行政院國家科學委員會補助專題研究計畫 ■ 期末進度報告

（計畫名稱）

Cerebral amyloid angiopathy (澱粉腦血管病變)-澱粉誘發基質金屬蛋白酵素-9 表現之轉錄機制

計畫類別：□ 個別型計畫　　■ 整合型計畫

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執行單位:臺北醫學大學

中　　華　　民　　國 97 年 10 月 30 日

一、中文摘要

乙型樣澱粉蛋白(β-Amyloid, Aβ) 被認為是引起許多神經退化性疾病的主要原因，而 Aβ和星狀細胞的交互作用對神經細胞產生的傷害會更進一步促進神經的退化。此外，星狀細胞又是構成血腦障壁的主要成分之一，因此星狀細胞凋亡對於中樞神經系統以及腦血管退化之病理發展過程是非常重要的。我們過去對 Aβ造成大腦血管內皮細胞的分子機制已經有充分的了解，但對於 Aβ對星狀細胞造成死亡的分子機制仍有許多未知之處。Aβ對非神經細胞的毒殺作用在於活化 nSMase，造成釋放出 ceramide, 然後活化 PP2A，在 Aβ造成腦血管內皮的死亡途徑中，PP2A 會使 Akt 和 FKHRL1 去磷酸化，使活化的 FKHRL1 進入細胞核中轉錄活化 Bim 基因，最後 BIM 蛋白引起細胞程式化死亡。本論文中，我們將探討 Aβ調控星狀神經膠質瘤細胞凋亡的詳細分子機轉。在 C6 星狀神經膠質瘤細胞中，Aβ誘導 FKHR 之 Ser256 的去磷酸化呈現時間相關性，Aβ也會時間相關性地誘導 IKKα/β Ser180/Ser181 的去磷酸化，此外, 在未處理藥物的 C6 星狀細胞中 IKKα/IKKβ/FKHR 形成一個蛋白複合體，一旦 Aβ處理細胞 30 分鐘後，PP2A 會結合到此複合體，使得 IKKα/β和 FKHR 去磷酸化，此複合體開始崩解，IKKα, IKKβ和 FKHR 彼此分離，FKHR 活化，進而啟動細胞程式化死亡，我們也發現除了 PP2A 被活化，PP1 也會被活化，但它的效應不如 PP2A。所以綜合以上實驗結果推測 Aβ可經由 PP2A/IKKα/IKKβ/FKHR/Bim 訊息路徑誘導 C6 星狀神經膠質瘤細胞死亡。

關鍵詞：乙型樣澱粉蛋白；神經退化性疾病；C6 星狀神經膠質瘤細胞；Bim_{EL}；FKHR；IKKα/β；PP2A；細胞凋亡

Protein complex dissociation in amyloid β peptide-induced astrocytic death

Abstract: Astrocytes, the most abundant cell type in the brain, provide metabolic and trophic support for neurons to modulate synaptic activity and for cerebral endothelial cells (CECs) to maintain blood-brain barrier (BBB) function. Amyloid-β peptiode (Aβ) has been implicated to cause neuronal degeneration, resulting in Alzheimer's disease and to induce death of non-neuronal cells in the brain, leading to cerebral amyloid angiopathy (CCA). CECs and astrocytes constitute the BBB that maintains the integrity of the brain. While the molecular mechanism of Aβ-induced CEC death has been well characterized by our group, how Aβ activates the death signaling processes in astrocytes remains partially unresolved. The balance of activities between protein kinases and phosphatases plays a key role in determining cell viability. In Aβ-induced death of non-neuronal cells including CECs, astrocytes, oligodendrocytes and VSMCs, an early death signaling event is Aβ activation of neutral sphingomyelinase (nSMase), resulting in the release of ceramide from membrane sphingomyelin. Ceramide then activates protein phosphatase 2A (PP2A), a member in the ceramide-activated protein phosphatase (CAPP)

compartments to interfere with the function of antiapoptotic Bcl-2 family members to initiate apoptosis (Huang and Strasser, 2000). Bim expression is regulated by transcription factors of the forkhead in rhabdomyosarcoma (FKHR) family, with includes FKHR (also known as FOXO1) and FKHRL1 (FOXO3a) (Burgering and Kops, 2002; Gilley et al., 2003). Forkhead protein activity, in turn, is negatively regulated via phosphorylation by the survival promoting kinase Akt (Brunet et al., 1999; Tang et al., 1999). Previous studies indicate that Akt regulates the activity of FKHRL1, a member of the Forkhead family of transcription factors. In the presence of survival factors, Akt phosphorylates FKHRL1, leading to FKHRL1's association with 14-3-3 proteins and FKHRL1's retention in the cytoplasm. Survival factor withdrawal leads to FKHRL1 dephosphorylation, nuclear translocation, and target gene activation. Within the nucleus, FKHRL1 triggers apoptosis most likely by inducing the expression of genes that are critical for cell death, such as the Fas ligand gene (Brunet et al., 1999; Burgering and Kops, 2002). In brain studies it has been reported that Aβ-induced apoptosis of Cerebral Endothelial Cells (CECs) is via Akt/FKHRL1 signaling pathway and *bim* expression (Yin et al., 2006). Recently, Biswas et al reported that Bim is elevated in Alzheimer's disease neurons and is required for β-Amyloid-induced neuronal Apoptosis (Biswas et al., 2007). They found that the cell cycle molecule cyclin-dependent kinase 4 (cdk4) and its downstream effector B-myb, are required for Abeta-dependent Bim induction and death in cultured neurons. However, the signaling pathway between Bim regulation and FOXO activation during astrocytic death by Aβ is still unknown. The signaling pathway of IκB kinase/NF-κB plays a critical role in many physiological responses and the pathology process. Transcription factor NF-κB widely distributes in each kind of cell, may activate expression of some specific genes to suppress the specific proteins involved in the apoptotic pathway to achieve the goal of cell survival. NF-κB may increase the transcripts of Bcl-2 protein family members like Bcl-X_L and A1/Bfl-1, which may inhibit the mitochondria-dependent apoptosis (Edelstein et al., 2003; Sevilla et al., 2001) ◦ The IKK complex consists of two catalytic subunits IKKα and IKKβ, and an essential regulatory subunit NEMO/IKKγ (Karin, 1999; Li et al., 2002). Two molecules of NEMO are believed to orchestrate the assembly of the IKKs into the high molecular weight signalsome complex partially by binding to specific carboxyl-terminally conserved residues of both IKKα and IKKβ termed the NEMO binding domain (NBD) (Li et al., 2001; May et al., 2000). NEMO may also facilitate the recruitment of Iκ

Bα to the IKK complex (Yamamoto et al., 2001). A large multiprotein complex, the IκB kinase (IKK) signalsome, is found to contain a cytokine-inducible IκB kinase activity that phosphorylates IκB-α and IκB-β ((Mercurio *et al*., 1997; Zandi *et al*., 1997). IKKβ is 50% identical to IKKα and like it contains a kinase domain, a leucine zipper, and a helix-loop-helix. Although IKKα and IKKβ can undergo homotypic interaction, they also interact with each other and the functional IKK complex contains both subunits. The catalytic activities of both IKKα and IKKβ make essential contributions to IκB phosphorylation and NF-κB activation. While the interactions between IKKα and IKKβ may be mediated through their leucine zipper motifs, their helix-loop-helix motifs may be involved in interactions with essential regulatory subunits (DiDonato et al., 1997; Karin, 1999; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). Although nuclear exclusion of the forkhead transcription factor FOXO3a by protein kinase Akt contributes to cell survival, more evidences shown that the negative regulation of FOXO factors by IKK as a key mechanism for promoting cell growth (Hu et al., 2004, Pardo et al., 2008).

Protein phosphorylation and dephosphorylation are the most important regulatory mechanisms governing many aspects of biology (Hunter, 1995). In eukaryotes, dephosphorylation at serine/threonine residues are executed by four major protein phosphatases, phosphatase-1 (PP-1), phosphatase-2A (PP-2A), phosphatase-2B (PP-2B), and phosphatase-2C (PP-2C) (Mumby and Walter, 1993; Moorhead et al., 2007) and several minor phosphatases including phosphatase-4 (PP-4), phosphatase-5 (PP-5), phosphatase-6 (PP-6), and phosphatase-7 (PP-7) (Mumby and Walter, 1993; Moorhead et al., 2007). Among these different serine/threonine phosphatases, PP-1 and PP-2A accounts for 90% of the intracellular protein serine/threonine phosphatase activities (Moorhead et al., 2007). Protein phosphatase 2A (PP2A), a member of the ceramide-activated protein phosphatase (CAPPs) family, regulates the activities of several major protein kinase families, including Akt to drive apoptotic processes (Millward et al., 1999; Silverstein et al., 2002). Moreover, recent studies have indicated that inhibition of PP2A phosphatases rescued FOXO1-mediated cell death by regulating the level of the pro-apoptotic protein BIM. Thus, they concluded that PP2A is a physiologic phosphatase of FOXO1 (Yan et al., 2008).

Despite rapid progress in elucidating the molecular mechanisms of activation of the kinase IKK, the processes that regulate IKK deactivation are still unknown. It has recently been demonstrated that CUE domain–containing 2 (CUEDC2) interacted with IKKα and IKKβ and

repressed activation of the transcription factor NF-κB by decreasing phosphorylation and activation of IKK. Notably, CUEDC2 also interacted with GADD34, a regulatory subunit of protein phosphatase 1 (PP1). They found that IKK, CUEDC2 and PP1 existed in a complex and that IKK was released from the complex in response to inflammatory stimuli such as tumor necrosis factor. CUEDC2 deactivated IKK by recruiting PP1 to the complex. Therefore, CUEDC2 acts as an adaptor protein to target IKK for dephosphorylation and inactivation by recruiting PP1 (Li et al., 2008). In our group we have reported that activation of PP2A is the intermediate step between the Aβ-ceramide cascade and the subsequent inactivation of Akt, activation of FKHRL1, and upregulation of *bim* (Yin et al., 2006). Therefore, we aimed to determine whether activation of the IKK/FKHR pathway contributes to Aβ-induced astrocytic death. Results from the present study provide experimental evidence to support the contention that activation of the IKKα/β-FKHR-Bim pathway contributes to Aβ-induced astrocytic apoptosis in a PP2A-dependent manner. We also found that IKKα/β and FKHR existed in a complex and that IKKα/IKKβ /FKHR complex was underwent time-dependent dissociation in response to Aβ treatment. These findings suggest that a protein complex may be dissociated for liberty and activation of FKHR downstream of PP2A in Aβ-induced astrocytic death.

Materials and Methods DMEM, fetal calf serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA); the enhanced chemiluminescence detection kit was from GE Healthcare (Little Chalfont, UK); Amyloid peptide ($A\beta_{25\text{-}35}$) was from AnaSpec (San Jose, CA, USA); protein A/G beads, anti-mouse, anti-goat and anti-rabbit IgG-conjugated horseradish peroxidase antibodies, and antibodies specific for FKHR, p-FKHR, IKKα/β, p-IKKα/β, IKKα, IKKβ, Akt, 14-3-3, PP2A-Cα/β, PP1α, GAPDH, α-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA); all reagents for SDS-PAGE were from Bio-Rad (Richmond, CA); okadaic acid (OA), a selective PP2A inhibitor, was from Upstate Biotechnology (Lake Placid, NK); 1,2-dioleoyl-sn-glycero-3-phosphate (PA), a selective PP1 inhibitor, was from Sigma (St, Louis, MO). And all other chemicals were from Sigma (St, Louis , MO).

Aβ preparation. Aβ was aggregated before experiments in the present study. For aggregation, amyloid peptide was dissolved in sterile double-distilled H_2O to a concentration of 1mM and then maintained for 3d at 37°C to allow polymerization.

Western blot analysis. To determine the expressions of FKHR, IKKα/β, FKHR-p and IKKα/β-p in C6 cells using α-tubulin , as the internal control, proteins were extracted and analyzed by Western blotting as described previously (Yin et al., 2002; Chen et al., 2004). Briefly, C6 cells were cultured in 6 cm dishes. After reaching confluence, cells were treated with vehicle or specific inhibitors followed by Aβ for various time intervals. After incubation, cells were washed twice in ice-cold PBS and solubilized in extraction buffer containing 10mM Tris, pH 7.0, 140 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 0.5% Nonidet P-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin. Samples of equal amounts of protein (60 μg) were subjected to SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane that was later incubated in TBST buffer (150 mM NaCl, 20 mM Tris-HCl, and 0.02 % Tween 20, pH 7.4) containing 5% nonfat milk. Proteins were incubated with first specific primary antibodies and then horseradish peroxidase-conjugated secondary antibodies. Specific bands were detected based on enhanced chemiluminescence per the instructions of the manufacturer. Quantitative data were obtained using a computing densitometer with scientific imaging systems (Eastman Kodak, Rochester, NY).

Coimmunoprecipitation. C6 cells were grown in 6 cm dishes. After reaching confluence, cells were treated with 20 μM Aβ for the indicated time intervals. The cells were harvested, lysed in 1 ml of PD buffer (40 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Nonidet P-40, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 300 μM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM dithiothreitol), and centrifuged. The supernatant was immunoprecipitated overnight with specific antibodies in the presence of protein A/G-agarose beads at 4˚C. The immunoprecipitated complex was washed three times with PD buffer. The samples were fractioned on 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and subjected to immunoblotting with antibodies specific for specific antibodies.

Gradient sedimentation. C6 supernatant lysate with Aβ or without Aβ was respectively subjected to sedimentation analysis on 10%-30% glycerol gradients in PD buffer. Gradients were centrifuged in SW40 at 37,000 rpm for 16h at 4℃ and collected in 0.42-mL fractions.

Gel-filtration chromatography. Cytoplasmic extracts from C6 cells without or with Aβ (20 mM) treatment were subject to size-exclusion

chromatography (Sephacryl S-400, flow-rate 0.25 ml/min and 1-mL fractions) at cold room. Fractions were analyzed by western blots.

Suppression of pp2a expression. For pp2a suppression, predesigned siRNAs targeting the mouse pp2a gene was also purchased from Ambion. The siRNA oligonucleotides targeting the coding regions of mouse PP2A catalytic subunit (PP2A-C) mRNA were as follows: pp2a siRNA-1 sense, 5'-ccauacuccgagggaaucatt-3' (siRNA ID#: 152168). The negative control siRNA comprising a 19 bp scrambled sequence with 3' dT overhangs was also purchased from Ambion.

Statistical analysis. Results are presented as mean±SEM from at least three independent experiments. One-way ANOVA, followed by Bonferroni's multiple range tests when appropriate, was used to determine the statistical significance of the difference between the means. A p value<0.05 was considered statistically significant.

Results:

FKHR activation in Aβ-treated C6 glioma cells

Although C6 astrocytic death induced by Aβ has been documented (Yang et al., 2004), it remains unclear whether FKHR activation involved in the signaling pathway. Phosphorylation of FKHRL1 by Akt leads to its sequestration by the 14-3-3 protein, preventing its translocation into the nucleus to act as a pro-apoptotic transcription factor (Brunet et al., 1999). We next examined whether FKHR dephosphorylation occurred in Aβ-treated C6 cells using Western blotting anlaysis. As illustrated in Figure 1, Aβ (20 μM) treatment decreased the level of phosphorylation of FKHR Ser256 unto the maximum of 30 min.

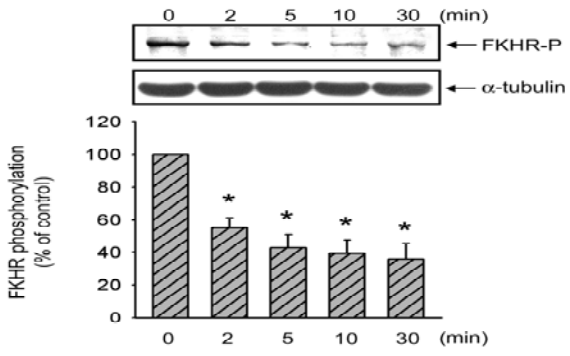

Figure 1. FKHR activation in Aβ-treated C6 glioma cell. C6 glioma cells were treated with 20 μM $A\beta_{25-35}$ for the indicated times, and FKHR-p was detected by Western blotting. Aβ peptides decreased the level of phosphorylation of FKHRSer256 in a time-dependent manner. The data shown are mean±SD of three separate experiments in triplicate.*p<0.05, significant difference from the oh group.

IKK activity was inhibited in Aβ-treated C6 glioma cells

As reported previously, in addition to Akt as the negative regulation of FOXO factors, IKK also seem to play the same role (Hu et al., 2004). To explore whether activity of IKKα/β was inhibited accompanying by activation of FKHR in Aβ-treated C6 cells, we examined the level of IKKα/β-p for the indicated times by Western blotting analyis. We observed the level of IKK-p decreased in a time-dependent manner. The lowest level was detected from 5 to 30 min.

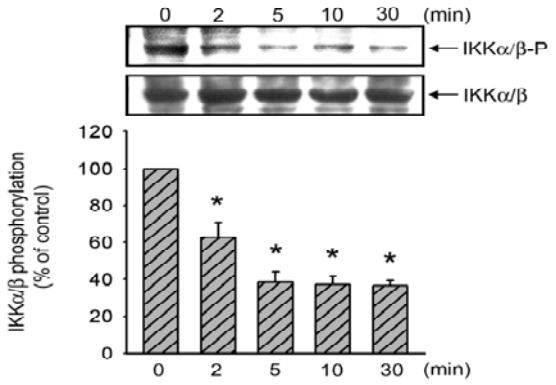

Figure 2. IKK activity was inhibited in Aβ-treated C6 glioma cells. C6 glioma cells were treated with 20 μM $A\beta_{25-35}$ for the indicated times, and IKKα/β-p was detected by Western blotting. Aβ peptides dephosphorylated IKKα/β in a time-dependent manner. The data shown are mean±SD of three separate experiments in triplicate.*$p<0.05$, significant difference from the oh group.

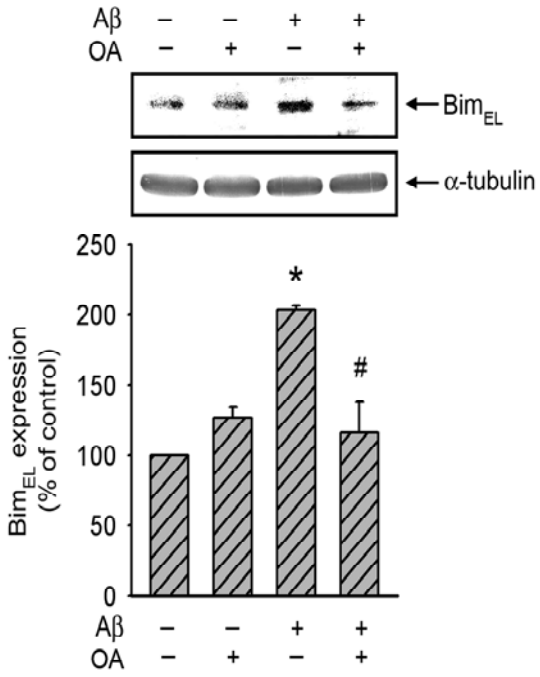

Figure 3. Aβ activate Bim expression via PP2A. C6 cells were pretreated with 0.1 nM OA for 30 min, and then treated with 20 μM Aβ for another 6h. Whole cell lysate were immunodectected with Bim and α-tubulin specific antibody. Data represent the mean±S.E. of three experiments performed in duplicate. *p<0.05 as compared with the control group. #p<0.05 as compared with the Ab-treated group.

Bim expression induced by Aβ via PP2A. Previous studies showed that PP2A as an early regulator of Aβ-induced bim expression in CEC (Yin et al., 2006). Hence, we suggested that PP2A may play the same role in C6 cells. To examine its role in Aβ-induced bim expression in C6 cells. We pretreated the C6 with PP2A specific inhibitor OA (0.1 nM) for 30 min, then treated cells with Aβ (20 μM) for another 6h. Whole cell lysates were prepared, and immunodetected with Bim, α-tubulin specific antibody. Equal loading in each lane is demonstrated by similar intensities of α-tubulin. The data showed that PP2A is the early regulator of Aβ-induced bim expression in CECs and C6 glioma cells.

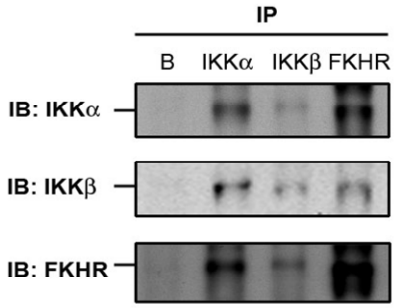

Figure 4. IKKα/IKKβ/FKHR associated each other in C6 cells. Coimmunoprecipitation applied to examine the interaction among IKKα, IKKβ and FKHR in steady state C6 cells.

IKKα, IKKβ and FKHR associated each other in C6 glioma cells, respectively. Cell extracts prepared from C6 glioma cells were precipitated with the anitibody IKKα, IKKβ and FKHR followed by Western blotting as shown in figure 4. The result suggested that IKKα, IKKβ and FKHR may form a complex in unstressed environment.

Aβ induced dissociation of IKKα, IKKβ and FKHR in C6 glioma cells. Cells were incubated with 20 μM Aβ for 0-60 min. Whole cell lysates were prepared, and then immunoprecipitated with an antibody specific for IKKα, IKKβ and FKHR, respectively. The immunoprecipitated complex was then subjected to immunoblotting with IKKα, IKKβ and FKHR, respectively. The results showed that IKKα, IKKβ and FKHR form a complex and Aβ induced dissociation of the complex in C6 cells. The similar events were reported (Tinel and Tschopp, 2004; Li et al., 2008)

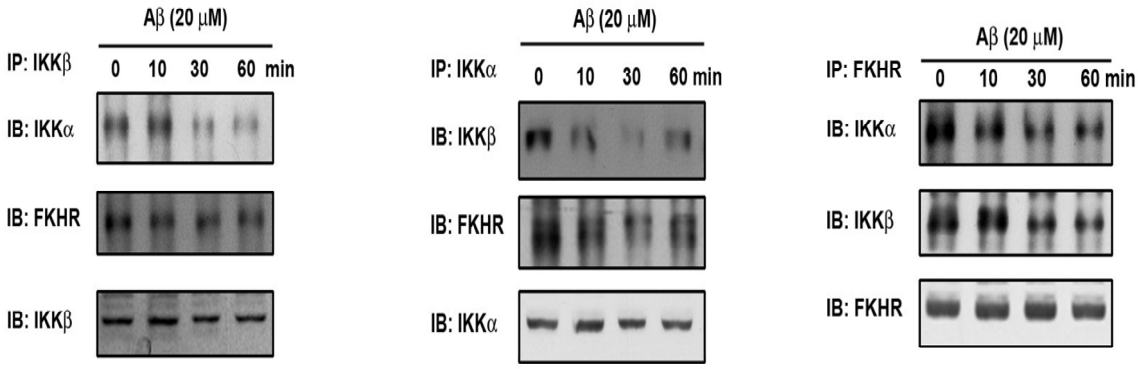

Figure 5. Aβ induced dissociation of IKKα, IKKβ and FKHR in C6 glioma cells. Reciprocal coimmunoprecipitation of IKKα, IKKβ and FKHR revealed that IKKα, IKKβ and FKHR dissociated each other under Aβ treatment.

Aβ induced association among PP2A with IKKβ and Akt. Previous study has demonstrated that Aβ induced interaction between PP2A and Akt (Yin et al., 2006). Thus, we further explore whether Aβ induced interaction between PP2A and Akt or IKKβ. The result indicated that Aβ induced not only interaction between PP2A and Akt, but also interaction between PP2A and IKKβ. The data suggest that PP2A may result in IKKα/IKKβ/FKHR dissociation.

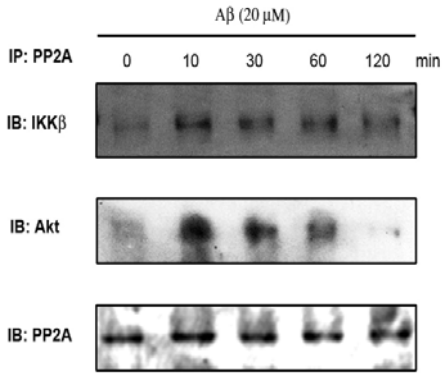

Fig 6. Aβ induced association among PP2A with IKKβ and Akt. C6 cells were incubated with 20 μM Aβ for 0-120 min. Whole cell lysates were prepared, and then immunoprecipitated with an antibody specific for PP2A, then was subjected to immunoblotting with IKKβ, Akt and PP2A.

Aβ induced the huge complex IKKα/β/FKHR dissociation which was examined by sedimentation analysis. IKKα/β and FKHR form a huge complex (MW > 900 kDa) may be examined by using 10-30% glycerol gradient sedimentation analysis. Moreover, the complex dissociation was observed.

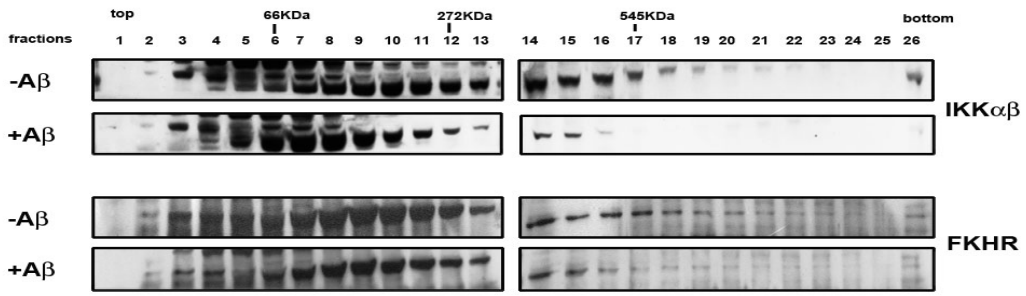

Figure 7. Aβ induced the huge complex IKKα/β/FKHR dissociation detected by sedimentation analysis. Using the 10-30 % glycerol gradient analysis we observed that IKKαβ and FKHR colocalized at the 26th fraction and Aβ induced the fraction disappearance.

Aβ induced the huge complex IKKα/β/FKHR dissociation which was examined by gel filtration chromatography. Using another technique we also observed that IKKa/IKKb/FKHR form a complex which was fractioned at the 41-37 fraction. Then, Aβ induced the complex dissociation to some smaller complexes.

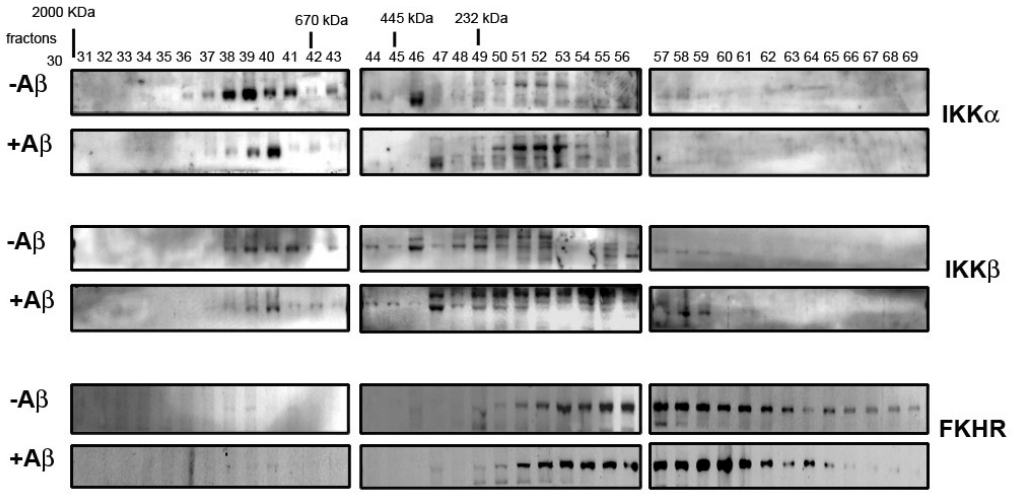

Figure 8. Aβ induced the huge complex IKKα/β/FKHR dissociation which was examined by gel filtration chromatography. IKKα/IKKβ/FKHR colocalized at the 37-41 fraction and Aβ induced the complex dissociation to the smaller complexes.

PP2A dephosphorylated IKKα/β and FKHR to result in the complex dissociation. OA, the specific PP2A inhibitor, may inhibit the dissociation of the complex by PP2A.

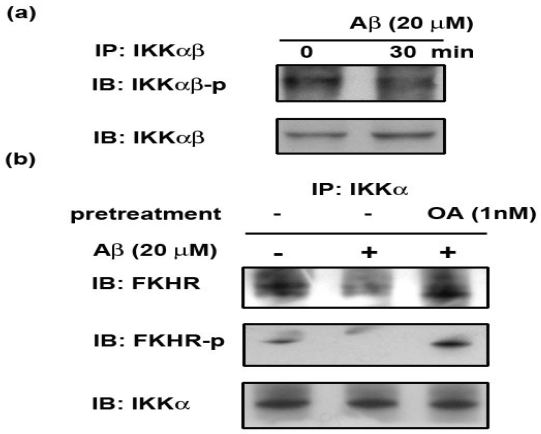

Fig 9 PP2A dephosphorylated IKKα/β and FKHR to result in the complex dissociation.

Activated PP2A induced by Aβ may dissociate the complex, but its action inhibited by OA. PP1 seems to participate in the action but mildly. PP1 is activated to a much less content in CEC treated with Aβ has been reported (Yin et al., 2006)

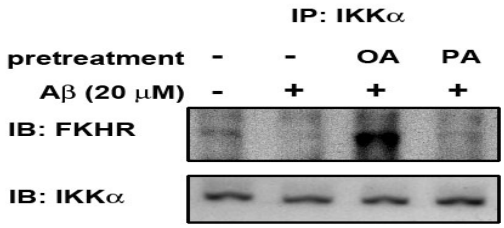

Fig 10 Activated PP2A induced by Aβ dissociated the complex inhibited by OA

Activated PP2A and PP1 by Aβ may dissociate the complex *in vitro*

Immunoprecipitated PP2A and PP1 were used to examined whether they executed the complex dissociation as *in vivo*.

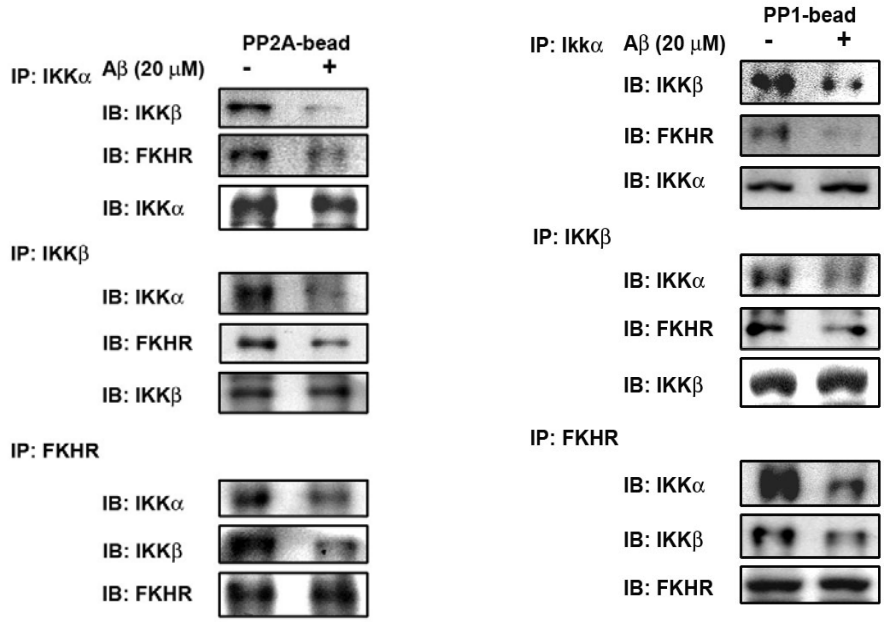

Figure 11. Activated PP2A and PP1 by Aβ may dissociate the complex *in vitro.* C6 cell lysates with or without Aβ treatment were immunoprecipitated by specific antibody PP2A or PP1, then PP2A-bead and PP1-bead were collected to execute the complex dissociation test.

Discussion and suggestion:

In conclusion, the present study suggests a novel complex Ikkα/IKKβ/FKHR exists in C6 glioma cell, which is dissociated by the effect of dephosphorylation of PP2A on IKKαβ-p/FKHR-p. However, the function of the complex is still unknown. Whether there are other unknown components existing , and who is the scaffold protein? The elucidation of the signaling events involved in Aβ-induced astrocytic death may be important for understanding molecular mechanisms of cerebrovascular degeneration to design effective strategies to attenuate the pathogenesis of CAA.

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