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Research article

IGF-1 protects against $A\beta_{25-35}$-induced neuronal cell death via inhibition of PUMA expression and Bax activation

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HIGHLIGHTS

- IGF-1 prevents $A\beta_{25-35}$-induced cell death through the activation of PI3K/Akt pathway.
- IGF-1 mediated neuroprotection against $A\beta_{25-35}$ toxicity via inhibition of PUMA expression.
- IGF-1 inhibits $A\beta_{25-35}$-induced PUMA expression via the PI3K/Akt/FOXO3a Pathway.
- IGF-1 mediated neuroprotection against $A\beta_{25-35}$ toxicity via inhibition of Bax activation.

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ABSTRACT

Amyloid-β-peptide (Aβ) is considered to be the toxic species in AD and causes cell death in the affected areas of patient's brain. Insulin-like growth factor 1 (IGF-1) has been reported to attenuate Aβ toxicity in neuronal cells. However, the molecular mechanisms involved in the neuroprotective function of IGF-1 remain largely unknown. In the present study, we for the first time demonstrated that IGF-1 protects against Aβ-induced neurotoxicity via inhibition of PUMA expression and Bax activation. We found that IGF-1 could activate Akt, which in turn inhibited Aβ-induced FOXO3a nuclear translocation and thus decreased the binding ability of FOXO3a to PUMA promoter, leading to decreased PUMA expression. In addition, IGF-1 inhibited the translocation of Bax to the mitochondria induced by Aβ. Notably, addition of wortmannin, a specific inhibitor of PI3K, significantly abolished the neuroprotective effect of IGF-1, suggesting that IGF-1 exerts its anti-apoptotic effect depend on PI3K activity. Our findings may provide new insights into molecular mechanisms mediated by IGF-1 in cell survival against Aβ-induced apoptosis.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by senile plaques, neurofibrillary tangles and neuronal loss which affecting elderly people worldwide [1]. Deposition of amyloid-β-peptide (Aβ) in the brain is a key factor contributing to the pathogenesis of AD [2]. Although there are symptomatic treatments for AD patients, currently there is no effective therapy to prevent this disease.

Insulin-like growth factor-1 (IGF-1), a trophic hormone, is structurally similar to insulin. IGF-1 is shown to have neuroprotective effect in many cell types [3–5]. Accumulating evidences have focused on the central role of the IGF-1 signaling pathway for lifespan regulation in *Caenorhabditis elegans*, *Drosophila*, *yeast*, and mammals [6–8]. IGF-1 decline has been related to age-dependent cognitive impairment and dementia [9]. Consistent with this finding, the pathogenesis of AD is attributed to IGF-1 deficiency [10]. It has also been demonstrated that IGF-1 prevents neuronal cell death in neurodegenerative diseases including Parkinson's disease (PD) [4,11], Huntington's disease (HD) [3], and AD [12]. Although the involvement of IGF-1 signaling pathway in cell survival has been identified in several models, its downstream targets are frequently cell type-specific. Hence, studies on its neuroprotective mechanism

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against Aβ toxicity could possibly provide a potential therapeutic strategy for AD.

The ability of IGF-1 to promote cell survival has been attributed in part to the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) signaling pathway [3,13,14]. Akt is a key mediator of survival signals in response to a range of extra- and intracellular stimuli. Once activated, Akt exerts anti-apoptotic effects by regulating the expression of targeted genes involved in cell death and survival. FOXO3a, a member of the O subfamily of the forkhead transcription factors, is a critical downstream molecule of the PI3K/Akt pathway [15]. In response to IGF-1, FOXO3a is inactivated by PI3K/Akt and excluded from the nucleus to cytosol, thereby preventing its binding ability to the promoters of the target genes.

Growing evidences indicate that the BH3-only proteins of the Bcl-2 family are involved in various cell death paradigms, including neurodegeneration [16–18]. In accordant with this notion, recent studies showed that p53 up-regulated modulator of apoptosis (PUMA) plays an essential role in Aβ-induced apoptosis, *in vitro* and *in vivo* [19,20]. Morevover, FOXO3a serves as a transcription factor, leads to the activation of proapoptotic signaling via transactivation of PUMA in Aβ-induced apoptosis [20]. So far, little is known about the relationship between IGF-1 and PUMA expression in Aβ-induced apoptosis. In the present study, we aimed to determine the downstream signaling pathways of IGF-1 that antagonizes Aβ-induced apoptosis in SH-SY5Y cells. We uncovered the possible mechanism and found that the activation of the PI3K/Akt and subsequent reduction of PUMA expression and Bax translocation are important for the survival of SH-SY5Y cells against Aβ neurotoxicity.

2. Material and methods

2.1. Materials

IGF-1 and $A\beta_{25\text{-}35}$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). $A\beta_{25\text{-}35}$ peptides were dissolved in ultrapure deionized distilled water at 1 mM as stock solutions. Before treatment, peptides were preincubated at 37 °C for 5 days to promote aggregation and then diluted with medium to desired concentrations (25 μM) [21]. $A\beta_{1\text{-}42}$ was purchased from Merck Millipore and the peptides were prepared as previously described [20]. Wortmannin was purchased from BIOMOL Research Laboratories, Inc. (Plymouth, PA). Phospho-Akt (Ser473), phospho-Akt (308), Akt, Bax, and phospho-FOXO3a (Ser253) antibodies were purchased from Cell Signaling Technology (Danvers, MA); Anti-PUMA was purchased from Epitomics (Burlingame, CA); Anti-β-actin was purchased from Santa Cruz (La Jolla, CA). DsRed-mito plasmid was obtained from Invitrogen (Carlsbad, CA); GFP-FOXO3a plasmid was a gift from Prof. Wolfgang Link [22].

2.2. Cell culture and transfection

The human neuroblastoma cell line SH-SY5Y was cultured in DMEM (GBICO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2, 95% air at 37 °C in a humidified incubator. The cells were transfected at 60–70% confluence with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for the overexpression experiments according to the manufacturer's instructions.

2.3. Cell viability assay

SH-SY5Y cells were cultured at a density of 5×10^3 cells/well in 96-well microplates. Cell viability was assessed with CCK-8 (Dojindo Laboratories, Japan) after Aβ oligomers and/or IGF-1 treatment according to the manufacturer's instructions. OD450, the absorbance value at 450 nm, was read with a microplate reader to determine the viability and proliferation of the cells.

2.4. Cell apoptosis assay

Cell apoptosis by Annexin-V/PI staining was performed as described previously [23]. After indicated treatments, apoptotic cell death was determined using the Annexin V, FITC Apoptosis Detection Kit (Dojindo Laboratories, Japan) according to the manufacturer's protocol. Flow cytometry was performed on a BD FACSCanto II flow cytometer (Becton Dickinson).

2.5. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis

RT-PCR was performed as described previously [24]. Puma primer sequence: sense: TTGTGCTGGTGCCCGTTCCA; antisense: AGGCTAGTGGTCACGTTTGGCT. β-actin primer sequence: sense: TCATGTTTGAGACCTTCAA; antisense: GTCTTTGCGGATGTCCACG.

2.6. Western blotting analysis

After indicated treatments, the cells were lysed in RIPA lysis buffer (50 mM Tris, 150 mMNaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail set I. Equal amounts of protein were loaded on SDS-PAGE, transferred to the PVDF membrane (Millipore). Blocked with 5% nonfat milk in TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 h, and the membrane were incubated with indicated primary antibodies, followed by secondary antibodies (Cell Signaling Technology). Detection was performed using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

2.7. Immunofluorescence

Cells were fixed in 3.7%–4% paraformaldehyde and then permeated with 0.1% Triton X-100. Samples were incubated in blocking buffer and then followed by incubation with the indicated primary antibodies. Finally, FITC-conjugated secondary antibodies were added for 2 h at a room temperature. Mitochondria were stained with MitoTracker Red. Slides were mounted and analyzed by confocal microscopy (Carl Zeiss).

2.8. GFP-FOXO3a translocation assay

To monitor nuclear translocation of FOXO3a, SH-SY5Y cells were transiently transfected with pGFP-FOXO3a before treatments. Subcellular GFP localization was assessed by using a 488 nm excitation light from an argon laser and a 500-550 band-pass filter (Carl Zeiss MicroImaging).

2.9. Chromatin immunoprecipitation analysis

CHIP was performed as previously described [20]. Brifely, the DNA was extracted, and the region of FOXO3a binding to the PUMA promoter was amplified using the following primers: sense, GCGCACAGGTGCCTCGGC and antisense, TGGGTGTGGCCGCCCCT.

2.10. Statistical analysis

All data represent at least three independent experiments and are expressed as the mean ± SEM. An unpaired, two-tailed Student's *t* test was employed to determine significant differences

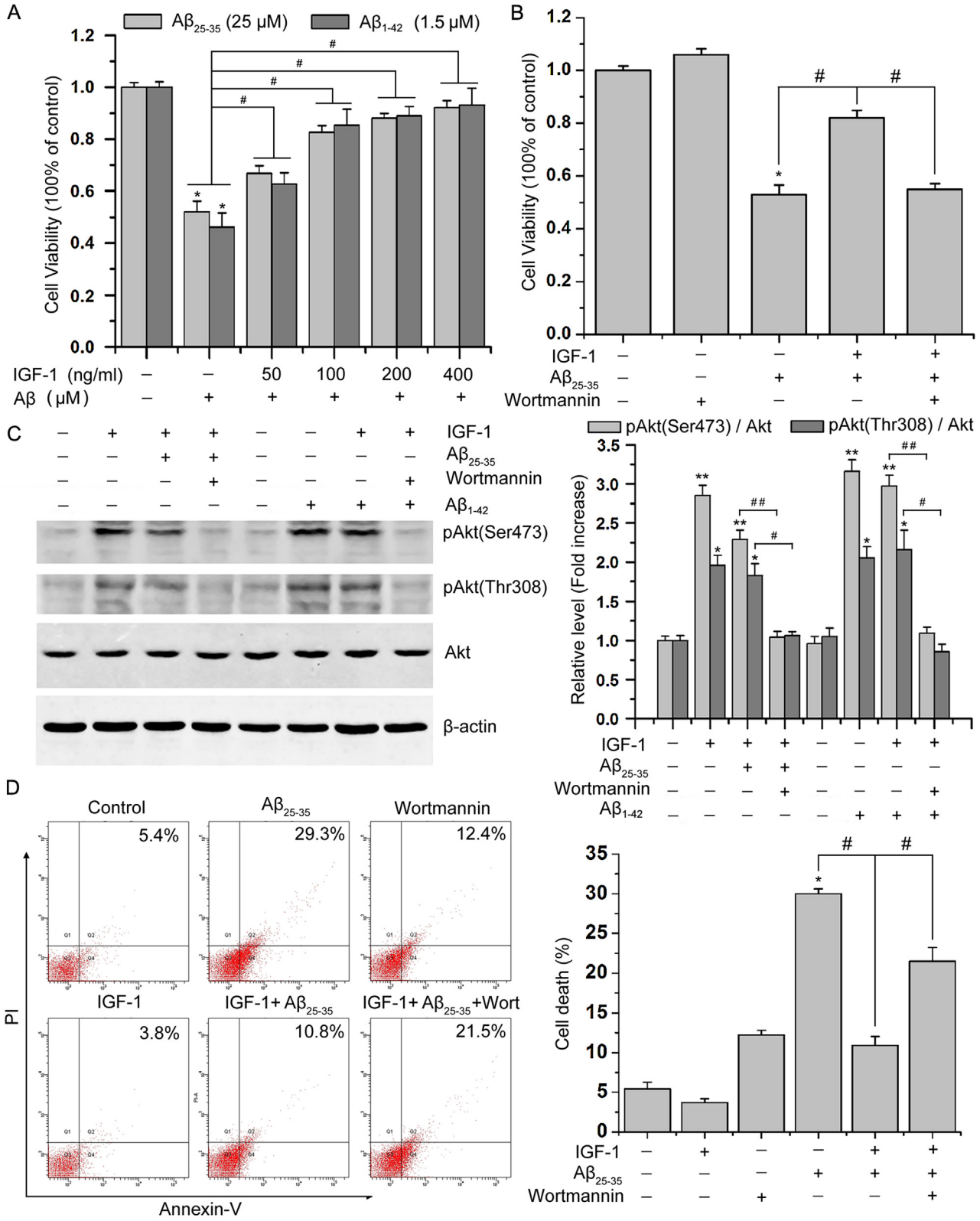

Fig. 1. IGF-1 rescued the SH-SY5Y cells from Aβ neurotoxicity through the activation of PI3K/Akt signaling pathway. (A) SH-SY5Y neuronal cells were treated with 25 μM $A\beta_{25\text{-}35}$ or 1.5 μM $A\beta_{1\text{-}42}$ for 24 h in the absence or presence of IGF-1 at indicated concentrations, and the cell viability was assessed by CCK8 assay. Data are expressed as fold increase relative to untreated cells. (B) Quantitative analysis of the effect of PI3K inhibitor wortmannin on the protective effect of IGF-1 on Aβ-induced neurotoxicity. SH-SY5Y cell viability was measured using the CCK8 assay. Pre-incubation with 1 μM wortmannin resulted in complete elimination of the IGF-1-induced neuroprotection. Wortmannin alone did not alter cell viability. Data are expressed as fold increase relative to untreated cells. (C) Western blotting analysis for Akt Thr308 and Ser473 phosphorylation. Akt and β-actin were used as a loading control. The blots were representative of three independent experiments. Experiments were performed three times and data are expressed as mean ± SEM. (D) H-SY5Y cells were treated with 25 μM $A\beta_{25\text{-}35}$ in the presence of 100 ng/ml IGF-1 or not, and cell apoptosis was performed by Annexin V-FITC/PI double staining using flow cytometric analysis. Quantitative data were expressed as mean ± SEM of three replicate values in three separate experiments. $^{*}P < 0.05$ *vs.* the control group, $^{\#}P < 0.05$ *vs.* the indicated group.

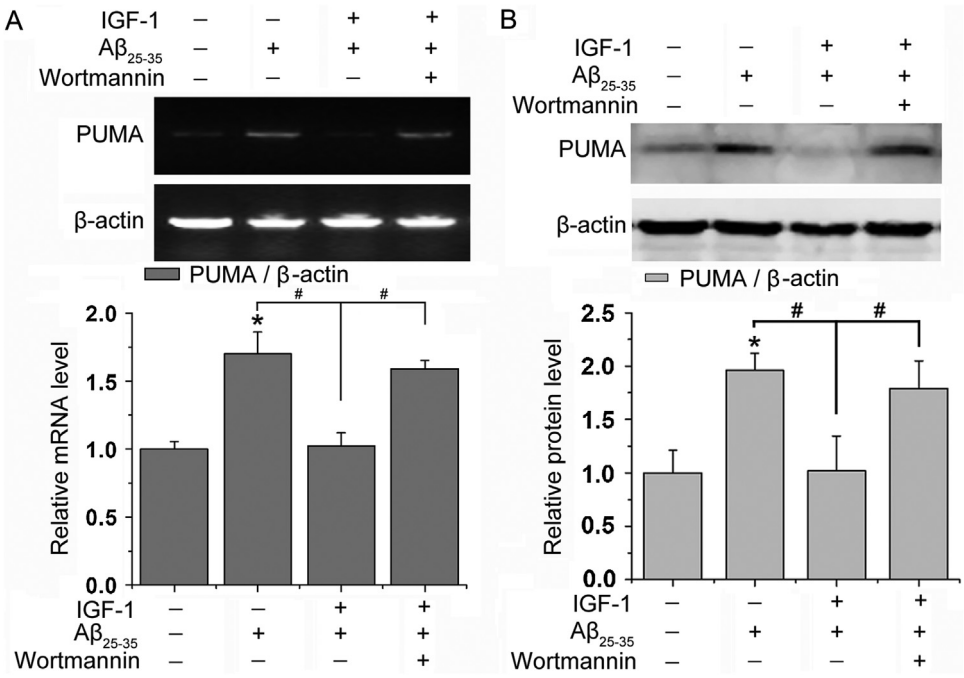

Fig. 2. IGF-1 mediated neuroprotection against $A\beta_{25-35}$ toxicity via inhibition of PUMA expression. (A) SH-SY5Y cells were treated indicated treatments. Total RNA was isolated to prepare cDNA. PUMA mRNA level was detected by RT-PCR and normalized to the loading control β-actin. Results are from a representative of three independent experiments and data are expressed as mean ± SEM. (B) Western blotting was performed to detect the expression of PUMA following $A\beta_{25-35}$ treatment for 24 h with or without IGF-1 and/or wortmannin in SH-SY5Y cells. The blots were representative of three independent experiments. Data were expressed as mean ± SEM of three replicate values in three separate experiments. $^{*}P < 0.05$ *vs.* the control group, $^{\#}P < 0.05$ *vs.* the indicated group.

between groups (SPSS software 22.0). Differences with $P < 0.05$ were considered significant.

3. Results

3.1. IGF-1 protects against Aβ-induced neuronal cell death through the activation of PI3K/Akt pathway

It has been shown that IGF-1 attenuates Aβ toxicity in lymphocytes [25] and hippocampal neurons [12]. To further examine the neuroprotective effect of IGF-1 against Aβ toxicity, we used the neuroblastoma SH-SY5Y cells, which is an *in vitro* model to mimic responses of neurons. SH-SY5Y cells were treated with 25 μM $A\beta_{25-35}$ or 1.5 μM $A\beta_{1-42}$ for 24 h with or without IGF-1. Data from CCK-8 assay showed that the cell viability significantly decreased in cells treated with Aβ oligomers compared to untreated cells, whereas the cells were found to be more resistant to Aβ oligomers after different concentrations of IGF-1 stimulation (Fig. 1A). These results suggest that, in the range (50, 100, 200 and 400 ng/ml), IGF-1 has a promotive effect on neuronal cell survival. Herein, IGF-1 at the dose of 100 ng/ml was applied in the subsequent experiments.

We next investigated the molecular mechanisms of how IGF-1 affects SH-SY5Y cells survival. Growing studies have reported that Akt signaling can be activated by IGF-1 in several models [3,13,14]. Using the CCK8 assay, we found that pre-incubation of SH-SY5Y cells with 1 μM PI3K inhibitor wortmannin 1 h before $A\beta_{25-35}$ treatment abolished the protective effects of IGF-1 (Fig. 1B), confirming that Akt signaling is underlying the neuroprotective effects of IGF-1. In according with these results, we observed that IGF-1 could effectively activate Akt even in cells exposed to Aβ oligomers. Addition of wortmannin significantly abolished the phosphorylation of Akt that was enhanced by IGF-1 (Fig. 1C).

Subsequently, we detected cell apoptosis in SH-SY5Y cells using flow cytometry (FACS) analysis followed by indicated treatments. Compared to the untreated group, the rate of cell death significantly increased under $A\beta_{25-35}$ treatment (Fig. 1D), which was markedly decreased by co-incubation with IGF-1 (Fig. 1D). These data thus suggest that PI3K/Akt signaling pathway may plays an essential role in the IGF-1-mediated neuroprotection against $A\beta_{25-35}$ toxicity.

3.2. IGF-1 mediated neuroprotection against Aβ toxicity via inhibition of PUMA expression

Accumulating evidence indicates that the BH3-only proteins of the Bcl-2 family are involved in various cell death paradigms, including neurodegeneration [16–18]. In line with these findings, recent studies showed that p53 up-regulated modulator of apoptosis (PUMA) plays an essential role in Aβ-induced apoptosis, *in vitro* and *in vivo* [19,20]. These prompted us to investigate whether IGF-1 exerts its anti-apoptotic effect through suppressing PUMA expression. We first measured PUMA mRNA levels in neuronal SH-SY5Y cells treated with various treatments by RT-PCR. We found that PUMA mRNA levels were significantly increased after 24 h of exposure to 25 μM $A\beta_{25-35}$, while in cell cultures administrated with $A\beta_{25-35}$ plus 100 ng/ml IGF-1 markedly reduced this increase, and the effect of IGF-1 was reversed by wortmannin (Fig. 2A). Next, we tested the effect of IGF-1 on PUMA protein levels and the results showed that PUMA protein expression was largely increased under $A\beta_{25-35}$ treatment, while this increase was almost blocked by IGF-1 treatment (Fig. 2B), suggesting that IGF-1 inhibits $A\beta_{25-35}$-induced PUMA expression. However, addition of wortmannin significantly inhibited the effect of IGF-1. Together, these results indicate that IGF-1 suppresses PUMA expression at both the transcriptional and translational levels through activating PI3K/Akt pathway.

3.3. IGF-1 inhibits $A\beta_{25-35}$-induced PUMA expression via the PI3K/Akt/FOXO3a pathway

Akt has been shown to modulate the activity of FOXO3a through phosphorylation at Ser253 [26]. Previous studies also

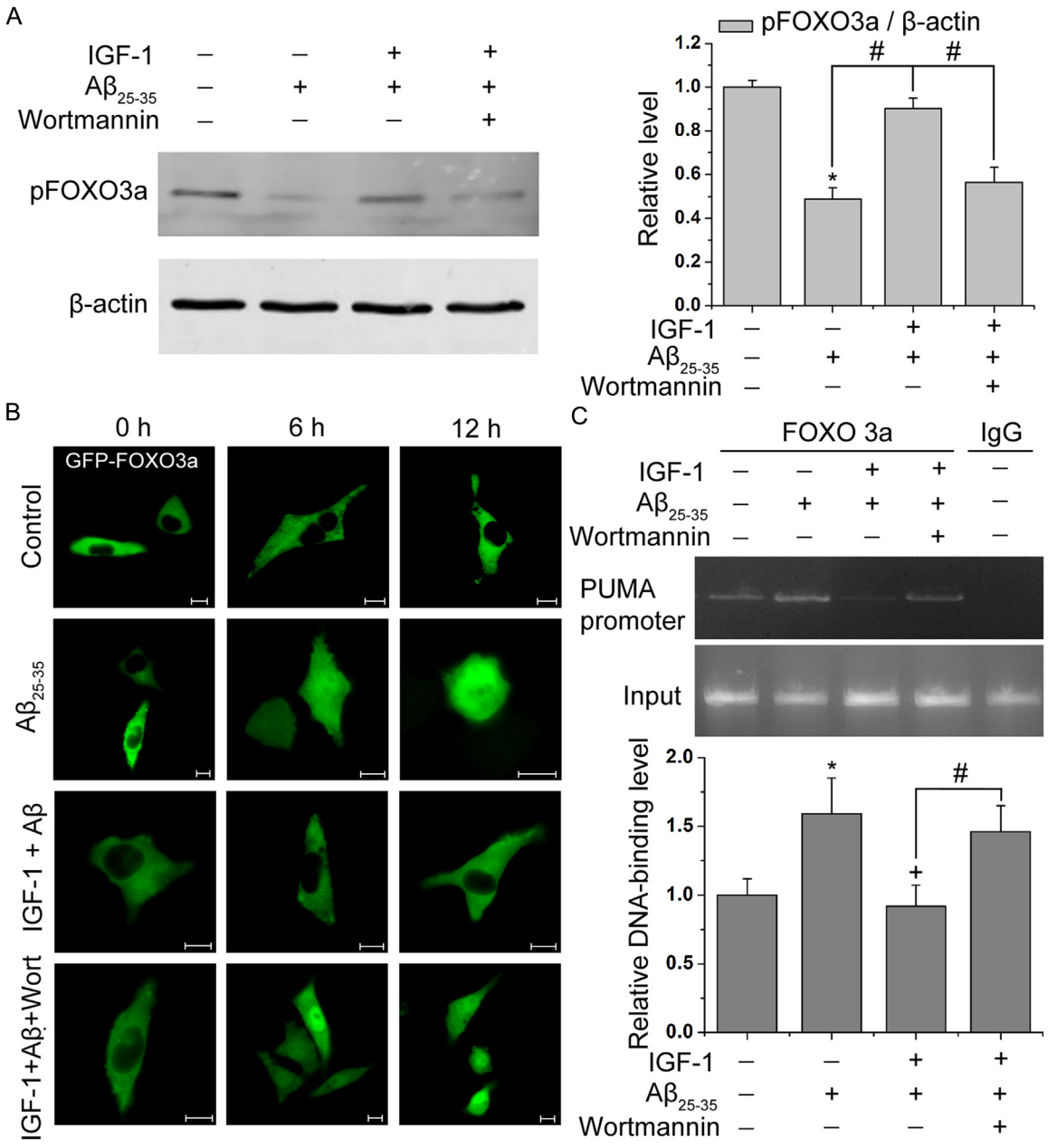

Fig. 3. IGF-1 inhibits $A\beta_{25-35}$-induced PUMA expression via the PI3 K/Akt/FOXO3a pathway. (A) Western blot of phosphorylated FOXO3a at Ser253 in SH-SY5Y cells exposed to $A\beta_{25-35}$ treatment for 12 h with or without 100 ng/ml IGF-1 and/or wortmannin. The blots were representative of three independent experiments. Data were expressed as mean ± SEM of three replicate values in three separate experiments. $^{*}P < 0.05$ *vs.* the control group, $^{\#}P < 0.05$ *vs.* the indicated group. (B) Representative images of FOXO3a nuclear translocation in SH-SY5Y cells transfected with GFP-FOXO3a treated with $A\beta_{25-35}$ for indicated times with or without IGF-1 and/or wortmannin. Scale bar = 10 μm. (C) ChIP was performed to examine the binding ability of FOXO3a to PUMA promoter in SH-SY5Y cells exposed to different treatments. Data were expressed as mean ± SD of three replicate values in three separate experiments. $^{*}P < 0.05$ *vs.* the control group, $^{+}P < 0.05$ *vs.* $A\beta_{25-35}$ group, $^{\#}P < 0.05$ *vs.* the indicated group.

proved that Aβ activated FOXO3a via inhibition of Akt, which subsequently increased the binding ability of FOXO3a to PUMA promoter, leading to increased PUMA expression [20]. We wondered whether and how FOXO3a participates in PUMA regulation in response to IGF-1. Initially, we found that the phosphorylation of FOXO3a at Ser253 was sharply reduced following $A\beta_{25-35}$ administration, while co-incubation with IGF-1 converted that (Fig. 3A). However, treatment with wortmannin significantly abolished the effect of IGF-1 (Fig. 3A). To further determine the effect of IGF-1, SH-SY5Y cells were transfected with GFP–FOXO3a to examine its subcellular localization following indicated treatments. As shown in Fig. 3B, we found that FOXO3a translocated from cytoplasm to nucleus in $A\beta_{25-35}$-treated cells, while after IGF-1 stimulation, FOXO3a re-localized to cytosol. However, after 1 h of pretreatment with wortmannin, FOXO3a was relocated to the nucleus. In summary, these results further demonstrate that, FOXO3a is activated in response to Aβ, while IGF-1 could inactivate it in a PI3K/Akt-dependent manner.

Next we performed CHIP to detect the binding ability of FOXO3a to PUMA promoter after indicated treatments. The results showed that the amount of DNA-binding FOXO3a was remarkably increased in response to $A\beta_{25-35}$ treatment, while co-incubation with IGF-1conversely decreased the amount of DNA-binding FOXO3a, and this effect could be inhibited by wortmannin (Fig. 3C). Taken together, our data indicate that IGF-1

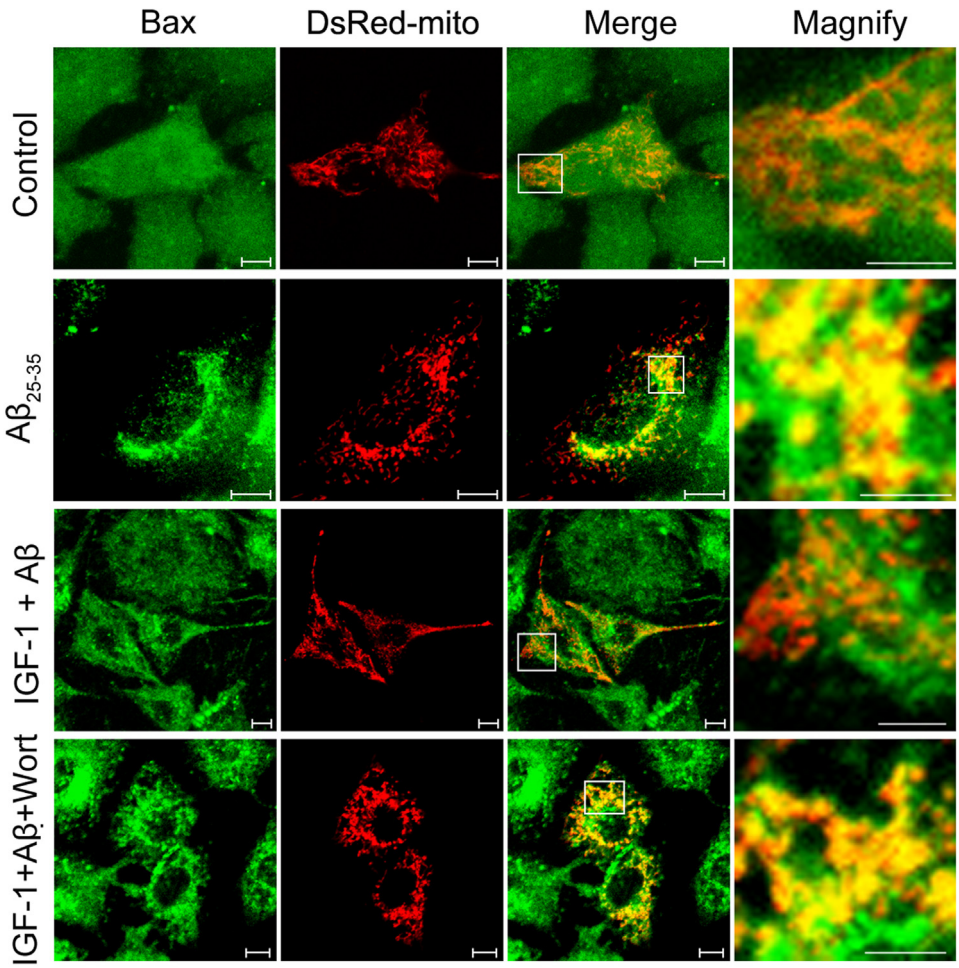

Fig. 4. IGF-1 mediated neuroprotection against $A\beta_{25-35}$ toxicity via inhibition of Bax activation. Representative immunofluorescence images of Bax translocation following $A\beta_{25-35}$ treatment for 24 h with or without IGF-1 and/or wortmannin in SH-SY5Y cells. The mitochondria were labeled with DsRed-mito to visualize mitochondria. Scale bar = 10 μm.

can activate Akt, which in turn inhibits $A\beta_{25-35}$-induced FOXO3a activation, thereby decreasing the binding ability of FOXO3a to PUMA promoter, leading to downregulation of PUMA expression.

3.4. IGF-1 mediated neuroprotection against Aβ toxicity via inhibition of Bax activation

Previous studies have demonstrated that Bax translocation is involved in the process of Aβ-induced apoptosis [27,28]. Since PUMA functions upstream of Bax translocation induced by Aβ stimulation [20], we speculated that IGF-1 may decrease PUMA expression, thereby inhibiting Bax activation induced by $A\beta_{25-35}$ treatment. To test this hypothesis, we performed immunofluorescent analysis to detect Bax activation by measuring the levels of Bax colocalizing with mitochondria in SH-SY5Y cells. SH-SY5Y was treated with indicated treatments, and then fixed, and stained by a specific antibody against Bax, and mitochondria were labeled with DsRed-mito. We found that $A\beta_{25-35}$-induced Bax mitochondrial translocation was inhibited by IGF-1, while addition of wortmannin significantly abolished the effect of IGF-1 (Fig. 4). Taken together, these results demonstrate that IGF-1 mediated neuroprotection against $A\beta_{25-35}$ toxicity via inhibition of Bax activation in a PI3K/Akt dependent manner.

4. Discussion

Deposition of Aβ in the affected areas of patient's brain is a pathological hallmark of AD. [29]. Thus, rescuing neuronal apoptosis induced by Aβ oligomers with pro-survival factors might present a potential therapeutic strategy for AD. IGF-1 is a potent neurotrophic and anti-apoptotic factor capable of promoting survival and preventing death in multiple cell types. Previous study has shown that IGF-1 protects hippocampal neurons against Aβ-induced toxicity [12]. However, the underlying mechanisms are not well understood. In the present study, we demonstrated that IGF-1 protects SH-SY5Y cells from Aβ-induced cell death through the activation of PI3K/Akt signaling pathway. Interestingly, the IGF-1 potentiated PI3K/Akt activity is found to negatively FOXO3a-mediated PUMA expression. In conclusion, our findings may provide a new insight into molecular mechanisms mediated by IGF-1 in cell survival against Aβ-induced neurotoxicity.

It has been shown that the PI3K/Akt signaling pathway is involved in protection of cells from various stimuli. Aβ induces neuronal cell death in part by inhibiting PI3K/Akt signaling pathway [19,20]. Furthermore, activation of PI3K/Akt pathway by IGF-1 produces an anti-apoptotic signal in SH-SY5Y cells against PrP (106–126) [30] and MPP^+-induced apoptosis [4]. These prompted us to investigate whether PI3K/Akt signaling is involved in the protective mechanism of IGF-1. We assessed the phosphorylation of Akt and found that IGF-1 induced Akt activation in SH-SY5Y cells.

The PI3K inhibitor, wortmannin, almost completely abolished the activation of Akt as well as the anti-apoptotic effects of IGF-1. Our results suggested that the PI3K/Akt signaling pathway is responsible for the neuroprotective effect of IGF-1 against $A\beta_{25-35}$-induced apoptosis in SH-SY5Y cells.

Several Akt substrates have been reported in recent years including GSK-3β and FOXO3a. FOXO3a is a transcription factor and can be inactivated through phosphorylation by Akt. FoxO3a has been shown to be involved in the neurodegenerative processes in AD pathogenesis, which represents a potential therapeutic target for blockage of Aβ-induced neuron death [31]. In the present study, we found that FOXO3a was phosphorylated and inactivated in response to IGF-1 stimulation and the inactivation is reversed when SH-SY5Y cells were pretreated with wortmannin. Furthermore, previous studies have demonstrated that activation of FOXO3a, which subsequently increased the binding ability of FOXO3a to the promoters of the target genes. Consistent with this notion, recent studies have shown that FoxO3a is activated by multiple post-translational modifications and mediates neuron death via PUMA in response to Aβ [19,20]. Given that PUMA functions downstream of FOXO3a signaling pathway in response to Aβ, we assessed the effect of IGF-1 on PUMA expression and found that IGF-1 decreased the binding ability of FOXO3a to PUMA promoter, leading to downregulation of PUMA expression, and such suppression could be reversed by wortmannin. Bax is a pro-apoptotic protein of the BCL-2 family that is localized in the cytosol until activated by stress stimuli to induce cell death. Since PUMA functions upstream of Bax translocation induced by Aβ stimulation [20], we further showed that IGF-1 mediated neuroprotection against Aβ toxicity via inhibition of Bax activation in a PI3K/Akt dependent manner.

Moreover, although we found that PI3K/Akt pathway plays an important role in IGF-mediated cell survival against Aβ-induced toxicity, we cannot rule out possible contributions from other signaling cascades. IGF-1 could also activate the MAPK/ERK signaling pathway, but the role of ERK activation in neuronal cell survival is still controversial. MAPK/ERK pathway can either promote the neuronal cell survival or induce apoptotic cell death, depending on the specific cell types and stimuli [11]. Whether MAPK/ERK pathway is involved in the anti-apoptotic effects of IGF-1 against Aβ neurotoxicity requires further investigation. As stated above, our data indicate IGF-1 protects against Aβ-induced neuronal cell death via inhibition of PUMA expression and Bax activation. Our findings may provide a new insight into molecular mechanisms mediated by IGF-1 in cell survival against Aβ-induced neuronal cell death.

Conflict of interest

The authors declare no competing financial interests.

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