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Focal Adhesion Kinase Activates NF-κB via the ERK1/2 and p38MAPK Pathways in Amyloid-$\beta_{25\text{-}35}$-Induced Apoptosis in PC12 Cells

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Abstract. Increasing evidence supports that amyloid plaques, comprised of amyloid-β (Aβ), are a key feature of Alzheimer's disease (AD). But the mechanism of Aβ in AD is not yet fully understood. Previous studies have demonstrated that in Aβ-induced apoptosis of nerve cells, differentiated rat pheochromocytoma (PC12) cells, and microglia, nucleus factor kappa B (NF-κB) is activated. Meanwhile, focal adhesion kinase (FAK) is also activated. However, the relationship between NF-κB and FAK remains unclear. Using differentiated PC12 cells, we investigated this relationship in $A\beta_{25\text{-}35}$-induced apoptosis. The results showed that FAK phosphorylation increased at 6–9 hours after Aβ treatment, slightly shorter than the activation of NF-κB (6–12 hours). In this process, both extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (p38MAPK) phosphorylation levels were increased. After FAK expression was inhibited by its siRNA, the activities of ERK1/2, p38MAPK, and NF-κB were all suppressed. When ERK1/2 and p38MAPK expressions were inhibited by their siRNAs respectively, NF-κB activity was also suppressed. But FAK phosphorylation was not affected. When NF-κB expression was inhibited, all of the phosphorylation levels of FAK, ERK1/2, and p38MAPK were not affected. These phenomena indicated that FAK is upstream of ERK1/2, p38MAPK, and NF-κB, and meanwhile both of ERK1/2 and p38MAPK are upstream of NF-κB. Co-immunoprecipitation results demonstrated that it is ERK1/2, but not p38MAPK, which directly interacts with IκB kinase. Taken together, our results suggest that FAK activates NF-κB via ERK1/2 and p38MAPK pathways in $A\beta_{25\text{-}35}$-induced apoptosis of differentiated PC12 cells.

Keywords: Amyloid-β, ERK1/2, focal adhesion kinase, NF-κB, p38MAPK, PC12

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INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disease in the elderly and afflicts over 5.0 million people in the United States alone [1]. Its main clinical manifestations include memory loss, cognitive dysfunction, and personality changes. Characteristic pathological changes are neurofibrillary tangles, extracellular senile plaques, and neuronal loss in the cerebral cortex and subcortical structure [2, 3]. The main component of senile plaques is amyloid-β protein (Aβ) aggregation. Jana and colleagues reported that Aβ plays a dominant role in AD occurrence and progress and leads to nerve cells apoptosis and

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necrosis. Therefore, the mechanism of abnormal Aβ deposition has become a focus of AD pathogenesis research [4].

In rat and human hippocampal neurons, Aβ treatment promotes nuclear translocation of the p65 and p50 subunits of nucleus factor kappa B (NF-κB) and leads to downstream apoptosis-related genes expressions. Before Aβ exposure, in rat primary neurons pre-treated with a double-stranded κB decoy oligonucleotide and a selective IκB kinase 2 inhibitor (AS602868), p50/p65 nuclear translocation and neuronal damage were completely inhibited [5]. In apoptosis of differentiated pheochromocytoma (PC12) cells induced by Aβ, the p38 mitogen-activated protein kinase (p38MAPK) and NF-κB were both activated. Reductions of NF-κB and p38MAPK activations suppressed Aβ-induced apoptosis by upregulating B-cell lymphoma/leukemia-2 protein (Bcl-2) expression [6]. Moreover, through inhibition of NF-κB activity and p38MAPK phosphorylation, both inducible nitric oxide synthase expression and nitric oxide production decreased in Aβ-induced differentiated PC12 [7]. In Aβ-induced PC12 cell apoptosis, cyclooxygenase-2 (COX-2) expression increased, while NF-κB was transiently activated. NF-κB inhibitor PDTC pretreatment eliminated the upregulation of COX-2 expression. It is possible that COX-2 expression upregulation occurs through NF-κB activation in Aβ-induced PC12 cell apoptosis. The use of pharmacologic inhibitors U0126 and SB203580 or knockout extracellular signal-regulated kinase (ERK) and p38MAPK, not only suppresses the activation of NF-κB, but also down-regulates COX-2 expression [8]. It is suggested that both ERK and p38MAPK are upstream of NF-κB. In addition, NF-κB signaling plays a critical role in microglia apoptosis induced by Aβ. Inhibiting the activation of NF-κB has strong neuroprotective effects [9]. However, the details of how ERK1/2 and p38MAPK activate NF-κB are still unclear.

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase, a member of the integrin pathway, and majorly expressed in cytoplasm. It plays an important role in cell adhesion, migration, survival, cell cycle regulation, embryo implantation, and tumor invasion. After interacting with extracellular ligands, the cytoplasm terminal of the β subunit of integrin interacts with the N-terminal of FAK and leads to a change in FAK conformation which activates its kinase domain. FAK then auto phosphorylates its Tyr397, eliminating the self-inhibition of Src. Activated Src catalyzes FAK Tyr407, Tyr576, Tyr577, and Tyr925 phosphorylation sites, fully activating FAK [10].

A previous study indicated that dissolved and fibrillar Aβ can replace the extracellular matrix bond to integrin and partially activate focal adhesion signaling FAK, thus can potentially leading to nerve impairment [11]. Integrin α1β1 participates in Aβ-induced neuron death of cultured aged hippocampal. Antibody blocking experiments showed that both integrin αvβ1 and α2β1 mediate the human cerebral cortex primary neuron bonding/aggradation and nerve toxicity by Aβ. *In vitro*, an inhibitor of integrin αv, SM256, suppressed cell adhesion mediated by integrin αv and nerve impairment caused by Aβ in cultured cerebral cortex neuron. *In vivo*, pretreatment with intraperitoneal injection of SM256 eliminated long-term effects of ventricle soluble and fibrillar Aβ injections [12]. Several previous studies demonstrated that overexpression of FAK induces the constitutive activation of NF-κB in human leukemic cell line HL-60 and in human breast cancer cell lines BT474 and MCF-7 [13, 14]. In particular, some other studies have proven that in Aβ-induced apoptosis, FAK is activated in primary human and rat brain cortical cultured cells, and in rat and human nerve cell lines B103 and SH-SY5Y [15–17].

Therefore, NF-κB can be activated during Aβ-induced PC12 cell apoptosis and can lead to downstream apoptotic events. Additionally, FAK is also activated in Aβ-induced apoptosis. However, to our knowledge, it is still uncertain whether there is a potential relationship between the activations of FAK and NF-κB in Aβ-induced apoptosis. This is an important issue because it relates to molecular mechanisms of AD. The present study aimed to investigate this relationship in Aβ-induced PC12 cell apoptosis.

In this project, we found that in the process of Aβ-induced PC12 cell apoptosis, NF-κB is activated. This is consistent with previous reports. In this process, NF-κB activity was mediated by FAK, ERK1/2, and p38MAPK. Meanwhile ERK1/2 and p38MAPK activities were mediated by FAK. Further experiments found that by phosphorylating IκB kinase (IKK), and then phosphorylating IKBα, ERK1/2 thereby directly activates NF-κB. However, p38MAPK does not interact with IKK directly, which activates NF-κB through other non-IKK pathway(s). Collectively, these findings indicated that in the process of Aβ-induced PC12 cell apoptosis, via ERK1/2 and p38MAPK pathways, FAK activates NF-κB. By activating IKK, ERK1/2 directly activated NF-κB. Through non-IKK pathway(s), p38MAPK activates NF-κB.

MATERIALS AND METHODS

Chemical and biochemical reagents

Dulbecco's modified Eagle's medium (DMEM) was provided by GIBCO (Grand Island, NY, USA). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Material Co., Ltd (Hangzhou, China). Amyloid β-protein fragment 25-35 (#A4559) and nerve growth factor beta (NGF-β) (#N2513) were supplied by Sigma-Aldrich (St. Louis, USA). l, 4-diamino-2,3-dicyano-l,4-bis [2-aminophenylthio] butadiene (U0126) (Cat. No. V1121), an ERK inhibitor, was purchased from Promega Biotechnology Co., Ltd. (Madison, WI, USA). Annexin V-FITC apoptosis detection kit I (Cat. No. 556547) and mouse anti human FAK (Py397) antibody (Cat. No. 611806) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit anti human NF-κB p65 (C-20) antibody (sc-372), rabbit anti human IκBα (C-21) antibody (sc-371), mouse anti β-actin (C-4) antibody (sc-47778), rabbit anti human FAK (C-20) antibody (sc-558), rabbit anti human IKKα/β (H470) antibody (sc-7607), and rabbit anti human Histone H3 (FL-136) antibody (sc-10809) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Rabbit anti human ERK1/2 (p44/42) antibody (#9102), rabbit anti human phospho-ERK1/2 (p44/42) (thr202/tyr204) antibody (#9101), rabbit anti human p38MAPK antibody (#9212), rabbit anti human phosphor-p38MAPK (thr180/tyr182) (3D7) antibody (#9215), rabbit anti human Bcl-2 (50E3) antibody (#2870), rabbit anti human Bax antibody (#2772) and normal rabbit IgG (#2729) were purchased from Cell Signaling Technology, Inc. (Danvers, USA). GoldView DNA dye (#HGV-1) was purchased from Beijing SBS Genetech (Beijing, China). Electrophoretic mobility shift assay (EMSA) kit (GMS30019.2) was purchased from GenMed Scientifics, Inc. (Arlington, USA). Transfection reagent Lipofectamine™ 2000 (Cat. No. 11668-019) was purchased from Invitrogen Co. (Carlsbad, USA).

Peptide preparation and cell culture

$A\beta_{25\text{-}35}$, the most toxic peptide fragment derived from amyloid-β protein precursor was dissolved in ultra-pure deionized distilled water at concentration of 1 mM at a stock solution and stored at −20°C until use. The stock solution was aggregated by incubation at 37°C for 72 h before use. Aggregated Aβ was directly added to cell culture medium.

Rat PC12 cells were cultured in a humidified (5% CO_2, 37°C) incubator in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and NGF-β (50 nM). To ensure maximum bioavailability, the medium was changed every other day, and cells were plated at a various densities according to each experimental protocol.

Flow cytometry analysis

After 24 h of exposure to $A\beta_{25\text{-}35}$, PC12 cells were harvested and treated with Annexin V-FITC apoptosis detection kit I for cell viability analysis. Cell apoptosis was measured by FACSCanto™ II flow cytometer (Becton Dickinson and Company, Mountain View, CA). All data were obtained by FACSDava 6.0. 10,000 cells were examined in each specimen tube.

Protein preparation

$A\beta_{25\text{-}35}$-treated cells (1×10^6 cells/mL in 60 mm dishes) were collected and washed three times with phosphate-buffered saline (PBS). After centrifugation at $2{,}000 \times g$, cell lysis was carried out on ice by vigorous shaking for 30 min in RIPA buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 100 mg/ml PMSF, 1 mM DTT, and protease inhibitors). After centrifugation at $13{,}200 \times g$ for 5 min, the supernatant was separated and stored at −80°C until use. The protein concentration was determined by Bradford assay. Nuclear proteins were prepared as following procedures. Cells were suspended with PBS and sedimented at $800 \times g$ for 10 min. The pellet was resuspended in a 5× packed cell volume of a hypotonic buffer (10 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, and 0.1 mM EGTA) and then sedimented at $300 \times g$ for 10 min. After removing the supernatant, the cells were homogenized by 3–5 strokes in a Dounce homogenizer and sucrose restoration buffer was added (1 vol of 500 mM HEPES, pH 7.9, 7.5 mM spermidine, 1.5 mM spermine, 2 mM EDTA, 2 mM EGTA and 10 mM DTT in 9 vol of 7.5% sucrose). After centrifugation for 30 s at $14{,}000 \times g$, the nuclei were sedimented. The nuclear protein was then extracted from the sediments pellets and resuspended with the same buffer as whole cell lysis.

Western blot analysis

After addition of sample loading buffer, protein samples were separated by 10% SDS-PAGE, and transferred to 0.45 μm polyvinylidene fluoride (PVDF) membrane (Pall Co., NY, USA). The membrane was first blocked with 5% skim milk for 1 h, followed by incubation with primary antibodies (dilution 1 : 1000) overnight at 4°C. Primary antibody incubation was performed in 5% bovine serum albumin in TBST (Tris base saline, pH 7.4, 0.1% Tween-20). A designated secondary antibody was diluted at the recommended ratio in TBST and 5% bovine serum albumin and was incubated for 60 min at room temperature. Washing with TBST was performed between all steps. The signals were detected by use of Odyssey® Infrared Imaging System (Li-Cor Biotechnology, Nebraska, USA).

Non-denaturing polyacrylamide gel electrophoresis

In the non-denaturing polyacrylamide gel electrophoresis procedure, gel concentration was 10% (Double distilled H_2O 5.25 mL, Gel Buffer (100 mM Tris pH 7.0, 1 M 6-aminocaproic acid) 9 mL, 50% polyacrylamide 3.75 mL, 10% Ammonium persulfate 72 μL, TEMED 7.2 μL). After addition of 10× sample loading buffer (5% Coomassie Brilliant Blue G-250, 0.5 M 6-aminocaproic acid, 0.1 mM PMSF, 100 mM Tris-HCl pH 7.0), protein samples were separated at 110 V constant voltage and then transferred to 0.45 μm PVDF membrane. The following procedure was same as normal western blot.

Electrophoretic mobility shift assay (EMSA)

EMSA method was used to characterize the binding activities of NF-κB in nuclear extracts. NF-κB oligonucleotide's sequence was 5′-GAG AGG CAA GGG GAT TCC CTT AGT TAG GA-3′ [18]. Unspecific binding was blocked by using 1 μg of poly (dI : dC) · poly (dI : dC). After the 5% polyacrylamide gel was prepared, 100 voltages pre-electrophoresis was performed in 0.5× TBE for the 30 min. The reaction mixture buffer contained 10× binding medium 2 μL, 25 mmol/L DTT containing 25% tween-20 2 μL, poly (dI : dC) · poly (dI : dC) 1 μL, 1% NP-40 1 μL, 100 mmol/L $MgCl_2$ 2 μL. The binding reaction was carried on at 30°C for 20 min after mixing the reaction mixture buffer and NF-κB oligonucleotide. Then nuclear protein 20 μg was added. Double-distilled water was made up the total volume to 20 μL. 10× Orange Loading Dye (infrared fluorescent dye) 2 μL was added. The nuclear protein with oligonucleotide complex was separated from free oligonucleotide by electrophoresis through a 5% native polyacrylamide gel in a running buffer containing 50 mM Tris, pH 8.0, 45 mM borate, and 0.5 mM EDTA. After separation was achieved, the gel was detected by use of Odyssey® Infrared Imaging System (Li-Cor Biotechnology, Nebraska, USA). Odyssey parameter settings were scan off focus 0.5 mm and laser wavelength 700 nm.

RNA interference (RNAi)

PC12 cells were transfected with 800 ng siRNA oligonucleotide according to the manufacturer's instructions. Rat ERK1/2, p38MAPK small interfering RNAs (siRNAs) sequences were 5′-GAC CGG AUG UUA ACC UUU AUU dTdT-3′ and 5′-GGA CCU CCU UAU AGA CGA AUU-3′, respectively [18, 19]. These siRNAs and their non-targeting sequences (negative controls) were synthesized by GenePharma Co., Ltd. (Shanghai, China). Both of NF-κB and FAK siRNAs and their non-targeting sequences were purchased from Santa Cruz Biotechnology, Inc. (sc-61876, sc-156037). Negative control siRNAs' nucleotide compositions are same as targeting siRNAs, but lack significant sequence homology to the genome. PC12 cells were transfected with siRNA oligonucleotides using Lipofectamine™ 2000.

Co-immunoprecipitation (Co-IP)

Cultured cells were washed twice with ice-cold PBS, scraped in ice-cold gentle lysis buffer (25 mM Tris-HCl, pH 7.5, 1% NP-40, 10% glycerol, 50 mM NaF, 10 mM NaH_2PO_4, 137 mM NaCl, 2 mM Na_3VO_4, 1 mM PMSF and Protease inhibitor Cocktail set I (Calbiochem®, Germany)), and centrifuged at 12,000 × g at 4°C for 15 min. Protein concentrations were determined by Bradford assay. After 50% Protein A Sepharose CL-4B beads (Amersham Biosciences, Sweden) were added to remove nonspecific interferential proteins, primary antibody (10 μg/mL) was added to protein lysates (500 μg) and incubated at 4°C overnight. Protein A Sepharose CL-4B beads were mixed with the immunoprecipitates for 1 h at room temperature. Immunoprecipitates were recovered by centrifugation at 12,000 × g for 5 s and washed three

times with ice-cold lysis buffer. Immunoprecipitated proteins were dissolved in 2× loading buffer, heated at 100°C for 10 min, then subjected to 10% SDS-PAGE and western blot analysis, respectively.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total mRNA was isolated from differentiated PC12 cells using TRIzol reagent (#15596-026, Invitrogen, Carlsbad, USA) and treated with RNase–free DNase. cDNA synthesis and amplification by RT-PCR was carried out using Mastercycler® ep gradient S PCR instrument (Eppendorf AG, Hamburg, Germany). The reverse transcriptase used was TIANScript RT kit (#KR104, Tiangen Biotech Co., LTD., Beijing, China). The PCR primers were as follows: FAK, 5′-ACT TGG ACG CTG TAT TGG AG-3′ (sense) and 5′-CTG TTG CCT GCT TTC TGG AT-3′ (antisense) [21]; p38MAPK, 5′-CAA CGA TGT GTA CCT GGT GAC-3′ (sense) and 5′-TCC ACT GTC TGG TTG TAG TGC-3′ (antisense) [22]; ERK1, 5′-GCT GAC CCT GAG CAC GAC CA-3′ (sense) and 5′-CTG GTT CAT CTG TCG GAT CA-3′ (antisense); ERK2, 5′-GCA GGT GTT CGA CGT GGG AAT-3′ (sense) and 5′-GTG CAG AAC ATT AGG TGA ATA-3′ (antisense); NF-κB p65, 5′-GAC CTG GCA TCT GTG GAC AAC-3′ (sense) and 5′-TCC GCA ATG GAG GAG AAG TCT-3′ (antisense) [23]; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GAC AAC TTT GGC ATC GTG GAA-3′ (sense) and 5′-TGC CAG TGA GCT TCC CGT TCA-3′ (antisense) [22]. All primers were purchased from Invitrogen Corporation Shanghai Representative Office (Shanghai, China). In PC12 cells, the expected sizes of the generated fragments were 833 bp for FAK, 318 bp for p38MAPK, 451 bp for ERK1, 394 bp for ERK2, 196 bp for GAPDH, 221 bp for NF-κB p65, respectively. Amplification products were analyzed by 1.0% agarose gel electrophoresis, stained with GoldView DNA dye, and photographed under ultraviolet light of a Universal Hood II (Bio-Rad, Milan, Italy).

Statistical analysis

When necessary, data from three independent experiments were expressed as means ± standard deviation. Statistical analysis was performed with Student's paired *t*-test. The criterion for statistical significance was $p < 0.05$.

RESULTS

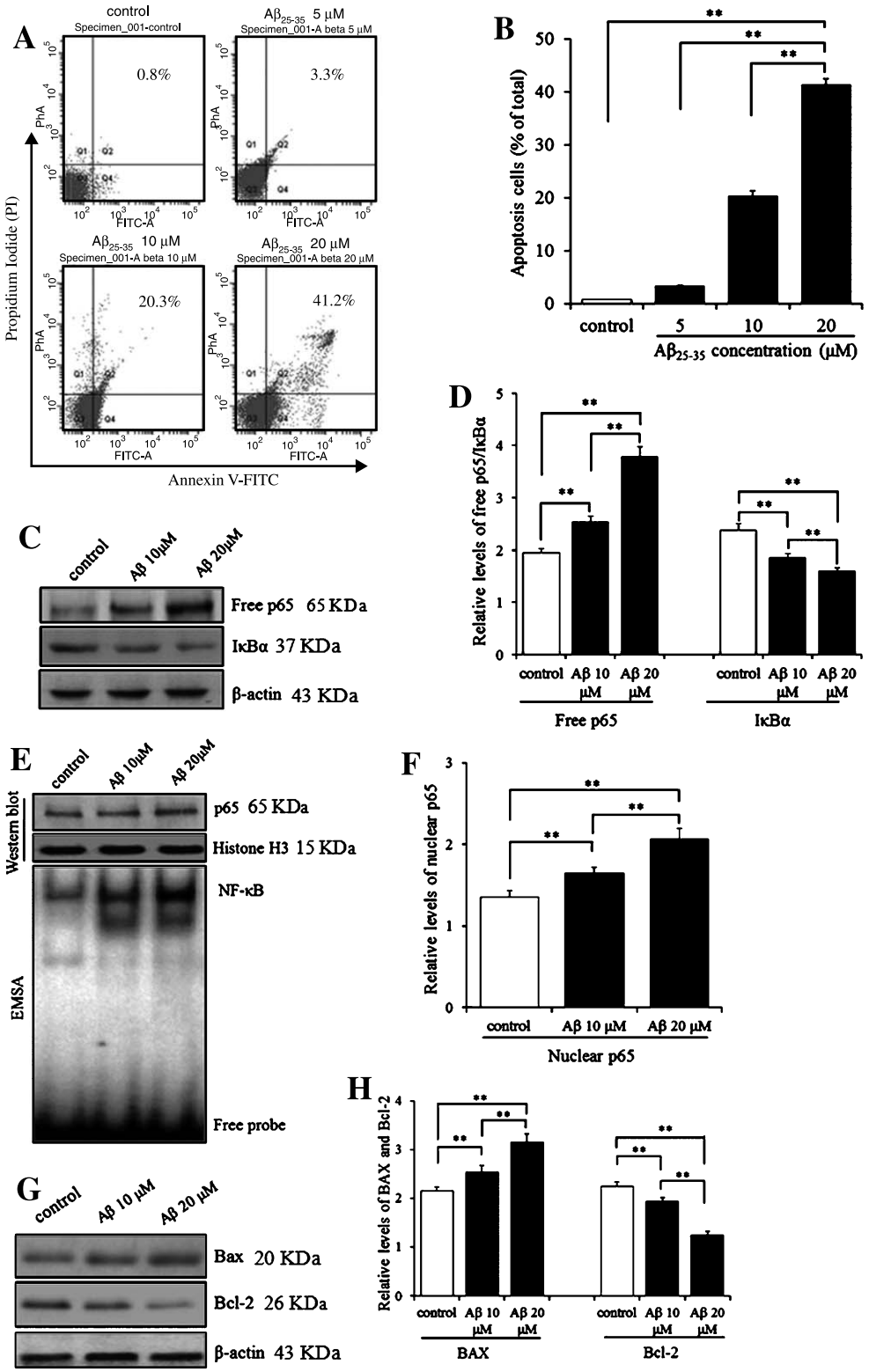
NF-κB is activated during Aβ$_{25\text{-}35}$-induced apoptosis of PC12 cell

After treatment with different concentrations of Aβ$_{25\text{-}35}$ (5, 10, 20 μM) for 24 h, PC12 cell apoptosis was measured by flow cytometry. The results indicated that PC12 cell apoptosis rate is dose-dependent (Fig. 1A, B). In order detect the free NF-κB p65 (not binding to IκB), we employed non-denaturing gel electrophoresis and western blot. The results indicated that with increasing doses of Aβ, free NF-κB p65 increased; meanwhile total IκBα correspondingly decreased (Fig. 1C, D). Western blot of nuclear protein showed that, compared to control, NF-κB p65 was significantly higher ($p < 0.01$) using 20 μM Aβ$_{25\text{-}35}$ treated (Fig. 1E, F). The EMSA result showed that the trend of NF-κB activity's change was consistent with that of p65 obtained by western blot (Fig. 1E). Both Bax and Bcl-2 are members of Bcl-2 family. Bax is a membrane-bound protein, a representative of pro-apoptotic protein. Meanwhile Bcl-2 is an anti-apoptotic protein. The ratio of Bax and Bcl-2 proteins is an important factor in regulation of cell apoptosis. Therefore these two proteins were measured by western blot. The results showed that compared to the control, after 20 μM Aβ$_{25\text{-}35}$ treatment, Bax expression was increased, while Bcl-2 was decreased (Fig. 1G, H).

With 20 μM Aβ$_{25\text{-}35}$ treatment, PC12 cell apoptotic rate reached more than 40%. In addition, NF-κB activity was greatly improved. The apoptotic rate and increased NF-κB activity are sufficient to our experimental purpose. Therefore, 20 μM Aβ$_{25\text{-}35}$ was used to induce PC12 cell apoptosis for the following experiments.

Activations of FAK and NF-κB are simultaneous

In order to determine when NF-κB was activated in Aβ$_{25\text{-}35}$-induced PC12 cell apoptosis, non-denaturing gel electrophoresis and western blot analysis were carried out. The results showed that the activity of free NF-κB p65 in PC12 cells was the highest 6 to12 h after Aβ$_{25\text{-}35}$ treatment (Fig. 2A, B). Meanwhile, the results of NF-κB p65 from the nucleus were consistent with those obtained from the whole cell (Fig. 2C, D). Similarly, the EMSA result showed that NF-κB activity reached the highest 6 to12 h after Aβ treatment, which was consistent with that of p65 obtain by western blot (Fig. 2C).


A
control
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0.8%
Aβ25-35 5 μM
Specimen_001-A beta 5 μM
3.3%
Aβ25-35 10 μM
Specimen_001-A beta 10 μM
20.3%
Aβ25-35 20 μM
Specimen_001-A beta 20 μM
41.2%
Propidium Iodide (PI)
Annexin V-FITC
FITC-A
PhA
B
Apoptosis cells (% of total)
control
5
10
20
Aβ25-35 concentration (μM)
C
control
Aβ 10μM
Aβ 20μM
Free p65 65 KDa
IκBα 37 KDa
β-actin 43 KDa
D
Relative levels of free p65/IκBα
control
Aβ 10 μM
Aβ 20 μM
Free p65
IκBα
E
Western blot
EMSA
p65 65 KDa
Histone H3 15 KDa
NF-κB
Free probe
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Relative levels of nuclear p65
Nuclear p65
G
Bax 20 KDa
Bcl-2 26 KDa
β-actin 43 KDa
H
Relative levels of BAX and Bcl-2
BAX
Bcl-2

To further determine the relationship between the activities of FAK and NF-κB, we made more detailed analysis of FAK phosphorylation. The carboxyl-terminal tyrosine (Y) 397 residue of FAK constitutes a major phosphorylation site located in a linker region connecting the regulatory and central kinase domain [24]. Moreover, the best-characterized FAK phosphorylation event is Y397 autophosphorylation, crucial for its kinase activity [25]. Therefore, Tyr-397 phosphorylation represents the status of FAK activity [10]. We measured the phosphorylation level of Tyr-397 (Y397). The results showed that in PC12 cells, FAK activity was highest 6 to 9 h after $A\beta_{25-35}$ treatment, similar to that of NF-κB. However, it decreased rapidly after 9 h treatment (Fig. 2E, F). By comparing the results of the above two western blots (Fig. 2C, E), it can be seen that the duration of FAK activation is slightly shorter than that of NF-κB. Flow cytometry results showed that the apoptosis rate increased rapidly 12 h after treatment by $A\beta_{25-35}$, slightly later than activations of FAK and NF-κB (Fig. 2G, H).

NF-κB activation is mediated by FAK

Because the following analysis used NF-κB and FAK siRNAs, we determined the effects of these siRNAs. The results showed that both of NF-κB and FAK siRNAs were efficient to inhibit their respective target protein expressions (Fig. 3A, B).

Since both NF-κB and FAK are activated in Aβ-induced apoptosis of PC12 cells, it is possible there is a correlation between their activations. To clarify this possibility, we inhibited the expression of NF-κB by its siRNA. But FAK phosphorylation level had no obvious change. However, when FAK expression was suppressed by its siRNA, the activity of NF-κB was correspondingly reduced (Fig. 3C, D). Flow cytometry results showed that using siRNAs inhibited the expressions of FAK and NF-κB, respectively, and the apoptotic rates both declined to different degrees (Fig. 3E, F). Meanwhile, the expressions of Bax and Bcl-2 changed correspondingly. However, the trends of expressions of Bax and Bcl-2 were exactly the opposite (Fig. 3G, H).

The results suggested that in Aβ-induced apoptosis in PC12 cells, increased activity of NF-κB is at least in part affected by FAK phosphorylation level.

With FAK activation, ERK1/2 and p38MAPK phosphorylation levels are also increased

FAK plays an important role via p38MAPK in stress-induced cell survival in cardiac myocytes and via ERK in TNFα-induced apoptosis in embryonic fibroblasts [26, 27]. So in Aβ-induced apoptosis in PC12 cells, does FAK also play a role through ERK and p38MAPK? Therefore, western blot analysis was performed to examine the changes in phosphorylation levels of ERK1/2 and p38MAPK during FAK activation. The results showed that with the increased FAK phosphorylation level, both the phosphorylation levels of ERK1/2 and p38MAPK were also increased (Fig. 4A, B).

Increased phosphorylation levels of ERK1/2 and p38MAPK have two possible sources: activation or increased expression. To find out the real source, RT-PCR experiments were conducted. The results showed that during 20 μM $A\beta_{25-35}$ treatment, mRNA levels of FAK, ERK1/2, p38MAPK, and NF-κB had no significant changes (Fig. 4C, supplementary Figure 1A; available online: http://www.j-alz.com/issues/32/vol32-1.html#supplementarydata02). In addition, there was no obviously increased expression of FAK, ERK1/2, p38MAPK, or NF-κB protein during Aβ treatment (Fig. 4D, supplementary Figure 1B). It was suggested that the real source of increased phosphorylation levels of ERK1/2 and p38MAPK is from activation.

FAK regulates the activities of p38MAPK and ERK1/2

To demonstrate the up- and downstream relationships of FAK, p38MAPK, and ERK1/2 in Aβ-induced apoptosis of PC12 cells, we used siRNA to inhibit the

Fig. 1. $A\beta_{25-35}$ induces apoptosis and NF-κB activation in differentiated PC12 cells. A) After treatment with different concentration $A\beta_{25-35}$ (5, 10, 20 μM) for 24 h, the cells were stained with annexin V-FITC and PI. The percentage of apoptotic cells was assessed by flow cytometry. The percentages refer to the levels of positive staining of annexin V-FITC. B) Effects of $A\beta_{25-35}$ on cell apoptosis rate from flow cytometry. C) After 12 h $A\beta_{25-35}$ treatment, proteins of whole cells were extracted with gentle lysis and separated with non-denaturing gel electrophoresis. Quantities of free NF-κB p65 (not bound to IκB) and IκBα were assessed by western blot. β-actin is a marker for whole proteins. D) Data analysis to C. E) Western blot analysis to NF-κB p65 in nucleus and EMSA analysis to NF-κB activity after 12 h $A\beta_{25-35}$ treatment. Histone 3 is a marker for nuclear proteins. F) Data analysis to the result of western blot in E. G) Expression quantity change analysis of Bax and Bcl-2 with different treatments by western blot. β-actin is a marker for whole proteins. H) Data analysis to G. Data in B, D, F, and H represent the mean of 3 independent experiments, $^{**}p < 0.01$. Control is specimen without treatment with $A\beta_{25-35}$.

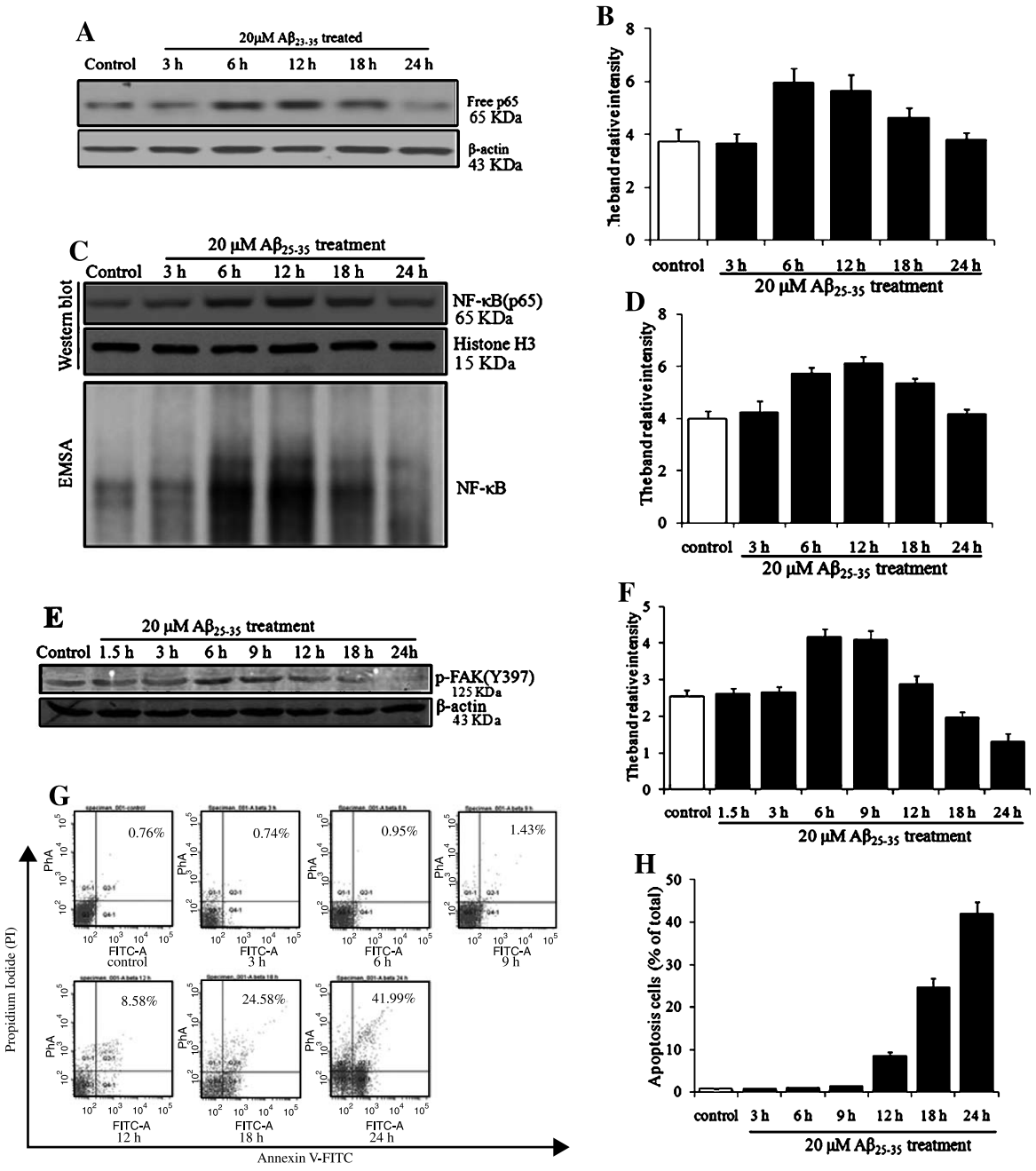


Fig. 2. Activation of FAK and NF-κB were simultaneous. A) Free NF-κB changes with different treatment time (h) were assessed by western blot. β-actin is a marker for whole proteins. After treatment for 6 to 12 h, free NF-κB reaches a peak. B) Data analysis to A. C) Western blot analysis to NF-κB p65 and EMSA analysis to NF-κB activity in nucleus after $A\beta_{25\text{-}35}$ treatment for 3–24 h. Histone 3 is a marker for nuclear proteins. After treatment for 6 to 12 h, both NF-κB p65 and NF-κB activity reaches a peak. D) Data analysis to the result of western blot in C. E) More detailed analysis (1.5–24 h) to FAK phosphorylation by western blot. FAK activity was the highest in 6–9 h after $A\beta_{25\text{-}35}$ treatment, but decreased rapidly after 9 h. β-actin is a marker for whole proteins. F) Data analysis to E. G) After PC12 cells treated with Aβ for different time (h), apoptosis rates were measured by flow cytometry. H) Data analysis to G. Cell apoptosis rate increased rapidly after 12 h treatment with $A\beta_{25\text{-}35}$. Data in B, D, F, and H represent the mean of 3 independent experiments. Control is specimen without treatment with $A\beta_{25\text{-}35}$.

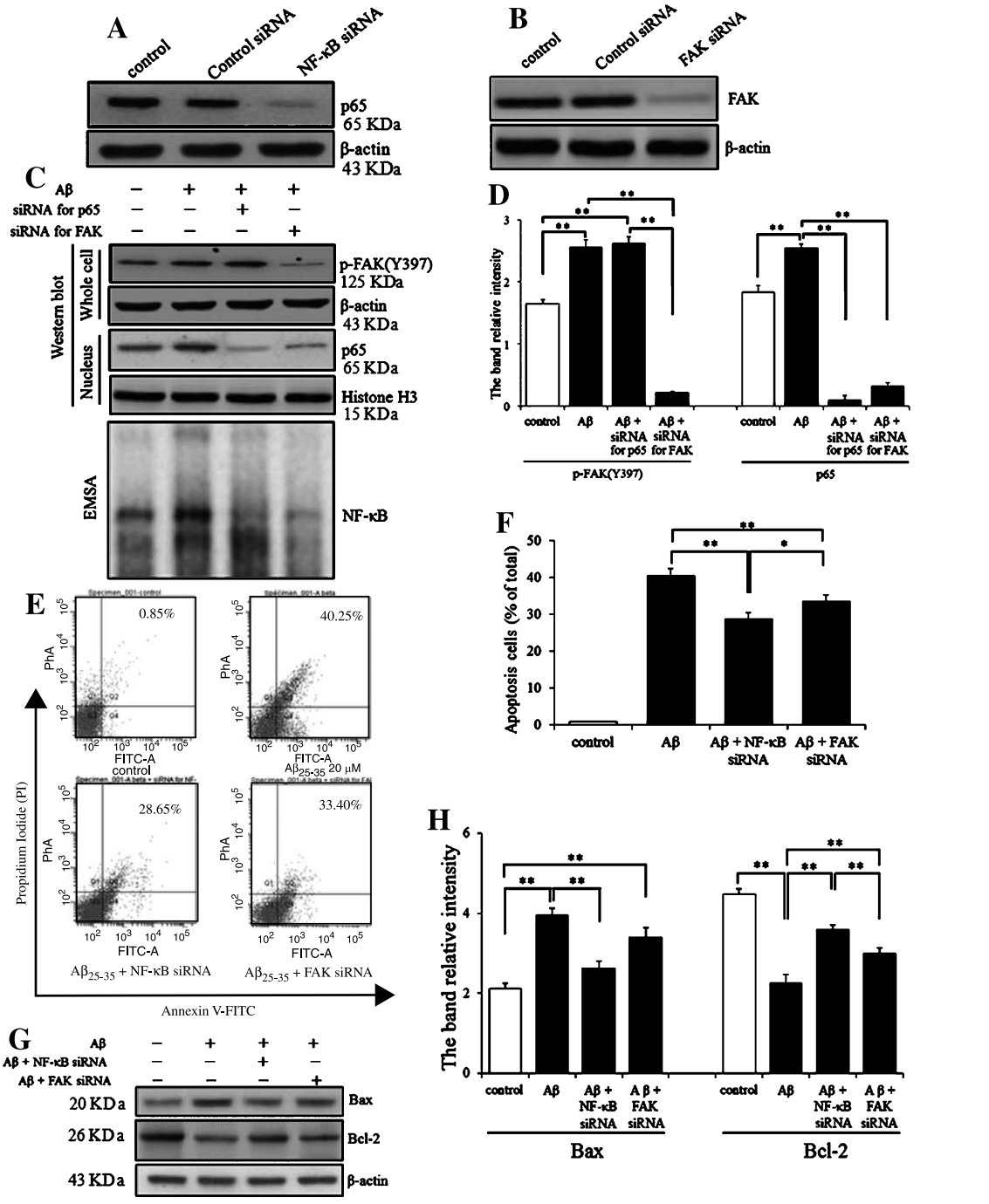


Fig. 3. NF-κB activation is mediated by FAK phosphorylation. A) NF-κB siRNA could effectively inhibit the expression of NF-κB. B) FAK siRNA could effectively inhibit the expression of FAK. C) After treatment with NF-κB and FAK siRNAs respectively, proteins of whole cells and of nuclear protein were extracted and separated with SDS-PAGE. Quantities of phosphorylated FAK (Y397) in whole cells and of NF-κB p65 in nucleus were assessed by western blot. β-actin is a marker for whole proteins, and histone H3, for nuclear protein. Meanwhile, EMSA was performed to analyze NF-κB activity. D) Data analysis to the result of western blot in C. E) After PC12 cells were treated with different siRNAs, apoptosis rates were measured by flow cytometry. F) Data analysis to E. Both NF-κB and FAK siRNAs treatments can decrease the apoptosis rate. G) Expression quantity change analysis of Bax and Bcl-2 with different siRNA treatments by western blot. β-actin is a marker for whole proteins. H) Data analysis to G. Data in B, D, F, and H represent the mean of 3 independent experiments, $^{**}p < 0.01$. Control is specimen without treatment with $A\beta_{25\text{-}35}$.

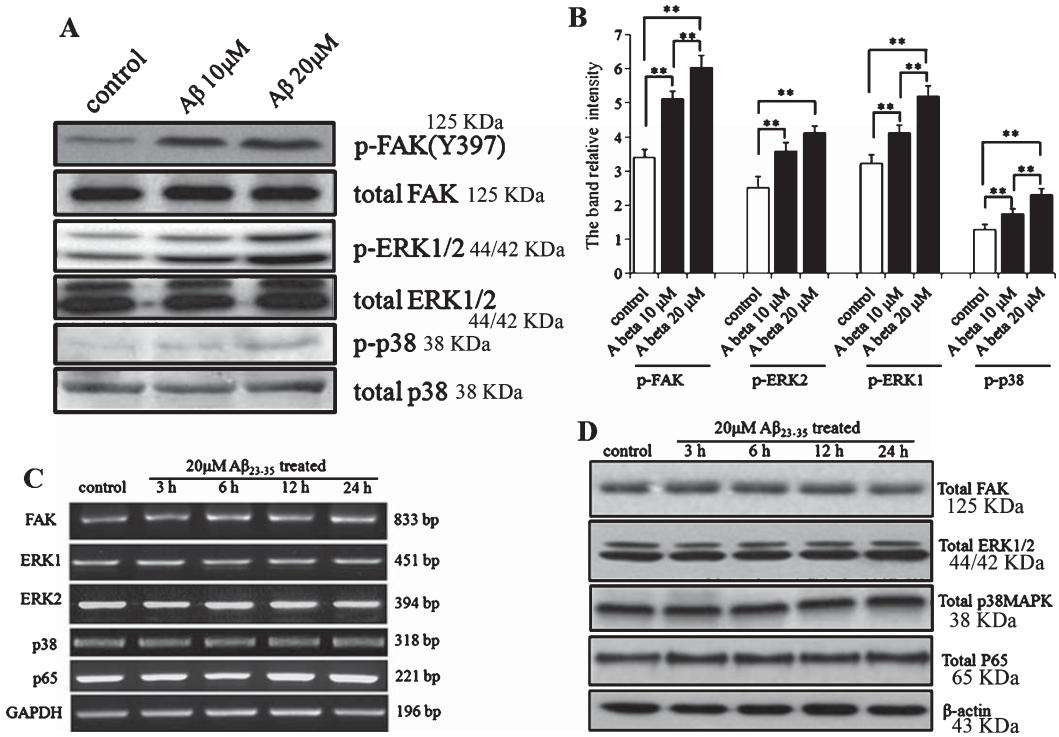
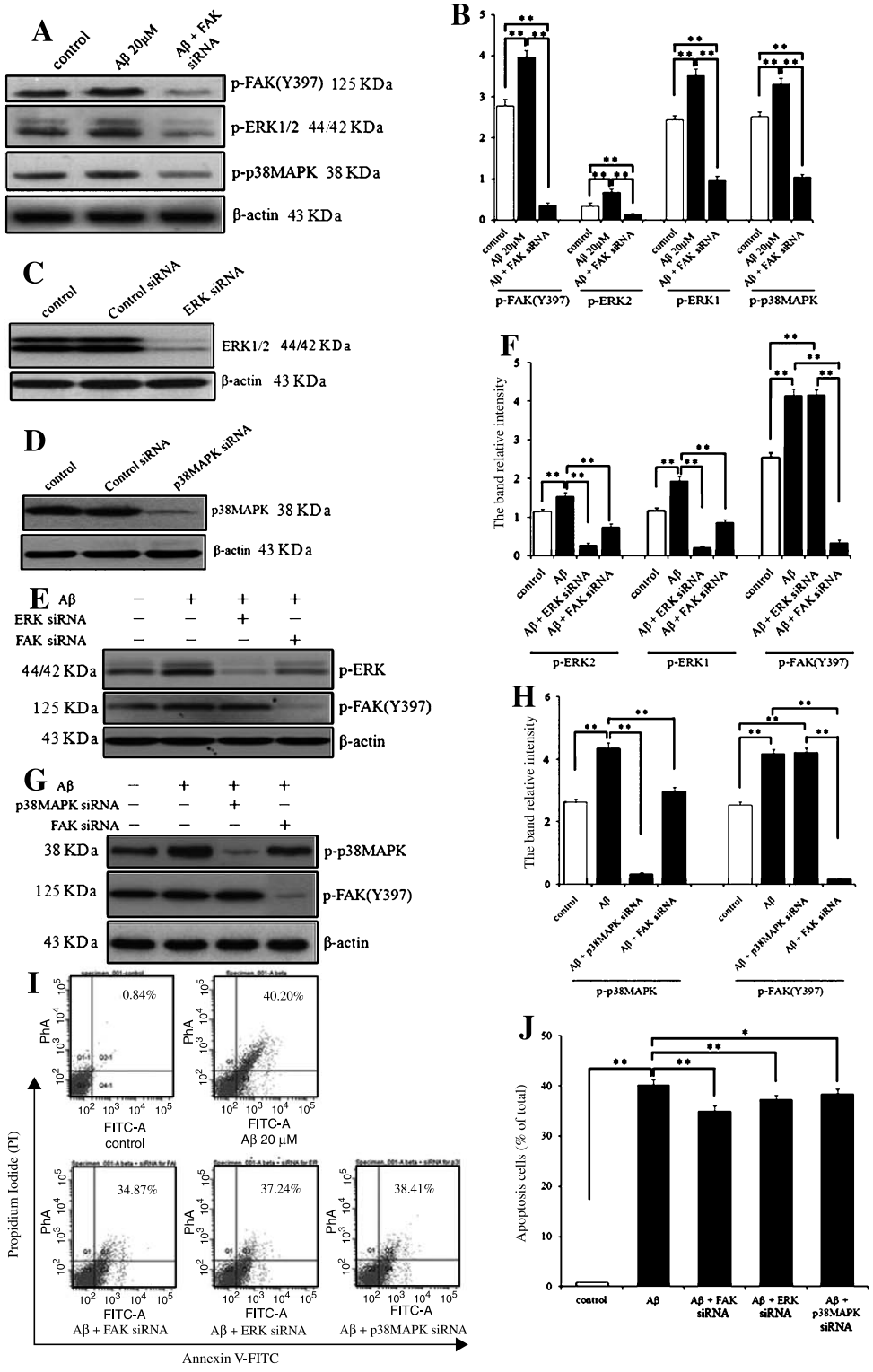


Fig. 4. With FAK activation, ERK1/2 and P38MAPK phosphorylation levels increased. A) After 6 h $A\beta_{25\text{-}35}$ treatment, proteins of whole cells were extracted and separated with SDS-PAGE. Quantities of phosphorylated FAK (Y397), total FAK, phosphorylated ERK1/2, total ERK1/2, phosphorylated p38MAPK, and total p38MAPK were assessed by western blot. β-actin is a marker for whole proteins. B) Data analysis to A. After 20 μM $A\beta_{25\text{-}35}$ treatment, phosphorylation levels of FAK, ERK1/2, and p38MAPK were increased, while the total FAK, total ERK1/2, and total p38MAPK had no significant change. C) After 6 h $A\beta_{25\text{-}35}$ treatment, mRNAs of whole cells were extracted with TRIZol reagent and separated with 1% agarose gel electrophoresis. Quantities of mRNAs of FAK, ERK1/2, p38MAPK, and NF-κB p65 were assessed by RT-PCR. GAPDH is a marker for whole mRNAs. mRNA levels of FAK, ERK1/2, p38MAPK, and NF-κB p65 had no significant change in process of Aβ-induced apoptosis in PC12 cells. D) Western blot analysis to the total protein levels of FAK, ERK1/2, p38MAPK, and p65 in process of Aβ-induced apoptosis. Total protein levels of FAK, ERK1/2, p38MAPK, and NF-κB p65 had no significant change in process of Aβ-induced apoptosis. The results of C and D suggested that increased NF-κB activity is caused by activation of ERK1/2 and p38MAPK, but not by increases in their expression levels. Data in B represent the mean of 3 independent experiments, $^{**}p < 0.01$. Control is specimen without treatment with $A\beta_{25\text{-}35}$.

expression of FAK, and then measured the p38MAPK and ERK1/2 phosphorylation levels. Western blot results showed that when FAK expression was inhibited, both p38MAPK and ERK1/2 activities were decreased as well (Fig. 5A, B). Because the following analysis will use ERK and p38MAPK siRNAs, we determined the effects of these siRNAs. The results showed that both of ERK and p38MAPK siRNAs were efficient to inhibit their respective target protein expressions (Fig. 5C, D).

When expressions of p38MAPK and ERK1/2 were inhibited by their siRNAs respectively, FAK

Fig. 5. FAK regulates the activities of p38MAPK and ERK1/2. A) After treatment with FAK siRNA, proteins of whole cells were extracted and separated with SDS-PAGE. Quantities of phosphorylated FAK (Y397), phosphorylated ERK1/2, and phosphorylated p38MAPK were assessed by Western blot. β-actin is a marker for whole proteins. B) Data analysis to A. When FAK expression was inhibited, phosphorylation levels of ERK1/2 and p38MAPK were decreased as well. C) ERK siRNA could effectively inhibit the expression of ERK1/2. D) p38MAPK siRNA could effectively inhibit the expression of p38MAPK. E) When ERK1/2 expression was inhibited by its siRNAs, FAK phosphorylation level had no significant change. F) Data analysis to E. It is suggested that FAK is upstream of ERK1/2. G) When expression of p38MAPK was inhibited by its siRNAs, FAK phosphorylation level had no significant change. H) Data analysis to G. It is suggested that FAK is upstream of p38MAPK. I) After PC12 cells treated with different siRNA treatments, apoptosis rates were measured by flow cytometry. J) Data analysis to I. All of FAK, ERK, and p38MAPK siRNAs treatments can decrease the apoptosis rate. Data in B, F, H, and J represent the mean of 3 independent experiments, $^{**}p < 0.01$. Control is specimen without treatment with $A\beta_{25\text{-}35}$.


A
control
Aβ 20μM
Aβ + FAK siRNA
p-FAK(Y397) 125 KDa
p-ERK1/2 44/42 KDa
p-p38MAPK 38 KDa
β-actin 43 KDa
B
control
Aβ 20μM
Aβ + FAK siRNA
p-FAK(Y397)
p-ERK2
p-ERK1
p-p38MAPK
C
control
Control siRNA
ERK siRNA
ERK1/2 44/42 KDa
β-actin 43 KDa
D
control
Control siRNA
p38MAPK siRNA
p38MAPK 38 KDa
β-actin 43 KDa
E
Aβ
ERK siRNA
FAK siRNA
44/42 KDa
p-ERK
125 KDa
p-FAK(Y397)
43 KDa
β-actin
F
The band relative intensity
control
Aβ
Aβ + ERK siRNA
Aβ + FAK siRNA
p-ERK2
p-ERK1
p-FAK(Y397)
G
Aβ
p38MAPK siRNA
FAK siRNA
38 KDa
p-p38MAPK
125 KDa
p-FAK(Y397)
43 KDa
β-actin
H
The band relative intensity
control
Aβ
Aβ + p38MAPK siRNA
Aβ + FAK siRNA
p-p38MAPK
p-FAK(Y397)
I
0.84%
40.20%
34.87%
37.24%
38.41%
PhA
FITC-A
control
Aβ 20 μM
Aβ + FAK siRNA
Aβ + ERK siRNA
Aβ + p38MAPK siRNA
Propidium Iodide (PI)
Annexin V-FITC
J
Apoptosis cells (% of total)
control
Aβ
Aβ + FAK siRNA
Aβ + ERK siRNA
Aβ + p38MAPK siRNA

phosphorylation levels had no significant change (Fig. 5E–H). Flow cytometry results showed that in Aβ-induced apoptosis, using siRNAs inhibited expressions of FAK, p38MAPK, and ERK1/2 respectively, and the apoptotic rates declined to different degrees (Fig. 5I, J). The results suggest that FAK is upstream of p38MAPK and ERK1/2, regulating the activities of p38MAPK and ERK1/2 in Aβ-induced apoptosis of PC12 cells.

Both of ERK1/2 and p38MAPK are upstream of NF-κB

Since the activity of NF-κB mediated by FAK, and FAK is also upstream of p38MAPK and ERK1/2, then, is there a relationship between ERK1/2, p38MAPK, and NF-κB? After using siRNAs to inhibit the expressions of ERK1/2, p38MAPK, and NF-κB, western blot results showed that when expression of ERK1/2 and p38MAPK were inhibited, NF-κB activity decreased as well. But when NF-κB expression was inhibited, the activities of ERK1/2 and p38MAPK had no significant change (Fig. 6A–D). After using siRNAs to inhibit the expressions of ERK1/2, p38MAPK, and NF-κB, Bax expression decreased correspondingly, while Bcl-2 increased (Fig. 6E–H). These results suggested that ERK1/2 and p38MAPK are upstream of NF-κB in Aβ-induced apoptosis of PC12 cells.

ERK1/2 directly interacts with IKK, but not IκB, to activate NF-κB; p38MAPK indirectly activates NF-κB

To demonstrate the relationship between activations of ERK1/2 and NF-κB, a Co-IP experiment was performed (using normal rabbit IgG as negative control). The results showed that during Aβ-induced apoptosis of PC12 cells, ERK1/2 directy interacts with IKK, but not with IκB or NF-κB (Fig. 7A). Here ERK1/2 is upstream of NF-κB and directly interacts with IKK; meanwhile IKK can activate NF-κB via phosphorylation of IκB. Therefore, via IKK, ERK1/2 activates NF-κB in Aβ-induced apoptosis of PC12 cells.

To demonstrate whether p38MAPK activates NF-κB via IKK similar to ERK1/2 in Aβ-induced apoptosis in PC12 cells, another Co-IP experiment was performed. Unfortunately, the results showed that p38MAPK does not directly interact with IKK, IκB, or NF-κB (Fig. 7B).

In order to further demonstrate that ERK1/2 can activate IKK, we inhibited ERK1/2 activity by 10 μM U0126 [28] and measured IκBα. The result showed that when cells were co-treated with $A\beta_{25\text{-}35}$ and U0126, the quantity of IκBα was higher than that treated with $A\beta_{25\text{-}35}$ alone (Fig. 7C, D). It was suggested that when ERK1/2 activity is inhibited, there is an increase in binding between NF-κB and IκBα. Reverse Co-IP (using ERK1/2 antibody to precipitate IKK) showed that when ERK1/2 activity was inhibited, the binding of IKK to ERK1/2 was reduced accordingly (Fig. 7E, F). From another point of view, these results indicated the role of ERK1/2 on IKK.

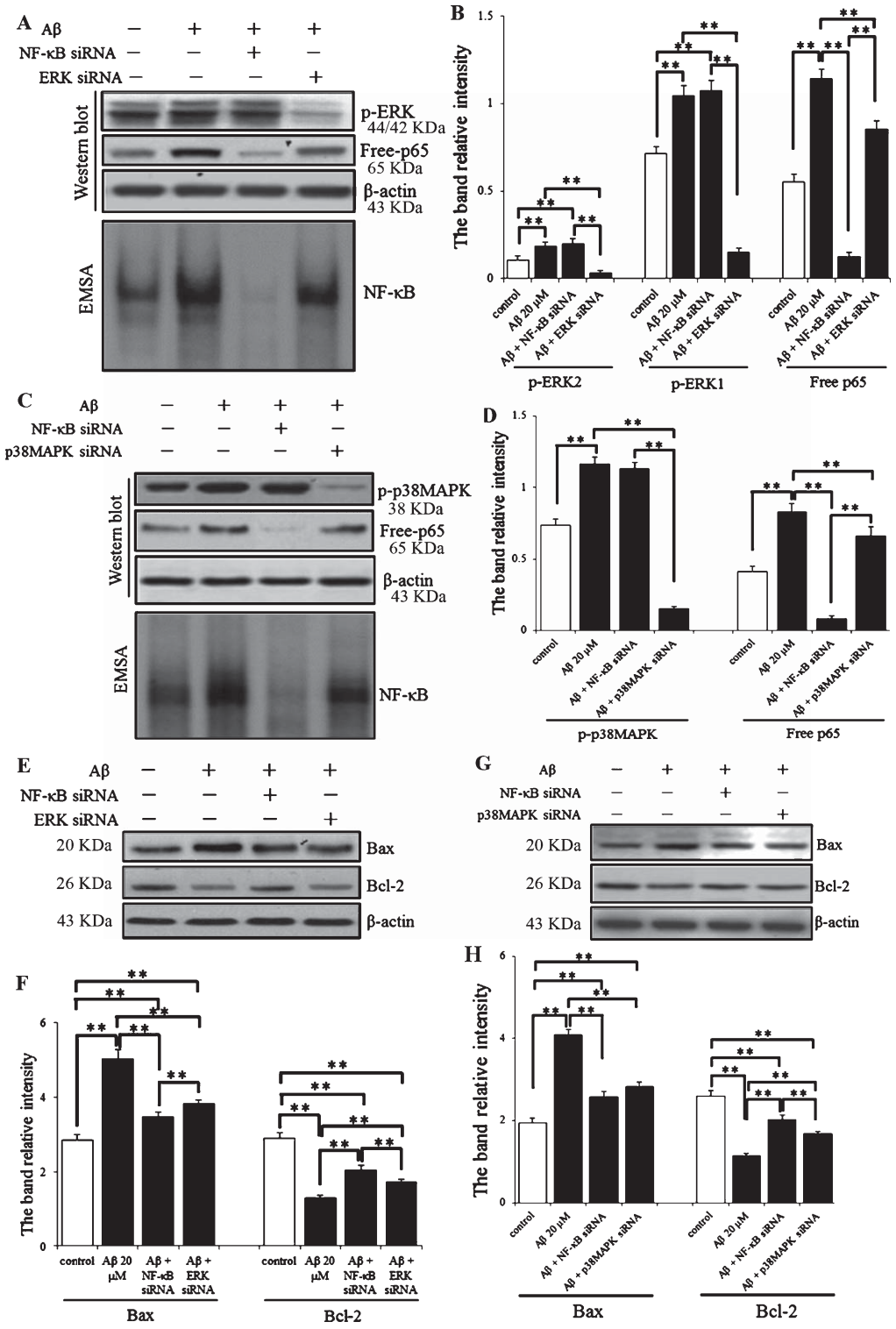
Considering p38MAPK is also upstream of NF-κB, it is suggested that p38MAPK indirectly activates NF-κB in Aβ-induced apoptosis of PC12 cells.

DISCUSSION

Aβ is a major component of senile plaques and has been considered causal in the development and progression of AD. The molecular mechanism of Aβ-mediated neurotoxicity is yet to be elucidated.

Through integrin aggregation, cell adhesion molecules mobilization and coordination with cell surface growth factor receptor signaling, integrin binds to Aβ and activates FAK [29–32]. But Aβ

Fig. 6. Both ERK1/2 and p38MAPK are upstream of NF-κB. A) After siRNA treatment, proteins of whole cells were extracted with gentle lysis and separated with SDS-PAGE. Quantities of phosphorylated ERK1/2 and of free NF-κB p65 (not bound to IκB) were assessed by western blot. β-actin is a marker for whole proteins. Meanwhile, EMSA was performed to analyze the nuclear NF-κB activity. B) Data analysis to the results of western blot in A. When NF-κB expression was inhibited by its siRNA, phosphorylation level of ERK1/2 had no significant change. But when expression of ERK1/2 was inhibited by its siRNA, both free p65 and NF-κB activity were decreased (Aβ versus Aβ + ERK siRNA). It is suggested that ERK1/2 is upstream of NF-κB. C) After siRNA treatment, proteins of whole cells were extracted with gentle lysis and separated with SDS-PAGE. Quantities of phosphorylated p38MAPK and of free NF-κB p65 were assessed by western blot. β-actin is a marker for whole proteins. Meanwhile, EMSA was performed to analyze the nuclear NF-κB activity. D) Data analysis to the results of western blot in C. When NF-κB expression was inhibited by its siRNA, phosphorylation level of p38MAPK had no significant change. But when expression of p38MAPK was inhibited by its siRNA, both free p65 and NF-κB activities were decreased (Aβ versus Aβ + p38MAPK siRNA). It is suggested that p38MAPK is upstream of NF-κB. E) After PC12 cells were treated with NF-κB and ERK siRNAs, expression quantity changes of Bax and Bcl-2 were analyzed by western blot. β-actin is a marker for whole proteins. F) Data analysis to E. G) After PC12 cells were treated with NF-κB and p38MAPK siRNAs, expression quantity changes of Bax and Bcl-2 were analyzed by western blot. H) Data analysis to G. Data in B, D, F, and H represent the mean of 3 independent experiments, $^{**}p < 0.01$. Control is specimen without treatment with $A\beta_{25\text{-}35}$.


A
Aβ
NF-κB siRNA
ERK siRNA
Western blot
p-ERK
44/42 KDa
Free-p65
65 KDa
β-actin
43 KDa
EMSA
NF-κB
B
The band relative intensity
control
Aβ 20 μM
Aβ + NF-κB siRNA
Aβ + ERK siRNA
p-ERK2
p-ERK1
Free p65
C
Aβ
NF-κB siRNA
p38MAPK siRNA
Western blot
p-p38MAPK
38 KDa
Free-p65
65 KDa
β-actin
43 KDa
EMSA
NF-κB
D
The band relative intensity
control
Aβ 20 μM
Aβ + NF-κB siRNA
Aβ + p38MAPK siRNA
p-p38MAPK
Free p65
E
Aβ
NF-κB siRNA
ERK siRNA
20 KDa
Bax
26 KDa
Bcl-2
43 KDa
β-actin
F
The band relative intensity
control
Aβ 20 μM
Aβ + NF-κB siRNA
Aβ + ERK siRNA
Bax
Bcl-2
G
Aβ
NF-κB siRNA
p38MAPK siRNA
20 KDa
Bax
26 KDa
Bcl-2
43 KDa
β-actin
H
The band relative intensity
control
Aβ 20 μM
Aβ + NF-κB siRNA
Aβ + p38MAPK siRNA
Bax
Bcl-2

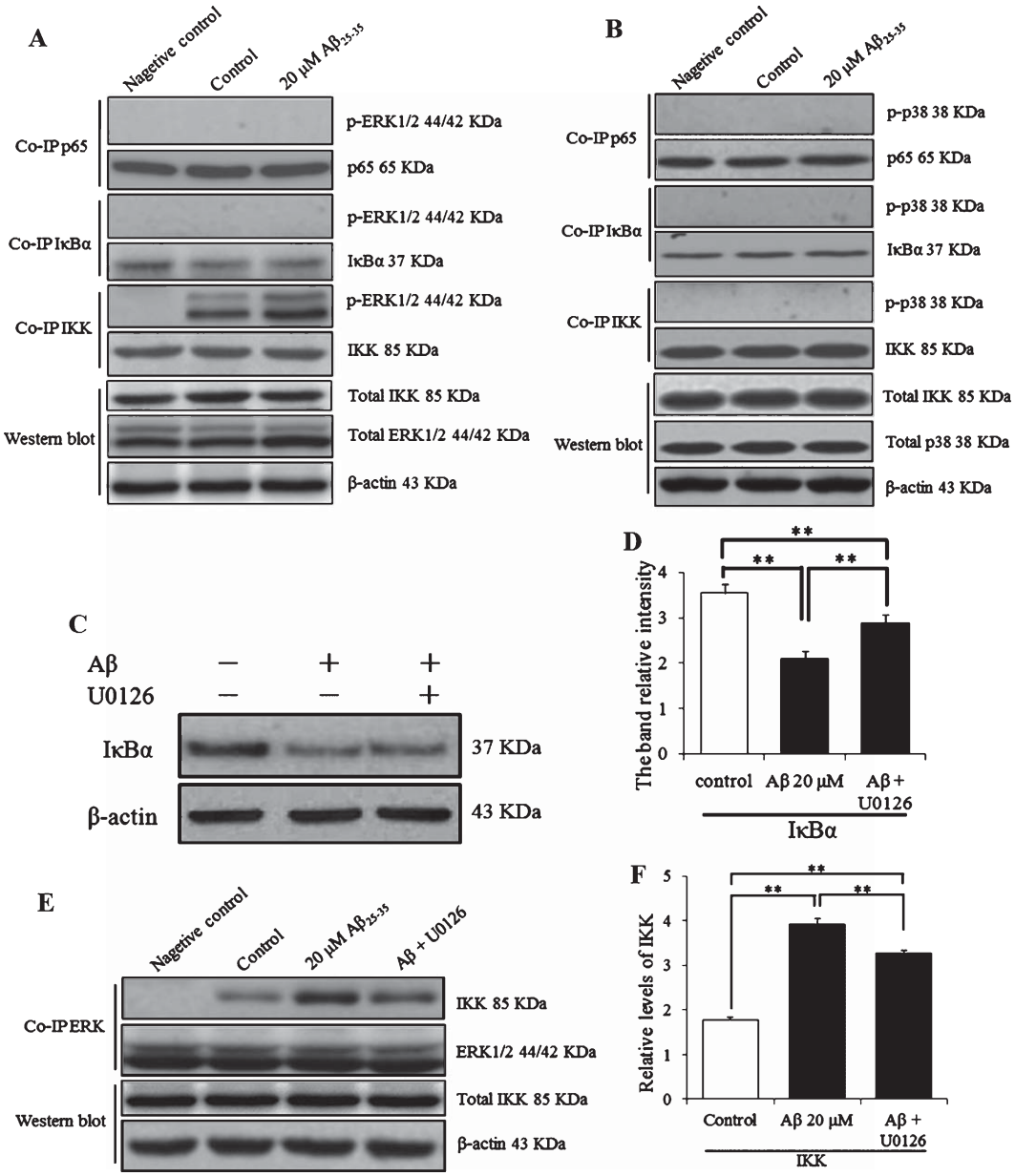


Fig. 7. ERK1/2 directly activates NF-κB via IKK, but not IκB; p38MAPK indirectly activates NF-κB. A) After 6 h $A\beta_{25\text{-}35}$ treatment, proteins of whole cells were extracted with gentle lysis. Interactions of ERK1/2 with NF-κB, IκB, and IKK were assessed by Co-IP. β-actin is a marker for whole proteins. There is an interaction between ERK1/2 and IKK, but no interaction between ERK1/2 and NF-κB or between ERK1/2 and IκB. B) Interactions of p38MAPK with NF-κB, IκB, and IKK were assessed by another Co-IP. β-actin is a marker for whole proteins. There is no interaction between p38MAPK and NF-κB, between p38MAPK and IκB, and between p38MAPK and IKK. C) Western blot analysis to IκBα with different treatment for 12 h. β-actin is a marker for whole proteins. D) Data analysis to C. Compared to treatment with Aβ alone, when ERK1/2 was inhibited by its specific inhibitor U0126, the level of IκBα was increased. E) Reverse Co-IP (using ERK1/2 antibody to precipitate IKK) analysis to interaction of ERK1/2 and IKK. F) Data analysis to C. It showed that when ERK1/2 activity was inhibited by U0126, IKK, which binds to ERK1/2, was reduced accordingly. Data in B, D, and F represent the mean of 3 independent experiments, $^{**}p < 0.01$. Negative control in Co-IP (but NOT in western blot) is using normal rabbit IgG to replace the primary rabbit antibody. Control is specimen without treatment with $A\beta_{25\text{-}35}$.

has no effect on total FAK protein level [16, 32]. By antibody binding of amyloid-β protein precursor on the cell surface, FAK phosphorylation levels in cytoplasm is reduced, thereby reducing neuronal damage [30]. Treated with fibrillar Aβ, FAK and its signal "integrator", paxillin, are both tyrosine phosphorylated before the death [29–32]. These phosphorylations may influence neuronal cytoskeletal architecture, gene expression, synaptic plasticity, and cell viability [34]. As a receptor, integrin receives Aβ signal and phosphorylates FAK by its β subunit [35]. When cells are treated with Aβ, the interaction stability of activated FAK with Src family member Fyn is increased. It is suggested that abnormal activity of Fyn and the interaction with FAK caused by Aβ may play a role in neural degeneration [36]. Activated FAK can cause the production of superoxide radicals in microglia, and this lead to oxidative damage in AD pathogenesis [33]. These studies indicate that FAK phosphorylation led by fibrillar Aβ results in downstream signaling events which regulate cell activity. Our results also showed that after PC12 cells were incubated with 20 μM Aβ for 6–9 h, FAK phosphorylation levels were significantly increased (Fig. 2E, F).

In Aβ-induced apoptosis in neuronal cells, ERK2 is activated [15]. Inhibition of ERK1/2 activity prevents nerve cell cycle and Aβ-induced cell death [37]. In cultured neural cells, through interaction of Aβ and integrin, FAK/Src are activated, and ERK1/2 phosphorylation is increased. This led to inhibitions of cell cycle and cell apoptosis [37]. These reports imply that in Aβ-induced apoptosis, FAK phosphorylation is related to ERK activation.

Serum amyloid A dependent IL-8 gene expression requires activation of ERK and p38MAPK in HT-29 cells [38]. Previous studies demonstrated that in Aβ-induced apoptosis in various types of nerve cells, NF-κB was activated. In these processes, p38MAPK or ERK was activated, or both of them were activated at the same time [8, 39, 40]. From the above studies, it can be seen that there is a relationship between NF-κB activation and p38MAPK/ERK pathways in Aβ-induced apoptosis.

In studies on neuroprotection *in vitro* (PC12 cells) and *in vivo* (mouse model), green tea extracts antagonize Aβ-induced apoptosis by inhibiting p38MAPK and ERK pathways [7, 41–44], or by inhibiting ERK/p38MAPK and NF-κB pathways [4, 28, 30]. In PC12 cells, Aβ upregulates expression of COX-2 by activating NF-κB. This activation is mediated by its upstream ERK and p38MAPK [8]. But unfortunately, there is still lack of information on the detailed relationship between ERK/p38MAPK pathways and NF-κB activation in Aβ-induced neurocyte apoptosis.

These reports have enhanced understanding of the Aβ-induced apoptosis mechanism in neural type cells. It is implied that phosphorylated FAK may mediate NF-κB activity through ERK1/2 and p38MAPK pathways in Aβ induced apoptosis in differentiated PC12 cells. Upon activation, NF-κB plays its role in apoptosis possibly through at least two pathways. One is by promoting Smad-7 expression to down-regulate the TGF-β pathway, thus reducing matrix metalloproteinase-2 production [28]. The other is by upregulating the expression of COX-2 to promote apoptosis [45].

Our results showed that, in Aβ-induced apoptosis of differentiated PC12 cells, FAK is activated, and the activated FAK regulates NF-κB activity through both ERK1/2 and p38MAPK pathways. ERK1/2 directly interacts with IKK and to promote IKK phosphorylation. p38MAPK has no direct interaction with IKK. It activates NF-κB by other means. There is no solid evidence yet though on how p38MAPK may promote NF-κB activity [46, 47]. The mechanism of interaction between the p38MAPK and NF-κB pathways is yet to be fully defined. A related study simply stated that the MKK3/6-p38MAPK pathway also mediates NTHi-induced NF-κB activation by an NF-κB nuclear translocation-independent mechanism [48].

Refering to pathways in other non-neural cells, ERK1/2 might be activated by phosphorylated FAK through Rac (a small G-protein in the Rho family)/Pak (p-21 activated kinase)/Raf-1 (Rapidly accelerated fibrosarcoma-1)/MEK1/2 pathways [49–51]. Through the tyrosine phosphorylation of βPIX (Pak interacting exchange factor-β), FAK activated Rac in fibroblasts [49]. Rac facilitates the assembly of a MAPK signaling complex required for ERK activation [50]. By phosphorylating Raf-1 on serine 338 (S338), Pak participates in Raf-1 activation [51]. Activated Raf-1 activates ERK1/2 via MEK1/2 [52]. As for the pathway of FAK activating p38MAPK, we assume it is possible that via the Ras/Raf/MEKK/MEK3/6 pathway, FAK activates p38MAPK, just like the pathway obtained in osteogenic precursor cells [53].

It should be noted that, although FAK regulates NF-κB activity through ERK1/2 and p38MAPK pathways, inhibition of ERK1/2 and p38MAPK expression cannot completely inhibit NF-κB activity (Figs. 5 and 6). This suggests that, in Aβ-induced

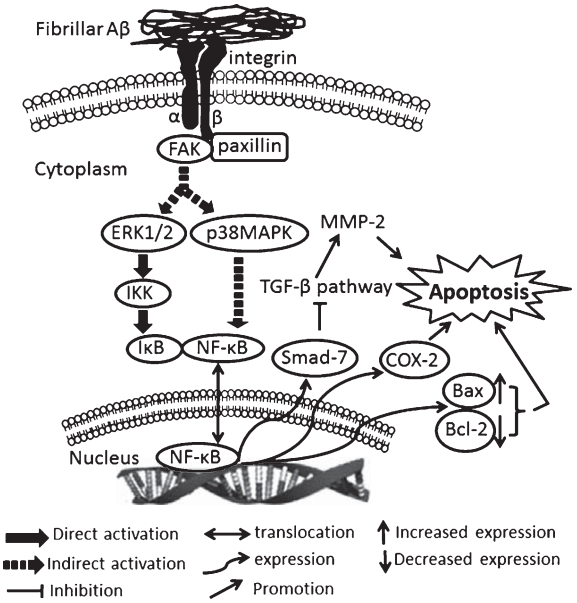


Fig. 8. Diagram of FAK, ERK1/2, p38MAPK, and NF-κB pathways relevant to this study. Fibrillar Aβ phosphorylates FAK and paxillin through integrin β subunit. Phosphorylated FAK promotes phosphorylation of ERK1/2 and p38MAPK. ERK1/2 direct affects on IKK, thereby mediating NF-κB activity via IκB. p38MAPK indirectly activates NF-κB activity. Activated NF-κB transfers into the nucleus and promotes apoptosis related genes expression, such as Samd-7, COX-2, etc., and upregulates pro-apoptotic Bax expression and downregulates anti-apoptotic protein Bcl-2 expression. As a result, differentiated PC12 cells are in apoptosis.

apoptosis of differentiated PC12 cells, additional pathway(s) exist to activate NF-κB. Similarly, inhibition of NF-κB expression cannot also completely inhibit the apoptosis induced by Aβ (Fig. 3E, F), indicating there are other pathway(s) to induce cell apoptosis besides NF-κB. In addition to the FAK/MAPK/NF-κB pathway, there are at least two pathways at work in Aβ-induced apoptosis. One is the PP2A/ASK1/p38MAPK/p53/Bax pathway [54, 55] and the other is the Ca^{2+}/GSK-3β/p-tau pathway [7, 56].

In summary, the results of the present study link up the pathways between FAK phosphorylation and NF-κB activation in Aβ-induced apoptosis of differentiated PC12 cells (Fig. 8). In this pathway, fibrillar Aβ phosphorylates FAK and paxillin through integrin β subunit. Phosphorylated FAK promotes phosphorylation of ERK1/2 and p38MAPK. ERK1/2 directly interacts with IKK, thereby mediating NF-κB activity via IκB. Phosphorylated p38MAPK activates NF-κB by other mean(s). The details of how p38MAPK regulate NF-κB activity require further study.

ACKNOWLEDGMENTS

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1344).

REFERENCES

[1] Varghese J (2010) *BACE: Lead Target for Orchestrated Therapy of Alzheimer's Disease*, Wiley Press, Hoboken, New Jersey, pp. 1-13.

[2] Castellani R, Hirai K, Aliev G, Drew KL, Nunomura A, Takeda A, Cash AD, Obrenovich ME, Perry G, Smith M (2002) Role of mitochondrial dysfunction in Alzheimer's disease. *J Neurosci Res* **70**, 357-360.

[3] Sullivan PG, Brown MR (2005) Mitochondrial aging and dysfunction in Alzheimer's disease. *Prog Neuropsychopharmacol Biol Psychiatry* **29**, 407-410.

[4] Jana A, Pahan K (2004) Fibrillar amyloid-beta peptides kill human primary neurons via NADPH oxidase-mediated activation of neutral sphingomyelinase. Implications for Alzheimer's disease. *J Biol Chem* **279**, 51451-51459.

[5] Valerio A, Boroni F, Benarese M, Sarnico I, Ghisi V, Bresciani LG, Ferrario M, Borsani G, Spano P, Pizzi M (2006) NF-kappaB pathway: A target for preventing beta-amyloid (Abeta)-induced neuronal damage and Abeta42 production. *Eur J Neurosci* **23**, 1711-1720.

[6] Song YS, Park HJ, Kim SY, Lee SH, Yoo HS, Lee HS, Lee MK, Oh KW, Kang SK, Lee SE, Hong JT (2004) Protective role of Bcl-2 on beta-amyloid-induced cell death of differentiated PC12 cells: Reduction of NF-kappaB and p38 MAP kinase activation. *Neurosci Res* **49**, 69-80.

[7] Esposito G, De Filippis D, Maiuri MC, De Stefano D, Carnuccio R, Iuvone T (2006) Cannabidiol inhibits inducible nitric oxide synthase protein expression and nitric oxide production in beta-amyloid stimulated PC12 neurons through p38 MAP kinase and NF-kappaB involvement. *Neurosci Lett* **399**, 91-95.

[8] Jang JH, Surh YJ (2005) AP-1 mediates beta-amyloid-induced iNOS expression in PC12 cells via the ERK2 and p38 MAPK signaling pathways. *Biochem Biophys Res Commun* **331**, 1421-1428.

[9] Chen J, Zhou Y, Mueller-Steiner S, Chen LF, Kwon H, Yi S, Mucke L, Gan L (2005) SIRT1 protects against microglia-dependent amyloid-beta toxicity through inhibiting NF-kappaB signaling. *J Biol Chem* **280**, 40364-40374.

[10] Hamadi A, Bouali M, Dontenwill M, Stoeckel H, Takeda K, Rondé P (2005) Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397. *J Cell Sci* **118**, 4415-4425.

[11] Ritchie W, Timothy S, Bruce RC, Graham G, Hugh R, Stuart K, Ian NB, Ian MV, Paul WS, Ian E, Brian HA (2002) Rapid tyrosine phosphorylation of neuronal proteins including tau and focal adhesion kinase in response to amyloid-β peptide exposure: Involvement of Src family protein kinases. *J Neurosci* **22**, 10-20.

[12] Rowan MJ, Klyubin I, Wang Q, Hu NW, Anwyl R (2007) Synaptic memory mechanisms: Alzheimer's disease amyloid β-peptide-induced dysfunction. *Biochem Soc Trans* **35**, 1219-1223.

[13] Sonoda Y, Matsumoto Y, Funakoshi M, Yamamoto D, Hanks SK, Kasahara T (2000) Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J Biol Chem* **275**, 16309-16315.

[14] Xu LH, Yang X, Bradham CA, Brenner DA, Baldwin AS Jr, Craven RJ, Cance WG (2000) The focal adhesion kinase suppresses transformation -associated, anchorage-independent apoptosis in human breast cancer cells: Involvement of death receptor-related signaling pathways. *J Biol Chem* **275**, 30597-30604.

[15] Williamson R, Scales T, Clark BR, Gibb G, Reynolds CH, Kellie S, Bird IN, Varndell IM, Sheppard PW, Everall I, Anderton BH (2002) Rapid tyrosine phosphorylation of neuronal proteins including tau and focal adhesion kinase in response to amyloid-beta peptide exposure: Involvement of Src family protein kinases. *J Neurosci* **22**, 10-20.

[16] Zhang C, Lambert MP, Bunch C, Barber K, Wade WS, Krafft GA, Klein WL (1994) Focal adhesion kinase expressed by nerve cell lines shows increased tyrosine phosphorylation in response to Alzheimer's A beta peptide. *J Biol Chem* **269**, 25247-25250.

[17] Grace EA, Busciglio J (2003) Aberrant activation of focal adhesion proteins mediates fibrillar amyloid beta-induced neuronal dystrophy. *J Neurosci* **23**, 493-502.

[18] Jung KJ, Lee EK, Kim JY, Zou Y, Sung B, Heo HS, Kim MK, Lee J, Kim ND, Yu BP, Chung HY (2009) Effect of short term calorie restriction on pro-inflammatory NF-kB and AP-1 in aged rat kidney. *Inflamm Res* **58**, 143-150.

[19] Li L, Fan D, Wang C, Wang JY, Cui XB, Wu D, Zhou Y, Wu LL (2011) Angiotensin II increases periostin expression via Ras/p38 MAPK/CREB and ERK1/2/TGF-β1 pathways in cardiac fibroblasts. *Cardiovasc Res* **91**, 80-89.

[20] Guner YS, Ochoa CJ, Wang J, Zhang X, Steinhauser S, Stephenson L, Grishin A, Upperman JS (2009) Peroxynitrite-induced p38 MAPK pro-apoptotic signaling in enterocytes. *Biochem Biophys Res Commun* **384**, 221-225.

[21] Jiang HQ, Zhang XL, Liu L, Yang CC (2004) Relationship between focal adhesion kinase and hepatic stellate cell proliferation during rat hepatic fibrogenesis. *World J Gastroenterol* **10**, 3001-3005.

[22] Mizuno S, Kato K, Asai S, Takahashi Y, Nagata T, Komuro S, Iwasaki A, Ishikawa K, Arakawa Y (2004) Gene expression analysis in the stomachs of water immersion-restraint stress rats using high-density oligonucleotide array. *J Gastroenterol Hepatol* **19**, 1264-1269.

[23] Liu Z, Zhang X, Hu X, Cheng M, Xu J, Du Y (2011) Effect of glucocorticoid on MIP-1α and NF-κb expressing in the lung of rats undergoing mechanical ventilation with a high tidal volume. *World J Emerg Med* **2**, 66-69.

[24] Rondas D, Tomas A, Halban PA (2011) Focal adhesion remodeling is crucial for glucose-stimulated insulin secretion and involves activation of focal adhesion kinase and paxillin. *Diabetes* **60**, 1146-1157.

[25] Parsons JT (2003) Focal adhesion kinase: The first ten years. *J Cell Sci* **116**, 1409-1416.

[26] Aikawa R, Nagai T, Kudoh S, Zou Y, Tanaka M, Tamura M, Akazawa H, Takano H, Nagai R, Komuro I (2002) Integrins play a critical role in mechanical stress-induced p38 MAPK activation. *Hypertension* **39**, 233-238.

[27] Huang D, Khoe M, Befekadu M, Chung S, Takata Y, Ilic D, Bryer-Ash M (2007) Focal adhesion kinase mediates cell survival via NF-kappaB and ERK signaling pathways. *Am J Physiol Cell Physiol* **292**, C1339-C1352.

[28] Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* **273**, 18623-18632.

[29] Kim TI, Lee YK, Park SG, Choi IS, Ban JO, Park HK, Nam SY, Yun YW, Han SB, Oh KW, Hong JT (2009) l-Theanine, an amino acid in green tea, attenuates beta-amyloid-induced cognitive dysfunction and neurotoxicity: Reduction in oxidative damage and inactivation of ERK/p38 kinase and NF-kappaB pathways. *Free Radic Biol Med* **47**, 1601-1610.

[30] Lee EO, Kang JL, Chong YH (2005) The amyloid-beta peptide suppresses transforming growth factor-beta1-induced matrix metalloproteinase-2 production via Smad7 expression in human monocytic THP-1 cells. *J Biol Chem* **280**, 7845-7853.

[31] Lee JW, Lee YK, Ban JO, Ha TY, Yun YP, Han SB, Oh KW, Hong JT (2009) Green tea (−)-epigallocatechin-3-gallate inhibits beta-amyloid-induced cognitive dysfunction through modification of secretase activity via inhibition of ERK and NF-kappaB pathways in mice. *J Nutr* **139**, 1987-1993.

[32] Xu YX, Wang HQ, Yan J, Sun XB, Guo JC, Zhu CQ (2009) Antibody binding to cell surface amyloid precursor protein induces neuronal injury by deregulating the phosphorylation of focal adhesion signaling related proteins. *Neurosci Lett* **465**, 276-281.

[33] McDonald DR, Brunden KR, Landreth GE (1997) Amyloid fibrils activate tyrosine kinase-dependent signaling and superoxide production in microglia. *J Neurosci* **17**, 2284-2294.

[34] Berg MM, Krafft GA, Klein WL (1997) Rapid impact of beta-amyloid on paxillin in a neural cell line. *J Neurosci Res* **50**, 979-989.

[35] Balleza-Tapia H, Peña F (2009) Pharmacology of the intracellular pathways activated by amyloid beta protein. *Mini Rev Med Chem* **9**, 724-740.

[36] Zhang C, Qiu HE, Krafft GA, Klein WL (1996) A beta peptide enhances focal adhesion kinase/Fyn association in a rat CNS nerve cell line. *Neurosci Lett* **211**, 187-190.

[37] Frasca G, Carbonaro V, Merlo S, Copani A, Sortino MA (2008) Integrins mediate beta-amyloid-induced cell-cycle activation and neuronal death. *J Neurosci Res* **86**, 350-355.

[38] Jijon HB, Madsen KL, Walker JW, Allard B, Jobin C (2005) Serum amyloid A activates NF-kappaB and proinflammatory gene expression in human and murine intestinal epithelial cells. *Eur J Immunol* **35**, 718-726.

[39] Costantini C, Rossi F, Formaggio E, Bernardoni R, Cecconi D, Della-Bianca V (2005) Characterization of the signaling pathway downstream p75 neurotrophin receptor involved in beta-amyloid peptide-dependent cell death. *J Mol Neurosci* **25**, 141-156.

[40] Gunn-Moore FJ, Tavaré JM (1998) Apoptosis of cerebellar granule cells induced by serum withdrawal, glutamate or beta-amyloid, is independent of Jun kinase or p38 mitogen activated protein kinase activation. *Neurosci Lett* **250**, 53-56.

[41] Chang CH, Chen CY, Chiou JY, Peng RY, Peng CH (2010) Astaxanthine secured apoptotic death of PC12 cells induced by beta-amyloid peptide 25-35: Its molecular action targets. *J Med Food* **13**, 548-556.

[42] Hu JF, Chu SF, Ning N, Yuan YH, Xue W, Chen NH, Zhang JT (2010) Protective effect of (−)clausenamide against

Abeta-induced neurotoxicity in differentiated PC12 cells. *Neurosci Lett* **483**, 78-82.

[43] Iuvone T, De Filippis D, Esposito G, D'Amico A, Izzo AA (2006) The spice sage and its active ingredient rosmarinic acid protect PC12 cells from amyloid-beta peptide-induced neurotoxicity. *J Pharmacol Exp Ther* **317**, 1143-1149.

[44] Lee SY, Lee JW, Lee H, Yoo HS, Yun YP, Oh KW, Ha TY, Hong JT (2005) Inhibitory effect of green tea extract on beta-amyloid-induced PC12 cell death by inhibition of the activation of NF-kappaB and ERK/p38 MAP kinase pathway through antioxidant mechanisms. *Brain Res Mol Brain Res* **140**, 45-54.

[45] Jang JH, Surh YJ (2005) Beta-amyloid-induced apoptosis is associated with cyclooxygenase-2 up-regulation via the mitogen-activated protein kinase-NF-kappaB signaling pathway. *Free Radic Biol Med* **38**, 1604-1613.

[46] Je JH, Lee JY, Jung KJ, Sung B, Go EK, Yu BP, Chung HY (2004) NF-kappaB activation mechanism of 4-hydroxyhexenal via NIK/IKK and p38 MAPK pathway. *FEBS Lett* **566**, 183-189.

[47] Papachristou DJ, Papadakou E, Basdra EK, Baltopoulos P, Panagiotopoulos E, Papavassiliou AG (2008) Involvement of the p38 MAPK-NF-kappaB signal transduction pathway and COX-2 in the pathobiology of meniscus degeneration in humans. *Mol Med* **14**, 160-166.

[48] Shuto T, Xu H, Wang B, Han J, Kai H, Gu XX, Murphy TF, Lim DJ, Li JD (2001) Activation of NF-kappa B by nontypeable Hemophilus influenzae is mediated by toll-like receptor 2-TAK1-dependent NIK-IKK alpha/beta-I kappa B alpha and MKK3/6-p38 MAP kinase signaling pathways in epithelial cells. *Proc Natl Acad Sci U S A* **98**, 8774-8779.

[49] Chang F, Lemmon CA, Park D, Romer LH (2007) FAK potentiates Rac1 Activation and localization to matrix adhesion sites: A role for βPIX. *Mol Biol Cell* **18**, 253-264.

[50] Eblen ST, Slack JK, Weber MJ, Catling AD (2002) Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. *Mol Cell Biol* **22**, 6023-6033.

[51] King AJ, Wireman RS, Hamilton M, Marshall MS (2001) Phosphorylation site specificity of the Pak-mediated regulation of Raf-1 and cooperativity with Src. *FEBS Lett* **497**, 6-14.

[52] Matallanas D, Birtwistle M, Romano D, Zebisch A, Rauch J, von Kriegsheim A, Kolch W (2011) Raf family kinases: Old dogs have learned new tricks. *Genes Cancer* **2**, 232-360.

[53] Buravkova LB, Gershovich PM, Gershovich JG, Grigor'ev AI (2010) Mechanisms of gravitational sensitivity of osteogenic precursor cells. *Acta Naturae* **2**, 28-36.

[54] Akterin S, Cowburn RF, Miranda-Vizuete A, Jimenez A, Bogdanovic N, Winblad B, Cedazo-Minguez A (2006) Involvement of glutaredoxin-1 and thioredoxin-1 in beta-amyloid toxicity and Alzheimer's disease. *Cell Death Differ* **13**, 1454-1465.

[55] Hsu MJ, Hsu CY, Chen BC, Chen MC, Ou G, Lin CH (2007) Apoptosis signal-regulating kinase 1 in amyloid beta peptide-induced cerebral endothelial cell apoptosis. *J Neurosci* **27**, 5719-5729.

[56] Cedazo-Mínguez A, Popescu BO, Blanco-Millán JM, Akterin S, Pei JJ, Winblad B, Cowburn RF (2003) Apolipoprotein E and beta-amyloid (1-42) regulation of glycogen synthase kinase-3beta. *J Neurochem* **87**, 1152-1164.