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Inhibition of $A\beta_{25-35}$-induced cell apoptosis by Low-power-laser-irradiation (LPLI) through promoting Akt-dependent YAP cytoplasmic translocation

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ABSTRACT

Deposition of amyloid-β-peptide (Aβ) in the brain is considered a pathological hallmark of Alzheimer's disease (AD). Our previous studies show that Yes-associated protein (YAP) is involved in the regulation of apoptosis induced by $A\beta_{25-35}$ through YAP nuclear translocation and its pro-apoptotic function is mediated by its interaction with p73. In the present study, we first found that Low-power laser irradiation (LPLI) promoted YAP cytoplasmic translocation and inhibited $A\beta_{25-35}$-induced YAP nuclear translocation. Moreover, the cytoplasmic translocation was in an Akt-dependent manner. Activated Akt by LPLI phosphorylated YAP on ser127 (S127) and resulted in decreasing the interaction between YAP and p73, and in suppressing the proapoptotic gene *bax* expression following $A\beta_{25-35}$ treatment. Inhibition of Akt expression by siRNA significantly abolished the effect of LPLI. More importantly, LPLI could inhibit $A\beta_{25-35}$-induced cell apoptosis through activation of Akt/YAP/p73 signaling pathway. Therefore, our findings first suggest that YAP may be a therapeutic target and these results directly point to a potential therapeutic strategy for the treatment of AD through Akt/YAP/p73 signaling pathway with LPLI.

1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease that is characterized pathologically by amyloid plaques, neurofibrillary tangles, and neuronal loss [1–4]. Previous studies demonstrate that apoptosis implicates in the pathogenesis of AD [5–7]. Aβ has been found to cause neurotoxicity and cell apoptosis *in vivo* and *in vitro* [8–10]. To date, various signaling pathways have been reported to account for Aβ-induced apoptosis [8–12]. Additionally, a large number of published studies indicate that targeting β-secretase, also known as BACE1 (β-site amyloid precursor protein (APP)-cleaving enzyme), may be a potentially safe and effective therapeutic approach for the treatment and/or prevention of AD [13–15]. Further studies are in progress to illustrate the mechanisms of AD and search for an effective therapeutic strategy.

Low-power laser irradiation (LPLI) can modulate various biological processes [16]. It has been used to treat diseases of regeneration limitation and to promote wound healing as a relatively noninvasive technique [17]. LPLI enhances both cell survival and proliferation, providing an effective means of ameliorating the long-term consequences of muscle injury [18]. Moreover, PKC kinases and Akt kinase can be activated stimulated by LPLI [19–21]. Apoptosis induced by $A\beta_{25-35}$ is significantly diminished with light irradiation [22]. Studies by Zhang et al. [23] have found that LPLI inhibits $A\beta_{25-35}$-induced apoptosis via PKC-mediated regulation of bax/bcl-xl mRNA ratio. However, the molecular mechanism associated with the stimulatory effect of LPLI inhibiting Aβ-induced apoptosis has not been clarified.

Yes-associated protein (YAP) is a transcriptional coactivator [24,25]. Previous studies indicate that YAP needs to bind its target transcription factors to stimulate gene expression [26]. YAP has been shown to positively regulate p73 in promoting apoptosis through mediating the expression of cell death-promoting genes, *bax* or *puma* [27–33]. Our previous studies have demonstrated that YAP accelerates $A\beta_{25-35}$-induced apoptosis through translocating from cytoplasm to nucleus to bind its target transcription factor p73 to stimulate *bax* gene expression and Bax activation [34]. Different from the ability of c-Abl to promote stabilization and activation of YAP [33], Akt phosphorylates YAP on serine 127 (S127) and promotes cytoplasmic localization of YAP by enhancing interaction of YAP with the cytosolic 14-3-3 scaffold proteins [28]. As a result, it loses its main function as a co-activator in the nucleus [32]. Hence, active Akt impairs YAP from associating with p73, leading to attenuation of p73's transactivation and apoptotic activity [28,29]. However, whether LPLI promotes YAP cytoplasmic localization through activating Akt is still unknown.

In the present study, we found that YAP translocated from cytoplasm to nucleus during $A\beta_{25-35}$-induced apoptosis and LPLI inhibited this translocation. LPLI promoted YAP cytoplasmic localization in an Akt-dependent manner and YAP phosphorylation on S127 was increased. Furthermore, LPLI inhibited $A\beta_{25-35}$-induced apoptosis through Akt activation. Therefore, our results suggest that YAP may be a therapeutic target. These results directly point to a potential therapeutic strategy for the treatment of AD through activating Akt/YAP pathway with LPLI.

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2. Materials and methods

2.1. Materials

Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA). $A\beta_{25-35}$ was purchased from Sigma-Aldrich (St. Louis, MO), $A\beta_{25-35}$ stock solution of 1 mM was prepared in distilled and deionized water and stored at −20 °C. Prior to a treatment, peptides were pre-incubated at 37 °C for 5 days to promote aggregation and then diluted with medium to desired concentrations (25 μM). To ensure maximum bioavailability, the medium was refreshed every other day, and cells were plated at appropriate densities according to each experimental protocol. The plasmids EGFP-YAP and EGFP-YAP-S127A were kindly supplied by Prof. Subham Basu and Prof. Julian Downward [28]. The pYFP–p73 was a gift from Prof. Hedeki Shimodaira [35,36]. Targeting sequence of YAP siRNA was described previously [28,37]. Akt siRNA [38,39] was used to inhibit Akt expression. YAP siRNA was synthesized by GenePharma Co., Ltd. (Shanghai, China). Akt siRNA was synthesized by RiboBio Co., Ltd (Guangzhou, China). The plasmids and siRNA were transfected into cells using Lipofectamine™ 2000.

2.2. Cell culture and transfection

PC12 cells were cultured in a humidified (5% CO_2, 37 °C) incubator in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.), supplemented with 15% fetal bovine serum, 5% horse serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). To obtain neuronally differentiated PC12 cultures, subconfluent cells were differentiated for up to 5 days in DMEM with 15% fetal bovine serum and 2.5 s NGF (100 nM) (Sigma-Aldrich, St. Louis, MO). 293 T cells were cultured in a humidified (5% CO_2, 37 °C) incubator in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.), supplemented with 10% fetal bovine serum. To ensure maximum bioavailability, the medium was refreshed every other day, and cells were plated at appropriate densities according to each experimental protocol. The plasmids EGFP-YAP, EGFP-YAP-S127A and YFP-p73 were transfected into PC12 or 293 T cells using Lipofectamine™ 2000.

2.3. Low-power laser irradiation

PC12 cells (monolayer cells at a density of 1×10^6 cells/dish) cultured for 24 h were treated with different chemicals and/or irradiated with He–Ne laser (632.8 nm, 5.4 mW, 6.89 mW/cm^2, HN-1000, Guangzhou, China) at a fluence of 2 J/cm^2. The chemicals were added to the culture medium 30 min before LPLI treatment. The entire procedure was carried out at room temperature. Throughout each experiment, the cells were kept either in a complete dark or a very dim environment, except when subjected to the light irradiation, to minimize the ambient light interference.

2.4. Cell viability assays and cell apoptosis assay

PC12 cells were cultured in 96-well microplates at a density of 5×10^3 cells/well for 24 h. The cells were then divided into four groups and exposed to 25 mM Aβ with/without He–Ne laser irradiation at fluence of 2 J/cm^2. Another group was pre-treated with wortmannin and then exposed to 25 mM Aβ plus 2 J/cm^2 LPLI. The irradiation was performed on monolayer cells. In all cases, control (non-irradiated) cells were kept in the same conditions as the treated cells. Cell viability was assessed with CCK-8 (Dojindo Laboratories) at 3 h, 6 h, 15 h, 24 h, and 48 h post-treatment according to the manufacturer's instructions. OD450, the absorbance value at 450 nm, was read with a 96-well plate reader (DG5032, Hua dong, Nanjing, China), to determine the viability of the cells.

Quantification of apoptosis by Annexin-V/PI staining was performed as described previously [34,40,41]. Apoptotic cell death was determined using the BD ApoAlert Annexin-V-FITC Apoptosis Kit (Becton Dickinson, Biosciences) according to the manufacturer's instructions. Flow cytometry was performed on a BD FACSCanto™ II flow cytometer (Becton Dickinson, Mountain View, CA).

2.5. Time-lapse confocal fluorescence microscopy

GFP was monitored confocally using a commercial laser scanning microscope (LSM 510/ConfoCor2) combination system (Carl Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40×/1.3 numerical aperture (NA) oil differential interference contrast (DIC) objective. Excitation wavelength and detection filter settings for each of the fluorescent indicators were as follows: GFP fluorescence was excited at 488 nm with an argon ion laser, and emission was recorded through a 500- to 520-nm band pass filter. For time-lapse imaging, culture dishes were mounted onto the microscope stage that was equipped with a temperature-controlled chamber (Carl Zeiss). During control experiments, bleaching of the probe was negligible.

2.6. GFP–YAP translocation assay

To monitor GFP–YAP translocation in living cells, PC12 cells were transfected with pGFP–YAP. Using an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), we imaged the distribution pattern of GFP–YAP simultaneously during Aβ-induced apoptosis. GFP–YAP translocation assay was performed as described [34].

2.7. Immunofluorescence (IF)

PC12 cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then were washed five times with PBS. Samples were incubated in blocking buffer (10% bovine serum albumin in PBS) for 1 h at room temperature, followed by incubation with anti-YAP antibody, or anti-Bax antibody 6A7 at 4 °C overnight. Cells were washed five times for 5 min each, after which FITC-conjugated secondary antibodies (Proteintech, Group Chicago) were added for 2 h at a room temperature. Nucleus was stained with PI (10 μg/ml). After five additional washes with PBS, slides were mounted and analyzed by confocal microscopy.

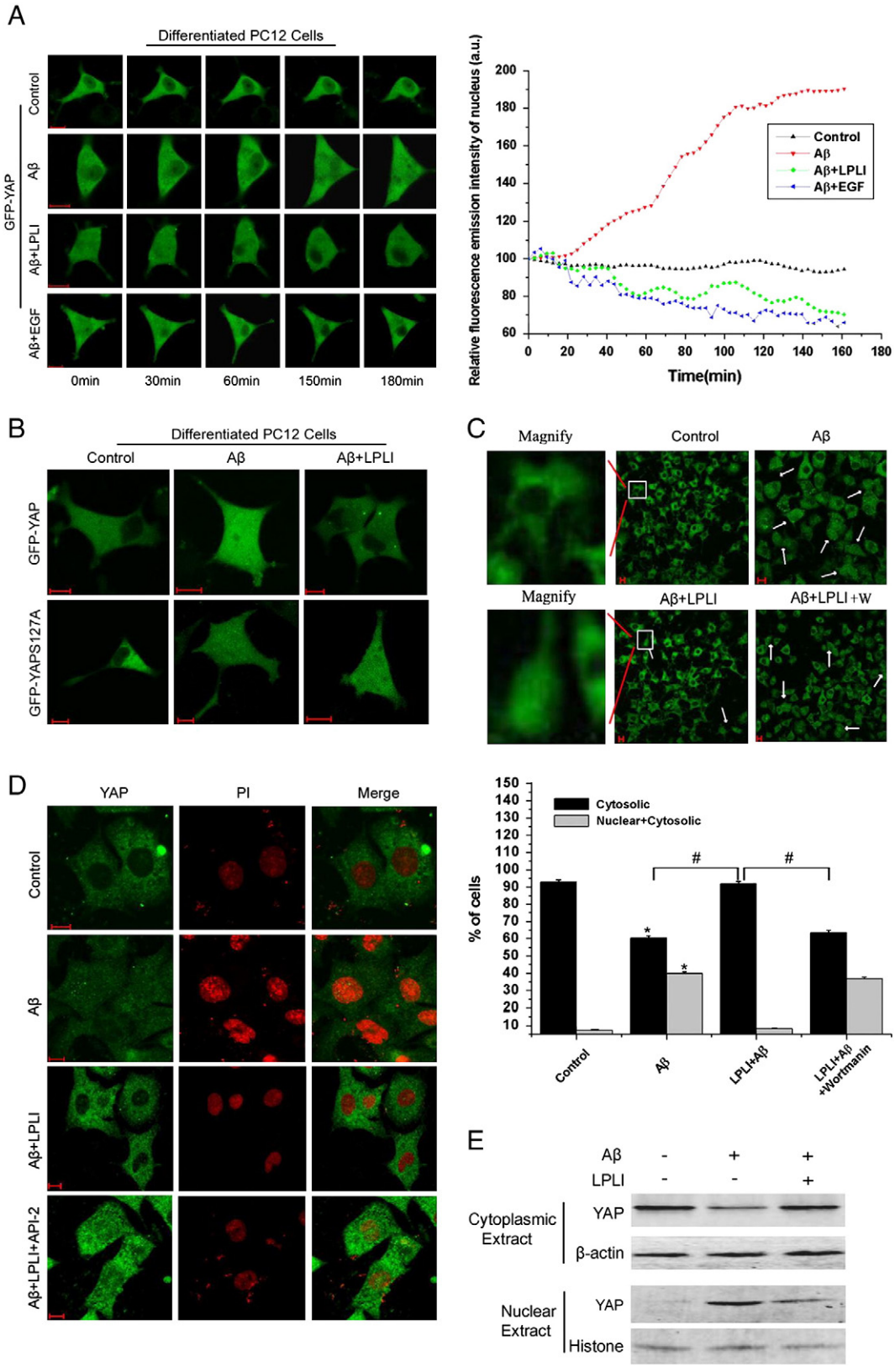
2.8. GFP–Bax translocation assay

To monitor Bax translocation in living cells, PC12 cells were transfected with pGFP–Bax and pDsRed-Mit (a marker for mitochondria). Using an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), we imaged both the distribution pattern of GFP–Bax and that of DsRed-Mit simultaneously during Aβ-induced apoptosis. Bax redistribution was assessed as described [34]. The cells exhibiting strong punctate staining of GFP, which overlapped with the distribution of DsRed-Mit, were counted as the cells with mitochondrially localized Bax.

2.9. Antibodies and western blotting

The antibodies used for western blotting include antibodies against Akt, phospho-Akt (Thr308), phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA), YAP (Bioworld Technology, Inc.), phospho-YAP (Ser127) (Cell Signaling Technology, Danvers, MA), Bax (Cell Signaling Technology, Danvers, MA), Bax-antibody 6A7 (Abcam plc, UK), full length/cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), and Histone H3 (Santa Cruz, CA). At the indicated times after $A\beta_{25-35}$ treatment, cells were harvested, washed with ice-cold PBS, pH 7.4, and lysed with ice-cold lysis buffer [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF)] for 30 min on ice. The lysates were centrifuged at 12,000 rpm for 5 min at 4 °C, and the protein concentration was determined. Equivalent

samples (40–100 μg of protein extract was loaded on each lane) were subjected to SDS-PAGE on 10% or 13% gel. The proteins were then transferred onto nitrocellulose membranes and probed with the indicated antibodies, followed by secondary antibodies: goat anti-mouse conjugated to Alexa Fluor 680 or goat anti-rabbit conjugated to IRDyeTM800. Detection was performed using the LI-COR Odyssey Infrared Imaging System (LI-COR, Inc. Lincoln, NE).



2.10. Cellular fractionation

To prepare cytoplasmic and nuclear fractions, cells were washed in ice-cold PBS, scraped, and homogenized in ice-cold hypotonic buffer (10 mM HEPES pH 7.4; 10 mM KCl; 1.5 mM $MgCl_2$; 1 mM EDTA; 1 mM DTT) containing 100 μg/ml PMSF. Cytoplasmic and nuclear fractions were obtained as described [8, 34]. Then the cellular fractions were analyzed by western blotting.

2.11. Coimmunoprecipitation (Co-IP)

Cells were extracted in lysis buffer (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1% TritonX-100, 100 lg/ml PMSF) supplemented with protease inhibitor cocktail set I for 60 min on ice. After centrifugation, the supernatant was incubated with the indicated antibody and subsequently with protein A-Sepharose (50% slurry, Roche Applied Sciences, Indianapolis, IN) at 4 °C overnight and then were washed three times. Pellet was resuspended with the same volume of SDS sample buffer, and boiled to remove Sepharose beads. Then the cells lysates and immunoprecipitates were analyzed by western blotting.

2.12. Statistical analysis

All data represent at least three independent experiments and are expressed as the mean ± SEM. Differences between groups were compared using Student's *T* tests by *SPSS* software, and significance was accepted at $P<0.05$.

3. Results

3.1. LPLI inhibits Aβ$_{25-35}$-induced YAP translocation from cytoplasm to nucleus through Akt activation

To determine the effect of LPLI, PC12 cells were transfected with GFP–YAP to examine its subcellular localization and were treated with laser irradiation after Aβ$_{25-35}$ stimulation. We found that YAP translocated from cytoplasm to nucleus in Aβ$_{25-35}$-treated cells, while after LPLI stimulation, YAP re-localized to cytosol (Fig. 1A, left panel). These results were further confirmed by the quantitative analysis of nuclear GFP fluorescence emission intensities (Fig. 1A, right panel).

Active Akt can promote YAP cytoplasmic localization through phosphorylation on S127 [28]. To determine whether Akt-dependent phosphorylation was indeed responsible for YAP translocation under LPLI, PC12 cells were transfected with GFP–YAP and GFP–YAP-S127A. As shown in Fig. 1B, GFP–YAP and GFP–YAP-S127A were localized mostly in the cytosol and both of them translocated to nucleus after cells treatment with Aβ$_{25-35}$, however, GFP–YAP showed almost all cytoplasmic localization under LPLI, while GFP–YAP-S127A was still in both cytosol and nucleus.

To examine the endogenous YAP distribution, PC12 cells were treated with Aβ$_{25-35}$ for 24 h. The location of YAP was determined by IF assay. As shown in Fig. 1C (upper panel and bottom), YAP re-localized to the cytosol after LPLI treatment, while YAP was still in nucleus after we added wortmannin, an inhibitor of PI3K that was upstream of Akt. Similar results were obtained by using an inhibitor of Akt, API-2 (Fig. 1D) and western blotting (Fig. 1E). Therefore, all the results above proved that LPLI inhibited Aβ$_{25-35}$-induced YAP translocation from cytoplasm to nucleus through Akt activation.

3.2. LPLI protects cell from Aβ$_{25-35}$-induced apoptosis through activation of Akt

In order to investigate the effect of LPLI on Aβ$_{25-35}$-induced apoptosis, we measured the cell viability with CCK-8. As shown in Fig. 2A, the cell viability significantly decreased in cells treated with Aβ$_{25-35}$ at the indicated time compared with that of non-treated cells. However, PC12 cells were found to be more resistant to Aβ$_{25-35}$ after LPLI stimulation. Moreover, wortmannin could abolish the effect of LPLI.

To observe the status of cell apoptosis directly, the cell apoptosis assay was performed by flow cytometric analysis. Approximately 31.2% of PC12 cells underwent apoptosis within 24 h under Aβ$_{25-35}$ treatment (Fig. 2B). However, the apoptosis rate was decreased to 21.8% after treated with LPLI ($P<0.005$) (Figs. 2B and 2C). More importantly, at least in part, wortmannin abolished the effect of LPLI (Fig. 2B).

To further determine the activation of Akt after LPLI treatment, western blotting was performed to detect the phosphorylation of Akt on ser473 and Thr308. As shown in Fig. 2D, LPLI could effectively activate Akt even in cells exposed to Aβ$_{25-35}$. Therefore, these results indicated that laser irradiation had a promotive effect on cell survival, which was dependent on Akt activity and Akt was involved in inhibiting Aβ$_{25-35}$-induced cell apoptosis by LPLI.

3.3. Activated Akt by LPLI promotes YAP phosphorylation on S127

YAP S127 phosphorylation has been reported to create a 14-3-3-binding site [28]. LPLI protected cell from Aβ$_{25-35}$-induced apoptosis through activation of Akt. To directly determine the effect of phosphorylation on S127 by LPLI, PC12 cells were treated by Aβ$_{25-35}$ without (−) or with (+) LPLI. Co-IP was firstly performed to detect the interaction between Akt and YAP. As shown in Fig. 3A, we found that Akt–YAP complex decreased significantly after treatment with Aβ$_{25-35}$, while the interaction increased in Aβ$_{25-35}$-treated cells plus LPLI. YAP S127 phosphorylation also increased after LPLI treatment alone. Conversely, depletion of Akt attenuated the level of the phosphorylation significantly (Fig. 3B). Moreover, LPLI promoted the YAP S127 phosphorylation even under Aβ$_{25-35}$-treatment, while the effect of LPLI was inhibited by wortmannin and API-2 (Fig. 3C). Similar results were obtained by Akt siRNA (Fig. 3D).

3.4. LPLI inhibits Aβ$_{25-35}$-induced expression and activation of Bax through Akt/YAP/p73 pathway

YAP accelerates Aβ$_{25-35}$-induced apoptosis through upregulation of Bax expression by interaction with p73 [34]. To confirm the interaction between YAP and p73 after cells treated by Aβ$_{25-35}$ with or without LPLI and API-2 treatment, we transfected

Fig. 1. LPLI inhibits Aβ$_{25-35}$-induced YAP translocation from cytoplasm to nucleus through Akt activation. (A) Differentiated neural PC12 cells transiently transfected with GFP–YAP were treated with Aβ$_{25-35}$ for 15 h with or without LPLI and wortmannin (an inhibitor of PI3K), then imaged by confocal microscopy. Quantitative analysis of relative GFP–YAP fluorescence emission intensities of nucleus in PC12 cells was subjected to different treatments. Data represent the mean ± SEM of 5 independent experiments. (B) Akt phosphorylation regulated YAP subcellular localization. Differentiated neural PC12 cells were transfected with either pEGFP–YAP or pEGFP–YAP-S127A and were further treated with Aβ$_{25-35}$ with or without LPLI. Cells GFP-protein expression visualized by confocal fluorescence microscopy. Representative images were shown. (C) Immunofluorescence analysis for the localization of endogenous YAP. Statistical analysis of endogenous YAP translocation was conducted and the percentage of cells showing YAP translocation to nuclear was assessed by counting the number of (Nuclear + Cytoplasmic) cells. Data represented the mean ± SEM and was collected from n = 150–200 cells per treatment. $^*P<0.005$, significant from the untreated cells. (D) PC12 cells were treated with Aβ$_{25-35}$ or/and API-2 (an inhibitor of Akt), then stained nucleus with PI to differentiate from the cytoplasm. Representative images were shown. (E) Western blotting of cytoplasmic and nuclear extracts were designed to examine the level of YAP. Cytoplasmic and nuclear fractions were obtained from the neural PC12 cells after Aβ$_{25-35}$ treatment without (−) or with (+) LPLI. Similar results were obtained from three independent experiments.

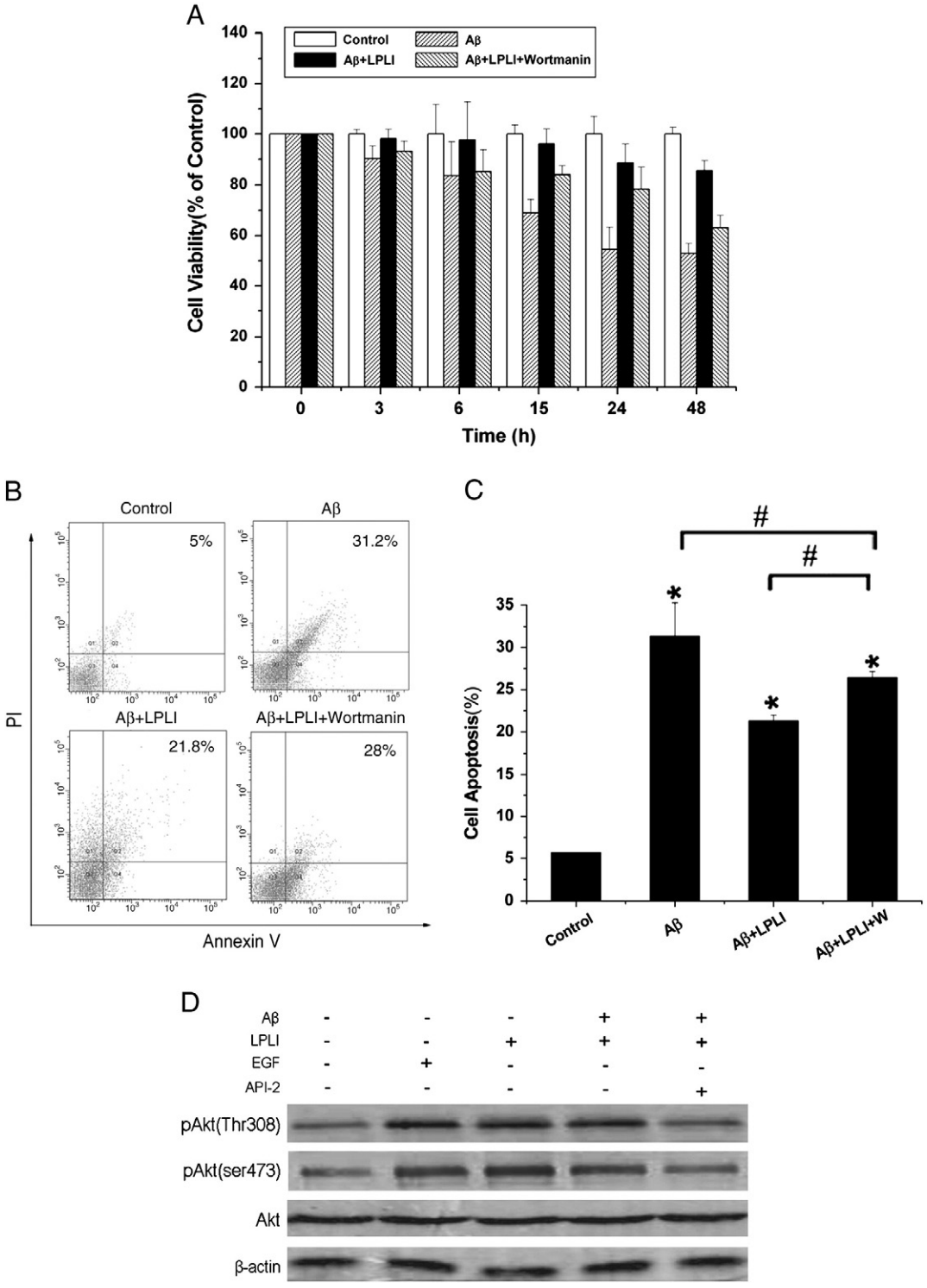


Fig. 2. LPLI protects cell from $A\beta_{25-35}$-induced apoptosis through activation of Akt. (A) Cell viability measured with CCK-8 assay. Data represent the mean ± SEM of 4 independent experiments (B) Apoptosis induced by $A\beta_{25-35}$ in PC12 cells decreased following LPLI treatment with or without wortmannin. Unfixed cells were stained with Annexin V and propidium iodide (PI) 24 h following $A\beta_{25-35}$ treatment, then analyzed by flow cytometry. Numbers refer to the percent Annexin V- and/or PI-positive cells in this representative experiment. (C) The percentage of apoptosis cells were used to calculate. $^{*}P<0.005$, significant from the untreated cells, # $P<0.005$, significant from the two indicated cells. Data represent the mean ± SEM of 3 independent experiments. (D) Western blotting analysis for Akt Thr308 and Ser473 phosphorylation. Data represent the mean ± SEM of 3 independent experiments.

YFP–p73 into 293 T cells and examined the YAP–p73 complex by Co-IP. As shown in Fig. 4A, we found that YAP–p73 complex increased significantly after treatment with $A\beta_{25-35}$ and decreased after LPLI. However, the complex was similar to $A\beta_{25-35}$-treated cells when wortmannin and API-2 added. Furthermore, Akt siRNA significantly abolished the effect of LPLI on YAP–p73 complex under $A\beta_{25-35}$-treatment (Fig. 4B).

Bax is an important transcriptional target of p73 [22,23,24,27]. To detect Bax redistribution during $A\beta_{25-35}$-induced apoptosis, PC12 cells were transiently transfected with GFP–Bax. The DsRed-Mit

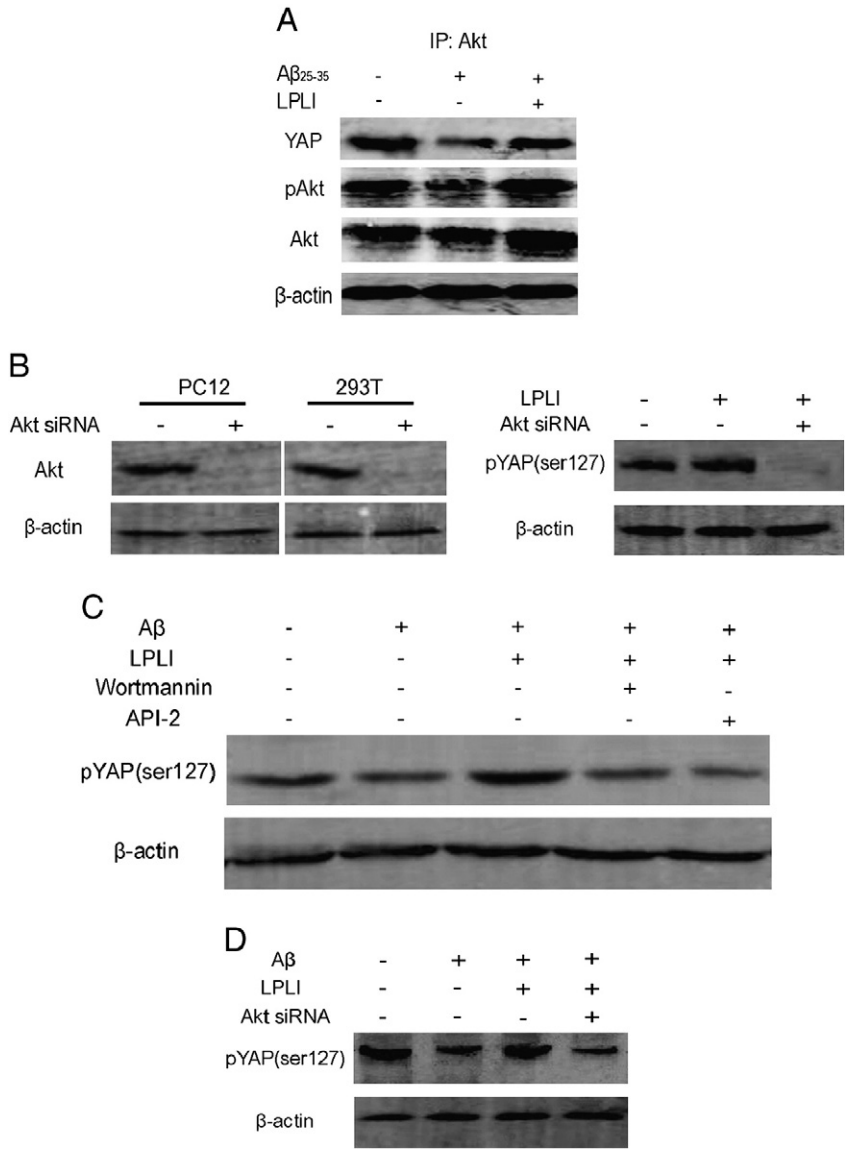


Fig. 3. Activated Akt by LPLI promotes YAP phosphorylation on S127. (A) Interaction between Akt and YAP increased after LPLI in cells exposed to $A\beta_{25-35}$. Co-IP was performed to detect the Akt–YAP complex after $A\beta_{25-35}$ treatment with LPLI. Similar results were obtained from three independent experiments. (B) The effect of LPLI on phosphorylation of YAP on S127 was evaluated by western blotting. Akt was suppressed by Akt siRNA. Similar results were obtained from three independent experiments. (C) Western blotting was performed to detect the phosphorylation of YAP on S127 after $A\beta_{25-35}$ with or without LPLI and wortmannin or API-2 treatment. Similar results were obtained from three independent experiments. (D) Knockdown of Akt decreased the level of the phosphorylation of YAP on S127 after $A\beta_{25-35}$ with (+) or without (−) LPLI and Akt siRNA treatment. Similar results were obtained from three independent experiments.

(a marker for mitochondria) also was transfected into PC12 cells to label the mitochondria. As shown in Fig. 4C, GFP–Bax had a diffuse distribution in the whole cell in the control cells. Under $A\beta_{25-35}$ treatment, GFP–Bax translocated from cytosol to mitochondria, however, GFP–Bax still had a diffuse distribution in $A\beta_{25-35}$-treated cells plus LPLI. More importantly, wortmannin inhibited the effect of LPLI (Fig. 4C, bottom). Similar results were obtained by IF using a conformation-specific anti-Bax monoclonal antibody (6A7) which selectively recognized the activated/proapoptotic form of Bax to directly detect Bax activation (Fig. 4D). Active Bax was not identified in both untreated cells and cells under LPLI treatment, but was readily detectable in $A\beta_{25-35}$-treated cells and cells after LPLI stimulation with wortmannin.

Western blotting was used to detect the stimulation of endogenous Bax expression by $A\beta_{25-35}$-treatment with (+) or without (−) LPLI. As shown in Fig. 4E and F, Bax expression was decreased significantly after treatment with LPLI, but was still similar to $A\beta_{25-35}$-treated cells when Akt was suppressed by siRNA. Moreover, knockdown of YAP by siRNA decreased $A\beta_{25-35}$-induced Bax expression which was consistent with previous studies [34] and LPLI further inhibited Bax expression (Fig. 4G).

4. Discussion

The deposition and accumulation of amyloid-β-peptide (Aβ) in the brain are considered as a pathological hallmark of AD [1–4]. Apoptosis, the general neuronal death pathway, has been implicated in the pathogenesis of AD [7,8,9,42]. Additionally, Aβ causes neurotoxicity and induces cell apoptosis *in vivo* and *in vitro*. Apoptosis induced by $A\beta_{25-35}$ is significantly diminished with light irradiation. LPLI can inhibit $A\beta_{25-35}$-induced apoptosis via PKC-mediated regulation of bax/bcl-xl mRNA ratio. Moreover, Akt is activated by LPLI [20,21]. Our former studies show that YAP accelerates $A\beta_{25-35}$-induced apoptosis through translocation and upregulation of Bax expression by interaction with p73 [34]. In the present study, we found that YAP increased significantly in nucleus when cells were exposed to $A\beta_{25-35}$, however, LPLI inhibited YAP nuclear translocation through Akt

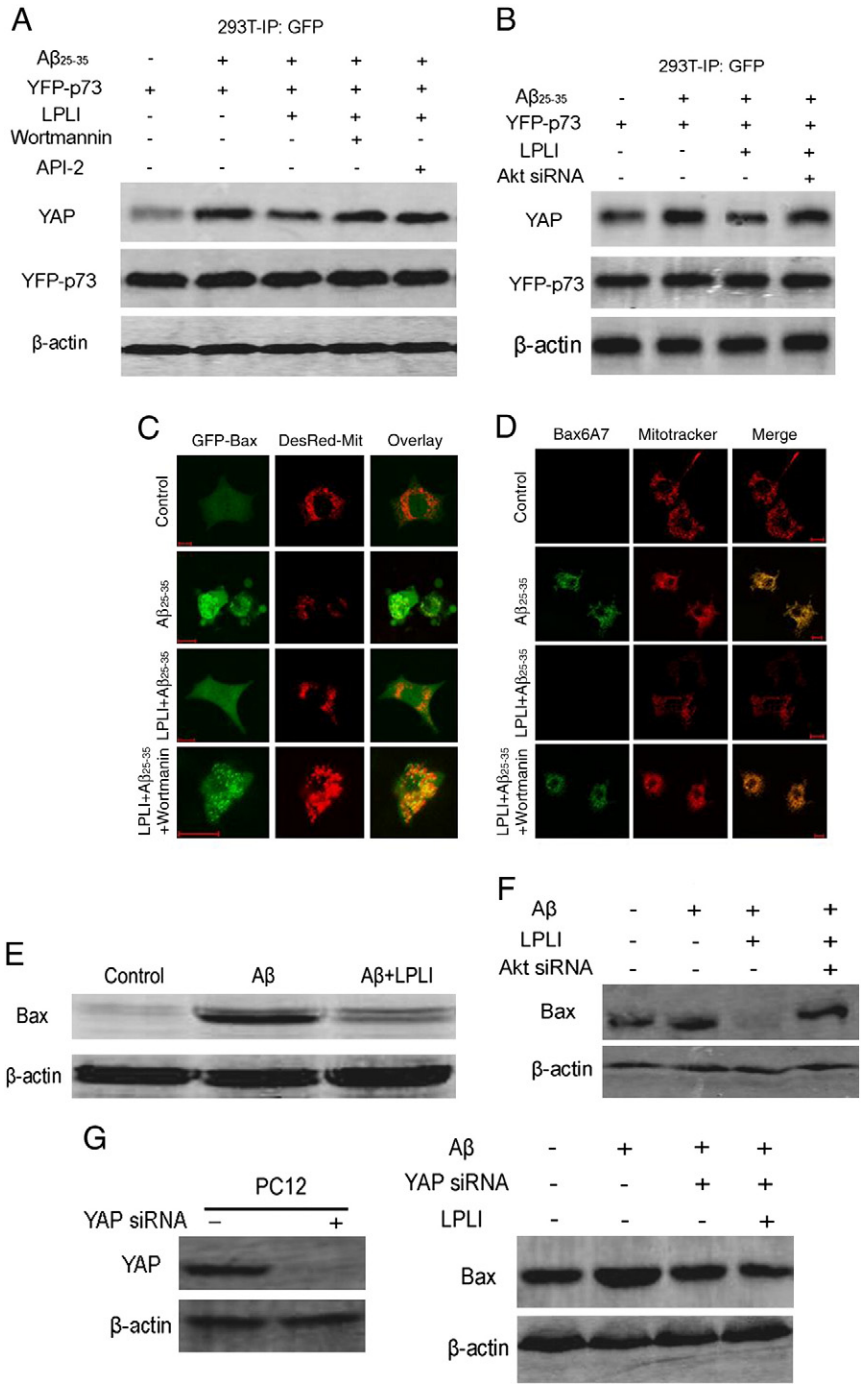


Fig. 4. LPLI inhibits $A\beta_{25-35}$-induced expression and activation of Bax through Akt/YAP pathway. (A–B) Interaction between YAP and p73 decreased after LPLI treatment in cells exposed to $A\beta_{25-35}$. (A) Co-IP was performed to detect the YAP–p73 complex after $A\beta_{25-35}$ treatment with or without LPLI and API-2. Similar results were obtained from three independent experiments. (B) The effect of LPLI on $A\beta_{25-35}$-induced YAP–p73 complex was inhibited by Akt siRNA. Similar results were obtained from three independent experiments. (C) LPLI inhibited Bax translocation from cytosol to mitochondria during $A\beta_{25-35}$-induced apoptosis. PC12 cells were transiently cotransfected with GFP–Bax and DsRed, and then treated with $A\beta_{25-35}$ plus LPLI or/and wortmannin. Representative images were shown. (D) Bax activation was inhibited by LPLI during $A\beta_{25-35}$-induced apoptosis. IF was performed to detect mitochondrial localization of conformationally activated Bax in cells treated by $A\beta_{25-35}$ plus LPLI or wortmannin. Similar results were obtained from three independent experiments. The mitochondria were stained with MitoTracker Red. (E–F) Western blotting was performed to detect the stimulation of endogenous Bax protein expression by $A\beta_{25-35}$-treatment plus LPLI with or without Akt siRNA. Similar results were obtained from three independent experiments. (G) Bax expression was still reduced by LPLI even YAP was suppressed by YAP siRNA. Western blotting was performed to detect the stimulation of endogenous Bax protein expression by $A\beta_{25-35}$-treatment plus LPLI with or without YAP siRNA. Similar results were obtained from three independent experiments.

activation. Activated Akt by LPLI phosphorylated YAP on S127 and resulted in suppressing the proapoptotic gene expression following $A\beta_{25-35}$ treatment. Our results showed that the activation of Akt by LPLI provided protection in the cells subjected to apoptosis induced by $A\beta_{25-35}$. Such protection was associated with a significant decrease in the apoptotic cells (%) and the down-regulation of the Bax expression. Furthermore, we found that wortmannin, an inhibitor of PI3K, which was upstream of Akt, and API-2 could significantly abolish the LPLI-induced protection from apoptosis in $A\beta_{25-35}$-treated cells. More importantly, similar results were obtained in the presence of Akt siRNA. Therefore, our findings suggest that LPLI can inhibit $A\beta_{25-35}$-induced apoptosis through activating Akt/YAP pathway and provided a potential

therapeutic strategy for the treatment of AD. $A\beta_{25-35}$-induced Bax expression is decreased during knockdown of YAP [34]. More interestingly, Bax expression was still reduced by LPLI even YAP was suppressed by YAP siRNA which suggested that other pathways could be also participate in inhibiting $A\beta_{25-35}$-induced apoptosis by LPLI [23]. More studies are needed to illustrate the mechanisms of the effect of LPLI.

Akt mediates distinct biological responses, including promoting proliferation and inhibiting apoptosis [43–46]. Dysregulation of Akt leads to diseases of major unmet medical need such as neurological diseases, cancer, diabetes and cardiovascular [39,47]. Previous studies indicate that Akt is a critical mediator of survival signals that protect cells from different apoptotic stimuli such as growth factor withdrawal, UV irradiation and cell-cycle discordance [48–52]. In addition, Akt phosphorylates and inhibits some pro-apoptotic protein activities, including that of Bad [53] and ASK1 [54], to promote survival. More importantly, Akt phosphorylating and inhibiting YAP has also been suggested to play a significant role in inhibiting apoptosis [28]. YAP localizes in both nucleus and cytoplasm, and its activity is tightly regulated [24]. YAP was originally recognized by virtue of its ability to bind to the SH3 domain of Yes at the plasma membrane [25]. Akt phosphorylates YAP on S127 and enhances the interaction of YAP with the cytosolic 14-3-3, leading to relatively long-term inactivation of YAP [28]. As a transcriptional coactivator, YAP needs to bind nuclear transcription factors to stimulate gene expression [26]. Our results demonstrated that YAP translocated from cytoplasm to nucleus during $A\beta_{25-35}$-induced apoptosis. LPLI attenuated the apoptosis induced by Aβ through Akt activation. Akt activation promoted YAP cytoplasmic distribution through its phosphorylation on S127. Thus, our results suggest that Akt was required for LPLI inhibiting Aβ-induced apoptosis and that this inhibition caused by LPLI was associated with a cytoplasmic translocation of YAP. As YAP has a proapoptotic function through interacting with p73 [33,34], this suggests that the potential inhibition of $A\beta_{25-35}$-induced apoptosis by LPLI is likely to be Akt/YAP/p73 pathway-dependent.

LPLI has been used to treat diseases of regeneration limitation and to promote wound healing [17]. LPLI (Low-energy laser irradiation, LELI), as a relatively noninvasive technique that enhances both cell survival and proliferation, might provide an effective means of ameliorating the long-term consequences of muscle injury [18]. LELI affects satellite cell proliferation and differentiation *in vitro* [55]. LELI enhances de novo protein synthesis via its effects on translation-regulatory proteins in skeletal muscle myoblasts [56]. Some studies have reported that the molecular mechanism associated with the stimulatory effect of LPLI. It has been indicated that PKC kinases and Akt kinase can be activated by LPLI [19–21,23]. In the present study, after treatment with $A\beta_{25-35}$, YAP translocated from cytoplasm to nucleus where it can bind to its nuclear target transcription factors. While LPLI could reverse this effect through Akt activation. We found that LPLI increased the YAP S127 phosphorylation which has been reported to create a 14-3-3-binding site. Moreover, phosphorylation was abolished by wortmannin, API-2 and Akt siRNA suggested that LPLI promoted YAP cytoplasmic localization through YAP S127 phosphorylation by Akt. Together, our investigation indicated that phosphorylation of YAP S127 by Akt was responsible for the nuclear-to-cytoplasmic translocation of YAP in response to LPLI. YAP's translocation from nuclear to cytoplasm likely resulted in YAP separating from p73 and attenuated p73 transcriptional activity and ultimately decreased $A\beta_{25-35}$-induced apoptosis (Fig. 5). The data also demonstrates that the activation of Akt protects cells from $A\beta_{25-35}$-induced apoptosis by blocking the YAP-dependent apoptotic pathways, suggesting a potential therapeutic role for LPLI in the treatment of Aβ-induced neuronal degeneration. Further studies are necessary on LPLI inhibiting Aβ-induced apoptosis and treating AD.

In summary, the current study demonstrates that LPLI realizes its anti-apoptotic effect via activating Akt/YAP signaling pathway and protects cells against $A\beta_{25-35}$-induced apoptosis. Specifically, Akt phosphorylates YAP on S127, leading to the YAP's nuclear-to-cytoplasmic translocation and results in YAP separating from its nuclear transcription factor p73. The attenuated p73 transcriptional activity and its target gene *bax* expression, ultimately decreases $A\beta_{25-35}$-induced apoptosis. The delineation of the Akt/YAP/p73/Bax signaling pathways involved in LPLI inhibiting $A\beta_{25-35}$-induced apoptosis will provide an insight into establishing LPLI as a novel approach to neuron-protection therapy. YAP protein may be a therapeutic target and our findings provide a potential therapeutic strategy for the treatment of AD through activating Akt/YAP/p73/Bax signaling pathway by LPLI.

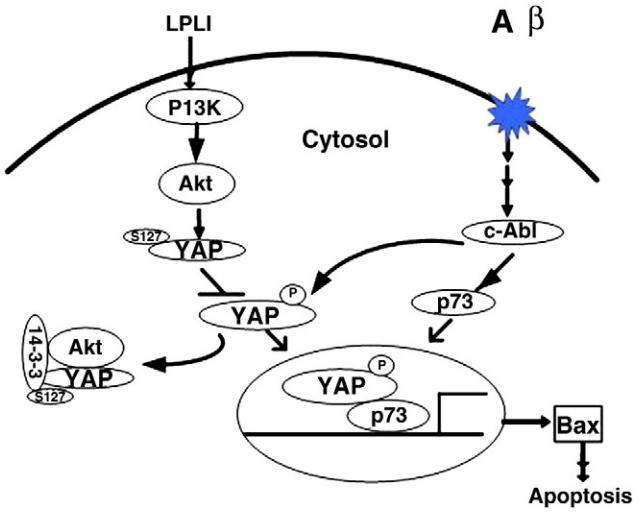


Fig. 5. A model of the signaling pathways for LPLI inhibiting $A\beta_{25-35}$-induced cell apoptosis.

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