Int J Clin Exp Pathol 2017;10(11):10770-10780
www.ijcep.com /ISSN:1936-2625/IJCEP0062322

Original Article

Retinoid X receptor ligand regulates RXRα/Nur77-dependent apoptosis via modulating its nuclear export and mitochondrial targeting

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Received March 10, 2017; Accepted September 28, 2017; Epub November 1, 2017; Published November 15, 2017

Abstract: Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder disease in elderly. It is characterized by the formation of amyloid plaques and nerve cells apoptosis in the brain. This study focuses on the association between nerve cells apoptosis and nuclear receptors within AD. Thus, we detected the changes of the expression and subcellular localization of RXRα/Nur77 and the apoptotic rate of neuroblastoma cells, SH-SY5Y cells and nerve cells in C57BL/6 mouse hippocampus in Alzheimer's disease pathologic condition, and investigated the effect of RXRα exporting inhibition caused by 9-cis-RA on the apoptosis of neurons. We demonstrated that Aβ peptide and H_2O_2 treatment could result in the translocation of RXRα and Nur77 from the nucleus to the mitochondria, and the ligand of RXR, 9-cis-RA, treatment can block the above phenomenon. More importantly, 9-cis-RA treatment could reduce the apoptotic rate of neurons caused by H_2O_2 or Aβ stimulation via enhancing the expression level of Bcl-2 protein. Therefore, our studies revealed a critical role of RXRα/Nur77 in 9-cis-RA-mediated anti-apoptosis in nerve cells and provided novel information for better management of AD.

Keywords: Alzheimer's disease, 9-cis-RA, RXRα, Nur77, mitochondria translocation, apoptosis

Introduction

Alzheimer's disease (AD) referred as progressive multifarious neurodegenerative disorder, is the main cause of dementia in elderly people [1]. The prevalence rate of AD is ten percent of older population in 60 years and this rate is develop into fifty percentage in 85 years older population. AD will lead to the progressive loss of mental, behavioral, functional decline and ability to learn. Since the time of Dr. Alois Alzheimer identified amyloid plaques and neurofibrillary tangles (NFTs) in the brains of people with AD, suggesting the potential cause of the disease, AD was confirmed as a neurodegenerative disease for one hundred years [2]. Approximately 3 million people with AD in the worldwide and over 1 million patients in China. As the aging of the population growing in China, this situation will be more serious, AD has already became the highest prevalence elderly disease in China [3]. Although it is cost a lot of time and energy to take care of patient, there is no cure for AD. Therefore, specific mechanism-based, more effective treatment strategies for AD still await to be developed.

In the pathology of AD, amyloid plaques is the feature which are extracellular deposits of Aβ in the brain parenchyma and in the cerebral blood vessels [4]. NFTs composed largely of paired helical filaments with hyperphosphorylated tau proteins and that caused neuronal and synaptic loss [5]. AD is associated with inadequate levels of this important neurotransmitter. Nuclear receptor distributed through cytoplasm or nuclei in cells with specific functions [6]. It can accept the stimulation of external signal and convert these signals into corresponding biochemical reaction which involved in regulating the growth, differentiation, metabolism, immunity reaction and cell death, almost all of the physiological processes of the eukaryotes [7]. As its structure and function nuclear receptors

can be divided into two groups. One is steroid hormone receptor family, such as the mineralocorticoid receptor, glucocorticoid receptors and sex hormone receptors. The other is non steroid hormone receptor family, such as thyroid hormone receptors, vitamin D receptor and retinoic acid receptors [8]. These nuclear receptors located in the nucleus, most of them were homologous dimers or heterologous dimers. When the ligands is activated and transferred into nucleus, the structure of nuclear receptor is changed and thus regulation the gene transcription process. Due to the important role of nuclear receptor in the progression of AD, we will focus our study on two famous nuclear receptors, RXR and Nur77.

RXR is a core member of the nuclear receptors and RXRs have extensive role in the embryonic development and sex determination. Recent years the research found that RXR can move back and forth between the cytoplasm and nuclei after the differential ligands activation, thus those cells are under deamination into proliferation, differentiation or apoptosis. This phenomenon is confirmed in the study of 4-HPR and CD437 [9]. Also the retinol compounds could induce RXR and nuclear orphan receptor TR3 form into heterologous dimers, thus the RXR gene sequences on the nuclear signal peptide is exposed which is causing the heterologous dimers out of the nuclear. Under the synergy of TR3, heterologous dimers are interacted with Bcl-2 on the mitochondria, making the Bcl-2 became into a promote apoptosis molecules [10]. After the cytochrome c is released from mitochondrial, the cell is under apoptosis procession.

Nur77 is an orphan nuclear receptor transcription factor protein and is a family member of steroid NGFI-B orphan receptors. It can combine with specific DNA response element and regulate the expression of specific gene in multicellular organisms, ultimately regulate the progression of cell growth, differentiation and apoptosis [11]. The researches have shown that Nur77 has a dual function. When the expression of Nur77 is stimulated with growth factors, as a nuclear transcription factors, it will active the expression of cell proliferation gene. While Nur77 is activated by apoptosis factors, it will also cause mitochondrial cytochrome c release [12]. It has been reported that Nur77 combines and forms heterodimer with RXR under the induction of apoptosis signal. Together they will induce the release of cytochrome c from mitochondrial and cause apoptosis. The migration of RXR from the nucleus to the cytoplasm is dependent on the nucleus output sequence (NES) and assistants with Nur77 which can block the function of RXR ligands [13].

One of the important pathological features of AD is the form of β-amyloid plaques. β-amyloid has the effects of nerve toxicity on several ways. First, when the β-amyloid plaques is formed, the immune system is activated and then the immune cells will attack self-normal cell. Second, β-amyloid will cause the apoptosis of nerve cell through the leak of mitochondrial cytochrome c and form of neurofibrillary tangles (NFT). Next, β-amyloid stimulates microglial activation and microglial expression of proinflammtory cytokines and directly activates the classical complement pathway leading to cell lysis [14]. In the etiology and pathogenesis of AD, oxidative stress plays an important role [15]. Under normal circumstances, reactive oxygen species (ROS) is generation for maintaining the dynamic balance in the normal cell, when the dynamic balance is broken, oxidative stress occurs. So the death of nerve cells could be caused by β-amyloid induced the oxidative stress.

In this study, we investigated the effect of RXR ligand, 9-cis-RA, on β-amyloid inducing the translocation of RXR alpha combine with Nur77 and found that 9-cis-RA could protection of β-amyloid caused nerve cell apoptosis. When we use 9-cis-RA, it can reduce the translocation of RXR alpha into cytoplasm in nerve cells which were stimulated with H_2O_2, a ROS stimulator. We also explore the mechanism of 9-cis-RA on the effect of anti-apoptosis on SH-SY5Y and found the effector molecules was Bcl-2. Dedicated to the research, we will be understanding about the relationship between the Nur77 nuclear receptors and RXR alpha in the Alzheimer's disease. Our data revealed a central role of Nur77 in 9-cis-RA-mediated anti-apoptosis in AD.

Materials and methods

Reagents and mice

Antibodies used in the immunoblot analysis, including those specific for phosphorylated

RXRα, Nur77, PARP, β-actin and α-tublin were from Cell Signaling Technology (Danvers, MA, USA) and Bax and Bcl-2 were from santa sruz (Texas, USA). Aβ 25-35 was purchased from sigma (Munich, Germany). C57BL/6 J mice were purchased from the Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). The mice were maintained under specific pathogen free conditions. The health of the mice was monitored periodically by veterinary professionals throughout the experiments, and all animal studies were approved by the Institutional Animal Care and Use Committee.

MTT assay

Cell viability was evaluated using the MTT assay. SH-SY5Y cells were cultured in a 96-well culture plate at a density of 4×10^4 cells for 24 h, and the supernatant was discarded. Each group was pretreated with 9-cis-RA (0.1 μmol/L) for 12 hours. Reagents and Aβ (25 μmol/L) or H_2O_2 (200 μmol/L) were added, 24 h later the media were removed. Medium (100 μL) with 10% MTT was added to each well, and kept at 37°C, 4 h later the media was discarded. DMSO was added and kept away from light on a shaker for 10 min. Absorption was measured at 570 nm using a Bio-Rad 400 microplate reader (Bio-Rad, Hercules, CA, USA). Experiments were repeated at least 3 times. Results were compared using one-way analysis of variance followed by Dunnett's t-test.

Flow cytometry

Cell apoptosis was detected using the Annexin V-FITC/PI apoptosis detection kit. SH-SY5Y cells were seeded into 6-well culture plates (4.0×10^4 cells) and cultured for 24 h. The medium was removed after these groups were treated, and the cells were rinsed once with PBS. Cells were passaged using 0.25% trypsinization for 2 min and collected in centrifuge tubes. Cells were centrifuged and supernatants were discarded. Binding Buffer (200 μL) was added to each tube and blended. Annexin V-FITC was added for 10 min at room temperature in the dark, and tubes were centrifuged. Supernatants were discarded, and 200 μL Binding Buffer was added to resuspend the cells. PI was added blended for testing. Detection of the double positive cells with flow cytometry.

Western blotting

The expression levels of Bax, Bcl-2, RXRα, Nur77 and tublin proteins were determined using western blotting. SH-SY5Y cells were plated in 6-well plates at a density of 1×10^6 cells per well and treated as described above. Cells were incubated with 9-cis-RA, and the medium was removed. SH-SY5Y cells were lysed in cell lysis buffer for 40 min at 4°C, and the lysate was centrifuged. Equal amounts of protein were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% (v/w) skimmed milk powder in Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature for 1 h. Membranes were washed three times with TBST and incubated with different primary antibodies overnight at 4°C. After washed three times with TBST the following day and incubated with a second antibody (anti-rabbit IgG) at room temperature for 1 h. then membranes were washed three times with TBST, and protein immuno-complexes were visualized using BeyoECL Plus. Blots were scanned and quantified with Quantity One software (4.62, Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

The tissue was cut into small pieces and fixed with 4% paraformaldehyde for 24 h at 4°C. Whole-mount staining was then performed. Briefly, the specimens were permeabilized with 1% Triton X-100, blocked with 1% BSA and 3% FBS in PBS, and then incubated with respective antibodies for the surface or nuclear molecules.

Statistical analysis

Data are presented as means ± S.E.M., and the significance was assessed by unpaired two-tailed t-test unless otherwise indicated. A value of $P < 0.05$ was considered as significantly different from the control.

Results

RXRα targets mitochondria is reversed through 9-cis-RA in response to Aβ stimuli

Nur77 migrates from the nucleus to mitochondria to induce apoptosis in response to certain stimulations [16]. Since RXRα heterodimerizes

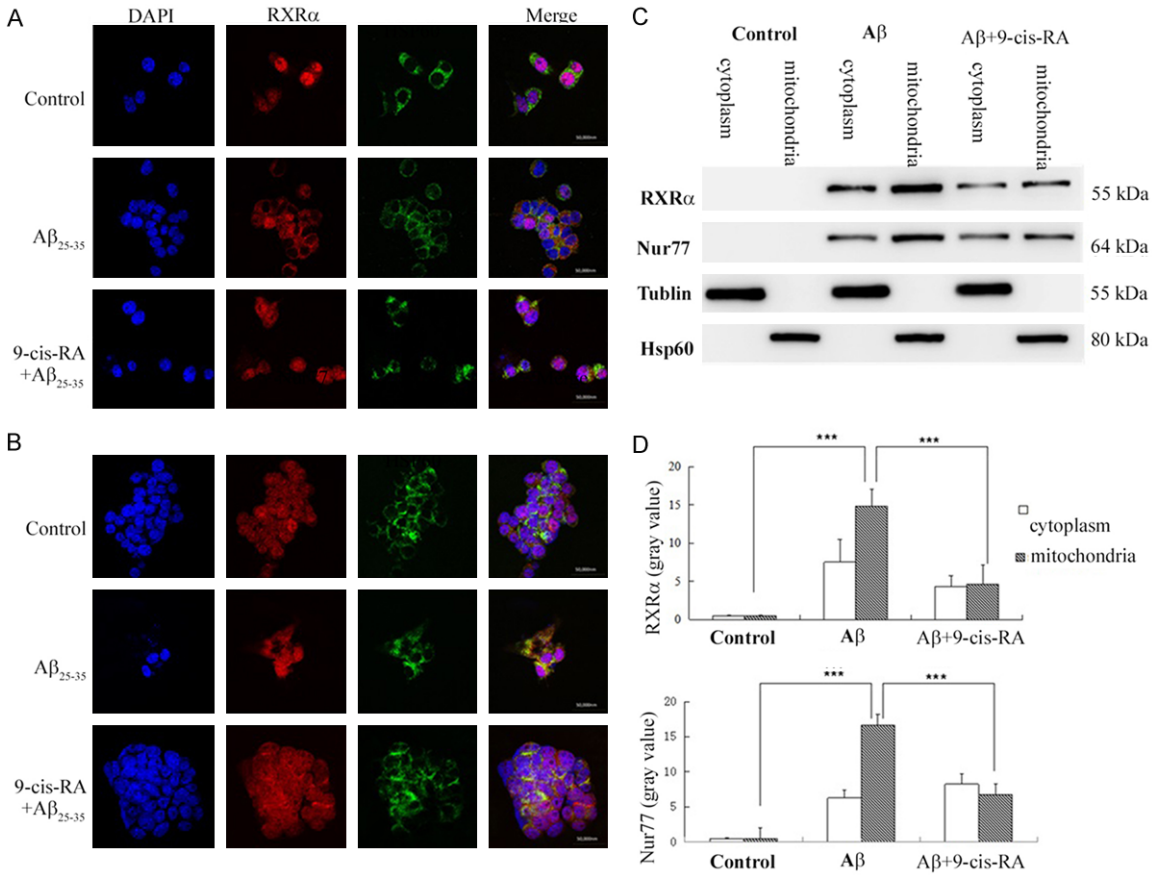

Figure 1. RXRα targets mitochondria is reversed through 9-cis-RA in response to Aβ stimuli. A, B: Immunohistochemical staining of RXRα and Nur77 in the N2a cells stimulated with Aβ (25 μmol/L) in the presence or absence of 9-cis-RA (0.1 μmol/L) for 24 hours. C: Immunoblot analysis of RXRα and Nur77 in lysates of N2a cells stimulated with Aβ (25 μmol/L) in the presence or absence of 9-cis-RA (0.1 μmol/L) for 24 hours. The N2a cells were then did nuclear cytoplasm separation and analyzed by immunoblotting. D: The quantification of RXRα and Nur77 in N2a cells. Protein levels were quantified using Image J software and was assessed by unpaired two-tailed *t*-test (n = 3). $*P < 0.05$; $**P < 0.01$.

with Nur77, we first studied whether RXRα also targeted mitochondria. Subcellular localization of RXRα in mouse neuroblastoma (N2a) cells in the absence or presence of Aβ 25-35, which potently induces N2a cells apoptosis, was examined by confocal microscopy analysis. Immunostaining showed that RXRα predominantly localized in the nucleus in the absence of Aβ 25-35 treatment (**Figure 1A**). However, when cells were treated with Aβ 25-35, RXRα was found in the cytoplasm. To study whether RXRα was associated with mitochondria, cells were stained for heat shock protein 60 (Hsp60), a mitochondrion specific protein (**Figure 1A**). The extensive overlap in the distribution patterns of Hsp60 and RXRα suggested the association of RXRα with mitochondria. Similarly, treatment with Aβ 25-35 also resulted in mitochondrial localization of Nur77 (**Figure 1B**), as previously reported [17]. The enhanced staining of Nur77 in Aβ 25-35-treated cells was due to induction of endogenous Nur77 expression by Aβ. We also analyzed the effect of 9-cis-RA which is the ligands of RXR and found that the localization of both RXRα and Nur77 in mitochondria were reversed in response to 9-cis-RA (**Figure 1A** and **1B**). The protein level of RXRα and Nur77 both in the nucleus and mitochondria in N2a cells which were stimulated with Aβ in the presence or absence of 9-cis-RA were also analyzed (**Figure 1C**). When we analyzed the quantification of the blot and shown that the level of RXR alpha together with Nur77 in mitochondria was respectively reduced in the response of 9-cis-RA, the number was dropped from 14.85 and 16.63 to 4.62 and 6.74 (**Figure**

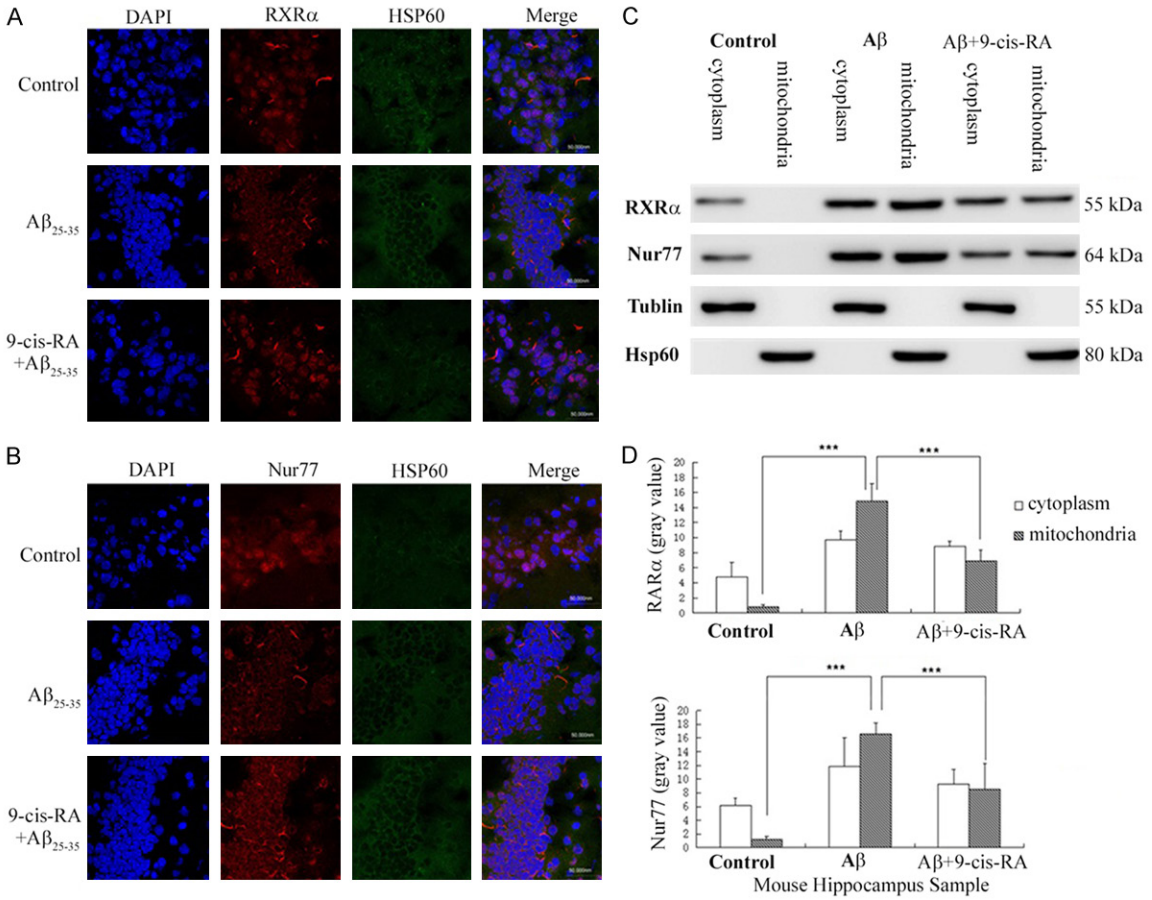

Figure 2. The migration of RXRα to mitochondria is inhibited via 9-cis-RA in AD mice. A, B: C57BL/6 mice were injected with 4 ug Aβ in hippocampus and the treatment group were administrated with 4.8 × 10^{-2} ug 9-cis-RA. The tissue of hippocampus were separated and analyzed. Immunohistochemical staining of RXRα and Nur77 in the tissue slice. C: Immunoblot analysis of RXRα and Nur77 in lysates of hippocampus cells purified from AD mice for 24 hours. The cells were then did nuclear cytoplasm separation and analyzed by immunoblotting. D: The quantification of RXRα and Nur77 were determinate using Image J software and was assessed by unpaired two-tailed *t*-test (n = 3). **P* < 0.05; ***P* < 0.01.

1D). Those results were confirmed with previous immunohistochemical staining.

The migration of RXRα to mitochondria is inhibited via 9-cis-RA in AD mice

To examine the effect of RXR ligands in vivo, we established the mouse AD model injection of Aβ in situ and treated with 9-cis-RA. We detected the location of RXR and Nur77 using immunofluorescence and found that the enhanced staining of RXRα and Nur77 in the Aβ 25-35-treated mice. When the AD mice were treated with 9-cis-RA, the level of RXRα and Nur77 in hippocampus were inhibited (**Figure 2A** and **2B**). To verify this observation, we isolated and digested nerve cells from the hippocampus in the brain of AD mice and did western blot analyzed. We also found a marked reduction in the translocation of RXR and Nur77 in the mitochondria in the 9-cis-RA treated mice (**Figure 2C**). Aβ treatment caused elevated mitochondria protein ratios of RXRα and Nur77 in hippocampus cells were decreased significantly after treated with 9-cis-RA (the gray value 14.83 vs. 6.9024 and 16.63 vs. 8.58) (**Figure 2D**). The data was convictive with the hypothesis that the RXRα heterodimerizes with Nur77 is one of the target in AD and the ligand of RXR can reverse of the translocation of Nur77.

The migration of RXRα to mitochondria is induced via ROS stress

In the pathogenesis of AD, oxidative stress plays an important role. A growing body of research suggests that oxidative stress and

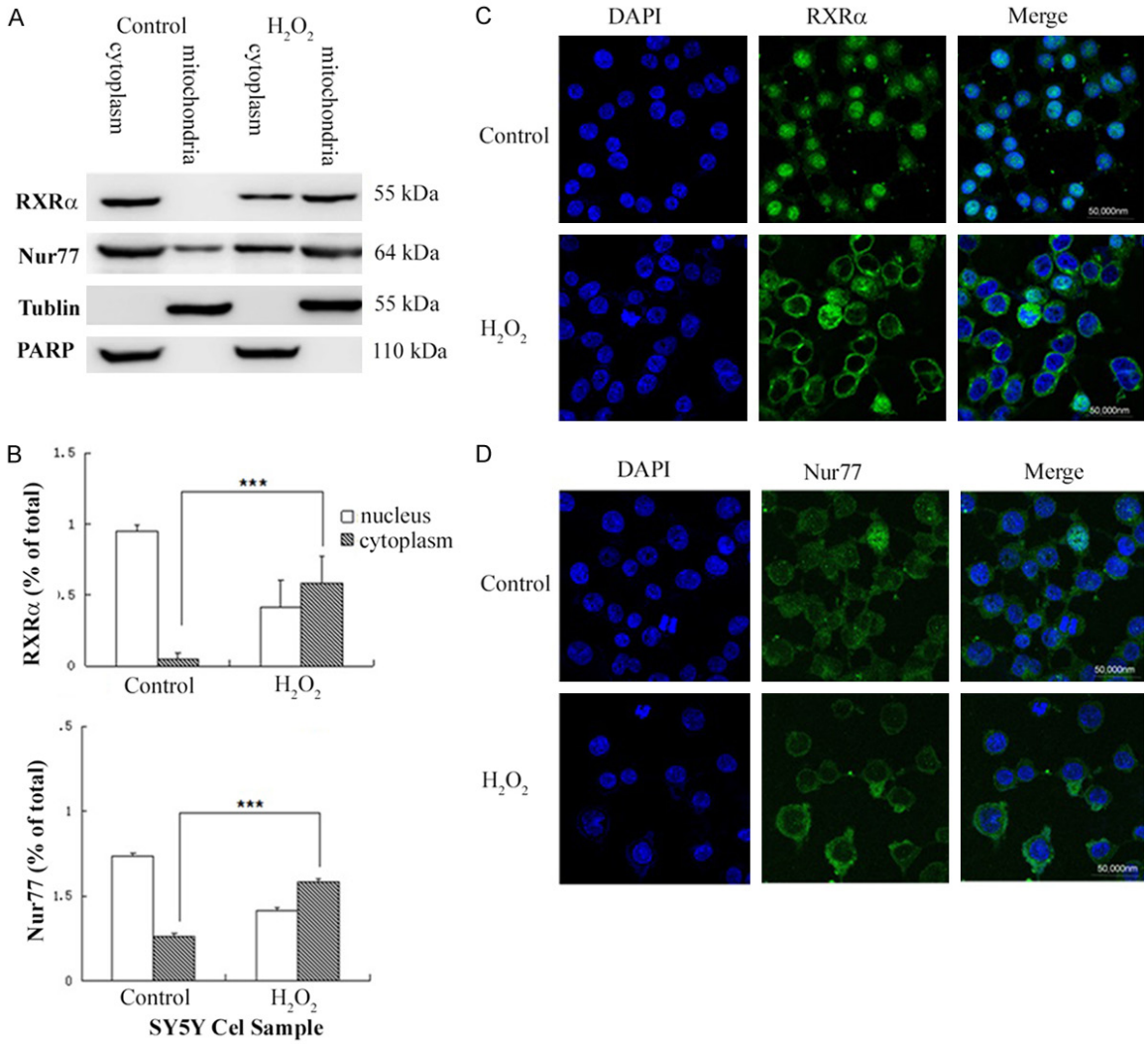

Figure 3. The migration of RXRα to mitochondria is induced via ROS stress. A: Immunoblot analysis of RXRα and Nur77 in lysates of SH-SY5Y stimulated with H_2O_2 (200 μmol/L) for 24 hours. B: The quantification of RXRα and Nur77 in SH-SY5Y. Protein levels were quantified using Image J software and was assessed by unpaired two-tailed *t*-test (n = 3). C, D: Immunohistochemical staining of RXRα and Nur77 in the SH-SY5Y stimulated with H_2O_2 (200 μmol/L) for 24 hours. $*P < 0.05$; $**P < 0.01$.

mitochondrial dysfunction may be the main triggering factor in the AD. To delineate the mechanism underlying the observed inhibitory effects of 9-cis-RA on nerve cells, the SH-SY5Y (human neuroblastoma cells) were stimulated with H_2O_2 for 24 hours. Comparing with control group, protein expression levels of RXRα and Nur77 in SH-SY5Y treated with H_2O_2 remained almost the same (Supplementary Figure 1A and 1B), however, the protein ratio of RXRα and Nur77 in the cytoplasm increased from 4.83% and 26.29% (in control group) to 58.35% and 58.36% (in H_2O_2 group) respectively (**Figure 3A** and **3B**). The immunofluorescence staining shows that RXRα and Nur77 were mostly located in the cytoplasm after the treatment of H_2O_2 (**Figure 3C** and **3D**). Those data declare that the stimulation of H_2O_2 could lead the RXRα with Nur77 out of the nucleus. Next we adopt the ligand of RXR to explore whether it can inhibit the phenomenon caused by H_2O_2 as the in vivo data. Comparing with control group without 9-cis-RA treatment in advance, SH-SY5Y treated with H_2O_2 for 24 h after 9-cis-RA treatment for 12 h had almost the same protein expression levels of RXRα and Nur77 (Supplementary Figure 2A and 2B), however, 9-cis-RA inhibited the shuttling of RXRα and Nur77 from the

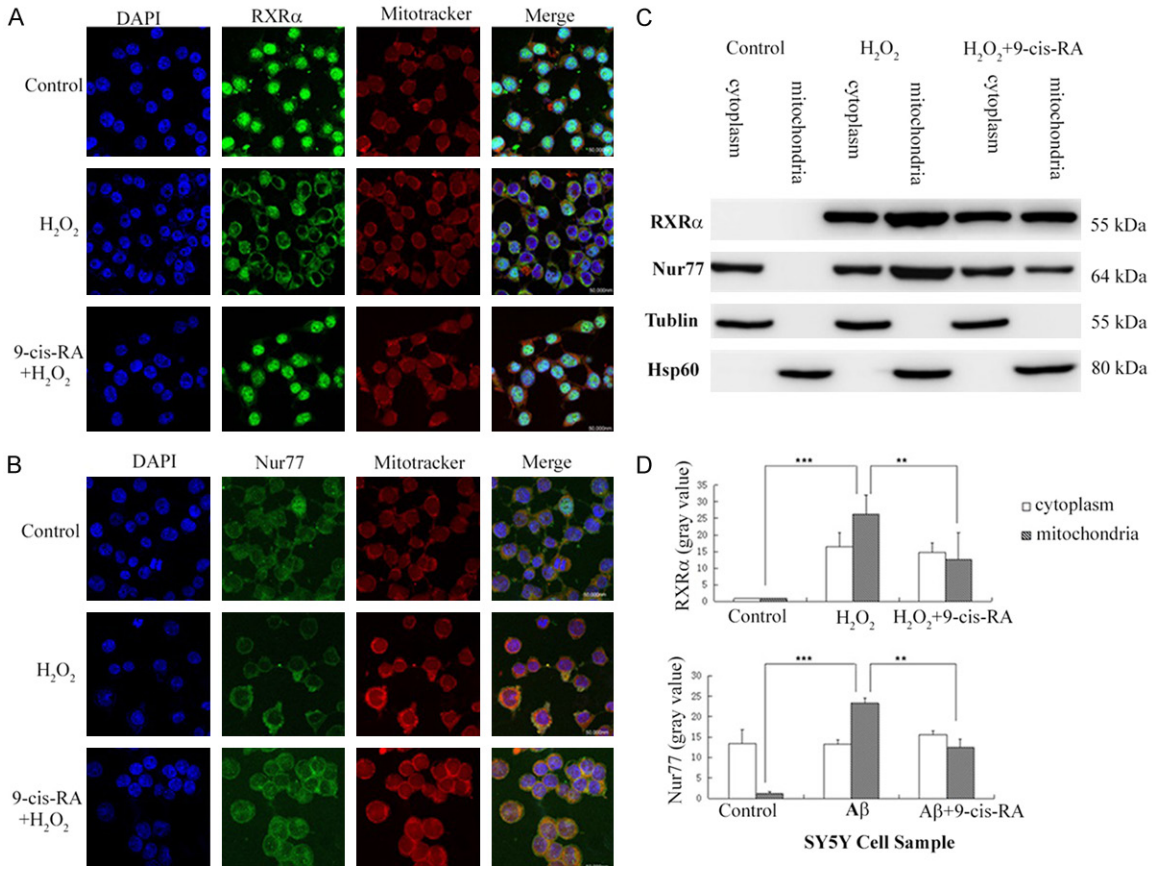

Figure 4. The migration of RXRα to mitochondria is inhibited via 9-cis-RA in SH-SY5Y. A, B: Immunohistochemical staining of RXRα and Nur77 in the SH-SY5Y stimulated with H_2O_2 (200 μmol/L) in the presence or absence of 9-cis-RA (0.1 μmol/L) for 24 hours. C: Immunoblot analysis of RXRα and Nur77 in lysates of SH-SY5Y stimulated with H_2O_2 (200 μmol/L) in the presence or absence of 9-cis-RA (0.1 μmol/L) for 24 hours. The SH-SY5Y were then did nuclear cytoplasm separation and analyzed by immunoblotting. D: The quantification of RXRα and Nur77 in SH-SY5Y. Protein levels were quantified using Image J software and was assessed by unpaired two-tailed *t*-test (n = 3). $*P < 0.05$; $**P < 0.01$.

nucleus to the cytoplasm (Supplementary Figure 3A and 3B). The protein ratio of RXRα and Nur77 in the cytoplasm reduced from 58.35%, 58.36% (in control group) to 11.54%, 35.65% (in 9-cis-RA group) respectively (Supplementary Figure 3C and 3D), while combined treatment with H_2O_2 and 9-cis-RA reduced mitochondria protein ratios of RXRα and Nur77 from 26.2709, 23.29 (in H_2O_2 group) to 12.6499, 12.5211 (in H_2O_2 +9-cis-RA group) respectively, 9-cis-RA inhibited the shutting of RXRα and Nur77 from the nucleus to the mitochondria (**Figure 4**).

Neuroprotective effect of 9-cis-RA against H_2O_2-induced apoptosis in SH-SY5Y

Several reports shown that Nur77 combines with RXR will induce the release of cytochrome c from mitochondrial and cause apoptosis. We used an annexin V-FITC assay to test whether the ligand of RXR could block the apoptosis of nerve cells caused by H_2O_2 treatment. We found that when the SH-SY5Y cells were treated with H_2O_2 for 24 hours the apoptosis rate was increased significantly, from 5.58% to 25.08%. While if the cells were pretreated with the ligand of RXR (9-cis-RA) in 12 hours the apoptosis rate was obviously reduced to 6.66% (**Figure 5A**). Cell viability was analyzed using the methylthiazolyltetrazolium (MTT) assays. Determined by MTT method, cell survival rate was significantly enhanced by 9-cis-RA pretreatment (**Figure 5B**). DAPI staining showed the nucleus morphology of apoptosis cells. From the **Figure 5C**, the apoptosis body caused by H_2O_2 was also remarkable decreased by

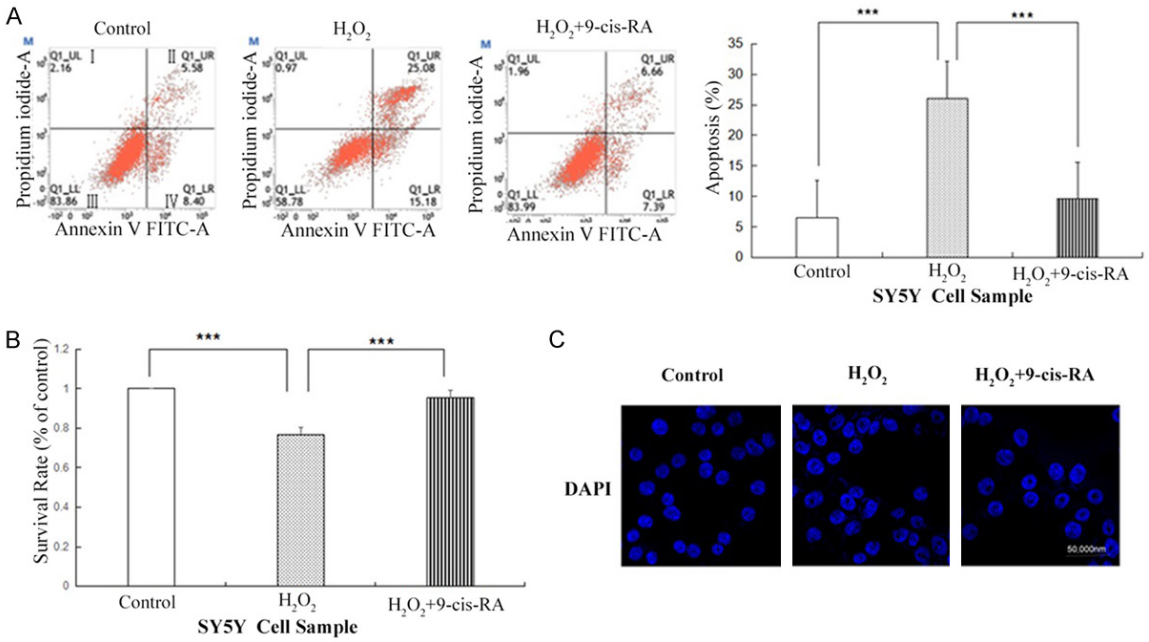

Figure 5. Effect of 9-cis-RA on H_2O_2-induced apoptosis in SH-SY5Y cells. A: Flow cytometry analysis of SH-SY5Y stimulated with H_2O_2 (200 μmol/L) in the presence or absence of 9-cis-RA (0.1 μmol/L) for 24 hours. The statistical result of annexin V/PI staining showed 9-cis-RA that can reduce SH-SY5Y apoptosis compared to H_2O_2-treated cells. B: Cell viability was evaluated using the MTT assay. SH-SY5Y stimulated with H_2O_2 (200 μmol/L) in the presence or absence of 9-cis-RA (0.1 μmol/L) for 24 hours. C: The nucleus morphology of apoptosis cells were shown as DAPI staining. Data are expressed as the mean ± S.E.M. $^{**}P < 0.01$ versus the 9-cis-RA treated alone group (n = 3).

9-cis-RA treatment. Thus, 9-cis-RA could protect the death of nerve cells leaded by ROS stress from anti-apoptosis ways.

The anti-apoptosis effect of 9-cis-RA is via BCL-2 pathway

To further explore the molecular mechanisms of neuroprotective effect of 9-cis-RA on H_2O_2-induced oxidative damage, we examined the expression of Bcl2 and Bax in SH-SY5Y cells by western blot. Apoptosis regulator Bcl-2 is a family of evolutionarily related proteins. These proteins govern mitochondrial outer membrane permeabilization and can be either pro-apoptotic (Bax) or anti-apoptotic (Bcl-2). In apoptotic cells, the level of Bcl-2 will be reduced and the expression of Bax will be increased. The results showed that treatment of cells with H_2O_2 alone caused a significant decrease in Bcl-2/Bax level as compared with control group. However, pretreatment of cells with 9-cis-RA could up-regulate Bcl-2/Bax level (**Figure 6**). Our results showed that 9-cis-RA is one of the effective neuroprotective compound that can mitigate oxidative stress and inhibit apoptosis in SH-SY5Y cells.

Discussion

Nuclear receptors are a big family of ligand dependent transcription factor with widely distributed. Nuclear receptor can combine with the modification drugs and then treat some related diseases such as cancer, metabolic disease, obesity, diabetes and osteoporosis [18]. Nuclear receptor is also very important in the procession of apoptosis, so it is key regulatory target of the Alzheimer's disease (AD) [19]. Retinoid receptors are widely distributed in the brain, the RARα are mainly found in the hippocampus [20]. This study discusses the emerging roles of the ligand of RXRα in the protection of nerve cell apoptosis and their possible implications in neurodegenerative disorders, as well as our current understanding of RXRα regulation via targeting the alternate binding sites on its surface.

There are currently three known isoforms (α, β, γ) that have been identified for the retinoid X receptor (RXR), and these receptors regulate transcription to achieve physiological responses, often in cooperation with other nuclear receptors [21]. RXR is an obligate in the central

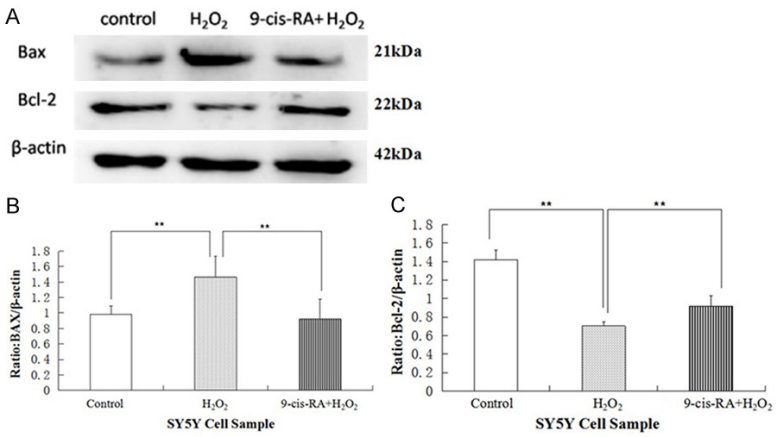

Figure 6. Effect of 9-cis-RA on Bcl2/Bax expression in SH-SY5Y cells. Protein expression was estimated using western blot. A: Effect of 9-cis-RA on Bcl2/Bax expression in SH-SY5Y cells. B, C: The quantification of blot were analyzed by Image J software and was assessed by unpaired two-tailed *t*-test (n = 3). The data are expressed as the mean ± S.EM. $^{**}P < 0.01$ versus the H_2O_2 group (n = 3).

position of the nuclear receptor family, it can form different source dimers with other nuclear receptors, thus be involved in cellular signal transduction [22]. RXR agonist can activate the dimers, collaborative adjusting balance of glucose and fat metabolism [23]. Now research on Alzheimer disease (AD) mainly focus on the relationship of the expression and activity of nuclear receptor, and our research is mainly on the study of translocation of nuclear receptor and the relationship between apoptosis and nuclear receptor during the process of AD.

Nur77 is a duality orphan nuclear receptor [24]. Within the nucleus, it could promote the growth of tumor cells, while the translocation to the mitochondria, it can induce cell apoptosis. Reports show that when RXR alpha with Nur77 formed heterodimer and located on mitochondria, the heterodimer could induce apoptosis in gastric cancer cells MGC803 [25]. Our study is investigated the relationship of RXR alpha and neuron cell apoptosis. With this premise, we investigated the effect of Aβ and H_2O_2 on neuron cell apoptosis with the relationship of the translocation of RXR receptor alpha in the vivo and vitro settings. By adopting the RXR alpha specificity ligand 9-cis-RA, we expect that we could reveal the causality of nuclear receptors change and neuronal apoptosis.

In our analysis, when the N2a cells and SH-SH5Y were stimulated with Aβ and H_2O_2 for 24 hours, cellular RXR alpha together with Nur77 were translocated from cytoplasm to mitochondria. In the consistence, the apoptosis rate of treated cells were significant enhanced (Supplementary Figure 2A) similar with the previous reports [26]. Thus, we speculate that the migration of RXR alpha and Nur77 to the mitochondria may cause the cell death. While we adopt the ligand of RXR, 9-cis-RA, to pretreat the nerve cells, it will protect the apoptosis caused by Aβ and H_2O_2 stress in SH-SY5Y. Those results were verified in the hippocampus of AD mice which were treated with RXR ligand, thus further confirmed with our conclusion. We assume that we can adopt by blocking the translocation of RXR alpha with Nur77 to reduce the apoptosis and to improve the symptoms of AD. In this study, the mouse model is an acute disease model, next we will explore whether the 9-cis-RA could have long term effect on chronic disease AD models.

To sum up, from the primary hippocampus cell and nerve cell lines we prove that β-amyloid could induce the translocation of RXR alpha combine with Nur77 on the mitochondria and also found that RXR ligand, 9-cis-RA, could protection of β-amyloid caused nerve cell apoptosis. In vitro settings, 9-cis-RA can inhibit the nerve cell apoptosis triggered by Aβ and H_2O_2 stress via enhance the expression of Bcl-2. While as exogenous ligands of RXR alpha, there are still a lot of limitations. The doses of 9-cis-RA in cells are very sensitive and its concentration for requirement need to be very accurate. It is a double-edged sword, high concentration will promote apoptosis, and low concentration inhibits cell apoptosis. These studies taken together suggest that RXRs are an excellent target for AD since their modulation may affect nerve cell apoptosis. Our data revealed a central role of RXR/Nur77 in 9-cis-RA-mediated anti-apoptosis in AD. Although we provide a new perspective target for the treatment of AD, we are still eager to find a better RXR alpha ligand for theoretical basis.

Acknowledgements

This work was supported by Professor Development foundation of Fujian Medical University (JS14003); National Natural Science Foundation of China (81241017). We thank the medicine department of Xiamen University for SH-SY5Y cells.

Disclosure of conflict of interest

None.

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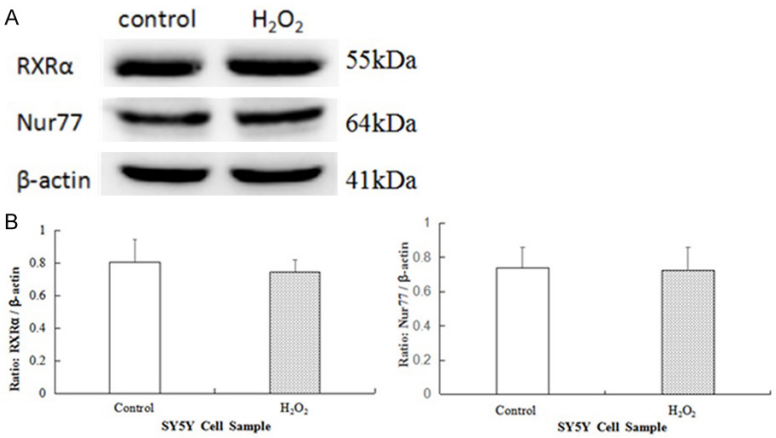
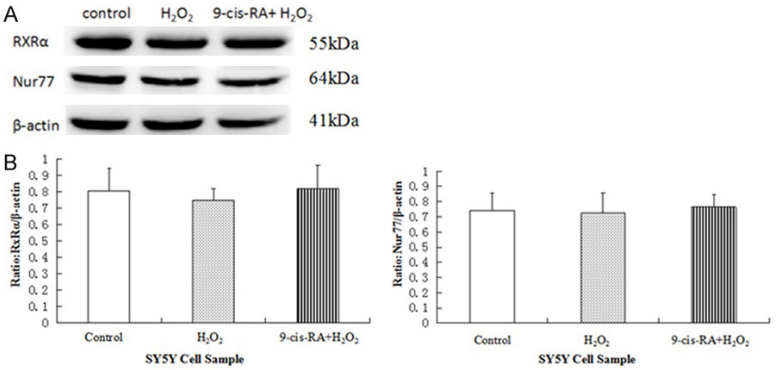
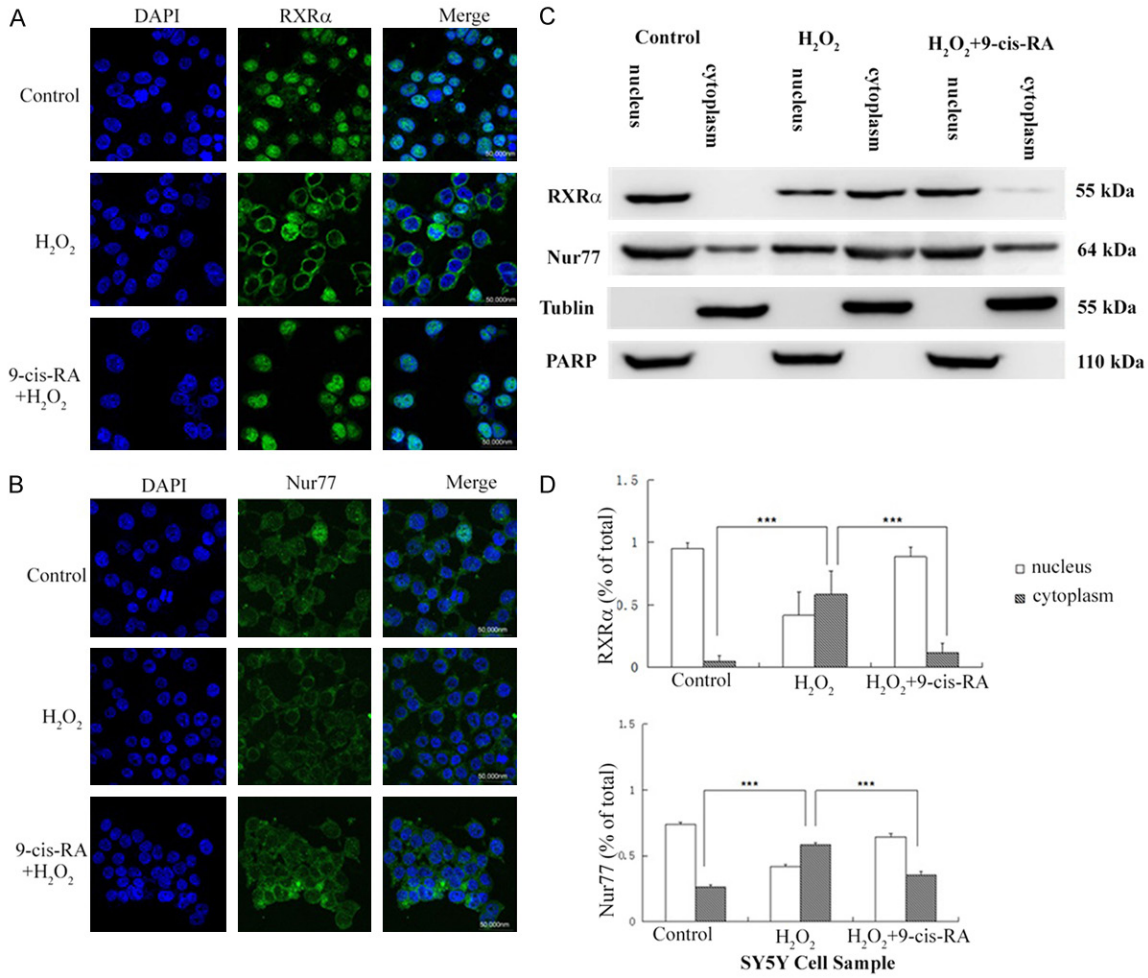


Supplementary Figure 1. Total level of RXRα and Nur77 in SH-SY5Y cells. Protein expression was estimated using western blot. A: Effect of H_2O_2 on RXRα and Nur77 expression in total SH-SY5Y cells. B: The quantification of blot were analyzed by Image J software (n = 3).



Supplementary Figure 2. Total level of RXRα and Nur77 were not altered via 9-cis-RA pretreatment in SH-SY5Y cells. Protein expression was estimated using western blot. A: SH-SY5Y treated with H_2O_2 for 24 h after 9-cis-RA treatment for 12 h had almost the same protein expression levels of RXRα and Nur77. B: The quantification of blot were analyzed by Image J software (n = 3).



Supplementary Figure 3. The migration of RXRα to cytoplasm is inhibited via 9-cis-RA. A, B: Immunohistochemical staining of RXRα and Nur77 in the SH-SY5Y stimulated with H_2O_2 (200 μmol/L) in the presence or absence of 9-cis-RA (0.1 μmol/L) for 24 hours. C: Immunoblot analysis of RXRα and Nur77 in lysates of SH-SY5Y stimulated with H_2O_2 (200 μmol/L) in the presence or absence of 9-cis-RA (0.1 μmol/L) for 24 hours. The SH-SY5Y were then did nuclear cytoplasm separation and analyzed by immunoblotting. D: The quantification of RXRα and Nur77 in SH-SY5Y. Protein levels were quantified using Image J software and was assessed by unpaired two-tailed t-test (n = 3). $*P < 0.05$; $**P < 0.01$.