rotor, Beckman) for 16 h at 4 °C. For lipid analyses, collected samples were lysed by suspension in 5 mM Tris–HCl, pH 8.5, followed by agitation for 90 min at 4 °C. After centrifugation at 43,700 × *g* for 20 min at 4 °C, the resultant pellet was collected. The neuritic terminals were collected 4 times and the combined resultant pellets were applied for the analyses of ganglioside, sphingomyelin, and phosphatidylcholine.

2.3. Liquid chromatography–electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) and multiple reaction monitoring (MRM) for the analysis of gangliosides

The analysis of gangliosides was performed by LC/ESI-MS/MS in combination with MRM as previously reported [14] with a slight modification. LC was performed by ultraperformance LC (UPLC) using ACQUITY UPLC system (Waters Corporation, Milford, MA, USA). The mobile phase was pumped at a flow rate of 50 μl/min and the column temperature was set at 40 °C. Resolutions were set at Q1 and Q3 "unit".

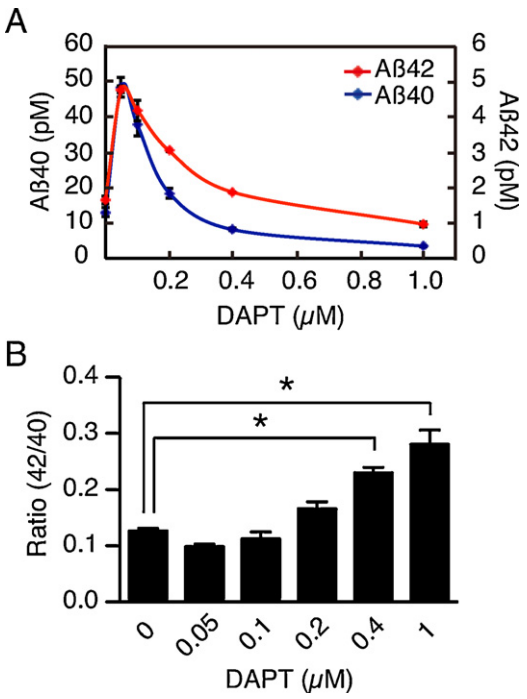

Fig. 1. Effect of suppression of γ-secretase activity on Aβ production. PC12N cells were treated with DAPT for 1 day at several concentrations from 0.05 to 1 μM and the levels of Aβ40 and Aβ42 in the media were determined by ELISA. (A) Aβ production at various concentrations of DAPT. Each point indicates mean + SEM of 4 values. (B) Ratio of production level of Aβ42 to that of Aβ40 at various concentrations of DAPT. Each column indicates mean + SEM of 4 values. $^*p < 0.05$ (one-way ANOVA combined with Dunnett's test).

The details of materials and methods are described in Supplementary methods.

3. Results

3.1. The effect of suppression of γ-secretase activity on Aβ production in PC12N cells

We treated PC12N cells with DAPT, a specific γ-secretase inhibitor [28], at several concentrations and measured the levels of Aβ, Aβ40, and Aβ42 production in the medium. In the range of the concentrations used for DAPT treatment, apparent cell death, cell-somal shrinkage, and neurite retraction were not observed in this study (data not shown). DAPT treatment at low concentrations paradoxically increased the level of Aβ production in contrast to the significant decrease for DAPT treatment at high concentrations up to 1 μM (Fig. 1A). The level of Aβ40 decreased more steeply than that of Aβ42 with increasing DAPT concentration; as a consequence, the ratio of the level of Aβ42 to that of Aβ40 gradually increased with DAPT concentration increased (Fig. 1B).

3.2. Characterization of neuritic terminals of PC12N cells

We attempted to separately obtain neuritic terminals, which are claimed as the initial Aβ deposition point in the brain [20] and on cultured neurons [30], by sucrose-density-gradient fractionation [21]. In Western blot analysis, proteins abundant in neuritic terminals, such as synaptophysin [17] and the mature amyloid precursor protein (APP) [1], were recovered in fractions 8–11 and fractions 8–14, respectively, whereas proteins abundant in cell somata, such as Bip/GRP78 (ER-localized protein), p115 and GM130 (Golgi-apparatus-localized proteins), and Tom20 (mitochondrial protein), were exclusively recovered in fractions 11–13 (Fig. 2A).