FEBS Letters 584 (2010) 3410–3414

journal homepage: www.FEBSLetters.org

# MEK inhibitors suppress β-amyloid production by altering the level of a β-C-terminal fragment of amyloid precursor protein in neuronal cells

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ARTICLE INFO

*Article history:*
Received 31 March 2010
Revised 4 June 2010
Accepted 24 June 2010
Available online 30 June 2010

Edited by Jesus Avila

*Keywords:*
Alzheimer's disease
Amyloid precursor protein
β-Amyloid
MEK inhibitor
Neuroblastoma
Proteasome

ABSTRACT

**β-Amyloid peptide (Aβ) is generated via sequential proteolysis of amyloid precursor protein (APP) by β- and γ-secretases. Cell-based screening experiments disclosed that the MEK (MAP kinase kinase) inhibitors, U0126 and PD184352, suppress Aβ secretion from human neuronal SH-SY5Y cells expressing Swedish mutant APP. These inhibitors did not affect the cellular levels of APP but significantly reduced those of the APP β-C-terminal fragment (β-CTF). Additionally, β-CTF levels were markedly reduced by these inhibitors in cells expressing the fragment in a γ-secretase-independent and proteasome-dependent manner. Our results suggest that MEK inhibitors reduce Aβ generation via secretase-independent alteration of β-CTF levels.**


## 1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative dementia disorder pathologically characterized by neuronal loss and formation of senile plaques and neurofibrillary tangles. β-Amyloid peptide (Aβ), the main constituent of senile plaques, is a major causative factor in AD pathogenesis according to the "amyloid cascade hypothesis", and thus an important therapeutic target [1]. Aβ is generated via sequential proteolysis of amyloid precursor protein (APP) by β-secretase (β-site APP cleaving enzyme 1: BACE1) and γ-secretase, which are recognized key proteases in amyloidogenic processing [2,3].

The mechanisms underlying the regulation of Aβ production have yet to be fully elucidated, in part because several factors other than secretases are also involved in Aβ generation. In this regard, Aβ production is modulated via both secretase-dependent and -independent mechanisms. For example, we recently reported that insulin-like growth factor-1 (IGF-1) promotes Aβ production via a secretase-independent mechanism involving APP phosphorylation at Thr668 [4]. One approach to resolving this issue is to test the ability of various kinase inhibitors to modulate Aβ production, since protein phosphorylation is critical in the modification of intracellular signaling.

The present study was undertaken to uncover novel mechanism(s) underlying the regulation of Aβ generation. Accordingly, we screened kinase inhibitor(s) able to modulate Aβ production using a cell-based assay employing human neuronal cells. Our data show that MEK inhibitors exert an Aβ suppression effect via a mechanism involving secretase-independent alteration of the APP β-C-terminal fragment (β-CTF) level.

## 2. Materials and methods

### 2.1. Cell culture and transfection

Human neuroblastoma SH-SY5Y cells stably expressing Swedish mutant APP (swAPP) or APP β-CTF (designated SH-swAPP and SH-β-CTF, respectively) were established in previous studies [4,5]. Cells were maintained in a humidified atmosphere of 5% $CO_2$/95% air in growth medium (DMEM/F12 supplemented with 10% fetal bovine serum) containing 200 μg/ml G418.

*Abbreviations:* AD, Alzheimer's disease; Aβ, β-amyloid peptide; APP, amyloid precursor protein; α- and β-CTF, α- and β-C-terminal fragment; swAPP, Swedish mutant APP

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0014-5793/$36.00 
doi:10.1016/j.febslet.2010.06.038

### 2.2. Antibodies and chemicals

A rabbit polyclonal antibody specific for the C-terminus of APP employed in this study is described in a previous report [6]. A rabbit polyclonal antibody (P-Thr668 antibody) specific for the Thr668-phosphorylated APP (P-APP) was obtained from Cell Signaling Technology (Beverly, MA, USA) while a rabbit polyclonal antibody against BACE1 and mouse monoclonal antibody against β-actin were purchased from Chemicon (Temecula, CA, USA) and Sigma (St. Louis, MO, USA), respectively. PD184352, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(*S*)-phenylglycine *t*-butyl ester (DAPT) and chloroquine were obtained from Alexis (San Diego, CA, USA), Calbiochem (San Diego, CA, USA) and Sigma, respectively. U0126, U0124 and Epoxomycin were acquired from Wako (Osaka, Japan). Kinase inhibitors used for screening (as listed in Supplementary Table 1) were provided by the Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from MEXT (Japan).

### 2.3. Screening of kinase inhibitors that modulate Aβ production

SH-swAPP cells were plated at a density of $2 \times 10^5$ cells/well on a 24-well plate. One day after plating, cells were incubated with growth medium containing each of the kinase inhibitors at 1 μM for 24 h. Aβ40 levels in the conditioned media were measured using sandwich ELISA, as described below.

### 2.4. Western blot analysis

Western blot analyses were performed as described previously [4,5,7]. Cells were lysed in RIPA buffer with protease inhibitors [7]. Proteins were separated on polyacrylamide gels, and blotted onto polyvinylidene difluoride (PVDF) membranes. Membranes were treated with PBS containing 0.05% Tween-20 and 5% non-fat dry milk to block non-specific binding sites, followed by probing with the appropriate antibodies. Membranes were subsequently incubated with anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase. Immunoreactive proteins were detected with chemiluminescence reagents (PerkinElmer, Boston, MA, USA). Densitometry was performed for quantitative measurement of protein expression intensity with an image analyzer, LAS-1000 (Fuji Film Co., Tokyo, Japan).

### 2.5. Aβ measurements

The concentrations of Aβ40 and Aβ42 in conditioned media were measured essentially as described previously using sandwich ELISA kits (Wako or IBL, Gumma, Japan) [4,5,8].

### 2.6. Statistical analysis

Statistical analyses were performed with one-way ANOVA, followed by Bonferroni multiple comparison test or Student's *t*-test.

## 3. Results

In the present screening experiments, we employed human neuroblastoma SH-SY5Y cells stably expressing swAPP (SH-swAPP cells) that secrete large amounts of Aβ40 and Aβ42 [4,5]. SH-swAPP cells were treated for 1 day with ~110 different kinase inhibitors listed in Supplementary Table 1 (final concentration of 1 μM), and the amounts of secreted Aβ40 in the conditioned media assayed using sandwich ELISA. Screening data revealed that a MEK1/2 (MEK: MAP kinase kinase) inhibitor, U0126 [9] has a potent Aβ suppression effect. The other inhibitors that significantly lowered Aβ secretion included IKK-2 inhibitor VI, but we focused on U0126 in this study. At concentrations of 1 and 2 μM, U0126 induced a significant decrease in Aβ40 and Aβ42 secretion by ~40% and ~45%, respectively, compared to that in untreated control cells (Fig. 1A). In contrast, U0124, an inactive analogue of U0126, had no effect on Aβ secretion (Fig. 1A). The MEK1/2 inhibitor, PD184352 (1 and 2 μM), with a chemical structure unrelated to that of U0126 [10], similarly reduced secretion of Aβ from SH-swAPP cells (Fig. 1A). U0126 and PD184352 also reduced Aβ secretion by ~14% and ~31% at 0.25 μM, by ~17% and ~33% at 0.5 μM, and by ~50% and ~50% at 5 μM, respectively (data not shown), suggesting that PD184352 suppresses Aβ secretion more potently than U0126. Another MEK inhibitor, PD98059, did not clearly affect Aβ secretion at a concentration of 1 μM, but induced significant decreases (by ~30% and ~35%) at 5 (data not shown) and 10 μM (Fig. 1A), respectively.

We analyzed MEK-dependent phosphorylation of ERK by Western blot analysis using an anti-phospho-ERK [9]. MEK-dependent ERK phosphorylation was significantly inhibited in SH-swAPP cells treated with U0126, PD184352, or PD98059, but not in cells treated with U0124, confirming the inhibition of intracellular MEK activity. PD184352 exhibited the most potent inhibitory effect on MEK (Supplementary data).

We employed U0126 and PD184352 for further analyses because of their potent Aβ suppression effect. Western blot analysis of cell lysates revealed that U0126 and PD184352 did not affect the cellular levels of APP and BACE1, but significantly suppressed those of β-CTF of APP (by ~40%) (Fig. 1B and C), compared to control cells. Marginal decreases in α-C-terminal fragment (α-CTF) levels were observed in cells treated with the MEK inhibitors (Fig. 1B and C). Additionally, these MEK inhibitors did not appear to alter APP phosphorylation at Thr668 (Fig. 1B).

Next, we investigated the mechanisms underlying the MEK inhibitor-induced decrease in β-CTF levels and Aβ production, using SH-SY5Y cells expressing β-CTF (SH-β-CTF cells) [4]. The β-CTF levels were significantly reduced in cells treated with U0126 or PD184352, compared with those in control cells (Fig. 2A and B). These inhibitors additionally suppressed Aβ40 secretion (Fig. 2C). As β-CTF-expressing cells secrete small amounts of Aβ, the Aβ42 levels were below the detection limit [4]. Our data suggest that the Aβ suppression effect of MEK inhibitors does not depend on β-secretase processing of APP.

To clarify whether the inhibitory effects of the MEK inhibitors on APP CTF and Aβ production are dependent on γ-secretase processing of APP, we employed a well-known γ-secretase inhibitor, DAPT [11]. DAPT treatment of β-CTF-expressing cells augmented the levels of β-CTF and α-CTF, the latter most likely representing endogenous α-CTF. Interestingly, both U0126 and PD184352 induced a significant decrease in the β-CTF level, even in the presence of DAPT (Fig. 3A and B), suggesting that these agents decrease β-CTF levels through γ-secretase-independent mechanisms.

MEK inhibitors suppress the β-CTF level via a mechanism that does not directly involve β- and γ-secretases. This decrease in availability of β-CTF, a γ-secretase substrate, in turn leads to reduced Aβ secretion. We hypothesized that MEK inhibitors affect secretase-independent degradation of β-CTF. To confirm this theory, we performed experiments with chloroquine, an agent that disrupts lysosomal function, and epoxomycin, a specific proteasome inhibitor. Upon treatment of SH-β-CTF cells with U0126 or PD184352 in the presence of chloroquine, β-CTF levels were effectively reduced, compared to those in cells treated with chloroquine alone (Fig. 4A and B), suggesting that the lysosomal system is not involved in the MEK-inhibitor-induced reduction of β-CTF levels. Interestingly, α-CTF levels were augmented in chloroquine-treated cells (Fig. 4A), which may be attributed to inhibition of lysosomal