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Original Article

Nicorandil inhibits oxidative stress and amyloid-β precursor protein processing in SH-SY5Y cells overexpressing APPsw

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Abstract: It has been demonstrated that ATP-sensitive potassium (K_{ATP}) channel activation has neuroprotective effects against neuronal damage induced by hypoxia, ischemia or metabolism stress. This study investigated the multiply protective effects of K_{ATP} channel opener nicorandil against neurotoxicity in SH-SY5Y cells transiently transfected with Swedish mutant APP (APPsw) and also the potential involvement of PI3K/Akt/GSK-3β pathway. Cells were treated with nicorandil (1 mM) for 24 h with and without glibenclamide (10 μM), a K_{ATP} channel inhibitor. Then the cells were collected for Hoechst33342, biochemical assays, real-time PCR, western blot and ELISA assay. Our results showed that nicorandil reduced apoptosis and decreased oxidative stress. Moreover, nicorandil down regulated APP695 mRNA and APP695 protein expression, also reduced $Aβ_{1-42}$ levels in the medium. In addition, nicorandil increased the protein levels of p-Akt and p-GSK-3β by PI3K activation. Applying a PI3K inhibitor, LY294002 blocked the protection. These findings suggest nicorandil to be a potential therapeutic agent to treat Alzheimer's disease (AD).

Keywords: Nicorandil, Alzheimer's disease, oxidative stress, APP processing, PI3K/Akt/GSK-3β

Introduction

Alzheimer's disease (AD) is a primary neurodegenerative disorder and the most common form of dementia among the elderly population. It is pathologically characterized by extracellular deposits of β-amyloid (Aβ) in senile plaques and intraneuronal neurofibrillary tangles. Aβ is produced from β-amyloid precursor protein (APP) by a series of proteases. Although the cause of AD has not been completely elucidated, there is increasing consensus that accumulation of Aβ plays a central role in triggering a cascade ultimately leading to profound neuronal death and memory defects [1, 2]. Due to limited effective therapeutic drugs in use, there is a great need to develop agents that can protect neurons and improve cognitive function.

ATP-sensitive potassium (K_{ATP}) channels are heteromeric protein complexes that directly couple the cellular energy metabolism to electrical activity. These channels are found throughout the brain. It is well documented that mitochondrial K_{ATP} (mitoK_{ATP}) channels play multifactorial roles in protecting against brain injury following hypoxia, ischemia or metabolic inhibition [3, 4]. The neuroprotective effects of mito-K_{ATP} opener diazoxide have been also demonstrated *in vitro* and *in vivo* models of AD [5, 6]. But the underlying mechanisms have not been completely elucidated. Nicorandil is a clinically available mitoK_{ATP} channel opener that exhibits a cardioprotective effect to be used for treating angina pectoris. Previous study showed that nicorandil prevented oxidative stress-induced apoptosis in neurons by preserving mitochondrial membrane potential (ΔΨm) [7]. We have reported that nicorandil could protect AD cellular models against apoptosis by activating PI3K/Akt pathway [8]. But the protective effects of mitoK_{ATP} opener will be involved complex

rather than a single mechanism. So we were interested in exploring other beneficial effects and clarifying the downstream signal transduction after Akt activation.

Recently in AD, apoptosis has been implicated as the mechanism underlying neuronal cell loss [9, 10]. Although multiple factors can contribute to the pathogenesis of AD, free radical overproduction-mediated oxidative stress has been proposed to be one of the major deleterious factors. Increasing evidence suggests that oxidative stress in neurons precedes and accompanies the accumulation of Aβ in AD [11]. Oxidative stress increases β- and γ-secretase activity and to produce Aβ [12, 13]. Therefore, oxidative stress has been considered either as causes or consequences of Aβ production [14].

The PI3K/Akt signaling pathway exerts a crucial role in promoting neuronal survival under a variety of circumstances [15]. Previous study has shown that insulin-PI3K/Akt signaling pathway is down-regulated in AD brain [16]. Glycogen synthase kinnase-3β (GSK-3β), a multi-task serine/threonine kinase, is a key regulatory component of a large number of cellular processes. GSK-3β activity can be mediated by multiple mechanisms including PI3K/Akt pathway [17-19], especially in neuronal survival [20]. GSK-3β also plays an important role in AD pathogenesis, which activity was implicated in tau phosphorylation, APP processing, Aβ production, and neurodegeneration [21].

In the present study, SH-SY5Y cells transiently transfected with Swedish mutant APP (APPsw) were applied as an *in vitro* model of AD to explore the multiply neuroprotective effects of nicorandil. We focused on the effects of nicorandil on cell apoptosis, oxidative stress and APP metabolism in APPsw cells. In addition, we investigated whether the PI3K/Akt/GSK-3β pathways were involved in the protective effects of nicorandil.

Materials and methods

Cell culture and transfection

Human neuroblastoma SH-SY5Y cells (Dep. Central Laboratory, Dalian Medical University 1st Affiliated Hospital) were cultured in DMEM (Cellgro, USA) supplemented with 10% fetal calf serum (Gibco, USA), 100 IU/ml penicillin and 100 ug/ml streptomycin in 95% air 5% CO_2 humidified atmosphere at 37°C. Transiently transfected SH-SY5Y cells with pcDNA3.1-APP695swe or empty vector (neo or control) pEGFPN1 were using lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Drug treatments

SH-SY5Y cells were transiently transfected with APPsw and empty vector. After 24 h, APPsw cells were treated with nicorandil (1 mM, Santa Cruz), K_{ATP} channel blocker glibenclamide (10 μM, Santa Cruz), or co-treated for 24 h (the selection of concentration and time was according to MTT results of preliminary experiment). PI3K inhibitor LY294002 (10 μM, Sigma) was added 1 h prior to nicorandil and then co-administered for 24 h. The stock solution of nicorandil was prepared in DMEM, glibenclamide and LY294002 in DMSO (Gibco, USA) and added to the culture medium at the indicated concentrations. The final DMSO concentration is less than 0.1%, which had negligible effects in all experiments.

Hoechst33342 apoptosis assay

SH-SY5Y cells transfected with APPsw and empty vector after 24 h were seeded in 6-well culture plates. 24 h after the treatment with indicated drugs, cells were fixed with cold 4% paraformaldehyde for 15 min, washed twice with PBS, and incubated with 5 μg/ml Hoechst 33342 for 30 min at 37°C in darkness. Images were obtained with a fluorescence microscope (Olympus, Japan). Apoptotic cells showed nuclear shrinkage, and chromatin condensation. At least 500 cells were counted and the percentage of apoptotic cells was calculated by the percentage of apoptosis in all cells.

GSH, MDA, Total-SOD content measurement

Glutathione (GSH), malondialdehyde (MDA) and total superoxide dismutase (SOD) in the cell homogenates were determined by colorimetry using kits (JianCheng Biology) according to the manufacturer's instructions. The absorbance was recorded at 412, 550, and 532 nm for GSH, total SOD, and MDA, respectively.

Western blot analysis

Total protein concentration of each experimental group was quantified using a BCA kit (Beyotime, China). Equivalent protein (30 ug)

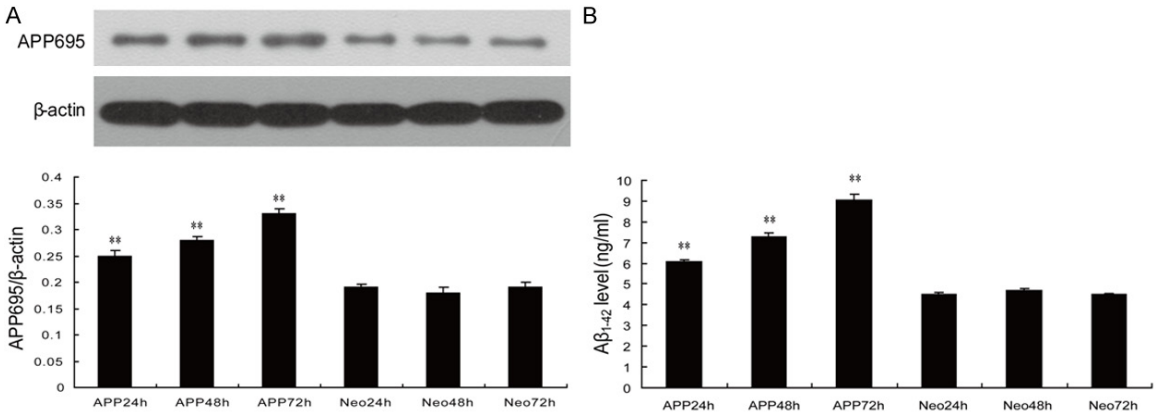

Figure 1. Expression of APP695 and $A\beta_{1-42}$ in APPsw transfected SH-SY5Y cells. A. APP695 protein expression in APPsw and neo cells transfected after 24 h, 48 h and 72 h were detected by western blot. B. $A\beta_{1-42}$ levels in the medium of APPsw and neo cells transfected after 24 h, 48 h and 72 h were measured by ELISA. $^{**}P < 0.01$ versus neo cells transfected in respective times.

was loaded onto 10% SDS-PAGE gels. Via electrophoresis, the proteins were transferred to PVDF membranes (Millipore, USA). Membranes were blocked with 5% non-fat dried milk constituted in TTBS (TBS with 0.1% Tween-20) for 1 h. The membranes were incubated with anti-APP695 (1:1000, BOSTER, China), anti-Akt, anti-p-Akt, anti-GSK-3β, anti-p-GSK-3β (1:1000, Bioworld, USA), and anti-β-actin-HRP (1:10000, KANGCHEN, China) overnight at 4°C. The membranes were then incubated with peroxidase-conjugated secondary anti-rabbit antibody (1:5000, Beyotime, China) at 37°C for 45 minutes. Signals were visualized using an enhanced chemiluminescence detection kit (Millipore, USA) and quantified using Quantitive One Image Analysis (BioRad, USA).

Quantification of Aβ using enzyme-linked immunosorbent assay (ELISA)

The supernatants of each test group were collected, centrifuged and diluted. The conditioned-medium supernatants were stored at -80°C for sandwich ELISA. Aβ production was measured via sensitive fluorescence-based sandwich ELISA using a human $A\beta_{1-42}$ colorimetric immunoassay kit (Cusabio, USA) according to manufacturer's instructions.

Real-time PCR analysis

Total RNA was prepared from cells using Trizol (Life Technologies). Reverse transcription was performed using PrimeScript RT reagent Kit (Takara). Total RNA concentration of each sample was determined by A260 measurement. β-actin served as a reference gene. APP695 primers were as follows: upstream, 5'GGACGATGAGGATGGTGATGAG3'; downstream, 5'GGTACTGGCTGCTGTTGTAGGA3'; the amplified fragment was 151 bp. β-actin primers were as follows: upstream, 5'CTTAGTTGCGTTACACCCTTTCTTG3'; downstream, 5'CTGTCACCTTCACCGTTCCAGTTT3'; amplified fragment was 156 bp. Primers were designed by Takara. And the efficiency of each primer pair was tested using the standard curve method. Real-time PCR was performed using ExicyclerTM96 (Bioneer) with SYBR Green I (Takara) as the detection system. The reaction consisted of the following: 1 μl of the cDNA product, 0.5 μl each primer, SYBR GREEN mastermix 10 μl and ddH_2O to a final volume of 20 μl. Additionally, the cycling parameters were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles (95°C for 10 s, 60°C for 20 s and 72°C for 30 s). Melting curve analysis was used to confirm the specificity of amplification. The expression levels of APP695 mRNA were standardized to β-actin mRNA, and data were expressed as a ratio: relative quantity of APP695 mRNA/relative quantity of β-actin mRNA.

Statistical analysis

All values were expressed as mean ± standard deviation (SD). The statistical analyses were then completed with one-way analysis of variance (ANOVA), or independent-samples t-test, with $P < 0.05$ significance.

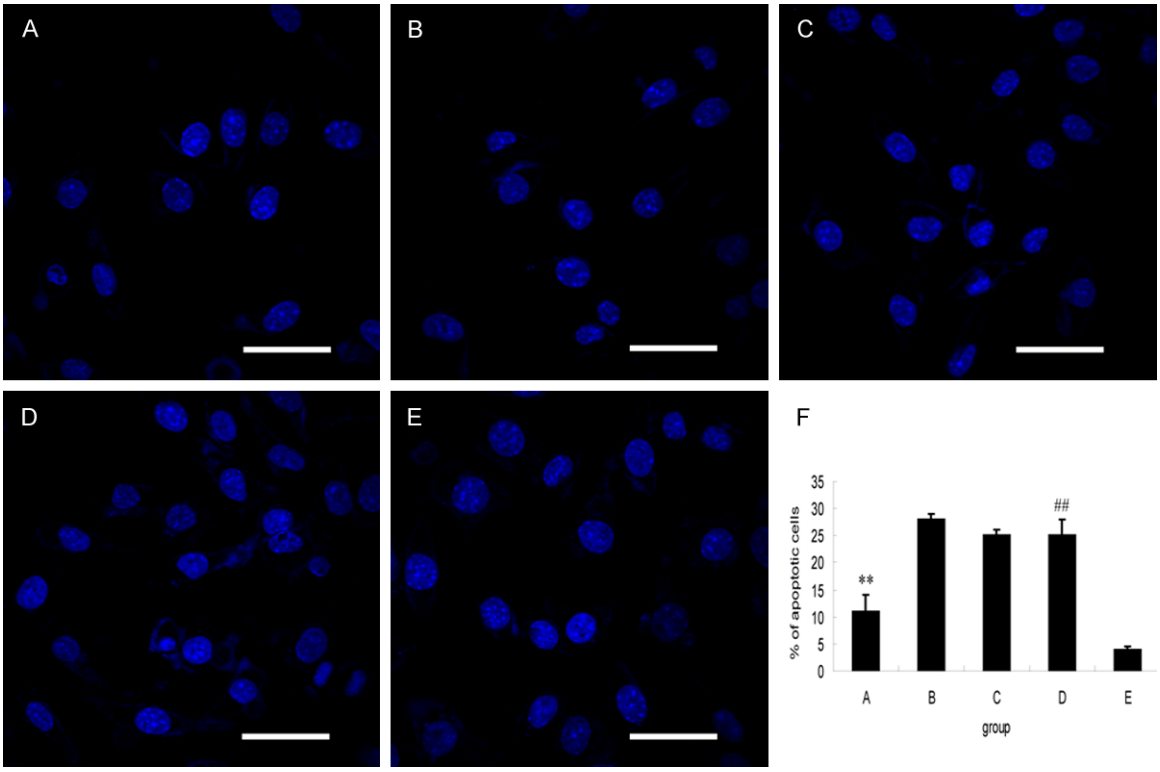

Figure 2. Effect of nicorandil on inhibiting cell apoptosis in APPsw cells. Apoptosis was detected by Hoechst33342 staining assay. Scale bar 20 μm. A. APPsw cells treated with nicorandil group; B. APPsw cells treated with glibenclamide group; C. APPsw cell treated with nicorandil and glienclamide group; D. APPsw cells without drug group; E. Neo cells group; F. The ratio of apoptotic cells in whole cells. $**P < 0.01$ versus APPsw cells without drug group, $##P < 0.01$ versus neo cells group.

Results

APP695 and Aβ$_{1-42}$ in SH-SY5Y cells increased in transiently expressed cells

To resemble APP expressing and Aβ secretion, SH-SY5Y cells were transiently transfected with APP695sw as an *in vitro* model. The transfection efficiency was observed to be roughly 90% by fluorescence microscopy. Western blotting for the APP695 protein revealed a major band at 86 kDa in both neo cells (SH-SY5Y transiently transfected with empty vector) and APPsw cells (SH-SY5Y transiently transfected with APP695sw). ELISA was performed to assess the Aβ$_{1-42}$ level in the conditioned medium. Consistent with previous report [8], APPsw cells showed significant increases in expression of APP695 compared with neo cells over time ($P < 0.01$) (**Figure 1A**). Concurrently, APPsw cells exhibited an increased Aβ$_{1-42}$ level compared with neo cells over time ($P < 0.01$) (**Figure 1B**).

Nicorandil reduced apoptosis of APPsw cells by Hoechst33342 staining

Apoptosis was detected using Hoechst33342 staining assay. As shown in **Figure 2**, there are few apoptotic cells in empty vector transfection group. In APP695sw plasmid transfection group, there are about 25% of apoptotic cells in whole transfected cells. Apoptotic nucleus shrank, showed condensed or fragmented fluorescence. However, nicorandil treatment significantly decreased the percentage of apoptotic cells in APPsw cells, and glibenclamide blocked the protective effect.

The antioxidative effects of nicorandil in APPsw cells

We analyzed the effect of nicorandil on modulating the activity of antioxidants and oxidative product in APPsw cells (**Figure 3**). The activity of lipid peroxidative product MDA in APPsw cells is significantly increased along with antioxidants

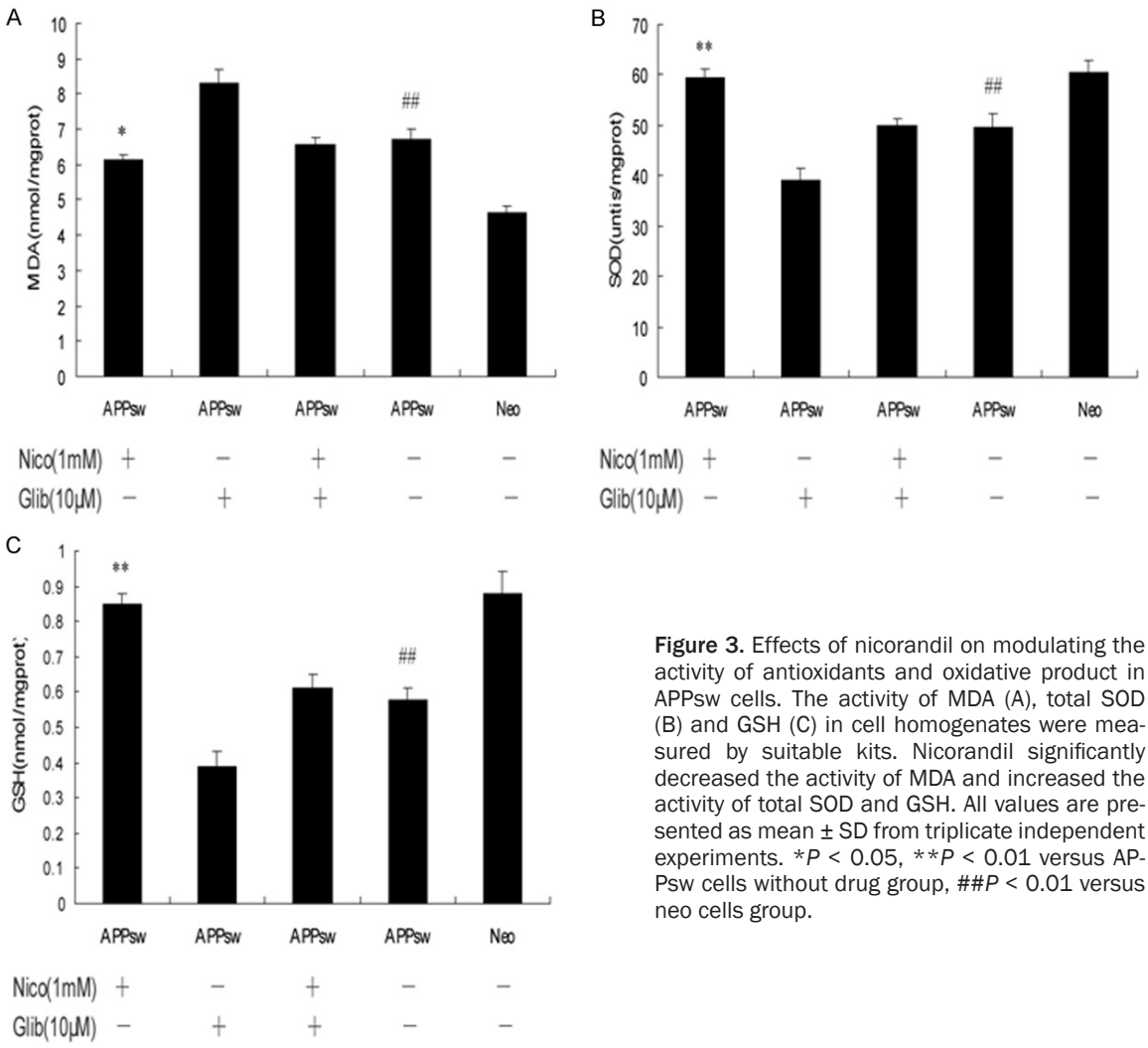

Figure 3. Effects of nicorandil on modulating the activity of antioxidants and oxidative product in APPsw cells. The activity of MDA (A), total SOD (B) and GSH (C) in cell homogenates were measured by suitable kits. Nicorandil significantly decreased the activity of MDA and increased the activity of total SOD and GSH. All values are presented as mean ± SD from triplicate independent experiments. **P* < 0.05, ***P* < 0.01 versus APPsw cells without drug group, ##*P* < 0.01 versus neo cells group.

of GSH and total SOD decreased compared with neo cells ($P < 0.05$). However, nicorandil could decrease the activity of MDA and increase the activity of total SOD and GSH with a significant difference ($P < 0.05$), all of the effects were attenuated by glibenclamide ($P > 0.05$).

Nicorandil regulates the metabolism of APP in APPsw cells

To explore effects of nicorandil on processing of APP in APPsw cells, we tested the mRNA and protein levels of APP695 and $A\beta_{1-42}$ by Real-time PCR (**Figure 4A**), APPsw cells treated with nicorandil resulted in a major decrease in APP695 mRNA level ($P < 0.01$), and the effect of nicorandil was attenuated by glibenclamide ($P > 0.05$). By Western blot (**Figure 4B**), APPsw cells treated with nicorandil resulted in a obvious decrease in APP695 expression ($P < 0.05$), and the effect of nicorandil was attenuated by glibenclamide ($P > 0.05$). By ELISA (**Figure 4C**), APPsw cells treated with nicorandil resulted in an obvious decrease in $A\beta_{1-42}$ level ($P < 0.01$), the effect was similarly attenuated by glibenclamide ($P > 0.05$).

Nicorandil inactivated GSK-3β via the PI3K/Akt pathway

PI3K kinase activates its downstream effector Akt to promote cell survival, which is achieved by phosphorylation of Akt residue Ser473 [20]. In **Figure 5A**, the level of p-Akt was significantly reduced in APPsw cells compared to neo cells ($P < 0.01$). Nicorandil treatment significantly increased p-Akt level in APPsw cells ($P < 0.01$). When PI3K inhibitor LY294002 (10 μM) was

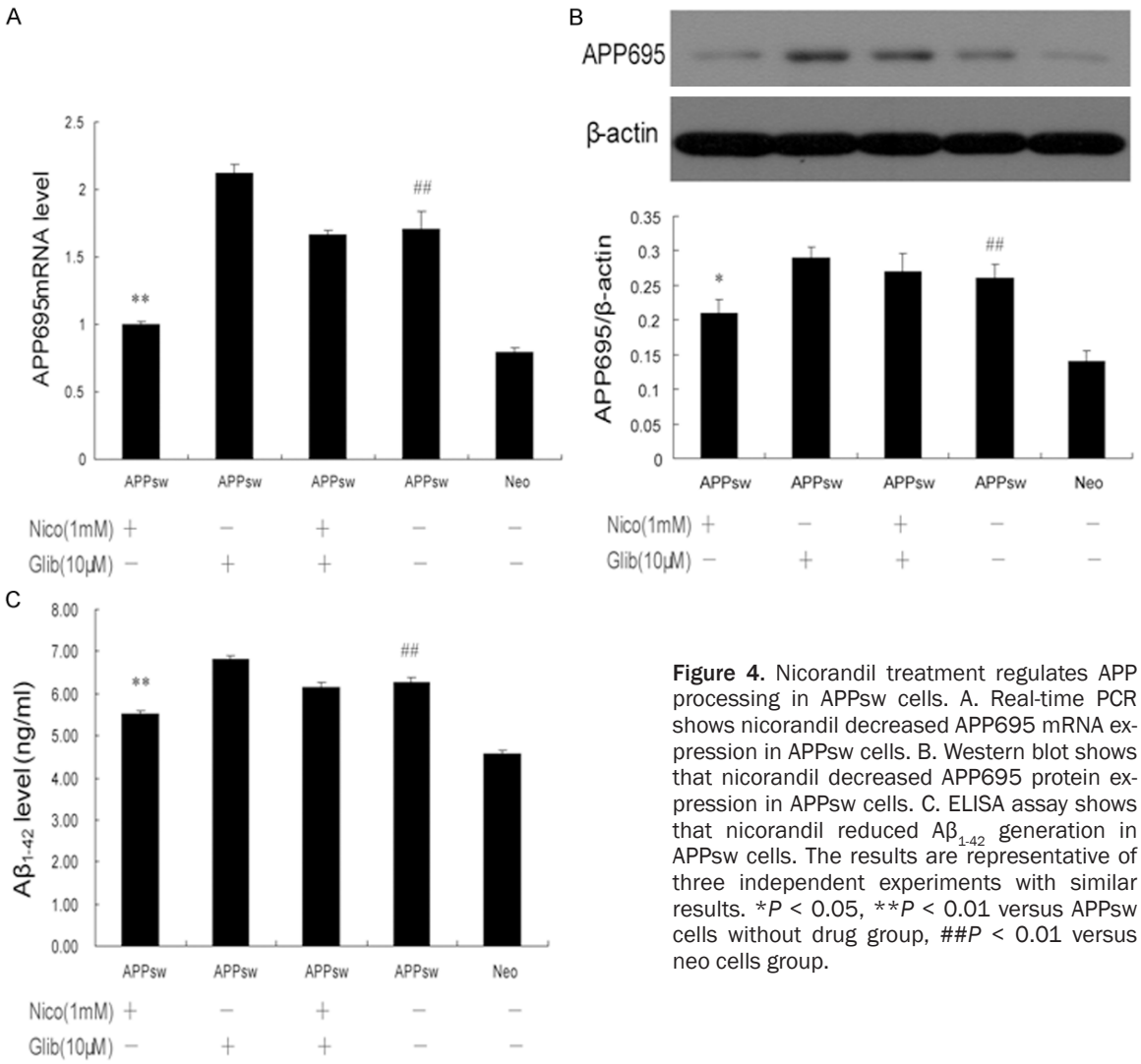

Figure 4. Nicorandil treatment regulates APP processing in APPsw cells. A. Real-time PCR shows nicorandil decreased APP695 mRNA expression in APPsw cells. B. Western blot shows that nicorandil decreased APP695 protein expression in APPsw cells. C. ELISA assay shows that nicorandil reduced $A\beta_{1-42}$ generation in APPsw cells. The results are representative of three independent experiments with similar results. *P < 0.05, **P < 0.01 versus APPsw cells without drug group, ##P < 0.01 versus neo cells group.

pretreated, LY294002 blocked the phosphorylation of Akt (P > 0.05), while the total Akt levels did not change (P > 0.05). These data suggest that activation of PI3K/Akt signaling might be an important upstream pathway in nicorandil-mediated neuroprotection for APPsw cells. Activation of PI3K/Akt signaling promotes Ser^9 phosphorylation and inactivation of GSK-3β [22]. In **Figure 5B**, the level of p-GSK-3β was significantly reduced in APPsw cells compared to neo cells (P < 0.01). Nicorandil treatment significantly upregulated p-GSK-3β level in APPsw cells (P < 0.01). Additionally, blockade of PI3K activity by LY294002 pretreatment also blocked the effect of nicorandil (P > 0.05). These findings suggest that down-regulation the activity of GSK-3β by stimulating the PI3K/Akt pathway in response to nicorandil may present a downstream neuroprotective mechanism.

Discussion

Aβ peptides, proteolytically excised from the APP, are considered the primary pathological agents in AD. APP transfected neuronal-like cells have robust expression of APP and make for a reliable system of Aβ production *in vitro*, this can model the pathological characteristics of AD. Human neuroblastoma SH-SY5Y lines are easy to transfect, whose biochemical properties are similar to normal neurons. They also have the insulin receptor and Kir6.2/SUR1 receptor of K_{ATP} channel [23]. To explore the neuroprotective effects of nicorandil, we established a cellular model of AD by overexpressing APP through transient transfection of APPsw into SH-SY5Y cells.

The present study focused on effect of mitoK_{ATP} opener nicorandil on anti-apoptosis, oxidative

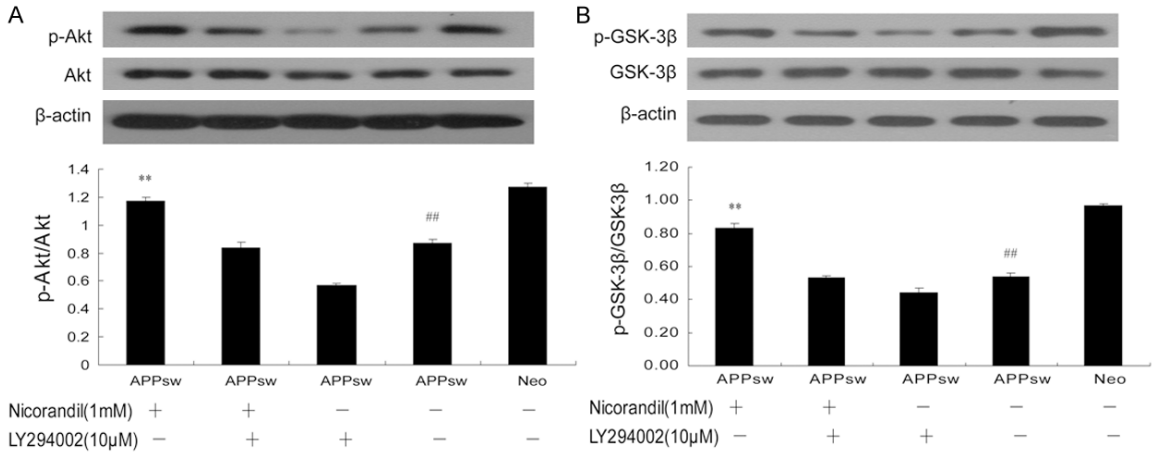

Figure 5. Involvement of PI3K/Akt/GSK-3β in the protective effects of nicorandil on APPsw cells. A. Western blot shows that nicorandil increased p-Akt/Akt, and LY294002 blocked the effect. B. Western blot shows that nicorandil increased p-GSK-3β/GSK-3β, and the effect was reversed by LY294002. The blots are representative of three independent experiments with similar results. $**P < 0.01$ versus APPsw cells without drug group, $##P < 0.01$ versus neo cells group.

stress, and APP processing in APPsw cells. We found that nicorandil significantly reduced apoptosis of APPsw cells. Simultaneously, nicorandil also increased the activity of antioxidants of SOD and GSH, and reduced the level of lipid peroxidation product of MDA in APPsw cells. It is indicated that nicorandil can obviously reduce oxidative stress injury by increasing antioxidant capacity accompanied by reducing lipid peroxidation in AD cellular models. We further found that nicorandil reduced expression of APP mRNA and APP695 protein, thereby inhibited the production of Aβ.

Apoptosis induced by Aβ has a critical role in the pathogenesis of AD. We previously reported that nicorandil reduced rate of apoptosis in APPsw cells by Annexin-V/PI staining, and we speculated that nicorandil achieved the effect of anti-apoptosis through mediating key proteins of evolutionally conserved pathway of apoptosis [8]. Consistent with these results, we further tested the effect of nicorandil on inhibition apoptosis from the morphology by Hoechst33342 staining. Mechanisms responsible for neuroprotection due to opening mito-K_{ATP} channel includes depolarization of intramitochondrial membrane as K^+ enters, which leads to matrix swelling, reduction in calcium overload and enhanced respiration.

APP mRNA expression and protein synthesis can be controlled in an activity-dependent manner in neurons [24]. A deprivation of membrane depolarization decreased both mRNA expression and protein synthesis of APP. Levels of APP mRNA expression and protein synthesis increased upon membrane depolarization by the influx of Ca^{2+} through L-type voltage-dependent calcium channels. Our experimental results can thus be explained according to the above mechanisms. Nicorandil caused the outflow of intracellular K^+ by activating K_{ATP} channel, neurons in a hyperpolarization state, thus reducing Ca^{2+} entry into the cell, all of which can reduce APP695 mRNA and protein expression. A decreased APP protein synthesis, there is less Aβ generation consequently.

Aβ deposition in the brain has been associated with increased oxidative stress, leading to elevated levels of oxidatively modified proteins and lipids in AD patients and transgenic mouse models of AD [25, 26]. Furthermore, it has been detected that lipid peroxidation precedes Aβ deposition in transgenic mouse models of AD [27], which suggests that oxidative stress plays a role in Aβ pathology. Oxidative stress contributes to Aβ production by increasing β-secretase (BACE1) expression and β/γ-secretase activity [28, 29, 13]. Aβ production has been shown to increase following events of oxidative stress and neuronal energy reduction [29, 30]. Similarly, Aβ reduction is seen as a result of manipulations to reduce brain oxidative stress, via caloric restriction or antioxidants [31-33]. Liu et al. believed that diazoxide may therefore reduce Aβ production in 3xTgAD

mice by decreasing neuronal excitability, activation of NMDA receptors, improvement of mitochondrial energy metabolism and reducing oxidative stress [6]. In the same way, our results suggested that nicorandil may regulate β/γ-secretase activity to reduce Aβ production through reducing oxidative stress and increasing capacity of antioxidants.

The PI3K/Akt signaling pathway plays a critical role in mediating neuronal survival [34, 35]. Activation of PI3K/Akt pathway has been reported to protect against Aβ-induced neuronal death [36-38]. Akt regulates survival at multiple sites by inactivate pro-apoptotic proteins or inducing the expression of survival genes. It is well established that GSK-3β is a key downstream of the PI3K/Akt pathway. PI3K activates Akt by phosphorylation of Akt residue Ser473, which phosphorylated GSK-3β at Ser^9 residue, inhibits the activity of GSK-3β [18, 19]. GSK-3β exerts an important role in the pathology of AD. Increased GSK-3β activity is mainly involved in neuronal cell death, tau over phosphorylation and Aβ production [21]. Overexpression of GSK-3β in a conditional transgenic model produced tau hyperphosphorylation and neuronal death [39, 40]. In the brains of GSK-3β transgenic mice, the level of Aβ was markedly increased [41]. LiCl, a selective inhibitor of GSK-3β, could suppress the cell apoptosis induced by GSK-3β, and the generation of $Aβ_{40/42}$ [42]. We investigated that nicorandil increased phosphorylation of Akt and GSK-3β, both of the neuroprotective effects of nicorandil were attenuated by a PI3K inhibitor. This strongly suggests a role for activating PI3K/Akt survival signaling and in consequence, phosphorylating and inhibiting the GSK-3β activity in nicorandil-mediated neuroprotection. Nicorandil stimulating PI3K/Akt/GSK-3β pathway, classically promoted the neuronal survival, in addition, reduced Aβ production by inhibiting GSK-3β activity in APPsw cells.

In conclusion, our data demonstrated that $mitoK_{ATP}$ opener nicorandil play important roles in anti-apoptotic and antioxidative effects on APPsw cells. Nicorandil can also down regulate the expression of APP mRNA and APP695 protein, eventually lead to reduction of Aβ in cellular models of AD. The activation of PI3K/Akt/GSK-3β pathway, classically assigned as pro-survival, could also influence the APP metabolism and Aβ generation. Taking these facts into account, as a clinically applicable $mitoK_{ATP}$ channel activator, nicorandil may represent a new therapeutic potential for drugs in treating AD.

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Disclosure of conflict of interest

None.

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